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Suppression of Photo-induced Sporulation in *Trichoderma viride* by Inhibitors

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ABSTRACT. The mycelium of *Trichoderma viride* grown in the dark under submerged conditions and transferred to membrane filters sporulated only after photoinduction. The optimum photoinduction of sporulation was reached when applying daylight for 3 min and near ultraviolet radiation (366 nm) for 10 to 30 sec. After the photoinduction pronounced synthesis of DNA, RNA and protein was observed. The photoinduced sporulation was partially or fully inhibited in the presence of phenethyl alcohol, actinomycin D, 5-fluorouracil, cycloheximide and ethidium bromide. The same inhibitors blocked also the photoinduced sporulation of surface growing colonies of *Trichoderma viride*. Various inhibitors of synthesis of nucleic acids and protein, inhibitors impairing the function of membranes and certain other compounds were also effective.

Light induces or stimulates both the sexual and asexual multiplication of numerous fungi (Leach, 1971). Sporulation of *Trichoderma viride* occurs after photoinduction with light of wave length from 350 to 510 nm. Two regions of maximum activity can be observed, one around 380 nm and another 440 nm (Gressel and Hartmann, 1968).

The photoinduced sporulation is a very attractive process, which is only little understood at present. Photoreceptors accepting the light "signal" inducing the sporulation are not known. It is also not known what mechanism facilitates transfer of this signal to the genetic information determining biochemistry and morphogenesis of the sporulation. The use of metabolic inhibitors is one of the ways that can be used for studying processes of the photoinduced sporulation. In the present paper we compared the photoinduced sporulation in the submerged grown mycelium and surface growing colonies of *Trichoderma viride*. The effect of inhibitors impairing biosynthesis of nucleic acids and protein on the photoinduced sporulation, as well as conditions under which sporulation is prevented, were also investigated.

MATERIALS AND METHODS

Organism. The strain *Trichoderma viride* 8-7 from the collection of this Institute was maintained on wort agar. According to the revision of the genus *Trichoderma* (Rifai, 1969) Dr. L. Marvanová of the Czechoslovak Collection of Microorganisms in

* A part of the results presented here was included in the dissertation of J.S. and defended at the Slovak Polytechnical University in Bratislava.

Brno classified this strain as *Trichoderma* sf. *viride* Pers. ex S. F. Gray (strain with smooth conidia; see Rifai, 1969).

Cultivation medium. A potato-glucose medium with or without agar (1.5%) was used in all experiments.

Submerged cultivation of the mycelium of Trichoderma viride. 100 ml of the liquid medium contained in 500 ml flasks were inoculated with 1 ml spore suspension (10^6 spores) of *Trichoderma viride* in distilled water with Tween 80 (0.1% v/v). The cultivation was in the dark for 27 h at 28 °C on a rotary shaker (240 strokes/min).

Preparation of colonies of Trichoderma viride. One drop of the spore suspension (10^6/ml) was added to the middle of the agar plate in a Petri dish (d = 10 cm) and the cultivation was in the dark for 48 h at 26 °C. Ten ml of the agarized medium were pipetted to a series of Petri dishes (d = 10 cm) and, after solidification, a sterile disc (d = 8 cm) of filter paper Whatman No. 3 was placed on each plate. Blocks (d = 0.8 cm) were cut out of the margin of the growing colony of *Trichoderma viride* and placed (mycelium downwards) in the middle of paper discs on agar plates. The incubation was in the dark for 48 h at 26 °C. During this time interval colonies of 5 to 6 cm in diameter were formed.

Photoinduction. The submerged grown mycelium from 3 ml volumes of the culture was harvested by means of filtration on membrane filters (d = 2.4 cm) under reduced pressure and washed with 5 ml distilled water. The procedure was performed in red light. Filters with the collected mycelium were placed on fresh plates with the agarized medium with or without inhibitors (5—7 filters per one plate of 10 cm in diameter).

Similarly, colonies grown on the paper discs were transferred in red light to fresh plates of the agarized medium with or without inhibitors. After placing the membrane filters with the mycelium or the paper discs with the colonies of *Trichoderma viride* the plates were kept in the dark for another 30—60 min. The colonies or mycelium on membrane filters were then subjected to daylight or ultraviolet radiation (366 nm) for various time intervals. The irradiation was with a UVIS (Desaga, Heidelberg) lamp in a dark room at room temperature. After the irradiation the incubation continued in the dark for 24 to 28 h at 26 °C.

On short-term treatment with the inhibitors the colonies on discs or the mycelium on membrane filters were placed before, during or after the photoinduction on agar plates with inhibitors and, after a required interval, placed on plates without inhibitors. All manipulations were carried out in red light and the incubation was always in the dark.

Counting of conidia. The sporulated mycelium on membrane filters or colonies on paper discs were transferred to dry beakers, washed with a standard volume of 10 ml distilled water with 0.1% Tween 80 and released by means of a rubber-coated rod. The homogenized suspension was filtered through 4 layers of gauze in order to collect fragments of the mycelium. Homogeneity was controlled microscopically. Absorbency at 650 nm was measured in a spectrophotometer SPECOL. Concentration of conidia was determined according to a calibration curve constructed for each independent experiment and relating absorbency with number of conidia determined by means of a haemocytometer.

Inhibitors. The studied inhibitors were dissolved in water, ethanol or dimethylsulfoxide (DMSO) and added to agarized media in such a way that the resulting concentration of ethanol or DMSO did not exceed 1%. The same volumes of solvents were used in controls.

Isolation of total DNA, RNA and protein. Both the sporulated and non-sporulated mycelium on membrane filters was subjected to fractionation using procedures

described by Betina and coworkers (1975). The complete material from 2 to 3 discs was always treated in parallel.

Determination of total DNA, RNA and protein. Total DNA (as deoxyribose) was determined by the diphenylamine method (Burton, 1956), total RNA (as ribose) by means of the orcinol method (Schneider, 1957) and proteins were determined by means of the biuret method using serum albumin as standard.

RESULTS AND DISCUSSION

Experiments with submerged grown mycelium

Submerged grown mycelium collected on membrane filters after the photoinduction sporulated evenly on the whole surface being physiologically identical during the photoinduction (Plate 1a). The mycelium on membrane filters placed on the potato-glucose agar was exposed for various time intervals to day light or radiation of 366 nm. Fig. 1 shows the relationship between conidiation and illumination. Under the used experimental conditions (illumination with the UVIS Desaga lamp) already a 1 min induction with daylight was sufficient for a very intensive sporulation. The intensity then slightly increased up to 3 min. Prolonged illumination did not lead to any further more intensive sporulation. The irradiation with light of 366 nm induced the sporulation only up to a 30 sec treatment; prolonged irradiation was undesirable. The cause is not known, but it may be related to other known effects of near ultraviolet light such as the inhibition of protein synthesis (Sulkowski *et al.*, 1964). The observed pronounced photoinduction of sporulation of *Trichoderma viride* at 366 nm is in agreement with the data of Gressel and Hartmann (1968), who found the maximum activity at 380 nm. Changes in the content of DNA, RNA and protein associated with the photoinduction were also followed. Whereas in the non-illuminated control only a minor increase could be detected, the content of DNA, RNA and protein increased considerably in the mycelium sporulating after photoinduction (Table I). This increase can apparently be attributed to fructification structures and spores.

TABLE I. Changes in the content of DNA, RNA and protein during the photoinduced sporulation of *Trichoderma viride*. The submerged grown mycelium collected on membrane filters was placed on agar plates 1 h before a 3 min photoinduction. It was then incubated in the dark for 24 h at 26 °C and analyzed. Values are compared with those at zero time of the photoinduction (t_0). Mean values of 2 parallel experiments are presented.

	Content					
	DNA		RNA		Protein	
Conditions	μM deoxyribose per disc	increase with respect to t_0 %	μM ribose per disc	increase with respect to t_0 %	mg albumin per disc	increase with respect to t_0 %
Control at t_0	420	—	1220	—	5.85	—
Non-illuminated discs at t_{24}	450	+7	1400	+15	6.40	+9
Illuminated discs at t_{24}	1210	+180	2000	+64	10.80	+85

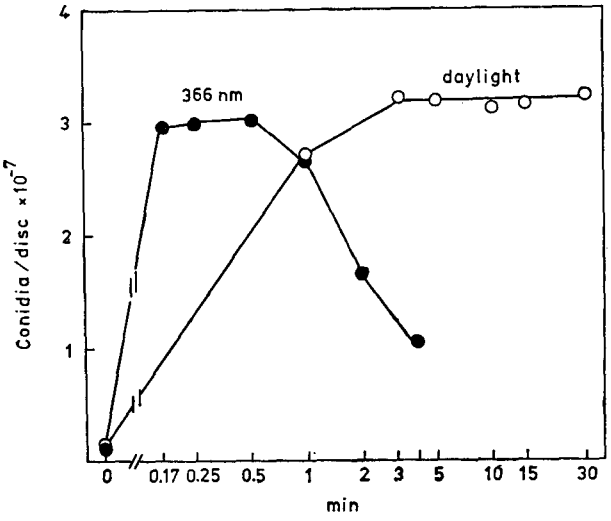

FIG. 1. Induction of sporulation of the submerged grown mycelium of *Trichoderma viride* with visible and near ultraviolet (366 nm) light.

It was assumed that the photoinduction leads to activation of genes bearing information for conidiation. Therefore, the effect of selected inhibitors that could intervene with various stages of transcription and translation of this information after the photoinduction was investigated in further experiments. We studied the effect of actinomycin D, which binds to DNA and thus inhibits RNA synthesis, 5-fluorouracil, which is incorporated in to RNA resulting in synthesis of defective proteins and cycloheximide, which blocks protein synthesis on 80S ribosomes. We also used the antibiotic ramihyphin A, which probably impairs the function of membranes (Baráthová and Betina, 1976). The mycelium on membrane filters was transferred to agar plates with the above inhibitors, one hour prior to the 3-min illumination with daylight. The effects of these inhibitors on the photoinduction of sporulation are shown in Fig. 2. Actinomycin D and ramihyphin A blocked the photoinduced sporulation only partially, whereas cycloheximide and 5-fluorouracil were practically fully inhibitory.

We also compared the long-termed and short-termed treatment with these inhibitors. The results are shown in Plate 1. It could be shown that after the treatment with the used concentrations of cycloheximide (5 μg/ml) and 5-fluorouracil (10 μg/ml) the sporulation is pronouncedly inhibited, irrespective of whether the inhibitors are present from 1 to 24 h or from 0.5 h to 2.5 h with respect to the photoinduction (at zero time). Actinomycin D inhibited sporulation when present during and 24 h after the photoinduction. A 3-h treatment of the mycelium, 5 to 2 h prior to the photoinduction, did not exhibit the inhibitory effect any more; metabolic disorders caused by the previous contact with the antibiotic were apparently repaired in the mycelium. Finally, the non-specifically acting antibiotic ramihyphin A blocked sporulation only on long-termed treatment of the mycelium. A 3 h contact, from −0.5 to 2.5 h, resulted only in a negligible effect.

However, the short-term treatment with the inhibitors was studied in more detail. The filtered dark-grown mycelium was maintained on agar with the inhibitors for 3 h intervals but at different time intervals with respect to the photoinduction. Table II presents quantitative data of sporulation of *Trichoderma viride* under these conditions. The long-term contact of the mycelium with cyclohexi-

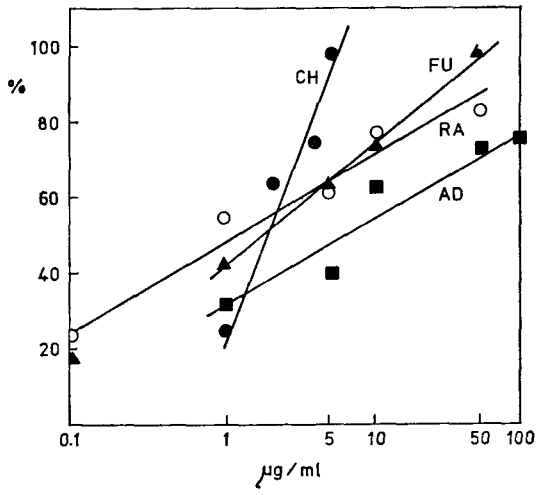

FIG. 2. Inhibition of the photoinduced sporulation (%) of the submerged grown mycelium of *Trichoderma viride* by actinomycin D (AD), cycloheximide (CH), 5-fluorouracil (FU) and ramihyphin A (RA) (in μg/ml).

mide and 5-fluorouracil, from −0.5 or from 0 to 24 h with respect to the photoinduction, inhibited sporulation practically completely, *i.e.* to the level of the non-illuminated control, whereas actinomycin D inhibited sporulation only to 73%. A 3 h contact with 5-fluorouracil from −5 to −2 h prior to the photoinduction did not inhibit the sporulation pronouncedly; the mycelium transferred to agar without the inhibitors could metabolically regenerate almost completely 2 h before the photoinduction. However, when the mycelium was in contact with 5-FU during the interval from −1 to +2 h, a partial inhibition (35.8%) was observed. Much more considerable inhibition was reached during contact with 5-FU at 3 h intervals beginning from −0.5 and 0 h and terminating from +2.5 to +3 h, *i.e.* when the photoinduction took place in the presence of 5-FU (inhibition was about 58%). On the other hand, when the contact with 5-FU took place only 2 h after the photoinduction, the inhibitor could not prevent induced metabolic processes that are essential for the sporulation. Pronounced differences between 3 h intervals of treatment with cycloheximide and actinomycin D at different time intervals with respect to the photoinduction could not be detected (compare data in Table II); nevertheless, the most pronounced inhibition of sporulation was reached at intervals identical with those of the 5-FU treatment. Similar results were obtained with 5-FU during the photostimulated conidiation of *Neurospora crassa* (Betina *et al.*, 1975). Gressel and Galun (1967), using different experimental conditions with colonies of *Trichoderma viride* growing on filter papers soaked with the liquid medium, slightly higher concentrations of 5-FU (10^{-4}M as compared with 7.6×10^{-5}M used by us) and lower density of the mycelium, found that a 1 h treatment with the inhibitor up to 7 h after the photoinduction could suppress the sporulation. In the experiments referred to here this was possible only up to 2 h after the photoinduction. However, both cases are analogous and resemble the well-known "point of no return" in differentiation of *Blastocladiella* (Cantino, 1961).

The effect of ramihyphin A, which induces very intensive branching of hyphae, probably due to its influence on membranes (Baráthová and Betina, 1976), was quite different in these experiments. On a 3 h treatment of *Trichoderma viride* it inhibited the photoinduced sporulation, mainly when applied to growing colonies 3 h prior to the photoinduction (from −5 to −2 h). The shorter the treatment

TABLE II. The effect of short and long-term treatment with cycloheximide (5 μg/ml), 5-fluorouracil (10 μg/ml), actinomycin D (100 μg/ml) and ramihyphin A (50/ml) on the photoinduced sporulation of *Trichoderma viride*. The submerged grown mycelium collected on membrane filters was maintained on the agarized medium without inhibitors and with inhibitors for various time intervals with respect to the photoinduction which took place at 0 time. The effect was evaluated after a 24 h incubation after the photoinduction. Mean values of 3 parallel experiments are presented.

Contact with inhibitor	Inhibitors							
	Cycloheximide		5-Fluorouracil		Actinomycin D		Ramihyphin A	
	10^6 spores per disc	% illuminated control	10^6 spores per disc	% illuminated control	10^6 spores per disc	% illuminated control	10^6 spores per disc	% illuminated control
From −5 to − 2 h	71.4	64.3	94.1	85.5	83.6	77.1	66.0	58.3
From −1 to + 2 h	60.3	53.0	73.2	64.2	72.2	66.0	70.5	65.3
From −0.5 to + 2.5 h	53.2	46.1	46.8	43.5	65.6	58.4	72.1	70.5
From −0.25 to + 2.75 h	58.8	51.0	44.0	41.5	69.8	65.0	79.4	74.1
From ±0 to + 3 h	58.1	51.3	45.2	42.8	70.5	65.3	106.1	98.0
From +2 to + 5 h	62.3	55.5	98.0	88.1	77.1	71.8	108.1	100.0
From −0.5 to +24 h	5.1	4.7	1.9	2.1	23.4	21.6	18.4	21.5
From ±0 to +24 h	6.25	5.4	2.3	3.5	29.5	26.8	21.5	23.5

Illuminated control without the inhibitor: 108.5×10^6 spores/disc = 100%;
Non-illuminated control without the inhibitor: 4.15×10^6 spores/disc = 3.83%.

before the photoinduction, the lower the inhibition of sporulation. Its addition simultaneously with the photoinduction or 2 h later did not suppress the sporulation at all (compare data in Table II). On long-termed treatment with this antibiotic at a concentration of 50 μg/ml the sporulation was inhibited by about 80% (Fig. 2, Table II). This observation can be considered as a non-specific effect. The photoinduced sporulation was inhibited also by phenethyl alcohol (up to 0.1%), aflatoxin B_1 (100 μg/ml) and ethidium bromide (100 μg/ml).

Experiments with growing colonies

Colonies of *Trichoderma viride* grown on discs of filter paper placed on agar plates and transferred during the photoinduction on fresh plates with or without inhibitors were used in further experiments. Colonies of *Trichoderma viride* maintained in the dark practically did not sporulate. A short-termed photoinduction resulted in fructification manifested by the occurrence of dark green rings of pigmenting conidia after 24—28 of further incubation in the dark. These rings had an outer diameter corresponding to that of colonies at the time of illumination. In the meantime the colonies grew further and after a new photoinduction another dark green sporulating ring originated (Plate 2). Forty-hour colonies on paper discs placed on agar plates and incubated in the dark at 26 °C were transferred to fresh plates in red light, 30 min prior to the photoinduction. Certain plates contained 5-FU (50 μg/ml) and were incubated again in the dark for 28 h after a previous 3 min photoinduction. The colonies were exposed to 5-FU, either the whole time after the photoinduction or for 3 h at various time intervals with respect to the photoinduction. They were then transferred to fresh plates without the inhibitor. Plate 2 demonstrates the result of this experiment. The long-term contact (28.5 h) and short-term contact (from −0.5 to +2.5 h) of colonies with 5-FU resulted almost in suppression of the sporulation. On the other hand, a 3 h contact with the inhibitor only after the photoinduction (from +2 to +5 h or from +3 to +6 h) was not sufficient for the inhibition of the photoinduced sporulation of colonies of *Trichoderma viride*. These results are generally in agreement with those of Galun and Gressel (1966) showing that the delayed exposure of illuminated colonies of *Trichoderma viride* to 5-FU cannot reverse the result of the photoinduction: sporulation. We also compared the inhibition of the photoinduced sporulation of colonies by phenethyl alcohol, which inhibits DNA synthesis and simultaneously impairs the function of membranes and of aliphatic alcohols and dimethylsulphoxide. Phenethyl alcohol was substantially more effective than ethanol and dimethylsulphoxide (Fig. 3). We tested more than 80 biologically active compounds of different type (antibiotics, fungicides, mycotoxins, synthetic inhibitors, cancerogens, membrane poisons *etc.*) and verified their effects on the photoinduced sporulation of *Trichoderma viride*. The inhibitory effect was exhibited by the following inhibitors (including those mentioned above): (a) Inhibitors of RNA synthesis: acriflavin, actinomycin D, aflatoxin B_1, 8-azaguanine, 6-azauracil, congocidin, ethidium bromide, 5-fluorouracil, gliotoxin and phenethyl alcohol. (b) Inhibitors of protein synthesis: cycloheximide, fluorophenylalanine, trichothecin and T-2 toxin. (c) Compounds impairing functions of membranes: azalomycin F, polymyxin B, dimethylsulphoxide, methanol, ethanol, *n*-propanol, *n*-butanol and *t*-butanol. (d) Compounds with unknown mechanism of action: euparen, fusaric acid, monorden, nybomycin, viridin and ramihyphin A.

More detailed data concerning their effects on growth and the photoinduced sporulation of *Trichoderma viride* will be published in further papers.

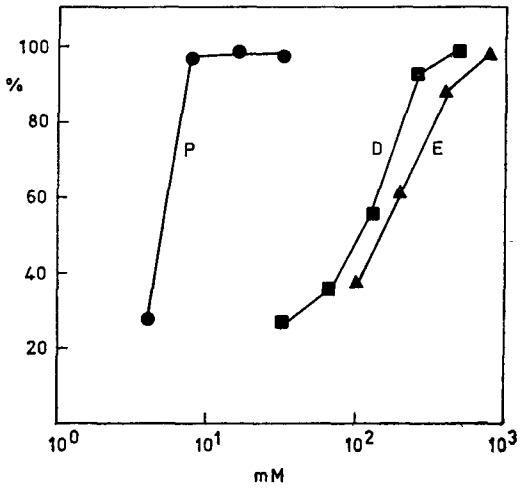

FIG. 3. Inhibition of the photoinduced sporulation of colonies of *Trichoderma viride* (by phenethyl alcohol (P), dimethylsulphoxide (D) and ethanol (E).

Trichoderma viride and other species of the genus *Trichoderma*, as soil saprophytes, produce antibiotics and play a role in the biological control of soil pathogenic microorganisms (Rodriguez-Kabana *et al.*, 1968). As producers of extracellular enzymes, cellulase in particular, these species are of industrial importance. With the exception of the Galun's group in the Weizmann Institute in Revohoth (see the original references for instance in Greenshpan and Galun, 1971); however, no systematic attention was devoted to problems of the photoinduced sporulation of this genus.

The results presented here indicate that the photoinduced sporulation of *Trichoderma viride* can be specifically inhibited by inhibitors of transcription and tranlation of genetic information. Inhibitors impairing the function of membranes, which can also intervene in the energetic metabolism, probably do not exhibit a direct effect. A similar indirect effect may also be exhibited by acriflavin and ethidium bromide, which react preferentially with the mitochondrial DNA resulting thus in disorders of metabolic functions of mitochondria.

NOTE ADDED IN PROOF

In addition to the effects of near UV light on sporulation of *T. viride* described in this paper the following recent findings should be mentioned. Near ultraviolet light was found to: a) inactive carotenoid-producing and albino strains of *Neurospora crassa* BLANC P. L , TUVESON, R. W., ARGENT, M. L.: Inactivation of carotenoid-producing and albino strains of *Neurospora crassa* with visible light, blacklight, and ultraviolet light. J. Bacteriol. *1 5*, 616, 1976) b) inactivate membrane transport in *Escherichia coli* (KOCH A. L , DOYLE R. J , KUBITSCHEK H. E.: Inactivation of membrane transport in *Escherichia coli* by near ultraviolet light. J. Bacteriol. *126*, 140, 976); and c) cause a shut-off of net RNA synthesis in *E. coli* RAMABHADRAN, T. V , JAGGER, J.: Mechanism of growth decay induced in *Escherichia coli* by near ultraviolet radiation. Proc. Nat. Acad. Sc . USA *73*, 59 1976).

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The plates will be found at the end of the issue.