Volatile Sexual Hormones in *Mucor mucedo*

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SUMMARY

A special technique permitted observation of sexual reactions between mycelia of *Mucor mucedo* without diffusion contact via the substrate. Morphogenetic phenomena (zygophore formation) appeared to be induced by volatile substances in a sex-specific way. Zygophores, induced via the air, showed chemotropic activity. The volatile stimuli were partly purified by thin-layer chromatography and appeared to have the same R_F values as precursors of trisporic acids. Trisporic acids are currently assumed to be responsible for the induction of zygophore formation via the substrate.

INTRODUCTION

In sexual reproduction in *Mucor mucedo* and related heterothallic species three reactions can be distinguished (Burgeff, 1924): a 'telemorphotic reaction', which involves the induction of aerial zygophore formation; a 'zygotropic reaction', in which directed growth of zygophores of 'plus' and 'minus' mating partners towards each other is observed; and a 'thigmomorphotic reaction', involving the morphogenetic events which occur after contact of the respective zygophores, such as gametangial fusion and septation.

Zygophore induction. There is evidence that zygophores are induced in a non-specific fashion by the trisporic acids B and C, low molecular weight substances produced by both mating partners and secreted into the culture medium (e.g. Gooday, 1973). In heterothallic species, such as *Blakeslea trispora* and *Mucor mucedo*, these hormones ('erogens': Machlis, 1972) are only synthesized when 'plus' and 'minus' mycelia are in continuous diffusion contact via the substrate (van den Ende & Stegwee, 1971). This synthesis in mated cultures is brought about by the production and secretion into the substrate of precursors of trisporic acids by each strain, and sex-specific conversion to the end products by the mating partners (Sutter, 1970; Sutter, Capage, Harrison & Keen, 1973). The production of precursors is stimulated by trisporic acids, indicating that hormone synthesis in these fungi is self-amplifying (Bu'Lock & Winstanley, 1971; Werkman & van den Ende, 1973). In these studies it became evident that these precursors also are potent zygophore-inducing agents. Unlike the trisporic acids, however, they act in a strictly sex-specific way. They are neutral materials of unknown chemical constitution that were partly purified by thin-layer chromatography (Werkman & van den Ende, 1973).

Apart from these studies there is a suggestion made by Burgeff (1924) (cf. also Gooday, 1973) that zygophore induction in *Mucor mucedo* could also be mediated by volatile substances. Trisporic acids are not volatile, but the precursors mentioned above might be.

Zygotropic reaction. When 'plus' and 'minus' zygophores are in close proximity, a directed outgrowth towards one another can be observed. It is generally assumed that this reaction is a typical chemotropic response, mediated by volatile substances conceivably

secreted by the zygomorphs themselves (Banbury, 1954). It is Banbury's opinion that besides the mutual attraction between zygomorphs of unlike mating type, there also exists a repulsive action between zygomorphs of the same type, but this has never been confirmed. Extensive work was published about zygotropism by Plempel (1960; 1962) and Plempel & Dawid (1961) who stated that the hormones involved in zygotropism are not the same as those inducing zygomorphs.

In this report we describe results which support the statement made by Burgeff (1924) and which indicate that zygomorph induction may be brought about not only by trisporic acids but also by volatile factors. They suggest a parallelism between volatile zygomorph-inducing and zygotropic action; zygomorph induction and zygotropism might even be mediated by the same hormonal factors.

METHODS

Organisms. The following strains were used: *Mucor mucedo* Brefeld, 'plus' (CBS 124.24); *M. mucedo* Brefeld, 'minus' (CBS 109.16); *Blakeslea trispora* Thaxter, 'plus' (NRRL 2456); *B. trispora* Thaxter, 'minus' (NRRL 2457). All strains were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Cultures. *Mucor mucedo* was grown in Petri dishes (10 cm diameter) containing about 15 ml of 20% (v/v) beer wort obtained from a local brewery, neutralized to pH 8 with KOH before sterilization. When appropriate, trisporic acids (mixture of trisporic acids B and C) were added to the culture medium, to a final concentration of about 15 µg/ml. This resulted in mycelia densely covered with relatively short zygomorphs. After a culture period of 3 to 4 days at 20 °C in the dark, the mycelial mats were removed with two pairs of sterile forceps, as shown in Fig. 1. The folded mat was laid down on one side of a slit cut in a solid substrate (2%, w/v, aqueous agar) in a Petri dish. By doing this, 'plus' and 'minus' mycelia were deposited facing each other, without diffusion contact via the substrate, with a minimum of manipulation. In order completely to eliminate the possibility of diffusion contact, the bottom of the plastic Petri dish was sometimes cut away under the slit. This never had any effect on the observed sexual reactions.

Cultures for bioassay of zygomorph induction and zygotropism were grown on a solid glucose-nitrate medium (van den Ende, 1968).

Isolation procedures. Neutral zygomorph-inducing substances were isolated from the liquid wort media of 'plus' and 'minus' cultures of *Mucor mucedo* (grown separately) by extraction from a total volume of 300 ml with peroxide-free diethyl ether after adjusting the pH to 9.0. The extract was washed several times with 5% (w/v) aqueous sodium bicarbonate and water (to remove traces of trisporic acids), dried over anhydrous sodium sulphate, and subjected to thin-layer chromatography; 20 × 20 cm plates, 0.25 mm thick, of silica gel H (Merck 7736) were used. The solvent system consisted of chloroform + diisopropyl ether + acetone + acetic acid (70:15:10:5, by vol.). After development, the chromatograms were divided into ten equal zones which were scraped off and eluted twice with ethanol. The solvent in the eluates was removed *in vacuo* and the residues taken up in 1 ml water or ether.

Neutral zygomorph-inducing substances were also isolated from *Blakeslea trispora* cultures as described by Werkman & van den Ende (1973).

Trisporic acids B and C, purified by chromatography over DEAE Sephadex, were obtained from *Blakeslea trispora* cultures as described earlier (van den Ende, Werkman & van den Briel, 1972).

Fig. 1. Folding and positioning of a mycelial mat on one side of a slit in an aqueous agar substrate.

RESULTS AND DISCUSSION

Evidence for volatile zygophore-inducing and zygotropic factors

Fig. 2 (a–d) shows *Mucor mucedo* at different times during one experiment. Fig. 2(a) shows the situation directly after the positioning of a 'plus' and a 'minus' mycelium, both covered with zygophores after growth in a medium containing trisporic acids, facing each other on either side of a slit in an agar layer. Zygophores are collapsed on account of the rather drastic change in microclimate. After 12 h erect zygophores are visible in large numbers, growing horizontally towards each other (Fig. 2b). Their length increases considerably, which is an additional feature of the zygotropic reaction (Plempel, 1962). Fig. 2(c) shows the situation after 20 h. Long zygophores (approx. 1.5 mm) of both 'plus' and 'minus' mycelia touch each other. The thigmomorphotic reaction after the contact of both mating types² is shown in Fig. 2(d).

From a large number of observations of the interactions between both types of mycelium of *Mucor mucedo*, the following conclusions can be drawn: (1) Zygophores are induced when two unlike *vegetative* mycelia (grown in the absence of trisporic acids) are on either side of the agar slit. This suggests that there are volatile zygophore-inducing substances other than the trisporic acids produced by vegetative mycelia. (2) When one of the two mycelia is vegetative, the other being covered with zygophores, these will grow in the direction of vegetative mycelium of the other mating type in a horizontal orientation, long before zygophores are visible on the latter. It may be concluded that the volatile zygotropic stimulus is secreted not only by zygophores, but also by vegetative mycelium. Fig. 2(e) shows the special case where three 'minus' zygophores are growing in the direction of a 'plus' sporangiophore. (3) No repulsion has ever been observed between zygophores of the same mating type. Sometimes two or more zygophores grow spiralling around each other. When

Fig. 2. Sexual reactions of *Mucor mucedo*, 'plus' and 'minus', positioned on either side of a slit in an aqueous agar substrate (left 'plus', right 'minus'). (a) Zero time, directly after positioning the mycelia; (b) 12 h later; (c) 20 h later; (d) 24 h later; (e) zygophores growing in the direction of a sporangiophore (arrow).

Table 1. *Zygophore-inducing activity of fractions derived from culture media of Mucor mucedo*

Fraction tested*	Test strain of <i>M. mucedo</i>	Zygophores induced†	
		via air	via substrate
Crude 'plus' extract	'plus'	—	—
<i>Idem</i>	'minus'	+	+
<i>Idem</i> ‡	'plus'	—	—
<i>Idem</i> ‡	'minus'	++	++
<i>Idem</i> ‡ $R_F = 0.0-0.5$	'minus'	—	—
$R_F = 0.5-0.7$	'minus'	++	++
$R_F = 0.7-1.0$	'minus'	—	—
Crude 'minus' extract	'plus'	+	+
<i>Idem</i>	'minus'	—	—
<i>Idem</i> ‡	'plus'	++	++
<i>Idem</i> ‡	'minus'	—	—
<i>Idem</i> ‡ $R_F = 0.0-0.1$	'plus'	++	++
$R_F = 0.1-0.6$	'plus'	—	—
$R_F = 0.6-0.8$	'plus'	++	++
$R_F = 0.8-1.0$	'plus'	—	—

* R_F values on thin-layer chromatograms of ether extracts of culture media. The thin layers were divided into ten zones, which were assayed separately.

† Zygophore induction via the substrate was accomplished by applying the solution (in water) in a well in the agar, in front of the respective mycelia. Zygophore induction via the air was effected with solutions (in water) in vials positioned on the bottom of the Petri dishes, after cutting away the agar along the front of the mycelium. —, No zygophores after 40 h; +, 1 to 10 zygophores after 40 h; ++, more than 40 zygophores after 40 h.

‡ The culture had grown in the presence of trisporic acids.

two mycelia of the same mating type, with zygophores, are positioned on either side of the slit, no reaction whatever can be detected. In due course a metamorphosis of zygophores to sporangiophores is observed. (4) The thigmomorphotic reaction can take place without prior zygotropic reaction. Two unlike mycelia containing zygophores can be positioned so that the zygophores exactly touch each other. The normal cell-fusion reaction is then observed within 2 h.

Isolation of volatile zygophore-inducing and zygotropic substances

Solid media in Petri dishes were eccentrically inoculated with single strains of *Mucor mucedo*. After three days the uncovered agar was cut away along the front of the mycelium, and small glass vials (4 mm depth, internal diameter 9 mm) were positioned about 4 mm from the front of the mycelium on the bottom of the dish. The vials were filled with crude ether extracts of liquid culture medium, or fractions thereof dissolved in water. In this way zygophore induction would be mediated unambiguously via the air. As is shown in Table 1, zygophores were induced on 'plus' and 'minus' mycelia of *M. mucedo* by fractions obtained from culture media of the sexual partner. The same results were obtained when extracts of culture media of *Blakeslea trispora* were used. Trisporic acid solutions never gave a response in this type of bioassay. Likewise, controls in which media or extracts were used which were derived from the same strain as used for the bioassay, were always negative. Sometimes, however, poorly reproducible responses were observed when fractions of 'plus' medium were applied to the 'plus' strain of *M. mucedo*. The structures induced had the appearance of zygophores, but without zygotropic features (see below). We tentatively designate these as pseudophores, as described by Plempel (1963).

Fig. 3. Orienting effect on zygothores of *Mucor mucedo* 'plus' by a piece of filter paper impregnated with an ether extract of culture medium of *Mucor mucedo* 'minus'. The photograph was taken 20 h after the start of the experiment. Bar marker = 1 mm.

There are two facts to be noted in particular: (i) biological activity was detected in the same fractions as found earlier to contain zygothore-inducing precursors of trisporic acids in *Blakeslea trispora* (Werkman & van den Ende, 1973) and *Mucor mucedo* (B. A. Werkman, unpublished); (ii) biological activity was much higher in fractions derived from cultures treated with trisporic acids and, in addition, 'minus'-culture extracts were more active than 'plus'-culture extracts. This suggests that airborne zygothore induction is mediated by these precursors of trisporic acids, the synthesis of which is stimulated by trisporic acids. Starting with equal culture volumes and keeping all circumstances as similar as possible, 'minus' extracts usually display more vigorous reactions than 'plus' extracts; this could be the result of more abundant production of active substances by the 'minus' strain, or of a higher sensitivity of the 'plus' strain. This at least rationalizes the observation of Gooday (1968) that in normal conjugations the 'plus' strain gives rise to zygothores both earlier and in larger numbers than the 'minus' strain.

All neutral fractions of culture media which induced zygothores, also had a marked effect on their morphology. Zygothores were longer and oriented towards the source of the agent. Fig. 3 shows a piece of filter paper, impregnated with an active fraction, placed in a vertical position near a mycelium containing randomly oriented zygothores induced by trisporic acids via the substrate. The zygothores nearest to the paper are more extended and grow in its direction, whereas the more distant ones keep their more-or-less vertical orientation. This suggests that volatile fractions which actively induced zygothores, also had zygotropic action. Whether we are dealing with substances having both the zygothore-inducing and zygotropic action, or with different (although related) substances exerting these two effects, cannot be decided from these experiments. However, the results do not preclude assigning this chemotropic role to precursors of trisporic acids, as isolated from the substrate.

The latter suggestion could imply that zygophore induction and zygophore orientation are based on the same mechanism. Bartnicki-Garcia & Lippman (1972) have suggested that enzymes that open bonds in the hyphal wall, leading to initiation of apical growth (e.g. branching or, more specifically, zygophore formation), are an integral part of the normal growth process. Stimulation and orientation of apical growth of zygophores could be visualized as a (localized) stimulation of these bond-splitting enzymes, thus changing the balance of wall softening and wall synthesis at the tips of the zygophore.

An analogous situation is found in *Achlya*, where the sex hormone antheridiol initiates the formation of antheridial branches and also elicits from them a chemotropic response (Barksdale, 1969).

Finally, the observation that the biologically active factors described here are not species specific with respect to *Mucor mucedo* and *Blakeslea trispora*, nor to *M. hiemalis* (unpublished), may provide the physiological basis for the understanding of the well-known interspecific conjugations which have been described by Burgeff (1924) and Blakeslee & Cartledge (1927).

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