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Department of Plant Protection, College of Agriculture,
Shiraz University, Shiraz, Iran;
and Department of Plant Pathology,
University of Wisconsin, Madison 53706, USA

Effect of Flavin Inhibitors on Photoactivation of Oospores of *Phytophthora cactorum*

Z. BANIHASHEMI and J. E. MITCHELL

Authors' addresses: Dr. Z. BANIHASHEMI, Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz (Iran). Dr. J. E. MITCHELL, Department of Plant Pathology, University of Wisconsin, Madison 53706 (USA).

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Abstract

When dark-grown mature oospores of *Phytophthora cactorum* were activated to germinate by exposure to 5 uW $cm^{-2}nm^{-1}$ of fluorescent light at 20—22 °C in the presence of certain flavin inhibitors such as KI, salicylhydroxamic acid and phenylacetic acid at 40, 1, and 0.1 mM respectively, photoactivation and hence subsequent germination of oospores were inhibited without appreciable irreversible effect on oospore viability. Likewise, when applied during the light period, NaN_3 and KCN at 1 mM reduced photoactivation but had a minimal effect on dark reactions. Diphenylamine, an inhibitor of certain carotenoids, had no effect on photoactivation of oospores. The data suggest that the photoreceptor pigment for activation of oospore germination is a flavin.

Zusammenfassung

Einfluß von Flavininhibitoren auf die Photoaktivierung von *Phytophthora cactorum*-Oosporen

Wenn in Dunkelheit angezogene, reife *Phytophthora cactorum*-Oosporen durch 5 uW · cm^{-2} · nm^{-1} fluoreszierendes Licht bei 20—22 °C und im Beisein von bestimmten Flavininhibitoren wie KI, Salicylhydroxaminsäure und Phenylessigsäure in den Konzentrationen 40, 1 bzw. 0,1 mM zum Keimen aktiviert wurden, wurde die Photoaktivierung und auch, als Folge davon, die anschließende Keimung der Oosporen gehemmt ohne nennenswerten, unwiderruflichen Einfluß auf die Oosporenviabilität ausgeübt zu haben. Ebenfalls, nach einer Behandlung mit 1 mM $NaNO_3$ oder KCN in der hellen Periode wurde die Photoaktivierung reduziert, Behandlungen, die die im Dunkeln stattfindenden Reaktionen kaum beeinflußten. Diphenylamin, als Inhibitor bestimmter Carotenoide bekannt, beeinflußt die Oosporenphotoaktivierung nicht. Die Ergebnisse deuten darauf hin, daß das Photorezeptorenpigment für die Oosporenkeimungsaktivierung ein Flavin ist.

Physiological response to blue and long-wavelength ultraviolet light has been found in many organisms such as bacteria, fungi, plants and animals (PRESTI and DELBRUCK 1978). In fungi such responses include phototropism in the sporangiophore of *Pilobolus kleinii* (PAGE 1956, PAGE and CURRY 1966), carotegensis in *Fusarium aquaeductum* (LANG-FEULNER and RAU 1975), phototaxis in *Dictyostelium discoideum* (POFF *et al.* 1974), condiation in *Neurospora crassa* (MUNOZ and BUTLER 1975), and photoactivation of oospores for germination in many species of Phytophthora (BANIHASHEMI and MITCHELL 1976, BERG and GALLEGLY 1966, CARDOSO and SCHMITTHENNER 1975, RIBEIRO *et al.* 1976 a, 1976 b). Although the exact wavelength for various maxima in the action spectra varies from organism to organism, the general shape of the curve is always the same. It is characterized by an action band between 400 and 500 nm with a maximum at about 450 nm. The resemblance of these action spectra to the absorption spectra of riboflavin and some carotenoids, especially B-carotene, suggested that these molecules would be the most likely candidates for the blue light photoreceptor(s). Both riboflavin and B-carotene are molecules of wide biological occurrence. Although both molecules have been long time candidates for the photoreceptor in organisms exhibiting physiological responses to blue light, it has been argued that the photoreceptor is a flavin rather than B-carotene (MUNOZ and BUTLER 1975, PAGE 1956, PAGE and CURRY 1966, POFF and BUTLER 1974, PRESTI *et al.* 1977, SCHMIDT and BUTLER 1976 a, SCHMIDT and BUTLER 1976 b, SCHMIDT *et al.* 1977, SONG and MOORE 1974).

It is not possible to distinguish between these two pigments on the basis of their action spectra of physiological responses because spectral characteristics of either of these pigments *in vivo* may be modified by their specific protein association and local environment (MUNZO and BUTLER 1975). Hence other criteria must be used for the identification of the photoreceptor pigment. These include the light-induced redox changes of a cytochrome (BORGESON and BOWMAN 1985, MUNZO and BUTLER 1975, SCHMIDT and BUTLER 1976 a, SCHMIDT and BUTLER 1976 b) and the use of specific inhibitors (BORGESON and BOWMAN 1985, CAUBERGS *et al.* 1978, MIKOLAJCZYK and DIEHN 1975, PAGE 1956, PAGE and CURRY 1966, SCHMIDT *et al.* 1977).

A photoresponsive pigment protein with an absorption maximum at about 450 nm isolated from the cells of the photoactive cellular slime mold, *D. discoideum*, was considered to be the photoreceptor for phototaxis in the pseudoplasmodium of this organism (POFF *et al.* 1974). The photoreceptor pigment was shown to be a flavin which is reduced upon irradiation and results in absorbance changes. This in turn causes the reduction of cytochromes b and c (BORGESON and BOWMAN 1985, KLEMM and MINNEMANN 1978, MUNOZ and BUTLER 1975, POFF *et al.* 1974, SCHMIDT and BUTLER 1976 b, SCHMIDT *et al.* 1977).

Information concerning the nature of the photoreceptor pigment in oospores of *Phytophthora* spp. is based on the action spectrum for the photoactivation and subsequent germination of oospores. CARDOSO and SCHMITTHENNER (1975) working with *P. cactorum* rejected a carotenoid or flavin as a photoreceptor candidate for the photoactivation of oospores, and the involvement of a phytochrome was suggested. RIBEIRO *et al.* (1976 a) postulated the presence of different photorecep-

tors in the production and germination of reproductive structures in several species of *Phytophthora*. A flavoprotein was suggested as a photoreceptor for sporangium production in *P. capsici* and *P. palmivora*. However, because of the lack of cytochrome-b in oospores of *P. capsici* (RIBEIRO *et al.* 1976 a) and the photoresponse in the far-red region, the proposal that a similar photoreceptor was involved in oospore production was not accepted (RIBEIRO *et al.* 1976 b).

The purpose of the present investigation was to study the nature of the photoreceptor pigment in the oospores of *P. cactorum* by employing different flavin inhibitors. A preliminary report of this work appeared earlier (BANIHASHEMI and MITCHELL 1981).

Materials and Methods

Free oospores of *P. cactorum* were obtained from cultures grown in V-8 broth in the dark for 2—4 months as reported earlier (BANIHASHEMI and MITCHELL 1976). Oospore germination studies utilized sterile distilled water (SDW) or dilute sterile soil extract at 20—22 °C. Soil extract was prepared by mixing 1 kg field soil and 1 liter of tap water. This was allowed to settle over-night at room temperature and the supernatant was passed through two layers of cheesecloth and then centrifuged at 4000 G for 15 minutes. The supernatant was collected and autoclaved for 20 minutes. This stock solution was diluted 1/100 when used. One drop of oospore suspension was placed in a 60 mm diameter plastic Petri plate containing 10 ml of the test solution. The inhibitors of flavin mediated reactions used were potassium iodide (CAUBERGS *et al.* 1978, MIKOLAJCZYK and DIEHN 1975, SCHMIDT *et al.* 1977), phenylacetic acid (SCHMIDT *et al.* 1977), salicylhydroxamic acid (BORGESON and BOWMAN 1985, CAUBERGS *et al.* 1978) and sodium azide (BORGESON and BOWMAN 1985, SCHMIDT and BUTLER 1976 a, 1976 b, SCHMIDT *et al.* 1977). Diphenylamine, an inhibitor of some carotenoids (PAGE 1956, PAGE and CURRY 1966), and KCN were also included.

The oospores were exposed for 34 hrs to the inhibitors dissolved in SDW either prior to or during the light period. After the exposure period, the inhibitors were removed from each plate in darkness by decanting the solution. Most of the oospores adhered to the surface of plastic (but not glass) Petri plates. The oospores were washed several times by filling the plates with SDW and decanting it. Oospores suspended (and subsequently washed) in SDW served as controls. Three plates were used for each treatment. All preparative processes were carried out in darkness or under dim red light. For the photoactivation test, oospores suspended in SDW were incubated under a cool-white fluorescent lamp (5 uW cm^{-2} nm^{-1}) for 34 h. Each plate finally received 10 ml of diluted soil extract and was incubated in the dark for 108 hrs unless otherwise indicated.

Three treatments were used. I. Exposure to the light treatment only without inhibitors determined the basic viability of the oospores (the control). II. Exposure to the inhibitors before the light treatment determined the irreversible inhibition of viability due to the inhibitor treatment, and III. Exposure to the inhibitors during the light period determined the sum of this and the inhibition of light induced photoactivation of the germination process. The percent inhibition of germination due to II and III were calculated relative to the results of I. The effects of the light treatment are evident from the difference between II and III.

The effect of the inhibitors on mycelial growth was determined by adding the inhibitors to corn meal agar melted and cooled to 40 °C. Plates were inoculated with mycelium of *P. cactorum* and incubated at 24 °C in the dark for 7 days. Inhibition of growth was calculated relative to the control growing in the absence of inhibitor.

The effect of the inhibitors during the post photo-activation period was determined by subjecting oospores to the photoperiod described above and then replacing the SDW with solutions of the inhibitors dissolved in dilute soil extract. The spores were then incubated in the dark in the presence of the inhibitors for 96 h at 22 °C.

Results

Flavin inhibitors significantly reduced photoactivation of germination of oospores of *P. cactorum* (Table 1). Potassium iodide at 40 mM and phenylacetic acid at 0.1 mM inhibited germination of oospores 97.5 and 96.8 % respectively, while having only a negligible effect on oospore viability during a similar exposure in the absence of light. Salicylhydroxamic acid at 1.0 mM also reduced germination 77 % with little effect on oospore viability in the absence of light.

The cytochrome inhibitors NaN_3 and KCN (both at 1.0 mM) reduced germination of oospores 71 and 51 % respectively. Again there was little persistent effect of a similar exposure to the inhibitor in the dark. Exposure of oospores to 0.1 mM diphenylamine, a known inhibitor of certain carotenoids, during the light period reduced subsequent germination by only 18 %. The results of these tests demonstrate that oospore germination is specifically inhibited by chemicals known to interfere with flavin mediated cellular reactions.

The effect of KI, salicylhydroxamic acid, phenylacetic acid and sodium azide on radial growth and germination of photoactivated oospores of *P. cactorum* was

Table 1
Effect of inhibitors on germination of oospores of *Phytophthora cactorum*[a]

Inhibitor	Concentration (mM)	Inhibition of germination when exposed to inhibitors (%)	
		before photoperiod[b]	during photoperiod[c]
KI	20	2.8	85.4
	40	9.4	97.5
	60	43.8	98.7
	80	91.2	100.0
	100	98.6	100.0
Salycylhydroxamic acid	0.1	4.8	29.5
	1.0	4.0	76.7
Phenylacetic acid	0.1	16.4	96.8
	1.0	100.0	100.0
NaN_3	1.0	8.8	71.4
	10.0	4.5	77.1
KCN	1.0	9.4	50.6
	10.0	8.3	82.4
Diphenylamine	0.1	7.3	18.0

[a] Effect of all treatments compared to controls (germination average 96 %) in which oospores were exposed for 34 hr at 22 °C to 5 uW cm^{-2} nm^{-1} cool white fluorescent illumination in sterile distilled water and then incubated in the dark in dilute soil extract (DSE) for 108 hr.

[b] Oospores were exposed to the inhibitors in the dark for 34 hr, washed free of the inhibitor and then exposed to light for 34 hr as noted above and incubated in the dark for 74 hrs in DSE.

[c] Oospores were exposed to the light treatment in the presence of the inhibitors for 34 hr, washed free of the inhibitor and then incubated in the dark in DSE for 108 hr.

Table 2
Effect of inhibitors on vegetative growth and on germination of photoactivated oospores of *Phytophthora cactorum*

Inhibitor	Concentration (mM)	% inhibition of	
		radial growth[a]	oospore germination[b]
KI	20	30	42
Salicylhydroxamic	1	59	53
Phenylacetic acid	0.1	0	64
NaN_3	1	100	87

[a] Growth from mycelial inoculum in Petri plates on corn meal agar containing the inhibitor was measured after incubation for 7 days in the dark at 24 °C.

[b] Oospores were exposed to cool white fluorescent illumination (5 uW cm^{-2} nm^{-1}) in SDW for 67 hr. The SDW was then replaced with solutions of the inhibitors dissolved in sterile dilute soil extract and oospores were incubated in the dark at 22 °C for 96 hr.
Inhibition was calculated relative to control treated as in a and b above but in absence of the inhibitors.

also studied (Table 2). NaN_3 at 1 mM completely inhibited vegetative growth of the fungus. Phenylacetic acid at concentrations which inhibited photoactivation of oospores had no effect on vegetative growth. KI salicylhydroxamic acid inhibited vegetative growth to some extent.

Presence of inhibitors also affected the germination of phytoactivated oospores. Microscopic examination of random samples of oospores photoactivated for 67 hr revealed that most of the oospores had not yet produced a germ tube or sporangium. When these photoactivated oospores were incubated in the dark in the presence of the inhibitors, germination was reduced by all inhibitors. Even phenylacetic acid which did not effect vegetative growth, considerably reduced the germination of oospores.

Discussion

Photoactivation of oospores of *P. cactorum* is a prerequisite for germination *in vitro*. Treatments such as aging, glusulase treatment (BANIHASHEMI and MITCHELL 1976), chilling during oospore maturation, light treatment during gametogensis (unpublished results) and the presence of a host (BANIHASHEMI and MITCHELL 1981) can not be substituted for the light effect. The fact that the wave length of the action spectrum resulting in maximum germination is about 450 nm suggests that riboflavin or some carotenoids may be involved. It has been argued that the action spectra for the physiological responses are not capable of distinguishing between these two candidates because the spectral characteristics of either of these pigments *in vivo* may be modified by their specific protein association and local environment (MUNOZ and BUTLER 1975). Because of these inadequacies of action spectra as the major criterion for identifying the photoreceptor pigment, Schmidt et al. (1977) used inhibitors such as potassium iodides, phenylacetic acid and

sodium azide to study the nature of photoreceptor pigment in corn coleoptiles which show phototropic response to blue light. A flavin was suggested as the photoreceptor pigment in this reaction.

Many of the photochemical reactions of riboflavin occur through the relatively long-lived triplet state (PRESTI 1977). Potassium iodide has been found to depopulate (quench) the flavin triplet state (SONG and MOOR 1968). Phenylacetic acid also covalently binds to irradiated flavins (HEMMERICH 1967). Sodium azide (SCHMIDT and BUTLER 1976 a, 1976 b, SCHMIDT *et al.* 1977) and salicylhydroxamic acid (BORGESON and BOWMAN 1985, CAUBERGS *et al.* 1978) have been reported to inhibit flavin-mediated photoresponses in *N. crassa* and in corn coleoptile respectively. Hydroxamic acid is reported to inhibit specificly the alternate pathway insensitive to cyanide (SCHNOBAUM *et al.* 1971). It has been shown that the inhibitors neither affect energy coupling nor electron transport through the cytochrome pathway (SHNOBAUM *et al.* 1971). The site of hydroxamic acid inhibition is located between the fluorescent high potential flavoprotein and oxygen (STOREY 1970).

In the present study it was shown that the chemicals found to be flavin inhibitors in other biological systems, inhibited the photoactivation of oospores of *P. cactorum*. The failure of diphenylamine to affect oospore photoactivation in our studies is in agreement with the previous report of work with *Pilobolus kleinii* (PAGE 1956, PAGE and CURRY 1966) which indicates that carotenoids may not be involved in the photoreaction.

Several reports indicated that oospores of some Phytophthora species (including *P. cactorum*) are stimulated to germinate by exposure to wave-length in the infra-red region to the spectrum (BERG and GALLEGLY 1966, CARDASO and SCHMITTHANER 1975, RIBEIRO *et al.* 1975, 1976 b). Certain flavins have been reported to absorb light at 450 and 700—1000 nm (cited in RIBEIRO *et al.* 1975). Since the inhibitors used in the present study inhibited photoactivation of oospores under fluorescent illumination (400 nm to 750 nm), it is possible that a similar mechanism might be operating in other portions of the spectrum.

Both cyanide and azide are known to be inhibitors of cytochrome oxidase. SCHMIDT *et al.* (1977) found that cyanide was a nonspecific inhibitor for both geotropism and phototropism in corn. Azide was more a specific inhibitor of phototropism than geotropism and has been described as an inhibitor of electron transfer involving flavins (SCHMIDT and BUTLER 1976 a).

The cytochromes inhibitors such as sodium azide and potassium cyanide used in the present study reduced photoactivation and subsequent germination of oospores. The chemicals had no effect during dark period prior to photoactivation. In other systems, it has been shown that photoexcitation of flavin resulted in the reduction of b-type cytochrome and it was assumed that cytochrome might be a part of the receptor complex (MUNOS and BUTLER 1975). Further studies regarding the characterization of cytochrome flavin complex in oospores of *P. cactorum* are needed before any conclusion could be drawn if cytochrom is a part of the receptor complex in the oospores.

The fact that photoactivation of oospores of *P. cactorum* is inhibited by the presence of certain flavin inhibitors without appreciably inhibiting vegetative

growth or reducing oospore viability, suggests that the photoreceptor pigment might be a flavin. We cannot however exclude the possibility that the inhibitors exert a specific inhibition of germination through separate effects on different metabolic processes, specific for photoactivation of oospores.

Further studies with other species of Phytophthora using photoactivation inhibitors and investigation on light-induced absorbance changes used in other biological systems (BORGESON and BOWMAN 1985, KLEMM and MINNEMANN 1978, MIKOLAJCZYK and DIEHN 1975, POFF *et al.* 1974, SCHMIDT and BUTLER 1976 b, SCHMIDT *et al.* 1977) and the isolation of the photoreceptor pigment involved are necessary before final conclusions can be reached.

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