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An Attempt to obtain Binucleate Pollen of Garlic, *Allium sativum* L.

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Introduction

Multivalent chromosomes at meiosis were observed in a number of clones of garlic, *Allium sativum* L.[1,3,5,7,8,16,17] It is well known that *A. sativum* L. is completely sterile, though many clones show the regular behavior of chromosomes at meiosis. The pollen grains of garlics degenerate in any case through the stages from tetrads to microspores, while the cause of the sterility has been still left unclarified. Concerning the sterility, some causes have been mentioned: the multivalent chromosomes[16], the nutrition competitions between the flower buds and the bulblets on the inflorescences[10], the hypertrophy of the tapetum[14], the microbiological factors such as virus, mycoplasma or rickettsialike organisms[2,7,9]. The scapes of garlics are usually laid down together with the withered leaves at the stage of microspores before anthesis, so that the deficiency of nutrients to the flower buds is supposed, besides the competitions between the flower buds and the bulblets. Environmental factors, principally, temperature and humidity, were mentioned with regard to the development of the sexual elements[11].

A few attempts were made to obtain fertile pollen, using the clones showing eight bivalents of sixteen chromosomes[7,9,15]. Antibiotics were examined, for the aim of obtaining them, successfully[7,9] or unsuccessfully[15]. It was assumed to be necessary to examine antibiotics with the clones having ring multivalents, which ought to show, at least, some pollen fertility, theoretically[18].

For these reasons, it was attempted to obtain binucleate pollen grains of cv. Shanhai-wase with ring octovalents.

Materials and Methods

Cultivar Shanhai-wase was originally introduced from China. It belongs, presumably, to *A. sativum* L. *f. pekinense* Makino[13], which has been looked upon as a mutant from the garlics cultivated in China or Japan[4]. The bulbs have white bulb coats and cloves in two groups. The leaves are broad and drooping. The scapes are well exserted, and the inflorescences have a number of flower buds together with many bulblets.

The scapes of cv. Shanhai-wase were cut off as tall as possible just before occurrence of the reduction division of pollen mother cells. Six scapes were cultured in each of the 300 ml Erlenmeyer flasks containing 100 ml of the liquid medium. In six flasks were contained Murashige-Skoog medium (pH 5.60–5.70), 0.3 M glucose and 0.1 ppm kinetin, respectively; while in other six were contained only distilled water. Both of the solutions were used after being autoclaved. In the respective six flasks of a solution were contained tetracyclinehydrochloride with the concentrations: 0, 5, 10, 20, 40, and 80 ppm. The spathes and the bulblets on the inflorescences were extirpated

from three of the six scapes in a flask. After extirpation, the inflorescences without bulblets were covered with transparent polyethylene bags. All the scapes in the flasks were put in a growth cabinet (20°C, natural day length). The solutions were renewed at the interval of two or three days, when the basal ends of the scapes were cut and renewed. The stages of the pollen mother cells and the pollen grains were examined every day. The pollen grains of the flower buds within polyethylene bags were counted for their stages after 22-day culture, just before the pollen grains got degenerated completely. The flowers opened after 35-day culture. The scapes were taken out when the flower buds or the scapes withered, entirely.

In addition to the experiment mentioned above, the other one was made, using the scapes at the different stage from that in the first experiment.

Six scapes at the degenerating stage of microspores were used to culture in the liquid medium: Murashige-Skoog medium supplemented with 0.3 M glucose, 0.1 ppm kinetin and 20 ppm tetracycline. The conditions for culture were made to be the same as those for the first experiment. The flowers opened after 17-day culture. The flower buds were fixed in Farmer's fluid, and their pollen grains were examined in acetocarmine solution.

Results

None of the flower buds of the scapes cultured in the distilled water developed so well as those in M–S media. At anthesis, the flowers in the former opened just a little, and their anthers hardly exserted. On the other hand, those in the latter opened widely (Fig. 1).

The stages of pollen after 22-day culture are shown in Table 1. The pollen mitosis and the binucleate pollen were observed, though they were small in number (Figs. 2, 3). They were observed more in M–S media than in the distilled water. Without tetracycline, the witherings of flower buds and the scapes in M–S media were earlier than those in the distilled water. And moreover, without tetracycline, neither the mitotic nor the binucleate pollen grains survived in M–S medium, though some of them survived in the distilled water. A few binucleate pollen grains were observed at 20 and 40 ppm tetracycline + M–S media, though the percentages of which were still under 0.1. The pollen mitosis was observed more at 40 and 80 ppm tetracycline + M–S media. However, the survival time of scapes in the same media was not so long as that at 20 ppm tetracycline + M–S medium (Table 2). Tetracycline at the concentrations of 40 or 80 ppm was assumed to be capable of checking the development of the flower buds or the scapes.

Average survival-days of three scapes without bulblets in each flask were longer than those with bulblets. The scapes in the distilled water without tetracycline survived for a long time, but their flower buds hardly increased in size, and their perianths became purple colored, earliest. The scapes at 5 ppm tetracycline + M–S medium survived long, and yet, in their flower buds the pollen mitosis was not observed and the total of binucleate pollen grains was only four. On the other hand, seven binucleate and eleven mitotic pollen grains were observed at 20 ppm tetracycline + M–S medium, and moreover, the scapes at the same medium survived longest. There were fourteen degenerating pollen grains out of 42 binucleate pollen grains, and even the normal ones were anticipated to degenerate, on account of their cytoplasm which was often uneven. Their nuclei were frequently distorted, though their exine and intine were always completed (Fig. 4).

At anthesis, the pollen grains of all the scapes examined were empty, except for a few degenerating pollen grains. No ripe, or fertile, pollen grains were observed at all. Therefore, in another experiment, the scapes were cultured again at 20 ppm tetracycline + M–S medium, as men-

Table 1. Stages of the pollen after 22-day culture of the scapes in the liquid media with tetracyclinehydrochloride

Media	Stages of the pollen					Numbers of pollen grains observed
	Microspores	Mitosis	Binucleate	Uncertain*1	Empty	
Distilled water +Tetracycline	%					
0 ppm	22.7 (21.6)	0.0 (—)	0.1 (0.0)	11.4	65.8	10,589
5	18.2 (17.8)	0.1 (0.0)	0.1 (0.0)	1.9	79.8	8,518*2
10	0.4 (0.3)	—	—	0.4	99.2	8,254*3
20	11.6 (9.9)	0.0 (—)	0.0 (0.0)	38.6	49.8	7,629*3
40	9.4 (5.8)	0.0 (0.0)	0.0 (0.0)	0.6	90.0	7,778*3
80	58.8 (56.6)	0.1 (0.0)	—	38.7	2.5	7,702*3
M–S media*4 +Tetracycline						
0 ppm	0.0 (0.0)	—	—	0.2	99.7	2,132
5	0.4 (0.2)	—	0.0 (0.0)	0.4	99.1	7,784
10	9.2 (8.7)	0.1 (0.0)	0.0 (—)	6.7	84.0	9,410*3
20	1.8 (1.5)	0.1 (0.0)	0.1 (0.0)	0.4	97.5	8,414
40	34.1 (31.1)	0.3 (0.1)	0.1 (0.0)	0.9	64.7	9,998
80	31.0 (30.4)	0.3 (0.1)	0.1 (0.0)	3.3	65.3	7,679

( ); Degenerating pollen grains
—; No pollen grains were observed.
*1 All of the pollen grains were degenerating.
*2 Two, of the three scapes, were obtained after 19-day culture because of their withering.
*3 One, of the three scapes, was obtained after 19-day culture because of their withering.
*4 Murashige-Skoog media+0.3 M glucose+0.1 ppm kinetin

Table 2. Survival days of the scapes in the liquid media with tetracyclinehydrochloride

		With bulblets	Without bulblets		With bulblets	Without bulblets
Distilled water				M–S media*		
Tetracycline	0 ppm	19.3	35.0		22.0	28.0
	5	22.0	24.3		25.0	35.0
	10	16.3	25.3		22.0	26.3
	20	21.0	30.0		28.0	35.0
	40	15.7	21.0		22.0	30.7
	80	14.0	23.3		25.0	30.7

* Murashige-Skoog media+0.3 M glucose+0.1 ppm kinetin

tioned in the item of Materials and Methods.

The culture began at the stage of microspores just before degeneration (Table 3). The pollen grains of three scapes were examined through their stages amounting to anthesis, 17 days after the beginning of the culture.

A number of pollen grains at mitosis and at the binucleate stage were observed. Their proportion to the total pollen grains was much higher than that of anyone obtained in the first experiment. The microspores and the mitotic pollen grains decreased with lapse of days, while none of the binucleate pollen grains increased. The binucleate pollen grains, when formed, developed a little

Table 3. Stages of the pollen of the flower buds on the scapes cultured in M-S medium*1 at the stage of microspores

Days of culture	Stages of the pollen					Numbers of pollen grains observed
	Microspores	Mitosis	Binucleate*2	Uncertain*3	Empty	
	%					
0	97.9 (65.1)	0.0 (—)	—	0.2	1.8	7,300
6	20.0 (9.7)	5.2 (0.5)	1.4 (0.5)	—	73.4	8,053
8	7.8 (4.2)	2.0 (0.4)	1.0 (0.4)	1.1	88.1	10,934
11	8.0 (3.2)	1.3 (0.3)	1.4 (0.5)	0.2	89.2	7,168
14	1.5 (0.3)	0.1 (0.0)	0.9 (0.3)	—	97.5	11,613
17	0.1 (—)	0.0 (—)	0.0 (0.0)	0.5	99.4	10,452

( ); Degenerating pollen grains
—; No pollen grains were observed.
*1 Murashige-Skoog medium+0.3 M glucose+0.1 ppm kinetin+20 ppm tetracycline
*2 Binucleate pollen grains contain multinucleate pollen grains.
*3 See Table 1.

more than those observed in the culture made from the stage of pollen mother cells (Fig. 5). The binucleate pollen grains, when normal, increased in their sizes. However, their cytoplasm was often uneven, and their nuclei frequently showed amoeboid shapes. At anthesis, few binucleate pollen grains were observed, and most of the pollen grains were empty. The pollen grains of garlic, at full maturity, were supposed to be binucleate, resembling those of leek which is the closest one to garlic among the fertile cultivated *Alliums* (Fig. 6). The microspores usually degenerated from the cytoplasm, suggesting a deficiency of nutrients (Fig. 7). At the stage of mitosis, few pollen grains degenerated (Fig. 8). Nine chromosomes, at pollen mitosis, were sometimes observed, though eight are haploid (Fig. 9). At the binucleate stage, the pollen grains with more-than-two-nuclei, including small nuclei, were often observed (Figs. 10, 11). The cytoplasm was frequently degenerated first around the germ pores (Fig. 12).

After all, the binucleate pollen grains of garlic were obtained, while the fertile ones were not obtained at all.

Discussion

Many clones of garlic do not produce the scapes; or the inflorescences are to be, partially, or not at all, exserted. Some clones develop their scapes, and the flowers wither in the stage of buds, although they are absent on the inflorescences in some other clones.

Konvicka[7] mentioned that the cultivation of inflorescence stems in a tetracycline solution induced a formation of fertile pollen grains in garlics. Novak and Havranek[15] argued that the development of pollen grains under the tetracycline-treatment ceased before the first pollen mitosis as well as in a control experiment. Konvicka et al.[9] mentioned that after a four-week-antibiotic-treatment (mixture of tetracyclinehydrochloride and tylocine) pollen fertility of garlic was successfully restored. Their materials showed chromosomal regularity at meiosis, with eight bivalents. Takenaka[16] concluded that the sterility in garlics was due to the multivalents observable at meiosis. However, some pollen grains must be fertile, if the chromosomal irregularity such as multivalents should be the only one cause of the sterility.

Cv. Shanhai-wase showed ring and chain octovalents[3]. The ring octovalents ought to have fertility of, at least, 12.5%[18]. In the chromosome configuration of cv. Shanhai-wase at meiosis, the frequency of 1_{VIII}(ring)+4_{II} was 42.8%[3]. If the pollen sterility in cv. Shanhai-wase is to be resulted from its multivalent chromosomes at meiosis, at least 5.4% of the pollen grains ought to be fertile. However, no fertile pollen grains were obtained in the present experiment, though some binucleate ones were observed. But for the alternative (zigzag) metaphase arrangement at meiosis, no binucleate pollen grains might have been observed.

In the present experiment, the pollen grains with multinuclei were often observed at the binucleate stage. Some of them might have been resulted from the abnormal components of their nuclei originated at meiosis. Some of the other, as well as the pollen grains with distorted or amoebic nuclei, might have been resulted from the incomplete poleward movement of chromosomes at the first pollen mitosis. If the chromosomal irregularity at meiosis were the only one cause of the sterility in cv. Shanhai-wase, most of the pollen grains with nuclei of abnormal components would have been degenerated before pollen mitosis. Hence, there must have been some other causes of the sterility rather than the chromosomal irregularity.

Majumdar[12] reported that substantial amount of pollen viability was restored when the flowers of the two complete male sterile species, *Haworthia fasciata* and *Astroloba aspera*, were grown in White's basal medium supplemented with IAA, kinetin and coconut milk. Konvicka[9] assumed that the pollen sterility in garlics occurred in consequence of nutritional disturbances, though with the additional assumption that the disturbances seemed to have been caused by rickettsialike organisms. Some nutrition competitions between the flower buds and the bulblets in the inflorescences were observed here, as suggested by Koul and Gohil[10]. The flower buds with the bulblets, as well as the scapes, withered earlier than those without bulblets. M–S medium was supposed to be containing sufficient nutrients for the development of microspores or pollen grains. Nevertheless, no fertile pollen grains were obtained here. By the removal of bulblets from the inflorescences, Novak and Havranek[15] obtained no binucleate pollen grains but obtained defective seeds, which did not germinate. Some attempts to culture the scapes or the flower buds aseptically might be necessary, for example, with the use of media other than these.

Should the cause of the sterility in the clones with eight bivalents be the same as that in the clones with multivalents? Konvicka[7] mentioned, as the cause, the microbial factor within the range of virus or mycoplasma, and Konvicka et al.[9] the richettsialike organisms. Etoh and Ogura[2] suggested that the morphological abnormality of the flower buds was similar to the symptoms caused by virus, and that the sterility might have been resulted from virus. In the present experiment, the effect of the antibiotics on the development of the pollen was not so clear as that mentioned by Konvicka et al.[9]. Therefore, for verifying the hypotheses mentioned above, it was assumed to be necessary to treat garlics by other kinds of antibiotics, or to culture, aseptically, the apical meristem for virus free garlics.

Besides this, it appears to be necessary to investigate the process of the pollen-degeneration in garlics, compared with those in other fertile *Allium*s.

Summary

The scapes of *Allium sativum* L. cv. Shanhai-wase having multivalents at meiosis were cultured with tetracyclinehydrochloride in distilled water, and in Murashige-Skoog media supplemented with glucose and kinetin. The mitotic and the binucleate pollen grains were observed more in the cul-

ture from the stage of the microspores than in the culture from the stage of the pollen mother cells just before meiosis. Tetracycline checked the development of the flower buds at the concentrations of 40 or 80 ppm. The binucleate pollen grains in culture degenerated and no fertile ones were obtained at anthesis.

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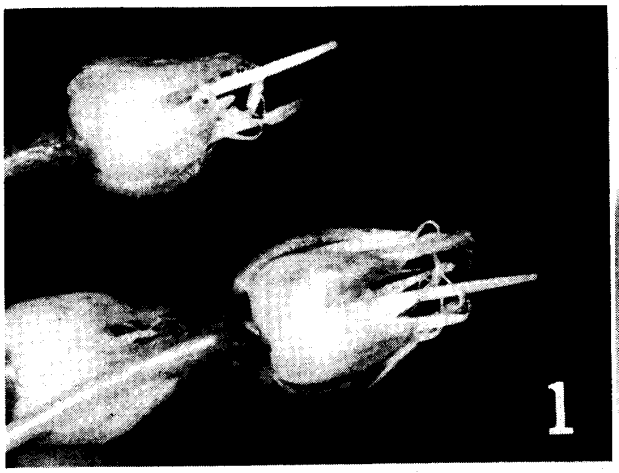
References

1) Etoh, T.: Variation of chromosome pairings in various clones of garlic, *Allium sativum* L. *Mem. Fac. Agr. Kagoshima Univ.*, **15**, 63–72 (1979)
2) Etoh, T. and Ogura, H.: A morphological observation on the formation of abnormal flowers in garlic (*Allium sativum* L.). *Mem. Fac. Agr. Kagoshima Univ.*, **13**, 77–88 (1977)
3) Etoh, T. and Ogura, H.: Multivalent chromosomes in garlic, *Allium sativum* L. *Mem. Fac. Agr. Kagoshima Univ.*, **14**, 53–59 (1978)
4) Jones, H. A. and Mann, L. K.: *Onions and their allies.* p. 36–38, Leonard Hill Books, London (1963)
5) Katayama, Y.: Chromosome studies in some Alliums. *J. Coll. Agr. Tokyo Imp. Univ.*, **13**, 431–441 (1936)
6) Konvicka, O.: Cytotaxonomische Studien von vier sterilen Arten der Gattung *Allium*. *Biol. Plant.* (*Praha*), **14(1)**, 62–70 (1972)
7) Konvicka, O.: Die Ursachen der Sterilitaet von *Allium sativum* L. *Biol. Plant.* (*Praha*), **15(2)**, 144–149 (1973)
8) Konvicka, O. and Levan, A.: Chromosome studies in *Allium sativum*. *Hereditas*, **72**, 129–148 (1972)
9) Konvicka, O., Nienhaus, F. und Fischbeck, G.: Untersuchungen über die Ursachen der Pollensterilität bei *Allium sativum* L. *Z. Pflanzenzüchtg.*, **80**, 265–276 (1978)
10) Koul, A. K. and Gohil, R. N.: Causes averting sexual reproduction in *Allium sativum* Linn. *Cytologia*, **35**, 197–202 (1970)
11) Krivenko, A. A.: A cytological study of garlic (*Allium sativum* L.). *Biologicheskij Zhurnal* (in Russian with English summary), **7**, 47–68 (1938)
12) Majumdar, S. K.: *In vitro* culture of flower buds of *Haworthia* and *Astroloba*. *Phyton*, **27**, 27–34 (1970)
13) Makino, T.: *Makino's new illustrated flora of Japan* (in Japanese). p. 840, Hokuryukan, Tokyo (1970)
14) Novak, F. J.: Tapetal development in the anthers of *Allium sativum* L. and *Allium longicuspis* Regel. *Experientia*, **28**, 1381–1382 (1972)
15) Novak, F. J. and Havranek, P.: Attempts to overcome the sterility of common garlic (*Allium sativum* L.). *Biol. Plant.* (*Praha*), **17(5)**, 376–379 (1975)
16) Takenaka, Y.: Further reports of the cytological investigations on the sterile plants. *J. Chosen Natural Hist. Soc.* (in Japanese with English summary), **12**, 25–41 (1931)
17) Takenaka, Y.: Ring formation in the meiosis of *Allium scorodoprasum var. viviparum*. *Ann. Rep. Nat. Inst. Genet.* (*Japan*), **4**, 41–42 (1953)
18) Yamashita, K.: H. Kihara (ed.), *Saibō-idengaku* [*Cytogenetics* — in Japanese] vol. 1, p. 123–146, Yōkendō, Tokyo (1951)

Explanation of figures

Fig. 1. Flowers in M–S medium at anthesis.
Fig. 2. Metaphase in the pollen mitosis. ×ca. 1,900
Fig. 3. A binucleate pollen grain. ×ca. 1,500
Fig. 4. Distorted nuclei at the binucleate stage. ×ca. 1,500
Fig. 5. A developed, but not fully mature, binucleate pollen grain. ×ca. 1,500
Fig. 6. Fertile pollen grains of leek at anthesis. ×ca. 1,100
Fig. 7. Degenerating microspores with vacuolate cytoplasm and contracted nuclei. ×ca. 1,100
Fig. 8. Degenerating pollen grains with small vacuoles at mitosis. ×ca. 1,900
Fig. 9. Nine chromosomes at metaphase of the first pollen mitosis. ×ca. 1,900
Fig. 10. A pollen grain with three nuclei at the binucleate stage. ×ca. 1,500
Fig. 11. A pollen grain with small nuclei at the binucleate stage. ×ca. 1,500
Fig. 12. A binucleate pollen grain with cytoplasm degenerating first around the germ pores. ×ca. 1,500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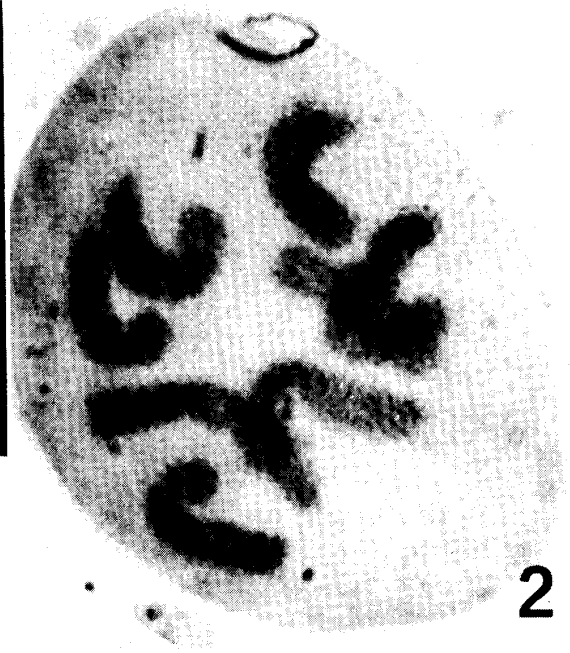
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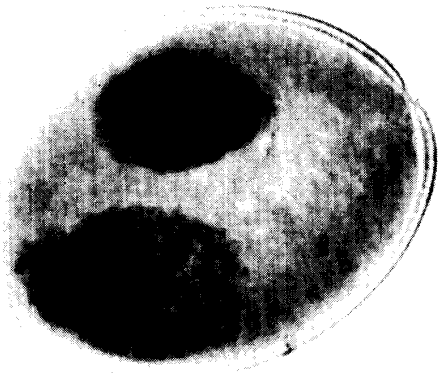
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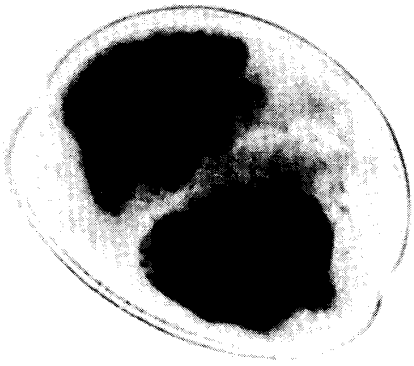
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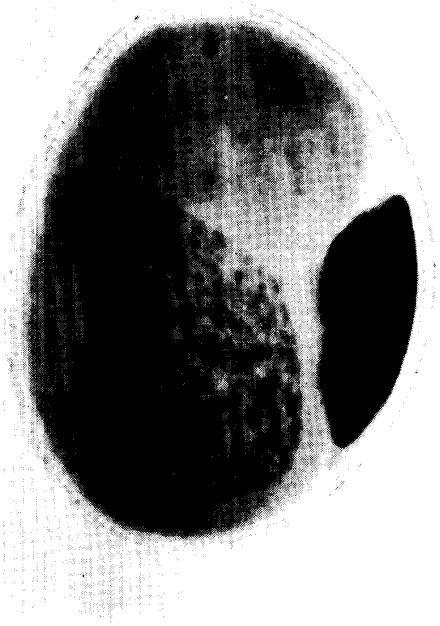
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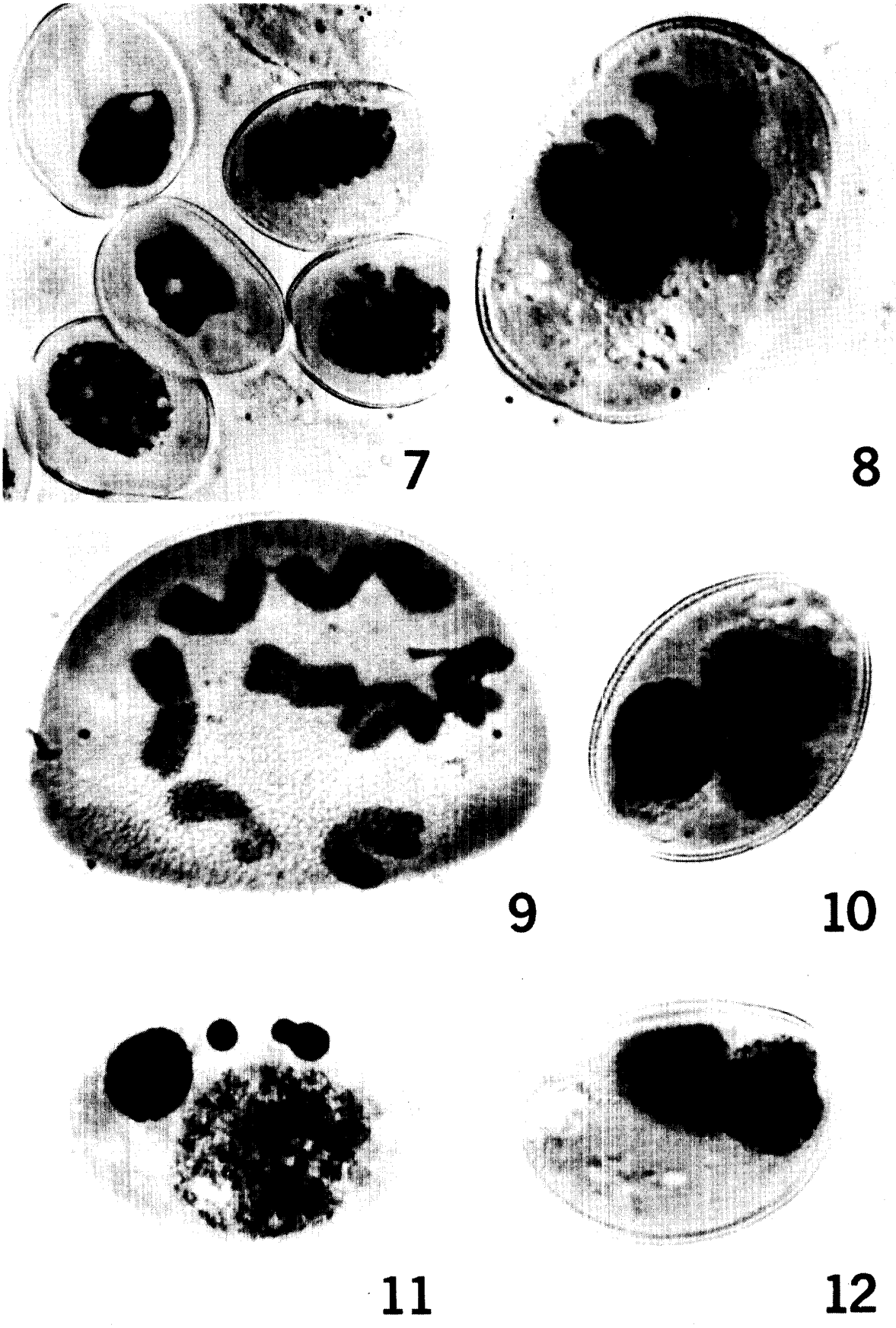
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