Distribution and Transport of Iron in Conidia of *Colletotrichum musae* in Relation to the Mode of Action of Germination Stimulants

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Comparative studies on germination of conidia of *Colletotrichum musae* with low and normal iron contents indicated that in addition to lacking the normal requirement for a germination stimulant low-iron conidia germinated substantially faster. Parallel investigations on the iron flux in conidia during germination demonstrated that the presence of germination stimulants did not significantly influence iron uptake or release by conidia. In addition no major intracellular movement of iron between the organelle, wall and soluble fractions of conidia was detected. These findings imply that the action of chelating agents in promoting germination in conidia of *C. musae* involves the complexing of inhibitory iron at a site within the conidia, so releasing the germination mechanism. Selective accumulation of a germination stimulant (EDTA) within the organelle fraction during induction of germination in iron-replete conidia suggests that the site involved is located in this fraction, possibly on ribosomes. However, the marked differences observed between the relative dimensions of low-iron and iron-replete conidia could implicate the conidial wall as the site of iron complexed by chelating agents involved in stimulation of germination.

INTRODUCTION

Swinburne (1976) demonstrated that anthranilic acid in leachates of banana fruit stimulated germination of conidia of *Colletotrichum musae*, a pathogen responsible for anthracnose lesions on bananas. Harper & Swinburne (1979) subsequently showed that anthranilic acid was metabolized by conidia of *C. musae* primarily to 2,3-dihydroxybenzoic acid (DHBA). The latter compound was found to be a more effective stimulant of germination than anthranilic acid both in terms of the concentration required to promote germination (Harper & Swinburne, 1979) and also the length of time before onset of germination in the presence of the compound (Harper *et al.*, 1980). It was therefore suggested that the activity of anthranilic acid as a germination stimulant could be attributed entirely to its conversion *in vivo* to DHBA rather than to any intrinsic activity.

Since DHBA and its conjugates are well known for their chelating properties, which enable them to play an important role in iron transport in bacteria (Lankford, 1973; Neilands, 1973), Harper *et al.* (1980) investigated the effect on germination of a number of synthetic metal-complexing agents. Such compounds produced stimulation of conidial germination comparable to that observed with DHBA, suggesting that the chelating properties of the latter were indeed important in its biological role in *C. musae*. Although all of the chelating agents effective in promoting germination had a high affinity for iron, and siderophores (ligands which form very stable Fe^{3+} complexes) produced by a number of bacteria were also found to be highly stimulatory to germination (McCracken & Swinburne, 1979, 1980), it was not possible on this

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evidence alone to conclude that the basis of their biological action *in vivo* was chelation with Fe^{3+} rather than another metal ion.

However, further investigations by Harper *et al.* (1980) convincingly established the involvement of iron in the germination process. It was observed that conidia of abnormally low iron content [$7 \mu\text{g (g dry wt)}^{-1}$] produced by growing mycelium on iron-depleted medium ($60 \text{ ng Fe}^{3+} \text{ ml}^{-1}$) germinated freely in water without the normal requirement for a germination stimulant. Increased iron content in conidia was linearly correlated with a decrease in percentage germination. This inhibition of germination by iron was relieved at all conidial iron contents by anthranilic acid, in the presence of which germination remained uniformly high at levels similar to those of conidia of abnormally low iron content.

Speculating on the mode of action of chelating agents in promoting germination, Harper *et al.* (1980) concluded that in order to stimulate germination, they must first be incorporated into conidia where they cause removal of iron from a binding site so releasing the germination mechanism. The studies described in this paper were directed towards confirming this hypothesis, locating the binding site and determining, with the aid of ^{59}Fe and ^{14}C -labelled germination stimulants, the fate of chelated iron, i.e. whether it is excreted from conidia, transported to a non-inhibitory site within the conidia or remains *in situ* in a chelated form which is ineffective in germination inhibition.

METHODS

Cultural conditions. *Colletotrichum musae* was isolated from anthracnose lesions on imported bananas and maintained on modified Cook's no. 2 agar medium (Swinburne, 1976). Conidia for experimental purposes were produced from mycelium grown in liquid shake cultures at 21°C on an asparagine/glucose-based medium of precisely defined iron concentration prepared as described by Harper *et al.* (1980). Conidia were harvested by filtering the contents of each flask through glass wool and centrifuging the resulting conidial suspension. Before use, conidia were washed by centrifugation and resuspension in water twice. The inoculum for liquid cultures was a conidial suspension harvested from mycelia grown for at least two generations in liquid culture medium containing $40 \text{ ng iron ml}^{-1}$.

Germination assay. Washed conidia were suspended (approximately 5×10^5 conidia ml^{-1}) in water or anthranilic acid (10^{-3} M) and duplicate drops ($10 \mu\text{l}$) were placed on glass slides and incubated in a moist chamber at 22°C for 24 h. Counts for germination were made on at least 250 randomly selected conidia in each drop. To examine the time-course of germination in DHBA, washed conidia were mixed with a solution of DHBA ($5 \times 10^{-4} \text{ M}$) or water on a series of glass slides and incubated in a moist chamber at 22°C . At regular intervals over the next 20 h, slides were removed from the chamber and counts for germination made in the usual manner.

Iron determination. The concentration of iron in culture medium was determined by flameless atomic absorption using a Perkin Elmer 306 atomic absorption spectrophotometer fitted with an HGA 72 heated graphite atomizer. Conidial iron content was assayed by replacement of $10 \text{ ng iron ml}^{-1}$ in the growth medium by $^{59}\text{FeCl}_3$ [sp. act. $5 \mu\text{Ci mg}^{-1}$ (185 kBq mg^{-1})]. Samples ($250 \mu\text{l}$) of suspensions of washed conidia or conidial fractions were added to NE 250 scintillation fluid (5 ml, Nuclear Enterprises, Edinburgh, U.K.) and counted using an ICN Tracerlab Coru/matic 200 scintillation counter. The iron concentration of the suspension could be calculated from such counts and the known specific activity of iron in the culture medium before inoculation, since localization of iron within conidia did not lead to any apparent increase in quenching. Dry weights of samples of suspension were determined by drying at 80°C to constant weight and the iron concentration in conidia was expressed on a dry weight basis.

Separation of conidial components. Conidia containing ^{59}Fe or [^{14}C]EDTA were disrupted by a modification of the glass-bead method of Van Etten & Freer (1978). Conidia (1 g dry wt) were suspended in 0.1 M phosphate buffer pH 6.5 (10 ml) in a boiling tube (45 ml) containing glass beads (5 g Jencons grade 4, $870\text{--}1275 \mu\text{m}$ diam.). After cooling to 4°C , the sample was homogenized for 3 min with a vortex mixer at maximum speed. Following decantation of the homogenate the glass beads were washed three times with buffer (5 ml). The homogenate and washings were combined, again cooled to 4°C and subjected to sonication for 1 min using an MSE Mk2 ultrasonic disintegrator. Microscopic examination established the complete disruption of conidia by this technique.

Conidial walls were removed from the suspension by centrifugation (500 *g*, 5 min). The pellet was washed in buffer ($2 \times 10 \text{ ml}$) by resuspension and centrifugation (500 *g*, 5 min). Sedimentation of the cell organelles (nuclear material, ribosomes and mitochondria) from the combined supernatant and washings was achieved by centrifugation at 95000 *g* for 30 min. The organelle pellet was again washed in buffer ($2 \times 10 \text{ ml}$) by resuspension and centrifugation (95000 *g*, 30 min). The washings were combined with the supernatant fraction to constitute the soluble fraction. Prior to determination of the iron or EDTA content by scintillation counting, the volume of each of the fractions was adjusted to 50 ml with buffer.

Measurement of conidial transport of iron and germination stimulants. Iron uptake during the initial stages of conidial germination was investigated by including ^{59}Fe in the germination medium. Conidia (approx. $5 \times 10^5 \text{ ml}^{-1}$) were suspended in a germination medium (100 ml) consisting of either distilled water or anthranilic acid (10^{-3} M), and $^{59}\text{FeCl}_3$ [sp. act. 250 nCi mg^{-1} (9.2 kBq mg^{-1})] was added to each medium to give a final ferric iron concentration of 100 ng ml^{-1} . The suspensions were incubated in conical flasks (250 ml) at 22°C on an orbital shaker (120 r.p.m., eccentricity 2.5 cm). Conidia were harvested by centrifugation (1000 g, 3 min) after various periods of incubation up to 8 h and washed with distilled water ($3 \times 30 \text{ ml}$) by resuspension and centrifugation. The radioactivities of the supernatant after incubation and of the conidial fraction following resuspension in distilled water (100 ml) were determined. Iron uptake was expressed as a percentage of total iron in the incubation medium and on a conidial dry weight basis.

Iron release from conidia and intraconidial transport during the initial stages of germination were monitored using ^{59}Fe -labelled conidia obtained by growth of the fungus on medium containing ^{59}Fe (see 'Iron determination'). Conidia were suspended ($5 \times 10^5 \text{ ml}^{-1}$) in distilled water or a solution of anthranilic acid (10^{-3} M) and incubated in conical flasks (250 ml) on an orbital shaker as previously described for various periods up to 8 h. Conidia were harvested, washed in distilled water ($3 \times 30 \text{ ml}$) by centrifugation (1000 g, 3 min) and resuspension. The radioactivities of the incubation medium and the conidia after resuspension in distilled water (100 ml) were measured and iron release expressed as a percentage of total conidial iron. In some experiments, before assay of iron, conidia were separated into components as described above.

Conidial uptake and localization of [^{14}C]EDTA employed as a germination stimulant were investigated by suspending conidia in a solution of [^{14}C]EDTA [100 ml, $5 \times 10^{-4} \text{ M}$, sp. act. 60 nCi mg^{-1} (2.2 kBq mg^{-1})]. Suspensions were incubated in conical flasks in the usual manner at 22°C for various periods. Conidia were then harvested and washed in water ($3 \times 30 \text{ ml}$) by centrifugation (1000 g, 3 min) and resuspension. The radioactivities of the incubation medium and the conidia after resuspension in distilled water (100 ml) were measured and the EDTA content of conidia expressed on a dry weight basis. Localization of [^{14}C]EDTA in conidia was determined by separation of cellular components and assay of each fraction individually.

Measurement of conidial dimensions. Conidial dimensions were examined using a Wild M20 photographic microscope. Duplicate drops of conidial suspensions stained with acid fuchsin were placed on glass slides and two photographs taken of each drop. Measurements on the developed film were taken of at least 50 randomly selected conidia in each drop and were standardized against a photograph of a calibrated slide. The experiment was repeated five times using conidia from different isolates of *C. musae*, and mean values of the dimensions of conidia grown under different iron regimens compared using a *t*-test.

Chemicals. $^{59}\text{FeCl}_3$ in solution in 0.1 M-HCl was supplied by Amersham. Stock solutions containing $20 \mu\text{g}$ iron ml^{-1} [sp. act. $5 \mu\text{Ci mg}^{-1}$ (185 kBq mg^{-1})] were prepared by dilution with 0.01 M-HCl. Ethylenediamine tetra-[2- ^{14}C]acetic acid [sp. act. $281 \mu\text{Ci mg}^{-1}$ (10.4 MBq mg^{-1})] was obtained as the sodium salt in aqueous solution from the same source. Aqueous solutions ($5 \times 10^{-4} \text{ M}$) for experimental use were prepared with a specific activity of 60 nCi mg^{-1} (2.2 kBq mg^{-1}) by dilution with unlabelled compound.

RESULTS AND DISCUSSION

Influence of iron concentration in the culture medium on conidial germination

Although Harper *et al.* (1980) clearly demonstrated an inverse relationship between conidial germination in water and the iron concentration of the culture medium on which the conidia were produced, the study was confined to conidia of one fungal isolate and media iron concentrations from 40 to 500 ng ml^{-1} . In view of possible variations in germination behaviour between different fungal isolates of *C. musae*, the investigation was extended to three different isolates of the fungus grown on media with iron concentrations from 60 to 1000 ng ml^{-1} . Figure 1 shows the percentage germination in water and anthranilic acid of conidia grown in media with various iron concentrations. The results confirm the previous observations of Harper *et al.* (1980) at iron concentrations below 500 ng ml^{-1} and it is evident that a similar germination pattern extends to iron concentrations up to 1000 ng ml^{-1} .

Since conidia grown in the presence of less than $100 \text{ ng iron ml}^{-1}$ germinate freely in water, any influence of anthranilic acid on metabolism in these conidia prior to germination cannot be attributed to the stimulatory effects of the compound on germination of the conidia. However, as the presence of anthranilic acid stimulates germination of conidia harvested from a medium containing $500 \text{ ng iron ml}^{-1}$, any biochemical changes in such conidia prior to germination that are not observed in conidia from the lower-iron medium are probably associated with the process by which germination is induced by the stimulant. Subsequent experiments described in this paper therefore comprise a comparative study of iron-replete conidia (produced on medium

Fig. 1

Fig. 2

Fig. 1. Influence of iron concentration in the culture medium on germination of conidia of *Colletotrichum musae* in water (O) and in 10⁻³ M-anthranilic acid (●). Values given are means $\pm$ S.E.M.

Fig. 2. Rate of germination of iron-replete and low-iron conidia from *Colletotrichum musae*. Data indicate rates for iron-replete conidia in 5 $\times$ 10⁻⁴ M-DHBA (●) and in water (O), and for low-iron conidia in 5 $\times$ 10⁻⁴ M-DHBA (■) and in water (□).

containing 500 ng iron ml⁻¹) and low-iron conidia (produced on medium containing 60 ng iron ml⁻¹) in relation to various aspects of germination and transport of both iron and germination stimulants.

Comparison of rate of germination of iron-replete and low-iron conidia

Rates of germination of iron-replete and low-iron conidia in water and in a solution of DHBA (5 $\times$ 10⁻⁴ M) are shown in Fig. 2. Conidia from low-iron medium showed a similar pattern of germination in both water and a solution of DHBA. Germination began after 3 h incubation and attained a maximum after 10 h. Iron-replete conidia in the presence of DHBA only began to germinate after about 5 h incubation and germination did not achieve the same percentage as that in low-iron conidia until 13 h had elapsed. As previously noted, such conidia only germinate poorly in water, germination not exceeding 15% after 14 h. The pattern of germination of iron-replete conidia is similar to that previously reported for conidia harvested from solid medium (Harper *et al.*, 1980), although in general conidia from liquid media only attained germination levels of about 75% of those found for their counterparts grown on solid medium. This difference can probably be attributed to some loss of viability through leaching of endogenous nutrients from conidia into the culture medium during growth under the former conditions.

The 2 to 3 h lag in germination between the low-iron conidia which germinate freely in water and the iron-replete conidia that only germinate in response to the presence of a germination stimulant suggests that, whilst a biochemical change (induced by the germination stimulant) must occur in iron-replete conidia before germination can ensue, such a change is not necessary or has previously occurred in low-iron conidia. This explanation is consistent with the conclusion of Harper *et al.* (1980) that iron acts as an inhibitor of germination and that germination stimulants release conidia from such inhibition by chelating iron.

Conidial iron uptake and release during germination

Horowitz *et al.* (1976) reported that, when conidia of *Neurospora crassa* were exposed to water with an activity of less than 0.95 they lost a substance essential for germination. This germination factor was identified as a siderophore (or iron-transport compound) and it was concluded that the germination process was delayed or inhibited as a result of iron deficiency if conidia had no mechanism for concentrating iron from the environment. If iron has an

Table 1. *Iron uptake during germination by conidia of Colletotrichum musae grown in media of normal and low iron status*

Incubation time (h)	Iron content of growth medium (ng ml ⁻¹)	Incubation medium*	Iron uptake by conidia [ng (mg dry wt) ⁻¹ †]
2	60	Fe	639
	60	Fe + AA	556
	500	Fe	324
	500	Fe + AA	333
4	60	Fe	685
	60	Fe + AA	685
	500	Fe	347
	500	Fe + AA	338

* Fe indicates FeCl₃ (100 ng ml⁻¹); AA indicates anthranilic acid (10⁻³ M).

† Means of three experiments using conidia from the same fungal isolate.

Table 2. *Iron release during germination by conidia of Colletotrichum musae from media of normal and low-iron status*

Incubation time (h)	Iron content of growth medium (ng ml ⁻¹)	Anthranilic acid (10 ⁻³ M)	Conidial iron released (%)*
4	60	—	0.9
	60	+	0.7
	500	—	1.3
	500	+	0.8
8	60	—	0.3
	60	+	0.4
	500	—	0.7
	500	+	0.5

* Means of three experiments using conidia from the same fungal isolate.

inhibitory role in germination of *C. musae* as postulated by Harper *et al.* (1980), it is difficult to envisage a basis for the stimulatory effect of chelating agents on germination which involves transport of iron from the environment into conidia as in *N. crassa*. Nevertheless it is possible that conidia from low-iron media germinate readily in the absence of germination stimulant simply because growth under low-iron conditions induces acquisition of an iron-uptake mechanism which replaces the normal requirement for a germination stimulant.

Uptake of iron from solution by iron-replete and low-iron conidia in the presence and absence of anthranilic acid was therefore measured after incubation periods of 2 and 4 h (Table 1). Both iron-replete and low-iron conidia absorbed substantial quantities of iron from their environment after 2 h incubation, but low-iron conidia incorporated almost twice that absorbed by iron-replete conidia. However, anthranilic acid did not significantly influence iron assimilation by conidia of either iron status. Thus stimulation of germination by anthranilic acid and chelating agents is apparently not a result of increased iron uptake. This conclusion is in accord with the inhibitory role for iron suggested by Harper *et al.* (1980) and also the observation of McCracken & Swinburne (1979) that only the desferri form of siderophores promoted germination. The greater iron uptake noted in low-iron conidia probably reflects an increased availability of free metal-ion binding sites in such conidia. Furthermore, double-labelling experiments with iron-replete conidia using both [³H]anthranilic acid and ⁵⁹FeCl₃ have indicated that the presence of iron influenced assimilation of anthranilic acid only to a comparatively minor extent (Graham, 1981). It is thus clear that, although iron uptake can occur prior to germination, it is entirely unrelated to the uptake and action of germination stimulants.

If chelating agents act as germination stimulants by removing iron from a binding site within the conidia, so initiating the germination process, such chelated iron may be transported out of conidia prior to germination. Losses of iron from washed ⁵⁹Fe-labelled conidia derived from media of either high or low iron status were therefore monitored during germination (Table 2).

Table 3. *Influence of conidial iron status on the iron content of conidial fractions from Colletotrichum musae*

Iron content of growth medium (ng ml ⁻¹)	Iron content of conidia [μg (g dry wt) ⁻¹]	Iron content of conidial fractions [μg (g dry wt conidia) ⁻¹]*		
		Wall	Organelle	Soluble
60	6.9	4.2 (60.9)	0.51 (7.2)	2.2 (31.9)
100	10.3	5.8 (51.7)	0.75 (6.8)	3.8 (36.9)
200	21.4	11.6 (54.2)	1.5 (7.0)	8.3 (38.8)
300	24.1	10.0 (41.5)	1.7 (7.0)	12.4 (51.5)
400	40.3	14.3 (35.5)	2.4 (6.0)	23.6 (58.5)
500	54.0	14.8 (27.4)	3.3 (6.1)	35.9 (66.5)
750	57.5	16.0 (27.8)	4.9 (8.5)	36.6 (63.7)
1000	73.0	19.4 (26.6)	6.1 (8.4)	47.5 (65.0)

* Means of three experiments using conidia from the same fungal isolate; figures in parentheses show iron content expressed as a percentage of total conidial iron.

Iron release prior to germination was relatively small and did not exceed 1.3% of total conidial iron. The iron lost during the first 4 h of incubation was partly reabsorbed during the next 4 h, possibly to satisfy the nutritional requirements of the germinating conidia. No significant difference was observed between iron release from conidia incubated in the presence and absence of anthranilic acid, nor did the iron status of the conidia appear to have any appreciable influence on the percentage of iron released. Thus, if stimulation of germination in iron-replete conidia by anthranilic acid and chelating agents is caused by removal of iron from an intraconidial binding site, the chelated iron is certainly not transported out of the conidia into the medium by such compounds. Therefore, either such transport must be restricted to intraconidial movement or alternatively inhibitory iron must remain *in situ* in a chelated form which is ineffective in inhibition of germination.

Distribution of iron within conidia of Colletotrichum musae

The distribution of iron between various fractions from conidia with different iron contents was measured (Table 3). As total conidial iron content increased so did the iron of each fraction, though not in identical proportions. In low-iron conidia, iron was found predominantly in walls, but as the total conidial iron content increased from 6.9 to 54.0 μg (g dry wt)⁻¹ so the proportion in this fraction decreased from 61% to 27%, probably as a consequence of the increasing degree of saturation of available binding sites associated with the cell-wall components. Chitin, which represents a major constituent of conidial walls, is known to be particularly effective at chelating iron (Muzzarelli, 1973).

The proportion of iron in organelles remained constant at about 7% of total conidial iron regardless of the overall iron status of conidia. Nuclear material and respiratory proteins in mitochondria are probably the major source of iron in this fraction. The soluble iron fraction, which corresponds to iron located in the cytoplasm (possibly as ferritin-like aggregates as described in *Phycomyces* sp. by David & Easterbrook, 1971) together with labile iron leached from walls or organelles during fractionation, increased from 32% of total conidial iron in low-iron conidia to 65% in iron-replete conidia.

The data in Fig. 1 and Table 3 were used to examine the effect of the iron content of each conidial fraction on germination of intact conidia in water (see Fig. 3) in an attempt to locate the site at which iron binds and inhibits germination. The iron content of each of the conidial fractions showed an inverse relationship with germination, a finding not unexpected in view of the observation by Harper *et al.* (1980) of a high inverse correlation between total conidial iron content and germination. As the correlation coefficient for each fraction exceeds 0.96 ($P < 0.001$) this treatment of the data does not provide any useful information as to the site of inhibition.

Fig. 3. Influence of iron content in conidial fractions of *Colletotrichum musae* on conidial germination. The data relate germination to iron contents of the organelles (○), walls (●), and soluble fraction (□).

Table 4. Movement of iron during germination of conidia of *Colletotrichum musae*

Incubation time (h)	Iron content of parent medium (ng ml ⁻¹)	Anthranilic acid (10 ⁻³ M)	Iron content of conidial fraction [μg (g dry wt conidia) ⁻¹]*		
			Wall	Organelle	Soluble
0	60	—	6.5	0.70	3.2
	500	—	15.5	3.8	32.0
2	60	—	6.4	0.71	3.3
	60	+	6.3	0.70	3.6
	500	—	15.3	3.7	33.2
	500	+	15.0	3.8	34.1
4	60	—	6.2	0.70	3.3
	60	+	6.2	0.69	3.5
	500	—	15.0	3.9	33.6
	500	+	14.2	3.8	34.8

* Mean of three experiments using conidia from the same fungal isolate.

Intraconidial movement of iron during germination

In order to ascertain whether intraconidial transport of iron occurred during induction of germination by germination stimulants, iron-replete and low-iron conidia were separated into cellular fractions at various stages of germination and the iron content of each fraction measured (Table 4). No major intracellular movement of iron in conidia of either iron status was observed during the first 8 h of incubation. A minor net movement of iron from the wall to the soluble fraction was apparent during the first 4 h incubation, but this occurred in both iron-replete and low-iron conidia regardless of the presence of anthranilic acid and was therefore not thought significant in relief of germination inhibition. Some of the loss from conidial walls can be accounted for by iron release into the incubation medium described in Table 2. These findings suggest that intraconidial transport of iron by chelating agents during germination does not occur. Instead it would appear that iron found at the presumed inhibitory site and displaced by complexing with a chelating agent remains *in situ* in a chelated form which cannot inhibit germination. Nevertheless, the results in Table 4 do not preclude the possibility that limited transport may occur within the conidial fraction concerned.

Distribution of EDTA employed as a germination stimulant in conidia of Colletotrichum musae

If iron involved in inhibition of germination in iron-replete conidia remains *in situ* after displacement from the binding site by a chelating agent, the distribution of the chelating agent within the conidia during germination induction should provide an indication of the site of

Table 5. *Uptake of EDTA into various fractions during germination of conidia of Colletotrichum musae*

Incubation time (h)	Iron content of parent medium (ng ml ⁻¹)	EDTA content of conidial fraction [μg (mg dry wt conidia) ⁻¹]*		
		Walls	Organelles	Soluble
2	60	—	0.2 (8)	2.4 (92)
	500	—	2.9 (37)	5.0 (63)
4	60	—	0.4 (11)	3.3 (89)
	500	0.5 (5)	3.7 (38)	5.5 (57)
8	60	0.9 (10)	1.9 (23)	5.6 (67)
	500	1.0 (8)	5.4 (46)	5.4 (46)

* Means of three experiments using conidia from the same fungal isolate; figures in parentheses show EDTA content expressed as a percentage of total conidial EDTA.

inhibition. Differences between chelating agent distribution in germination stimulant-requiring, iron-replete conidia and freely-germinating, low-iron conidia should be of particular significance in this respect. During the first 4 h of conidial incubation the wall fraction did not contain appreciable quantities of EDTA in either iron-replete nor low-iron conidia (Table 5). Only 5% of total conidial EDTA was detected in the walls even in iron-replete conidia after this period of time, despite the comparatively large proportion of total conidial iron present in this fraction in conidia of both normal and low iron status (see Table 3). However, this observation must be considered in the context of the likelihood that much of the iron, and indeed other cations, in the wall is complexed with chitin and is not available for chelation with EDTA (Muzzarelli, 1973). The absence of EDTA in walls makes it unlikely that iron in this fraction was involved in inhibition of germination, though results reported below do not entirely eliminate this possibility.

During the first 2 h incubation, EDTA uptake in low-iron conidia was mainly into the soluble fraction, with only 8% passing into the organelle fraction. Little change in this ratio was apparent after 4 h incubation. In contrast, although after 2 h incubation of iron-replete conidia the concentration of EDTA in the soluble fraction was only double that of the low-iron conidia, the concentration of EDTA in the organelle fraction exhibited an approximately 15-fold increase relative to that of the low-iron conidia, resulting in almost 40% of the total conidial EDTA being found in this fraction. This increase is especially significant when considered in the light of the observations in Table 3 which demonstrate that the percentage of total conidial iron present in the organelle fraction is similar in iron-replete and low-iron conidia. Such selective accumulation of EDTA in the organelle fraction in iron-replete conidia which require a germination stimulant suggests that iron in this fraction is particularly susceptible to binding by EDTA and so may represent the iron involved in inhibition of germination.

The involvement of iron in mitochondrial metabolism and the reported control by iron of ribosomal translation (Downer *et al.*, 1970; Zahringer *et al.*, 1976) suggest potential sites within the organelle fraction where control of germination by iron might be exercised. Regulation at the ribosomal level represents a particularly attractive possibility, as RNA transcription and *de novo* synthesis of proteins are the first detectable biochemical changes occurring in germinating conidia of various fungal species (Barash *et al.*, 1967; Shepherd *et al.*, 1980).

Hall & Axelrod (1977) reported that including EDTA during homogenization of young mycelia of *Aspergillus nidulans* increased the susceptibility of DNA to hydrolysis with hot perchloric acid, and they considered that this was due to the complexing of ferric ions in the homogenate which would otherwise inhibit hydrolysis. Removal of ferric ions from DNA of *A. nidulans* was shown to occur *in vivo* immediately prior to cell division during sporulation (Hall & Axelrod, 1978). A similar effect might explain the requirement for germination stimulants in *C. musae*. However, Graham (1981) demonstrated that whilst EDTA did increase the susceptibility of conidial DNA to acid hydrolysis in this organism, there was no change in

Table 6. Influence of iron status on dimensions of conidia of *Colletotrichum musae*

Dimension of conidia	Conidial iron status		S.E.M.	Significance
	Low (from medium with 60 ng Fe ³⁺ ml ⁻¹)	Replete (from medium with 500 ng Fe ³⁺ ml ⁻¹)		
Width (µm)	4.55	4.03	0.410	***
Length (µm)	14.21	15.45	2.86	***
Length:width ratio	2.98	4.11	0.05	***

*** Values differ significantly ($P < 0.001$).

hydrolysability during germination. It seems unlikely therefore that the effect of germination stimulants is at the DNA level.

Influence of conidial iron status on dimensions of conidia of Colletotrichum musae

Conidia with higher iron contents were significantly longer and narrower than their lower iron counterparts (Table 6) though the dimensions of both were compatible with values reported by Sutton & Waterston (1970) for conidia of this species i.e. $3-6 \times 11-17 \mu\text{m}$. The value for the axial ratio (length:width) of 2.98 for low-iron conidia contrasts with 4.11 for iron-replete conidia and is indicative of the markedly more spherical shape of the former. Whether this morphological difference is important with regard to the relative responses of such conidia to germination stimulants is uncertain. It is possible to envisage iron acting as a crosslinking agent between the polymeric chains of chitin comprising the wall and so strengthening the structure in a manner analogous to stabilization of pectin gels by calcium ions (deMan, 1980). Greater mechanical strength associated with higher iron content would permit the attainment of the more cylindrical form observed in iron-replete conidia. Stimulation of germination in high-iron conidia by chelating agents could then be explained in terms of the removal of chitin-bound iron during passage of the chelating agent through the wall, a process resulting ultimately in a decrease in the mechanical strength sufficient to allow germ-tube emergence. An enzyme-mediated weakening of the spore coat has previously been considered an essential event in germination of spores of other fungi (Powell & Strange, 1953; Leighton & Stock, 1970). The small proportion of iron released from the walls prior to germination would seem to militate against such a hypothesis. However, Swinburne (1976) reported that germ tubes normally emerge from specific sites which constitute only a small fraction of the conidial surface. Transport of iron from such localized morphologically distinct sites only might therefore explain the comparatively small release of iron during germination. Nevertheless, if induced weakening of the wall by removal of iron is confined to such small areas it seems unlikely that it could affect the dimensions of conidia grown under different iron regimens to the extent found.

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