J. Mycopathol. Res. 30(1) : 57-64 (1992) *Printed in India*

Retinol-induced zygospore formation in five species of *Rhizopus*

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All the five species of *Rhizopus* under study formed zygospores on the addition of retinol @ 167.67 i.u/ml. Though doses higher than this were also found to induce zygospore formation but minimal amount of the chemical was used in order to get the desired result. Retinol as such is be ng used for the first time in such type of studies and it is also freely available in the market.

Key Words : Retinol, Zygospore formation, *Rhizopus* spp.

INTRODUCTION

Blakeslee (1904) demonstrated the phenomenon of sexuality in Mucorales. He encountered heterothallism, a condition in which the mycelia from a single sporangium could not mate and rquired two compatible strains for zygospore formation. These compatible strains were designated as plus (+) and minus (-) by him. Mucorales also exhibit homothallism in which case the mycelia drawn from a single spore could from zygospores. Burgeff (1924) implicated a chemical which is responsible for this sexual mating. The (+) and (-) strains of *Mucor mucedo*, even when separated by a membrane could induce one another to form zygospores. Raper (1952) defined the aboxe chemical as a substance produced in one portion of the organism and transported by another means including diffusion to other portion of the same species, where they induce specific responses. These hormones/chemicals are classified on the basis of their specific mode of action into 'erotactins', where movement are induced like spore attractants. Erotrofins are those which induce the directional growth of the sexual structures in response to chemotrophic agents while 'erogens' are those which control the induction and differentiation of sexual

Publication No. 2150 MPP IARI New Delhi-12

structures (Machlis, 1972). The mucoralean hormone is, therefore, an erogen. Since the process of mating may involve the movement of mycelia over considerable distances, this movement was named as zygotropin by Gooday (1973). Burgeff and Plempel (1956) could obtain the cell free activity of this hormone, but did not characterise its completly. Prieto *et al.* (1964) detected an acidic fraction from the hyphae of mixed cultures of *Blakeshlea trispora*. Later, this fraction was considered akin to trisporic acid, comprising of three fractions, A, B & C in the proportion of 1-2, 15 and 80 per cent respectively (Cagliotti *et al.*, 1967). Amongst the fractions, B & C are required to accomplish mating in *Mucor mucedo* (van den Ende, 1968). Austin (1969) and his coworkers, isolated a hormone from *M. mucedo* and *B. trispora*, which was identical to trisporic acid.

In case of *Rhizopus*, out of the fifty one names assigned, only eleven are attributed to form zygospores. Of these, *B. chinensis*, *R. chiuniang*, *R. echinulata*, *R. nodosus*, *R. microsporus*, *R. oryzae*, *R. stolonifer* are heterothallic, while the present *R. artocarpi*, *R. homothallicus*, *R. oligosporus* and *R. sexualis* are homothallic. In investigation five species and their strains were used for the production of intra-specific zygospores with the help of retinol (vitamin A) a precursor of trisporic acid.

MATERIAL AND METHODS

Three different media viz., potato Dexrtose Agar, Synthetic *Mucor* Agar, Hay Extract Agar and Glucose Peptone Agar were used for the production of zygospores. The following five different *Rhizopus* species viz. *R. circinans* van Tieghem, *R.homothallicus* Hesseltine and Ellis., *R.microsporus* var. *nucrospores* van Tieghem, *R. oryzae* Went et Geerlings and *R. stolonifer* var. *stolonifer* (Fr. ex. Eh.) Lind. were used in mating experiments. The standard mating strains obtained from Central Bureau voor Schimmelcultures, Baarn, Holland : *R. circinans* (CBS 319.35 and 320.31) ; *R. microsporus* var. *microsporus* (CBS 699.60 and 700.64) ; and *R. stolonifer* var. *stolonifer* (CBS 107.76, 108.76 and 109.76) were also included.

Retinol (Vitamin A) a precursor of trisporic acid was used to induce zygospore formation in *Rhizopus* species. It was incorporated into the solid media @167.67 i.u./ml. Besides, it was also supplied as an aqueous solution on surface of solidified agar media. One ml of the hormone solution containing 1250 i. u/ml., 2500 i.u/ml, 5000 i. u/ml and 10,000 i. u/ml was added to each plate at an interval of 0,2,4,6 and 8 days from the time of inoculation, using disposable plastic syringes 0/one ml capacity obtained from Mo!s Plastipek Inc. (U. S. A.). The petriplates were incubated at the desired temperatures and observations were recorded at 4,7,11,15 and 20 days after inoculation.

RESULTS AND DISCUSSIONS

Fiftyseven crosses were attempted for zygospores formation using thirty different strains of above mentioned *Rhizopus* species. It was recorded that retinol @83.33 i. u/ml was unable to produce the formation of zygospores whereas 167.67 i. u/ml and higher concentrations were effective in achieving the desired results (Table 1).

Table 1. Showing optimal doses of retinol for zygospore formation in *Rhizopus* species

Doses in I. u ml	*R. circinans*	*R. microsporus var. microsporus*	*R. oryzae*	*R. oryzae*	*R. stoloni fer var solani fer*
83.33	—	—	—	—	+
167.67	+	+	+	+	+
2.50	+	+	+	+	+
333.33	+	+	+	+	+

(—) no desirable mating reaction observed
(+) zygospore formation

1. *R. microsporus var. microsporus*: The (+) and (-) strains from CBS, Holland, mated successfully at 37 C on PDA after eleven days of incubation. The designated (+) and (-) strains were later crossed with five strains (22, 93, 122, 147 and 152) individually in all eleven different crosses were made. Of these, 147 and 152 formed zygospores measuring 80-110 μm. While the other two (22 and 122) only exhibited incomplete eonjugation as evident by the intermingling of hyphae, swollen tips and accumulation of fat globules in the mycelia. However, strain No.93 showed no discernable approach to the sexual stage. On addition of Retinol @ 167.67 i. u./ml, the later two strains (22 and 122) also formed zygospores (Table 2).

Table 2. Interaction between designated (+) and (—) and local strains of *Rhizopus microsporus* var. *microsporus*

CBS Strains	22		93		122		147		152	
	R-	R+	R-	R+	R-	R+	R-	R+	R-	R+
699 69 (+)	*	+	—	—	+	+	+	+	+	+
700.67 (—)	—	—	—	—	—	—	—	—	—	—

R- where no ratinol was added
R+ Where retinol was added
* Incomplete conjugation
\+ Complete mating reaction
— no discernable approach to the sexual stage

2. *Rhizopus oryzae* : Thirteen strains (6, 8A, 9, 15, 19, 26, 29, 49, 54, 59, 70 and 150) were used for the induction of zygospores (Table 3a & b). Of these 9, 19, 54, 59 were initially designated as *R. arrhizus*. In all thirtyeight crosses were attempted, but only eight were successful in the production of zygospores measuring between 100-150 μm by the addition of retinol @ 167.67 i. u./ml. In the remaining cases incomplete conjugation was observed.

Table 3A. Mating reactions between five strains of *Rhizopus oryzae (=R. arrhizus)*

Concentration of Retinol	Strain MS	R−	R+	R−	R+	R−	R+	R−	R+	R−	R+
Conc. of 167.67	9	0	0	—	—	—	—	—	—	—	—
Retinol	14	—	—	0	0	0	—	—	—	—	—
	19	—	—	*	+	—	0	—	*	*	*
	54	—	—	—	—	—	—	—	0	0	—
	59	—	—	—	—	—	—	*	—	0	0

Table 3B. Mating reactions between eight strains of *R. oryzae*

Strains Nos	R+		R+		R+		R+		R+		R+		R+		R+	
6	0	0	*	+	—	—	—	—	—	—	—	—	—	—	—	—
8A	*	+	0	0	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	0	0	—	—	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	0	0	—	—	—	—	—	—	—	—
29	—	—	—	—	—	—	+	—	0	0	—	—	—	—	—	—
49	—	—	—	—	—	—	—	—	—	—	0	0	—	—	—	—
70	—	—	*	*	—	—	—	—	—	—	—	—	0	0	—	—
150	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0

R− where retinol was not added
R+ where retinol was added
* incomplete conjugation
\+ zygospores observed
— no discernable mating reaction
0 not attempted

3. *R. circinans* : The standard CBS strains (CBS nos. 319.20 and 320.20) were able to produce zygospores ranging from 90-110 μm at 20 Celcius on glucose peptone agar after 15 days of incubation. No addition of retinol was required to induce mating reaction.

4. *R. stolonifer* var. *stolonifer* : The designated (+) and (−) strains for CBS Holland 107.70 (−), 109.76 (+), 108.76 (+) were employed in zygospore production. These strains formed zygospore at 20°C of GPA, after eleven days of incubation. Five strains collected during this investigation were used in order to form zygospores. Only in two instances the zygospores were observed, zygospores size 120-140 μm while in others either incomplete conjugation or swelling of hyphal tips was noted (Table 4).

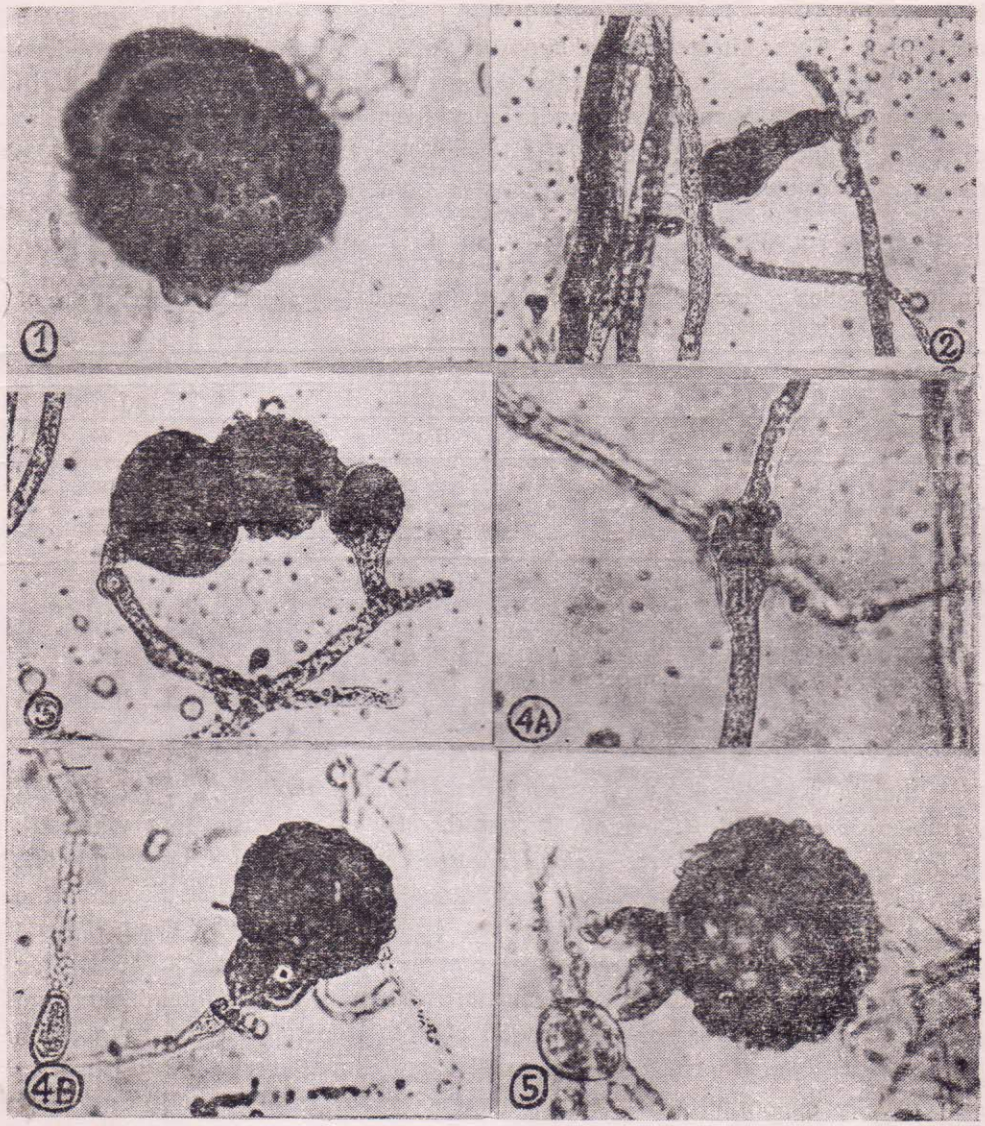
Table 4. Mating reaction between designated (+) and (—) strains and local strains of *Rhizopus stolonifer var. stolonifer*

Designated (+) & (—) strains	Local strains									
	2		3		13		50		60	
	R		R		R		R		R	
109.76 (+)	*	+	—	+	*	+	—	—	—	—
108.76 (+)	*	*	—	*	*	*	—	—	—	—
107.76 (—)	—	—	—	—	—	—	—	*	—	—

R where retinol was added
* incomplete conjugation
\+ zygospores formed
— no discernable mating reaction ocserved

From the above studies it was revealed that *R. homothallicus*, *R. microsporus* var. *microsporus* and *R. oryzae* preferred. Potato dextose agar while *R. circinans* and *R stolonifer* var. *stolonifer* formed zygospores on Glucose peptone agar. It has been well established that nutrients have a profound effect on the mating processes. Hesseltine and Fennell (1955) working with *Circinella* and Gams *et al.* (1972) with *Mortierella* noted that Malt extract, Synthetic Mucor agar and Starch agar etc. do not usually support mating. Media containing natural products or complex carbon & nitrogen sources generally induce zygospore formation. Kuhlman (1972) also reported that hempseed and Pavlum extract agar usually supported the maximum production of zygospores in the genus *Mortierella*, whereas Schipper (1978) found bear wort agar ideal for *Mucor* Similarly, Sarbhoy (1984) reported yeast extract agar for zygospores formation in *Absidia*.

Temperature has been regarded as an important factor in the mating reaction by many investigators (Hesseltiv and Benjamin, 1959 ; Schipper 1978, 1984) in the Mucorales. It has been confirmed from the present study that two different species of *Rhizopus* viz. *R. homothallicus*, *R. microsporus* var. *microsporus* supported 20° Celcius for the production of zygospores.



Explanation of Figures :

1. Zygospore of *Rhizopus circinans* X 400
2. Developing zygospore of *R. homathallicus* X 400
3. Mature zygospore with suspensors of *R. homothallicus* X 400
4 (A) Zygospore of *Rhizopus microsporus* var *microsporus* X 400
(B) Initiatiin of zygospore formation in *R. microsporus* var *microsporus* X 400
5. Zygospore of *R. oryzae* X 400

Although the production of zygospores is not clearly understood from a genetical point of view, zygospores are an excellent means of confirming the phylogenetic relationship between two strains. However, an incomplete mating reaction does not indicate a separate genetic line. This may be due to lack of suitable temperature, nutrient factor or compatable strains and age of culture (Schipper, 1978 ; Baijal & Mahrotra, 1980), though similarity in morphology of zygospores is related to phylogenetic entity.

All the five species of *Rhizopus* under study formed zygospores on the addition of retinol (@ 167.67 iu/ml). Though doses higher than this were also successfully, it was decided that minimal amount of the chemical be used in order to get the desired results. There is another advantage of using retinol instead of trisporic acid, as it ia freely available in the market. Retinol as such is being used for the first time in the present study and no earlier data is available.

It was also revealed in some strains isolated by the authors, as well as others which are know to be standard (+), (—) strains of *Rhizopus circinans*, *R. microsporus* var. *microsporus* and *R. stolonifer* var. *stolonifer*. There was no need to provide retinol as these spontaneously formed zygospores when proper mating conditions were available. However, in other crosses only the addition of retinol could induce zygospore production. Conclusion ferreted out from the studies that the efficiency of zygospore formation depends upon the mating partners as corroborated by Blackeslee *et al.* (1927) and Hasseltine & Anderson (1956). Schipper (1978) was of the opinion that in some of the *Mucor* species a fewer number of zygospores are found even by using standard (+) and (—) strains. Few of the strains under study, which did not from zygospores even with the designated (+) and (—) strains are considered as neutral. Similar were the findings of (Blakeslee *et al.*, 1927) and Schipper (1984) in case of some of the *Rhizopus* spp. as well.

ACKNOWLEDGEMENTS

The authors are grateful to the Head, Division of Mycology & Plant Pathology, for providing laboratory facilities during the course of the work. Our thanks are also due to the Director, Central Bureaux Voor Schimmel cultures, Baarn, Holland for supplying the standard mating strains of *Rhizopus microsporus* var. *microsporus* and *R. stolonifer* var. *stolonifer* and *R. circinans*.

REFERENCES

Austin, J. D., Bulock, J. D. and Gooday, G. W. (1969). Trisporic Acid : Sexual hormones from *Mucor mucedo* and *Blakeslea trispora*. *Nature* (Lond.) **223**, 1178-1179.

Baijal, U. and Mehrotra, B. S. (1980). The genus *Cunninghamella*-a reassessment. *Sydowia* **33**, 1-13.

Burgeff, H. (1924). Untersuchungen uber sexualitat und Parasitisumus bei Mucorineen I. *Botan. Abhamdl.* (Goebel) **14**, 1-135.

Burgeff, H. and Plemple, M. (1956). *Natuurwissen schaften* **43**, 473.

Blakeslee, A. F. (1904). Sexual reproduction in the Mucorineae. *Proc. Am. Acad. Arts. Sci.* **40**, 205-319.

Blakeslee, A. F. and Cartledge J. L. (1927). Sexual dimorphism in Mucorales. II. Interspecific reactions. *Beet. Goaz* **72**, 185-219.

Cagliotti, L., Cainelli, B. G., Camerina, R., Moldelli, R., Preito, A., Quiluo, A., Salavatnion, T.R. and Selva, A. (1967). The structure of trisporic-acid. *Tetrahedron suppl.* **7**, 175-187.

Games, W., Chein, Chiu-Yuan and Domsch, K. H. (1972). Zygospore formation by the heterothallic *Mortierella elongata* and a related homothallic species, *M. epigamma* sp. nov. *Trans Br mycol Soc* **58**, 5-13.

Gooday, G. W. (1974). Fungal sex hormones. *Ann. Rev. Biochem* **43**, 35-50.

Hesseltine, C. W. and Anderson, R. F. (1956). The genus *Thamnidium* and a study of the formation of its zygospores. *American J. Bot.* **43**, 696-703.

Hesseltine, C. W. and Benjamin, R. K. (1959). Microbial production of carotenoids VI some factors affecting sporulation and growth in the Choanephoraceae. *Mycologia* **51**, 881-901.

Hesseltine, C. W. and Fennell. D. I. (1955). The genus *Circinella*. *Mycologia* **47**, 193-212.

Kuhiman, E. G. (1972). Variation in zygospore formation among species of *Mortierella*. *Mycologia* **64**, 325-341.

Machlis, L. (1972). The coming of age of sex hormones in plants. *Mycologia* **64**, 235-247.

Raper, J. R. (1952). Chemical regulation of sexual processes in the Thallophytes. *Bot. Rev.* **18**, 447-545.

Prieto, A., Spalla, C., Bianela, M. and Biffi, G. (1964). Biosynthesis of β-carotene by strains of Chaonephoraceae, p. 38. Common. 2nd Intern. Fermentation Symp., London.

Sarbhoy, A. K. (1984). Scanning Electron Microscopic studies of Zygospores of the genus *Absidia*. *Bibl.Mycol* **91**, 224-238.

Schipper, M. A. A (1973). On certain species of *Mucor* with a key to all accepted species. *Stud Mycol* **17**, 1-71.

Schipper, M. A. A. (1984). A revision of the genus *Rhizopus* 1. The *Stolonifer* - group and *R. oryzae*. *Stud. Mycol* **25**, 1-19.

Van den Ende, H. (1968). Relationship between sexuality and carotene synthesis in *Blakeslea trispora*. *J Bacteriol* **96**, 1298-1303.

(Accepted for publication 3 March 1991)