Effect of the availability of magnesium ions in κ-carrageenan gels on the formation of conidia by *Coniothyrium minitans*

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An isolate of *Coniothyrium minitans* did not sporulate on media to which 0·05 mM magnesium was added when κ-carrageenan was used to solidify the medium. Normal conidiation was observed when a technical grade agar or agar-agar was used as the gelling agent. The κ-carrageenan and agars used in this study contained significant amounts of Mg. In the agar media all the Mg present was available to the fungus. By contrast, in the κ-carrageenan gel a large portion of the Mg was bound by the gel and not available. Sporulation of *C. minitans* was only observed on media containing $\geqslant$ 0·17 mM of unbound Mg. The possible role of Mg in the initiation of conidium formation by *C. minitans* is discussed.

Most microbial cultivations in industry are done in submerged culture. For some processes, however, cultivation on solid substrates in the absence of free-flowing water (solid-substrate cultivation) is advantageous. The production of high quantities of *Coniothyrium minitans* W. A. Campb. conidia, for instance, in stirred-tank reactors has heretofore not been reported. Numerous conidia, however, can easily be obtained when *C. minitans* is grown on various solid substrates (McQuilken & Whipps, 1995). These conidia are a promising biocontrol agent to treat *Sclerotinia sclerotiorum* contaminated soils and plants (Whipps & Gerlagh, 1992). The prospects of a biocontrol agent based on *C. minitans* are promising, provided that an efficient production process is developed.

Optimization of the solid substrate with respect to spore formation is essential for the development of a cost-effective biocontrol agent. Quantification of biomass dry weight is important in such optimization studies. Direct gravimetric quantification of biomass is usually not possible when natural solid substrates are used, due to the close association of the microorganism with the substrate. During the development of an alternative model system based on κ-carrageenan which facilitates the quantitative recovery of biomass dry matter (Weber, Tramper, & Rinzema, 1999), we found that magnesium is very important for sporulation. The present study evaluates the effect of several minerals in combination with various gelling agents on sporulation of *C. minitans*. We demonstrate that the amount of freely diffusible magnesium strongly affects the spore yield. Furthermore, we demonstrate that the gelling agent used to mimic solid substrate cultivation may produce artefacts in medium optimization studies.

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MATERIALS AND METHODS

Microorganism and inoculum preparation

Coniothyrium minitans, isolate IVT 1 (CBS 148.96), was kindly provided by M. Gerlagh of IPO-DLO, Wageningen, The Netherlands. The strain was routinely cultured on potato-dextrose agar (PDA) (Oxoid, Basingstoke, England) at 20 °C. A stock solution of spores was obtained by flooding a PDA-dish with a sterile saline solution (0·8% w/v NaCl) and gently scraping the plate with a bent glass rod. Glycerol (20% w/v, final concentration) was added to the conidial suspension, which was stored at −80° in 1 ml aliquots (approx. 10^7 conidia ml^{-1}).

Media and growth conditions

A defined growth medium containing minerals, thiamine, and carrageenan (MMTC) was used to cultivate *C. minitans*. The composition of the defined medium was as follows (l^{-1} demineralized water): 20 g glucose, 5 g $(NH_4)_2SO_4$, 16·2 g $Na_2HPO_4 . 2H_2O$, 2·6 g KH_2PO_4, 5·5 g $NaH_2PO_4 . H_2O$, 20 mg thiamine, 0·1 g $MgCl_2 . 6 H_2O$, 10 mg EDTA, 2 mg $ZnSO_4 . 7 H_2O$, 1 mg $CaCl_2 . 2 H_2O$, 5 mg $FeSO_4 . 7 H_2O$, 0·2 mg $Na_2MoO_4 . 2 H_2O$, 0·2 mg $CuSO_4 . 5 H_2O$, 0·4 mg $CoCl_2 . 6 H_2O$, 0·1 mg $MnCl_2 . 2 H_2O$ and 500 g 4% (w/v) κ-carrageenan.

To determine the effects of medium composition on the growth and sporulation of *C. minitans*, the following modifications were made to the MMTC medium. The effect of the concentration of various cations on growth and sporulation was determined on MMTC medium in which the amounts of Mg, Ca, Fe or Mn present were altered individually by a factor 1/100, 1/10, 1/3 or 10. To assess the influence of magnesium present in various solidifying agents on sporu-

lation, $MgCl_2$ was omitted from the previous formulation. The κ-carrageenan (Genugel, Copenhagen Pectin, Denmark) in this medium was replaced by either technical agar (grade III, Oxoid), bacteriological agar (grade I, Oxoid), or agar–agar (Merck, Darmstadt, Germany) with final concentrations of, respectively, 1·2, 1·0 and 1·2% (w/v). Whether increased amounts of other cations could induce conidiation of *C. minitans* growing on medium containing a reduced amount of Mg, was tested on modified MMTC media containing only 0·01 g l^{-1} $MgCl_2 . 2 H_2O$ (0·05 mM), and an increased concentration (0·5 mM) of either the Ca, Zn, Fe or Mn salt.

For all media tested, a concentrated solution with glucose and a separate solution containing all the nutrients except glucose and thiamine were prepared and sterilised in an autoclave (20 min, 121°C). Afterwards these solutions were aseptically mixed, and thiamine, which was sterilized by filtration through a 0·22 μm filter, was added. Medium (20 μl) was dispensed into Petri dishes (9 cm diam.). After solidification, these dishes were inoculated with *C. minitans* by spreading 100 μl of a 10 × diluted spore stock solution with a sterile spatula. The dishes were incubated at 20° in the dark.

Assessment of biomass formation and conidia production

At regular time intervals, Petri dishes were removed from the incubator and the carrageenan matrix was divided into two equal parts. One half of the carrageenan disc was placed in an Erlenmeyer flask and demineralized water was added up to 100 ml. After 1 h of gentle mixing at room temperature (18–25°C), the carrageenan was completely dissolved, and the solution was filtered over pre-dried and weighed glass-fibre filters (APFC04700, Millipore, Etten-Leur, The Netherlands). Dry weight was determined after drying for 48 h at 80°. The other half of the carrageenan disc was used to determine the number of conidia. The conidia were liberated by blending the gel for 1 min in 100 ml of demineralized water in a laboratory blender (Waring, New Hartford, U.S.A.). The number of conidia in this solution was either determined by microscopy using a Neubauer improved counting chamber, or using an electronic particle counter (Casy 1, Schärfe-System, Reutlingen, Germany). Sporulation on agar was assessed with the above method, except that a mixing time of 2 min was used to liberate the conidia.

Magnesium quantification

In order to determine the total amount of magnesium which was present in the agars and carrageenan, 2 g of the gelling agent was extracted by continuously mixing the powder with 0·8 M H_2SO_4 at room temperature. After 18 h, the mixture was filtered through a 0·45 μm filter, and the Mg content of the filtrate was determined as described below. Some of the Mg is bound by the matrix and is, therefore, unavailable to the fungus. The quantity of Mg which could freely diffuse in the matrix was determined by dialysis of the complete medium. The agar or carrageenan media containing all the components, except glucose, were prepared in the usual way. After autoclaving, 25 ml of the medium was poured into a tubular dialysis membrane (19 mm diam., M_w cut off 12–14 kDa, Medicell; London, England) and placed in 50 ml of phosphate buffer (same concentrations as present in the medium: 16·2 g $Na_2HPO_4 . 2 H_2O$, 2·6 g KH_2PO_4, and 5·5 g $NaH_2PO_4 . H_2O$ l^{-1} demineralized water) at 4°C with gentle mixing. After 5 d, the Mg content in the phosphate buffer was determined. Magnesium was quantified as described previously (Walinga *et al.*, 1995), by means of atomic absorption spectrometry (SpectrAA 300, Varian, Palo Alto, U.S.A.) using an air-acetylene flame.

RESULTS

Effect of cations on growth and sporulation

Sporulation occurred on all media except those containing a low magnesium concentration (Table 1). On media to which 0·005 mM or 0·05 mM Mg was added *C. minitans* did not form conidia and a reduction in the growth rate and total amount of biomass formed was observed (Fig. 1). On the medium containing 0·17 mM $MgCl_2$, growth and glucose consumption were equal to the medium with 0·5 mM, but sporulation was retarded. On media containing 0·5 or 5 mM $MgCl_2$, similar growth and sporulation profiles were observed (Fig. 1). The observed effect of Mg on sporulation in *C. minitans* was not anticipated: at concentrations below 0·17 mM, sporulation was completely suppressed, and at concentrations above 0·17 mM profuse sporulation occurred. We did not observe intermediate levels of sporulation at any Mg concentration.

Effect of other solidifying agents on sporulation

On media to which $\leqslant$ 0·03 mM Mg was added, the formation of conidia by *C. minitans* was highly dependent on the solidifying agent used. *C. minitans* produced large numbers of spores on technical agar and on agar-agar, whereas no sporulation was observed on bacteriological agar and κ-carrageenan (Table 2). Normal conidiation was observed on all media to which 0·42 mM $MgCl_2$ was added (Table 2). The Mg content of all four gels was determined by means of atomic-absorption spectroscopy. The Mg content of bacteriological agar, technical agar, agar-agar and κ-carrageenan was, respectively, 0·07, 0·35, 0·45 and 0·44 mg g^{-1}. Subsequently, the Mg concentration of the various media was calculated

Table 1. Effect of the cation concentration on the formation of conidia by *C. minitans*. The number of conidia formed (± S.D., $n = 3$) on the defined growth medium (MMTC) with only 1/10 or 1/100 times the original amount of either $MgCl_2$, $CaCl_2$, $ZnSO_4$ or $FeSO_4$ is shown

	Conidia formed (no. per Petri-dish) after		
Cation content	8 d	14 d	26 d
1/100 Mg	$< 10^5$	$< 10^5$	$< 10^5$
1/10 Mg	$< 10^5$	$< 10^5$	$< 10^5$
1/100 Ca	$< 10^5$	$(1·9 \pm 0·6) \times 10^9$	$(2·9 \pm 0·9) \times 10^9$
1/10 Ca	$(3·1 \pm 1·4) \times 10^8$	$(1·5 \pm 0·7) \times 10^9$	$(3·2 \pm 0·7) \times 10^9$
1/10 Zn	$(1·9 \pm 1·0) \times 10^8$	$(2·0 \pm 0·4) \times 10^9$	$(2·2 \pm 1·1) \times 10^9$
1/10 Zn	$(2·7 \pm 1·4) \times 10^8$	$(2·1 \pm 0·4) \times 10^9$	$(2·5 \pm 0·8) \times 10^9$
1/100 Fe	$< 10^5$	$(2·3 \pm 0·5) \times 10^9$	$(2·2 \pm 0·8) \times 10^9$
1/10 Fe	$< 10^5$	$(2·6 \pm 0·6) \times 10^9$	$(3·3 \pm 0·7) \times 10^9$

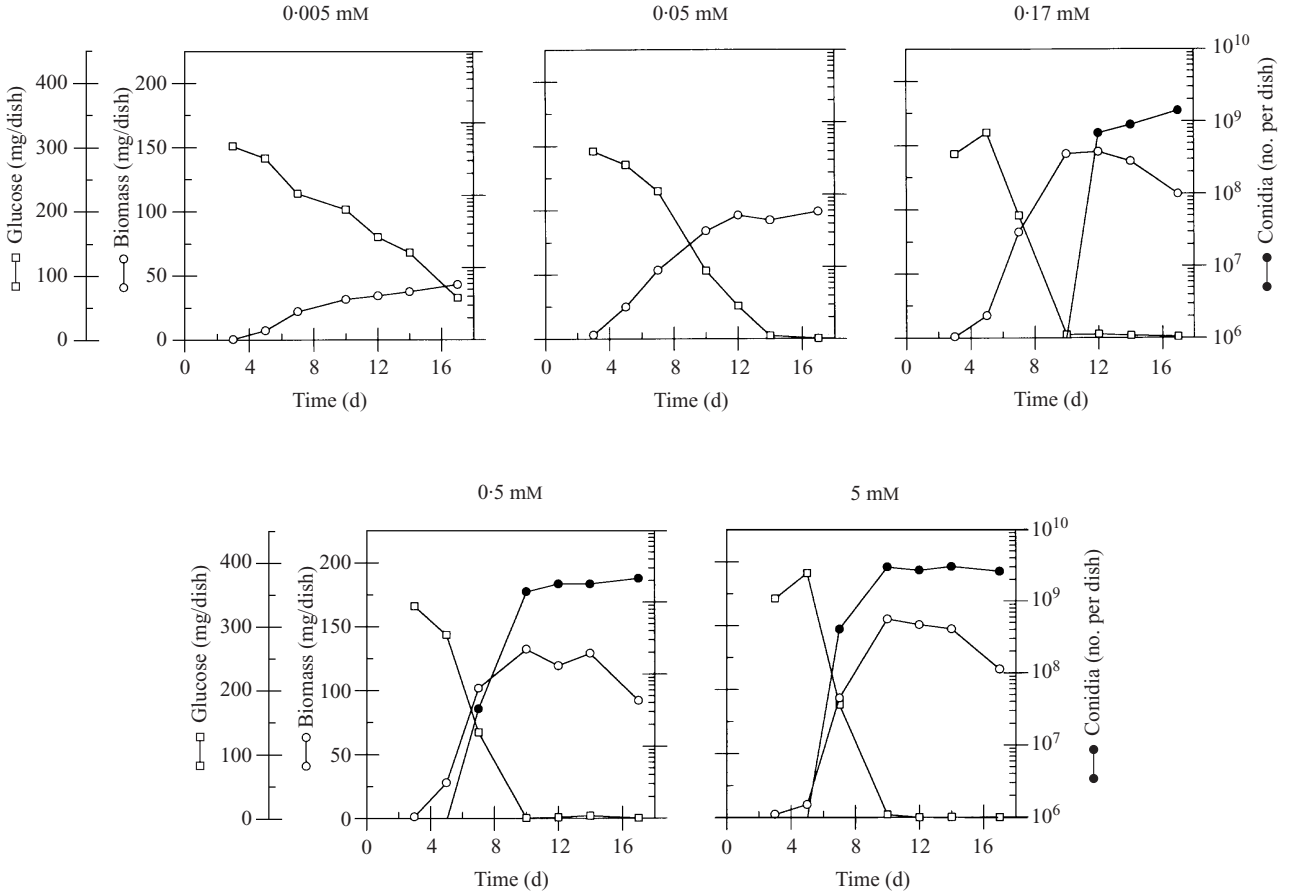

Fig. 1. Effect of magnesium on biomass formation, glucose consumption, and sporulation in *C. minitans*. The amount of magnesium in the defined growth medium (MMTC) varied between 1/100 and 10 × the amount of the standard medium (= 0·5 mM $MgCl_2$). Mean values from duplicate measurements are shown.

Table 2. Effect of the magnesium content (total & available) of various solid media on the conidiation by *C. minitans*. The number of conidia (±S.D., $n = 3$) formed was determined after 21 d of cultivation

	Mg added mM	Total Mg mM	Diffusable Mg mM	Conidia (no. per Petri-dish)
Agar bacteriological	0·00	0·04	0·02	$< 10^5$
	0·03	0·06	0·06	$< 10^5$
	0·42	0·45	0·47	$(3{\cdot}9 \pm 0{\cdot}8) \times 10^9$
Agar technical	0·00	0·18	0·17	$(4{\cdot}1 \pm 0{\cdot}8) \times 10^9$
	0·03	0·21	0·21	$(3{\cdot}9 \pm 1{\cdot}6) \times 10^9$
	0·42	0·59	0·63	$(4{\cdot}9 \pm 0{\cdot}8) \times 10^9$
Agar-Agar	0·00	0·22	0·22	$(5{\cdot}9 \pm 1{\cdot}7) \times 10^9$
	0·03	0·26	0·25	$(5{\cdot}4 \pm 2{\cdot}6) \times 10^9$
	0·42	0·64	0·66	$(6{\cdot}3 \pm 1{\cdot}2) \times 10^9$
κ-Carrageenan	0·00	0·37	0·12	$< 10^5$
	0·03	0·40	0·09	$< 10^5$
	0·42	0·78	0·47	$(2{\cdot}2 \pm 0{\cdot}6) \times 10^9$

(Table 2). Although the κ-carrageenan media contained a high concentration of Mg, its availability to the fungus was limited because little could freely diffuse through the gel. For all the agar media tested all Mg in the gel was available to the fungus (Table 2).

Effect of other cations

Conidiation of *C. minitans* on κ-carrageenan medium containing only 0·05 mM $MgCl_2$ could not be induced by increasing the concentration of Ca, Zn, Fe or Mn (data not presented).

DISCUSSION

Direct determination of biomass grown on a solid substrate is usually not possible due to the close association of the microorganism with the substrate. When κ-carrageenan is used to solidify liquid media, direct quantification of biomass grown on this medium is feasible, as the solid carrageenan

matrix is easily dissolvable in demineralized water (Weber *et al*., 1999). When κ-carrageenan was used instead of agar, however, the concentration of micronutrients affected the sporulation of *C. minitans* IVT1. *C. minitans* did not sporulate when only 0·05 mM $MgCl_2$ was added to the defined growth medium (Fig. 1). We also observed differences in the sporulation of *C. minitans* when various agars were used instead of κ-carrageenan. When no magnesium was added to the medium, *C. minitans* sporulated on technical agar and on agar-agar, whereas on bacteriological agar and on κ-carrageenan no sporulation of *C. minitans* was observed (Table 2). As both agar and carrageenan are polysaccharides extracted from algae, it is expected that they contain impurities. For the agars, the correlation between their Mg content and the initiation of conidiation by *C. minitans* is clear (Table 2). On κ-carrageenan media containing the highest amount of Mg, however, no sporulation of *C. minitans* was observed. κ-Carrageenan is a polysaccharide composed of two sugars: 3,6-anhydro-D-galactopyranose and D-galactopyranose-4-sulphate. The ester sulphates of D-galactopyranose-4-sulphate are known to bind various cations (Guiseley, 1989). The gelling of κ-carrageenan depends on the ionic binding between certain metallic cations and the negative charge of the ester sulphate groups. Besides potassium, magnesium has also been reported to cause gelling of κ-carrageenan solutions (Zabik & Aldrich, 1968; Guiseley, 1989). Indeed a large quantity of the Mg was shown to be bound by the carrageenan (Table 2). Due to this binding, the fungus very likely is exposed to much lower free Mg concentrations in κ-carrageenan media, than expected on basis of the total quantity. This explains why *C. minitans* did not sporulate on these media.

Gels like agar and carrageenan are generally used to mimic solid substrates and used to perform medium optimization studies. These optimization studies are problematic with natural substrates, as direct gravimetric quantification of biomass is impossible when natural solid substrates are used. The results obtained from optimization studies using gels are, therefore, frequently used to improve cultivations on other solid substrates. The high quantity of impurities present in agar or carrageenan, however, and the ability of carrageenan to bind cations, might produce artefacts. When, for instance, technical agar media are used to optimize sporulation by *C. minitans*, the importance of Mg would not be detected. By contrast, the role of Mg would have been recognized when κ-carrageenan were used. The optimal concentration would, however, probably be overestimated as the fungus is exposed to a lower Mg concentration than the amount added to the κ-carrageenan. These examples clearly illustrate the pitfalls that could occur when the results obtained on gels are used to optimize the composition of other solid substrates.

Sporulation of *C. minitans* was only observed on those media containing $\geqslant$ 0·17 mM of unbound Mg (Table 2). A reduction in the amounts of $CaCl_2$, $FeCl_2$ and $ZnSO_4$ had no significant effect on the final number of conidia produced by *C. minitans* (Table 1). Furthermore, increased concentrations of other cations could not induce sporulation of *C. minitans* growing on media with low Mg. Sporulation is initiated above a certain threshold level of Mg and, once started it continues and high numbers of spores are formed. The fact that a threshold concentration of Mg must be exceeded for the induction of sporulation suggests that this cation may play an important regulatory role in *C. minitans*.

Although the specific effects of inorganic salts on fungal reproduction were already recognized in the previous century by Klebs (1896), the mechanisms behind these effects are still poorly understood. Only for calcium-induced conidiation in *Penicillium cyclopium*, and several other penicillia, has part of the underlying mechanism been understood (Roncal, Ugalde & Irastorza, 1993). The role of magnesium in the sporulation of *C. minitans* is still unclear. Magnesium is known to participate in a large array of metabolic processes. For instance, most of the cellular ATP and ADP are present as Mg salts. Furthermore, Mg is involved in the transfer of phosphate groups and numerous enzymes, such as isocitrate dehydrogenase, phosphofructokinase or DNA polymerase require Mg as a cofactor (Stryer, 1981; Garraway & Evans, 1984).

Further elucidation of the role of magnesium in the conidiation of *C. minitans*, will be a challenging subject for investigation. This research may be useful in enhancing the efficiency of a solid-substrate cultivation process used for the mass production of *C. minitans* conidia.

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