The Role of a Bacterial Siderophore and of Iron in the Germination and Appressorium Formation by Conidia of *Colletotrichum acutatum*

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Pseudomonas sp. isolate UV3, when added to conidial suspensions of *Colletotrichum acutatum*, stimulated germination and appressorium formation on glass slides and on host surfaces. A siderophore (S_A), purified from low-iron culture of the bacteria, stimulated germination and appressorium formation to a significantly greater extent than the bacterial cells alone. The fully chelated siderophore (S_AFe) and free ferric iron were inhibitory *in vitro* to germination and appressorium formation but inhibited only the latter *in vivo*. The S_A is compared with nutrients at similar concentrations, and the role of the phytosphere microflora and associated siderophores is discussed in relation to fungal infection.

INTRODUCTION

The formation of an appressorium, a cushion-like structure from which the infection peg develops, is a prerequisite for infection by many plant pathogenic fungi. Species of *Colletotrichum* produce both light- and dark-pigmented appressoria, the latter being resistant structures with an innate dormancy.

Blakeman & Parbery (1977) demonstrated that appressorium formation by *Colletotrichum acutatum*, an anthracnose pathogen of strawberry, was greatly enhanced by the presence of washed cells of *Pseudomonas* spp. in the inoculum drop. They attributed this phenomenon to nutrient competition, implicating in particular competition for amino acids. Blakeman & Brodie (1977), Blakeman & Parbery (1977) and Brodie & Blakeman (1976) concluded that appressoria were formed in response to nutrient stress and drew an analogy with resting structures such as chlamydospores. Although nutrient competition from phylloplane microflora is almost certainly an important factor, other modes of action may be involved in the stimulation of fungal germination and appressorium formation, notably iron chelation.

Conidia of *Colletotrichum musae* do not germinate freely in water but do so readily in washings from banana fruit. This was ascribed to the presence in such washings of anthranilic acid (Swinburne, 1976). It was later shown that anthranilic acid is readily converted to 2,3-dihydroxybenzoic acid (DHBA) and that this was the primary stimulant. The mode of action of DHBA appeared to be related to its iron-chelating properties. McCracken & Swinburne (1979), using the same isolate of *Pseudomonas* sp. as Blakeman & Parbery (1977), showed that a siderophore produced in iron-deficient medium by this isolate (UV3) was highly stimulatory to both germination and appressorium formation in *C. musae*. Such siderophores stimulate plant infection processes as a whole in *C. musae* (Brown & Swinburne, 1981), *Glomerella cingulata* (Adikaram *et al.*, 1982) and *Botrytis cinerea* (Brown & Swinburne, 1982).

The aim of this study was to determine if bacterial siderophores could account for the previously observed stimulation of *C. acutatum* by *Pseudomonas* sp. isolate UV3.

METHODS

Culture, maintenance and preparation of spore suspensions. *Colletotrichum acutatum* Simmonds was isolated from black-rot symptoms on strawberry fruit, cv. Brighton, which had been grown at East Malling Research Station from runners imported from the USA. The fungus was cultured on modified Cook's no. 2 medium (Swinburne, 1976) at 18 °C under continuous illumination by fluorescent light. Conidial suspensions were prepared in sterile distilled water by removing slime spores from 14-d-old cultures with a sterile loop. They were washed three times by centrifugation and resuspension in sterile distilled water, and diluted to give a final concentration of 10^5 ml⁻¹, for use in germination assays.

Pseudomonas sp. isolate UV3 was maintained on King's B medium (King *et al.*, 1954) at 18 °C under constant illumination. Suspensions of bacteria from the edge of a 3-d-old colony were prepared in sterile distilled water. These were washed by centrifugation and resuspension in sterile distilled water three times and diluted to a concentration of 10^7 bacteria ml⁻¹, for fungal germination assays.

Preparation of low-iron water and glassware. Water used for washing glassware and preparation of test solutions was double glass-distilled and treated by passage down an ion-exchange resin column (4 × 25 cm Chelex 100, Na⁺ form, 200–400 mesh, BioRad) to remove all traces of contaminating iron. Glassware for all experiments was soaked in 6 M-HCl overnight, rinsed in water and then soaked in 1 M-NaOH for 2 h before a final rinse in water. Low-iron glass microscope slides were stored under Analar ethanol.

Siderophore production. Siderophores were separated from culture filtrates after growth of the bacteria in a low-iron mineral salts/glucose medium as previously described (McCracken & Swinburne, 1979). UV absorption spectra of a component, designated S_A, were recorded with increasing concentrations of added iron and over a pH range of 4 to 9. The absorbance maximum of the major peak was pH dependent (398 nm at pH 7) and addition of iron directly increased absorbance at 450 nm. This suggested that S_A was a member of the pseudobactin (Teintze *et al.*, 1981; Teintze & Leong, 1981) and pyoverdine (Meyer & Hornsperger, 1978; Meyer & Abdallah, 1978) family of siderophores produced by *Pseudomonas* species. Fully chelated S_A (S_AFe) was prepared by titration with 1 M-Fe₂(SO₄)₃ in a spectrophotometer cuvette until the absorbance at 450 nm did not rise further. This brown complex contained approximately equimolar S_A and Fe³⁺, with no excess iron, and had lost most of the bright yellow-green fluorescence of the S_A. Neither the molecular weight nor the molar absorption coefficient of S_A was accurately determined. It was assumed they were close to the figures given for pseudobactin as values for all the siderophores in the group are similar. S_A and S_AFe concentrations, calculated by using the extinction coefficient of pseudobactin (Teintze *et al.*, 1981), were adjusted to 10⁻⁴ M and stored at -18 °C.

Spore germination assays. Germination was assayed on glass slides, with 10 µl droplets containing 5 × 10³ conidia suspended in test solutions, water, or the bacterial suspension. Spores in at least five fields of view were counted in 12 replicate drops per treatment after 18 h incubation at 18 °C in a humid chamber.

Stolons from healthy, mature plants of field-grown strawberry cv. Cambridge Favourite, were removed and cut into 10 cm lengths and suspended within a humid chamber. These were inoculated with 10 µl drops of conidial suspensions, one or more per piece of stolon. Germination and appressorium formation were assessed by examining thin longitudinal sections of stolons stained with cotton blue in lactophenol (BDH) under a light microscope. Experiments were repeated at least once and counts of at least 300 conidia per treatment were analysed statistically.

RESULTS AND DISCUSSION

Germination and appressorium formation by C. acutatum conidia in vitro

The germination of *C. acutatum* conidia in distilled water ranged between 14% and 26%, with a mean value $\pm$ SE of $19 \pm 3\%$. In 5×10^{-5} M-S_A germination ranged from 37% to 93%, with a mean value of $60 \pm 17\%$. Although the S_A was always stimulatory, the extent of the stimulation was variable. This was not easily explained and may have been due to small differences in spore age and absolute slide cleanliness between the experiments. Certain conidial characteristics may favour the action of S_A, although there was no evidence to suggest what these may be as all experiments were done under the same specified conditions. The presence of live *Pseudomonas* sp. isolate UV3 stimulated germination but only by half as much as by S_A (Table 1). This differs from previous results in which bacteria marginally inhibited germination (Blakeman & Parbery, 1977). Appressorium production was stimulated in a manner similar to that described earlier except that dark (mature) appressoria were not produced on glass in that study. This may have been partly due to the particularly clean glass surfaces used during the present investigation, since it was noted that fewer appressoria were formed on new slides than on those that had been acid-washed. The percentage of germinated conidia that produced appressoria was not

Table 1. Effect of S_A and *Pseudomonas* UV3 on the germination and appressorium formation of *C. acutatum* conidia on glass slides

The results are means $\pm$ SE.

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of all conidia with dark appressoria
Control (H_2O)	14 $\pm$ 5	55 $\pm$ 21	9 $\pm$ 4	35 $\pm$ 18	7 $\pm$ 4
5×10^{-5} M- S_A	93 $\pm$ 3	97 $\pm$ 2	91 $\pm$ 3	56 $\pm$ 11	53 $\pm$ 11
<i>Pseudomonas</i> UV3 (10^7 cells ml $^{-1}$)	47 $\pm$ 11	99 $\pm$ 1	46 $\pm$ 10	95 $\pm$ 4	42 $\pm$ 8

Table 2. Effect of S_A , S_AFe and Fe^{3+} on the germination and appressorium formation of *C. acutatum* conidia on glass slides

The results are means $\pm$ SE.

Treatment*	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of all conidia with dark appressoria
Control (H_2O)	26 $\pm$ 2	47 $\pm$ 7	10 $\pm$ 2	6 $\pm$ 1
S_A	51 $\pm$ 3	68 $\pm$ 4	35 $\pm$ 3	18 $\pm$ 2
S_AFe	11 $\pm$ 2	5 $\pm$ 3	1 $\pm$ 1	<1
$Fe_2(SO_4)_3$	13 $\pm$ 2	34 $\pm$ 7	5 $\pm$ 1	3 $\pm$ 1

* All at a concentration of 5×10^{-5} M.

Table 3. Effect of S_A , glucose, glutamine and alanine on germination and appressorium formation by *C. acutatum* conidia on glass slides

The results are means $\pm$ SE.

Treatment*	Percentage germination	Percentage of all conidia with appressoria	Percentage of all conidia with dark appressoria
Control (H_2O)	16 $\pm$ 2	14 $\pm$ 2	13 $\pm$ 1
S_A	37 $\pm$ 3	26 $\pm$ 2	25 $\pm$ 4
Glucose	12 $\pm$ 2	11 $\pm$ 1	10 $\pm$ 2
Glutamine	37 $\pm$ 4	18 $\pm$ 2	14 $\pm$ 2
Alanine	28 $\pm$ 3	15 $\pm$ 2	12 $\pm$ 2

* All at a concentration of 5×10^{-5} M.

significantly different between the S_A and bacterial treatments, whilst in the presence of bacterial cells more of the appressoria were dark. Thus, although the percentage of total conidia that produced appressoria was lower in the bacterial treatment because of poorer germination, the percentage of conidia with dark appressoria did not differ significantly between S_A and bacterial treatments. When considering the effect of a bacterial siderophore on *C. acutatum* conidia in a field situation, the stimulation of appressorium formation is particularly important since it is from these structures that the fungus penetrates and infects the host plant.

Germination was inhibited by 5×10^{-5} M- $Fe_2(SO_4)_3$ and S_AFe , the fully chelated form of S_A (Table 2). The failure of S_AFe to stimulate germination reinforces the view that the biological effect of S_A results from its capacity to bind iron. Few appressoria of any kind were produced in the presence of free Fe^{3+} and even fewer in S_AFe . That the relatively small number of appressoria which were produced in the presence of iron were predominantly hyaline is in accord with results obtained with *C. musae* (Swinburne & Brown, 1983).

S_A was compared with glucose, glutamine and alanine, each at concentrations of 5×10^{-5} M (Table 3). The amino acids significantly increased germination, glutamine to an extent

Table 4. *Effect of S_A, S_AFe, Fe³⁺, Pseudomonas UV3 and bacteria plus Fe³⁺ on the germination and appressorium formation by C. acutatum conidia on strawberry stolons*The results are means $\pm$ SE.

Treatment*	Percentage germination	Percentage of all conidia with appressoria	Percentage of appressoria that were dark	Percentage of all conidia with dark appressoria
Control (H ₂ O)	58 $\pm$ 5	38 $\pm$ 4	75 $\pm$ 5	28 $\pm$ 4
S _A	89 $\pm$ 2	78 $\pm$ 3	84 $\pm$ 4	66 $\pm$ 4
S _A Fe	73 $\pm$ 3	37 $\pm$ 3	38 $\pm$ 7	13 $\pm$ 3
Fe ₂ (SO ₄) ₃	50 $\pm$ 6	24 $\pm$ 4	35 $\pm$ 8	10 $\pm$ 2
<i>Pseudomonas</i> UV3	64 $\pm$ 4	47 $\pm$ 4	95 $\pm$ 2	44 $\pm$ 4
<i>Pseudomonas</i> UV3 + Fe ₂ (SO ₄) ₃	69 $\pm$ 3	55 $\pm$ 4	78 $\pm$ 6	43 $\pm$ 5

* S_A, S_AFe and Fe₂(SO₄)₃ were added at a concentration of 5×10^{-5} M. Bacteria were added at a concentration of 10^7 cells ml⁻¹.

comparable with S_A. However, this stimulation did not extend to appressorium formation. There was no significant difference in germination or appressorium formation between 5×10^{-5} M-glucose and water. Blakeman & Parbery (1977) used concentrations higher than those tested here and found that nutrients inhibit appressorium formation, and that germination in water was 100% and could not be stimulated. To simulate competition from bacteria they induced nutrient deprivation by leaching conidia between polycarbonate and cellulose acetate membranes. Such conidia germinated well and produced mostly dark appressoria. However, leaching may also have reduced the conidial iron content (or moved it from its site of action) which would have given rise to the observed effects in a way similar to that described here, and similar also to conidia grown under low-iron conditions (Graham & Harper, 1983). However, nutrients must be involved in part since leaching with a nutrient solution, which, unless contaminated by iron, would also lower the iron content, does not stimulate appressorium formation. Excess exogenous nutrients induce saprophytic growth with conidia producing long ramifying germ tubes and few appressoria (Van Burgh, 1950; Skoropad, 1967). Although some micro-organisms may be able to utilize siderophores as a nutrient source, the stimulation of *C. acutatum* by S_A cannot be wholly explained on a nutritional basis. The stimulatory effect of S_A on germination and appressorium formation is much greater than that exhibited by equivalent concentrations of the nutrients tested.

Germination and appressorium formation by C. acutatum conidia in vivo

Percentage germination was significantly higher on strawberry stolons than on glass slides. S_A had a marked stimulatory effect on germination but S_AFe was not inhibitory (Table 4). The stimulation of germination by cells of isolate UV3 was diminished compared with the situation *in vitro*, resulting from a higher control value rather than a lowered efficacy of the bacterial treatment. Free Fe³⁺ in the inoculum drop had no significant effect on germination in either the presence or absence of added bacteria. The absence *in vivo* of the inhibitory action of free iron *in vitro* is not readily explained, but may be related to the complex interaction of factors on the stolon surface which may have buffered the effect of the iron.

S_A increased the percentage of conidia which formed appressoria (light plus dark), but the S_AFe was not inhibitory, although it had none of the stimulatory effects of S_A alone. The lack of inhibition by S_AFe also differed from the situation *in vitro* and must be related to factors on the plant surface. Free Fe³⁺ reduced the number of appressoria formed, and *Pseudomonas* UV3 slightly increased it, but addition of iron to the bacteria had no effect on the stimulatory properties of the latter. S_AFe and Fe³⁺ markedly reduced the percentage of dark appressoria produced by germinating conidia, while S_A and isolate UV3 stimulated their formation compared with the control. The proportion of appressoria that were dark was significantly lowered by S_AFe and Fe³⁺ and raised by the S_A and isolate UV3 treatments, but there was no

significant difference between the water control and the bacteria with iron. The total number of conidia which produced dark appressoria *in vivo* was stimulated by S_A and inhibited by S_AFe and by free Fe^{3+} . *Pseudomonas* UV3 in water and in Fe^{3+} increased the number of dark appressoria but significantly less than did S_A . In general, the biological effects of free Fe^{3+} are more thoroughly reversed by the addition of live bacteria than by S_A . Blakeman & Brodie (1977) showed that appressorium formation by *Colletotrichum dematium* f. sp. *spiniciae* on beetroot leaves was stimulated by *Pseudomonas* sp. isolate 14 while germination was unaffected. Although this is similar to the observed effect of *Pseudomonas* UV3 on *C. acutatum* it was attributed to nutrient competition. *Pseudomonas* 14 is also a siderophore-producing isolate (unpublished data) and it is probable that siderophores are involved in fungal infection processes.

A single factor operating alone is unlikely to be the sole determinant of germination and appressorium formation *in vivo*; rather, a number of different effects interacting at the plant surface are likely to influence the development of a fungal propagule landing on the phytplane. A variety of factors can influence *Colletotrichum* spp., including temperature, light intensity, relative humidity, surface wetness, thigmotropic effects, spore age, nutrient status and host susceptibility (Emmett & Parbery, 1975). The results reported in this study and by Swinburne (1981) add iron and siderophores to this list. The greater germination of *C. acutatum* *in vivo* than *in vitro* is not readily explained without further study but some of the factors outlined above may be involved in the complex interaction on the stolon surface and be absent from the germination assays on glass slides. Perhaps the most important feature of the effect of S_A on *C. acutatum* *in vivo* is the stimulation of the formation of dark appressoria, the proportional occurrence of which is a critical factor in determining the success of *C. musae* infection of banana, a related anthracnose disease (Swinburne, 1976; Swinburne & Brown, 1983).

The stimulation of germination and appressorium formation of *C. acutatum* by the bacterial siderophore S_A heightens the importance of the role of phytplane micro-organisms and their products in the complex relationship between a fungal pathogen and its host. It also indicates that the previously observed stimulation of fungal germination and appressorium formation by certain bacteria may be mediated, at least in part, by siderophores.

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REFERENCES

- ADIKARAM, N. K. B., BROWN, A. E. & SWINBURNE, T. R. (1982). Rotting of immature *Capsicum frutescens* L. fruit by iron-depleted *Glomerella cingulata* (Stonem.). *Physiological Plant Pathology* **21**, 171–177.
- BLAKEMAN, J. P. & BRODIE, I. D. S. (1977). Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beetroot leaves. *Physiological Plant Pathology* **10**, 29–42.
- BLAKEMAN, J. P. & PARBERY, D. G. (1977). Stimulation of appressorium formation in *Colletotrichum acutatum* by phylloplane bacteria. *Physiological Plant Pathology* **11**, 313–325.
- BRODIE, I. D. S. & BLAKEMAN, J. P. (1976). Competition for exogenous substances *in vitro* by leaf surface microorganisms and germination of conidia of *Botrytis cinerea*. *Physiological Plant Pathology* **9**, 227–239.
- BROWN, A. E. & SWINBURNE, T. R. (1981). Influence of iron and iron chelates on formation of progressive lesions by *Colletotrichum musae* on banana fruits. *Transactions of the British Mycological Society* **77**, 119–124.
- BROWN, A. E. & SWINBURNE, T. R. (1982). Iron-chelating agents and lesion development by *Botrytis cinerea* on leaves of *Vicia faba*. *Physiological Plant Pathology* **21**, 13–21.
- EMMETT, R. & PARBERY, D. G. (1975). Appressoria. *Annual Review of Phytopathology* **13**, 147–167.
- GRAHAM, A. H. & HARPER, D. B. (1983). Distribution and transport of iron in conidia of *Colletotrichum musae* in relation to the mode of action of germination stimulants. *Journal of General Microbiology* **129**, 1025–1034.
- KING, E. O., WARD, M. K. & RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine* **44**, 301–307.
- MCCRACKEN, A. R. & SWINBURNE, T. R. (1979). Siderophores produced by saprophytic bacteria as stimulants of germination of conidia of *Colletotrichum musae*. *Physiological Plant Pathology* **15**, 331–340.
- MEYER, J. M. & ABDALLAH, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical pro-

- perties. *Journal of General Microbiology* **107**, 319–328.
- MEYER, J. M. & HORNSPERGER, J. M. (1978). Role of pyoverdine_{pf}, the iron-binding fluorescent pigment of *Pseudomonas fluorescens*, in iron transport. *Journal of General Microbiology* **107**, 329–331.
- SKOROPAD, W. P. (1967). Effect of temperature on the ability of *Colletotrichum graminicola* to form appressoria and penetrate barley leaves. *Canadian Journal of Plant Science* **47**, 431–434.
- SWINBURNE, T. R. (1976). Stimulants of germination and appressoria formation by *Colletotrichum musae* (Berk. & Curt) Arx. in banana leachate. *Phytopathologische Zeitschrift* **87**, 74–90.
- SWINBURNE, T. R. (1981). Iron and iron chelating agents as factors in germination, infection and aggression of fungal pathogens. In *Microbial Ecology of the Phylloplane*, pp. 227–243. Edited by J. P. Blakeman. London: Academic Press.
- SWINBURNE, T. R. & BROWN, A. E. (1983). Appressoria development and quiescent infections of banana fruit by *Colletotrichum musae*. *Transactions of the British Mycological Society* **80**, 176–178.
- TIENTZE, M. & LEONG, J. (1981). Structure of pseudobactin A, a 2nd siderophore from plant growth promoting *Pseudomonas* B10. *Biochemistry* **20**, 6457–6462.
- TIENTZE, M., HOSSAIN, M. B., BARNES, C. L., LEONG, J. & VAN DER HELM, D. (1981). Structure of ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. *Biochemistry* **20**, 6446–6457.
- VAN BURGH, P. (1950). Some factors affecting appressorium formation and penetrability of *Colletotrichum phomoides*. (Abstract.) *Phytopathology* **40**, 29.