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Effects of chemical components and nitrogen sources on zygospore development in *Phycomyces blakesleeanus*

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We examined the effects of chemical components and nitrogen sources on zygospore development, using 62 different ingredients based on Sutter's synthetic medium SI, which has been widely used for studies of sexual physiology in *Phycomyces.* An increase of inorganic microelements such as $ZnSO_4$, $NaMoO_4$ and $CaCl_2$ promoted an increase in the number of zygospores per unit area. Glutamate (Glu) contained in SI as the sole nitrogen source was indispensable for sexual development, and replacement of Glu with NH_4^+(Am) strongly inhibited it, mainly because of growth inhibition. However, zygospore production was enhanced 1.8-fold by equivalent amounts of both Glu and Am as compared with Glu alone. A newly developed medium, mSI+Am, enriched with Am and the above-mentioned effective microelements doubled the number of zygospores formed per unit area (density), compared with Sutter's original SI, and increased both the density and the weight (volume) of zygospores, 1.6- and 2-fold, respectively, compared with potato-dextrose-agar medium enriched with yeast extract and casitone (PDAYC). Sexual stimulation by mSI+Am was also observed in the mating of a pair of β-carotene-deficient mutants. Methionine sulfoxime, an inhibitor of glutamine synthetase, strongly inhibited the progress of mating without significant growth inhibition.

Key Words——ammonium; mating reaction; nitrogen source; *Phycomyces blakesleeanus;* zygospore.

The heterothallic fungus *Phycomyces blakesleeanus* Burgeff possesses two mating types, (+) and (−), and develops zygospores as the final product when their hyphae meet on a solid medium (Blakeslee, 1904; Sutter, 1987). The mating process has been divided into seven steps (A-G), with a somewhat ambiguous classification (Hocking, 1967). Sutter (1975) clearly classified the process into eight developmental stages (S1-8): formation of zygophores as swollen and highly-branched hyphae (S1); intertwining of two zygophores of different mating types (S2); progametangium formation upright in the air after enlargement of the paired zygophores (S3); looping of progametangial cells due to splitting in the middle region of the enlarged tongs (S4); formation of gametangia and suspensors along both progametangia by delimitation with furrowing septa (S5); appearance of thorn-like appendages on the suspensors (S6); zygote formation by fusion of two gametangia (S7); and maturation of zygote to zygospore with the maximum size and a thick black coat (S8).

The mating of *Phycomyces* is greatly influenced by medium composition as well as external conditions such as temperature, light, pH, moisture, and aeration (Robbins and Schmitt, 1945; Hocking, 1967; Sutter, 1975; Yamazaki and Ootaki, 1996; Yamazaki et al., 1996). Many different kinds of media for both growth and zygospore development have been examined using cultures of different *Phycomyces* strains (Burkholder and McVeigh, 1940; Leonian and Lilly, 1940; Robbins and Schmitt, 1945; Ødegård 1952; Benjamin and Hesseltine, 1959; Komarova et al., 1972). Almost all investigations, in particular those for zygospore production, have been qualitative. The most popular synthetic minimal media for systematic study of *Phycomyces* were developed based on refinements and modifications introduced by Sutter (1975) to the medium proposed by Ødegård (1952): S-media (SI, SII, SIII and SIV)-SI for the study of sexual physiology and morphology and SIV as a medium useful for the study of vegetative growth and sporangiophore differentiation. Abundant zygospores are formed on SI solid medium, but very few on SIV. The difference between SI and SIV is only in nitrogen source: SI and SIV contain 5.3 mM glutamate and 13.3 mM asparagine, respectively. Given the above, there is a possibility of further improvement of the medium, in particular, for promoting the mating response.

In the present study, we systematically investigated the effects of chemical elements, different kinds of nitrogen sources and their combinations on sexual development of the most general strain of *P. blakesleeanus* wild type. By surveying 62 different test media, we confirmed that modifications of the composition greatly improved the zygospore development of this fungus, when compared with complete media such as PDAYC

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and SIYC enriched by yeast extract and casitone, a hydroxylized casein. The improvement was generated in not only the wild type but also its β-carotene-deficient mutants, implying that the new medium could be very useful for genetic analysis and for elucidating mechanisms of mating reactions of *Phycomyces* and Mucorales as well.

Materials and Methods

Strains and culture conditions The standard wild types, IGE1101 (=NRRL1555) (−) and IGE1103 (=A56) (+) (Alvarez and Eslava, 1983), and β-carotene-deficient mutants, C2 [*carA5* (−)] and C3 [*carA5* (+)], were used in this study. Mutants of *carA* gene are white and produce very small amounts of β-carotene, but no accumulation of other intermediate carotenes (Meissner and Delbrück, 1968; Ootaki et al., 1973). To obtain mycelial inocula for mating experiments, each strain was grown on PDAYC at 20°C under continuous illumination with white light at 0.1 W/m^2 at the mycelial level (Yamazaki et al., 1996). Rectangular strips of mycelial mat (2×20 mm) cut out from the mycelial front were used as the inocula. The (+) and (−) strains were inoculated 4 cm apart at confronting positions on a petri plate 6 cm in diam containing 10 ml of various kinds of solid test media whose surfaces were covered with dialysis membranes. To investigate the effect of $CaCl_2$, Ca-free material (polyurethane foam) was used instead of agar, because agar presumably contains high Ca contents. These plates, four per treatment, were placed and kept in humidified dark boxes at 16°C.

Measurement of zygospores The number of zygospores which developed in a unit area (10 mm^2) on the test media was measured 20 d after the onset of mating as described previously (Yamazaki and Ootaki, 1996; Yamazaki et al., 1996). Zygospores were counted in one 5-mm^2 area per plate. Then the total of four counts for each treatment was calculated. Deviation between the four plates was usually small, being within 5. To measure the weight, 10 zygospores were harvested from the plates and attached suspensors, thorns and extra walls surrounding zygospores were removed with fine tweezers. The weight of 10 zygospores was determined using a super precision balance (d=0.001 mg; Sartorius GHBH, Göttingen, Germany). The weight (mg per zygospore) was calculated and expressed by indexes: $B' < 0.015$, $0.015 < B < 0.025$, $0.025 < A' < 0.04$, $0.04 < A < 0.05$, $0.05 < A^+$.

Chemicals All the media tested in the present study were modifications of Sutter's original SI. PDAYC was made from commercial Difco-Potato Dextrose Agar (Lot No, 765090) supplemented with Difco-Yeast Extract (1g /l) and Difco-Casitone (1g/l). All the other chemicals were purchased from Nacalai Tesque, Kyoto, Japan or

Table 1. Chemical composition of SI and test media 01-21.

Medium[a]	Glc (mM)	Glu (mM)	VB_1 (μM)	KH_2PO_4 (mM)	$MgSO_4$ (mM)	$CaCl_2$ (mM)	$Fe(NO_3)_3$ (μM)	$ZnSO_4$ (μM)	$MnSO_4$ (μM)	$CuSO_4$ (μM)	$NaMoO_4$ (μM)
SI	110	5.3	6	37	2	0.25	3.7	3.5	2	0.2	0.2
01	**0**	5.3	6	37	2	0.25	3.7	3.5	2	0.2	0.2
02	**220**	5.3	6	37	2	0.25	3.7	3.5	2	0.2	0.2
03	110	5.3	**0**	37	2	0.25	3.7	3.5	2	0.2	0.2
04	110	5.3	**24**	37	2	0.25	3.7	3.5	2	0.2	0.2
05	110	5.3	6	**0**	2	0.25	3.7	3.5	2	0.2	0.2
06	110	5.3	6	**148**	2	0.25	3.7	3.5	2	0.2	0.2
07	110	5.3	6	37	**0**	0.25	3.7	3.5	2	0.2	0.2
08	110	5.3	6	37	**8**	0.25	3.7	3.5	2	0.2	0.2
09[b]	110	5.3	6	37	2	**0**	3.7	3.5	2	0.2	0.2
10[b]	110	5.3	6	37	2	**0.25**	3.7	3.5	2	0.2	0.2
11[b]	110	5.3	6	37	2	**1**	3.7	3.5	2	0.2	0.2
12	110	5.3	6	37	2	0.25	**0**	3.5	2	0.2	0.2
13	110	5.3	6	37	2	0.25	**14.8**	3.5	2	0.2	0.2
14	110	5.3	6	37	2	0.25	3.7	**0**	2	0.2	0.2
15	110	5.3	6	37	2	0.25	3.7	**14**	2	0.2	0.2
16	110	5.3	6	37	2	0.25	3.7	3.5	**0**	0.2	0.2
17	110	5.3	6	37	2	0.25	3.7	3.5	**8**	0.2	0.2
18	110	5.3	6	37	2	0.25	3.7	3.5	2	**0**	0.2
19	110	5.3	6	37	2	0.25	3.7	3.5	2	**0.8**	0.2
20	110	5.3	6	37	2	0.25	3.7	3.5	2	0.2	**0**
21	110	5.3	6	37	2	0.25	3.7	3.5	2	0.2	**0.8**

a) Amount of glucose (Glc), vitamin B_1 (VB_1) or inorganic elements was changed in each medium as compared with SI medium as shown by bold, underlined figures.

b) Ca-free wet material was used instead of agar.

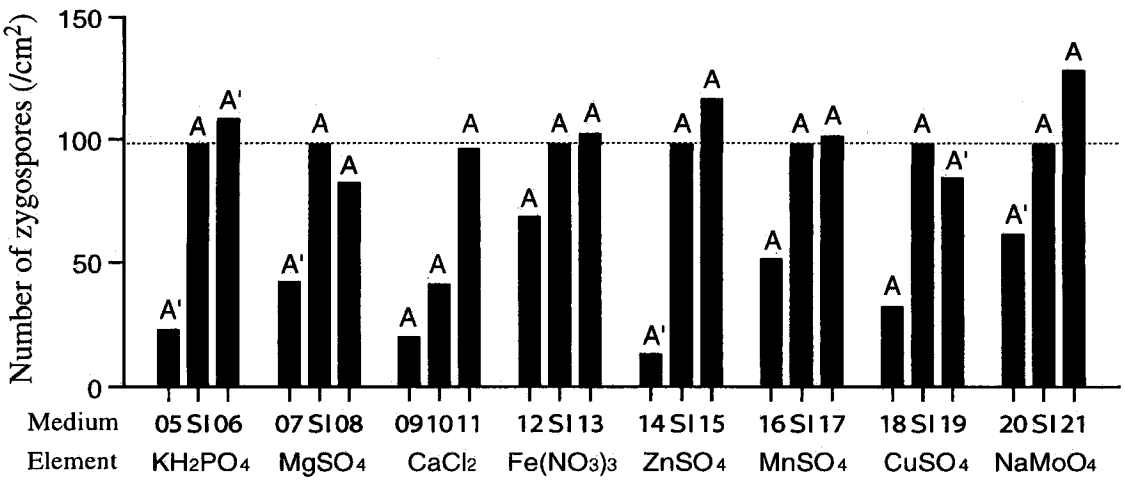

Fig. 1. The number and weight of zygospores on SI and test media 05–21 modified with inorganic components. Weight (mg per zygospore) shown above each column is expressed by the following indexes: $B' < 0.015$, $0.015 < B < 0.025$, $0.025 < A' < 0.04$, $0.04 < A < 0.05$, $0.05 < A^+$.

Sigma Chemicals, St. Louis, MO, USA. Stock solutions of nitrogen sources such as L-glutamic acid monosodium salt (Glu), L-glutamine (Gln), L-aspartic acid sodium salt (Asp), and L-asparagine (Asn) were autoclaved and chilled before use. NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ were filter-sterilized and then added to autoclaved media. Microelements such as $Fe(NO_3)_3$, $ZnSO_4$, $MnSO_4$, $CuSO_4$ and $NaMoO_4$ were first dissolved in distilled water containing a drop of 0.5 mM citric acid, which allowed a high solubility, then sterilized through membrane filters. A solution of 0.05% thiamin (vitamin B1) was also filter-sterilized. Methionine sulfoxime (MSX) was dissolved in distilled water at a concentration 100 times that of the working solution, then filter-sterilized.

Results

Effects of glucose, vitamin B_1 and inorganic elements

Sixty-two test media modified from Sutter's original SI were investigated as to their effects on zygospore formation (Tables 1, 2; Figs. 1–3). Around 100 zygospores per unit area were formed on SI under the culture conditions employed. An increase in glucose content (2%; 110 mM) as the sole carbon source in SI did not significantly promote sexual organ development; the addition of two times more glucose had no significant effect. Vitamin B_1 was indispensable and its optimal concentration in the medium was 6 μM (2 mg/l), the same level as that of Sutter's original SI. Removal of any inorganic nutrient resulted in a decrease in the number, dependent on chemicals, of zygospores formed on the media compared with those formed on SI (Fig. 1). The zygospores formed on such element-free media were small and/or colorless (data not shown). With increasing concentrations of $CaCl_2$, zygospore production was promoted by

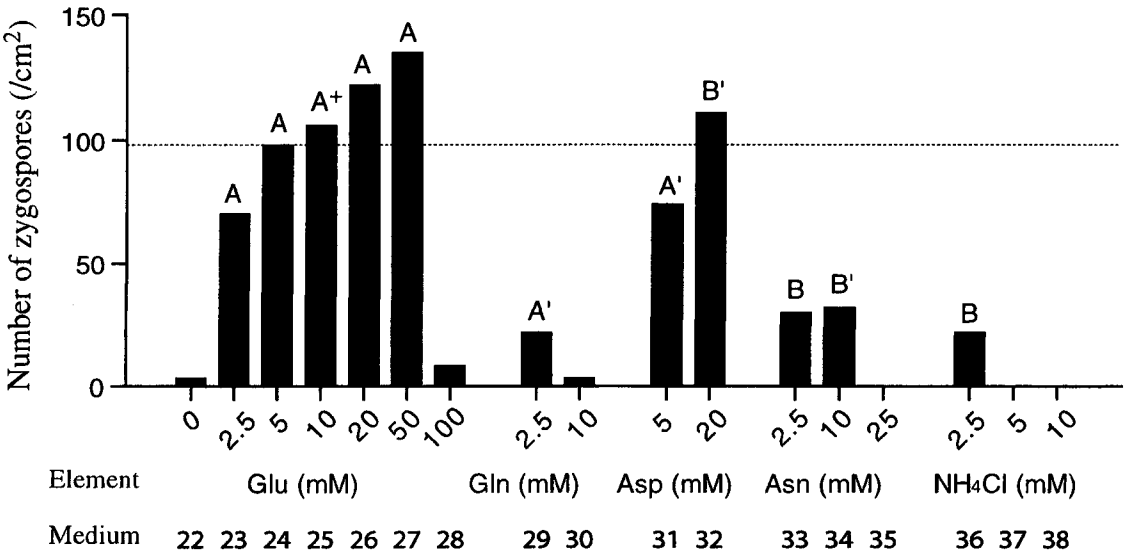

Fig. 2. The number and weight of zygospores on test media 23–38 modified with nitrogen sources. The index for weight is the same as in Fig. 1.

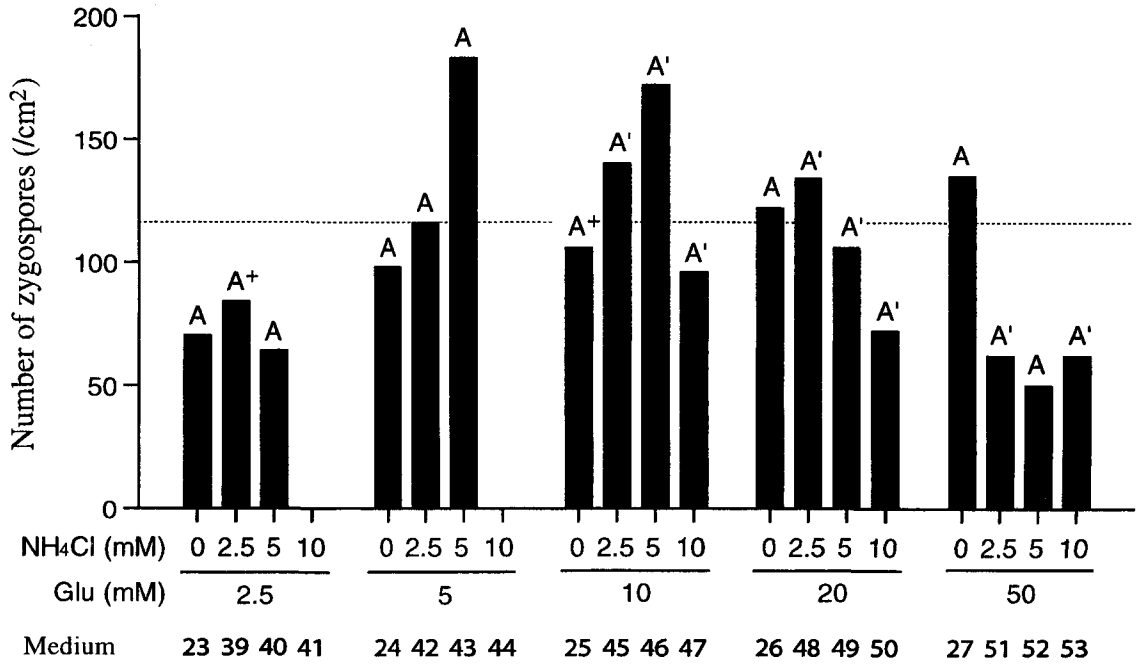

Fig. 3. The number and weight of zygospores on test media 23–27 and 39–53 modified with the ratio of glutamate and NH_4Cl. The index for weight is the same as in Fig. 1.

up to fourfold (Fig. 1). The addition $CaCl_2$ equivalent to that in SI on the agar-free plate resulted in only 42% of the zygospore formation of the agar culture. Nutritional components $ZnSO_4$ and $NaMoO_4$ stimulated zygospore formation (Fig. 1). An increase of the other components, KH_2PO_4, $MgSO_4$, $Fe(NO_3)_3$, $MnSO_4$ and $CuSO_4$, did not significantly affect the number, or was possibly inhibitive (Fig 1).

Effects of nitrogen sources Glu, the sole nitrogen of SI, seemed to be a key component in the sexual reproduction of *Phycomyces*. In fact, Glu was the most effective nutrient for the formation of zygospores, increasing the number until 50 mM, at which a 40% increase was observed (Fig. 2). To investigate the importance of Glu, it was replaced with the metabolically related amino acids Gln, Asp and Asn (Fig. 2). Gln and Asn amido group amino acids were less effective. Asp, probably functioning as a NH_3 acceptor, showed a similar effect to Glu, but a higher concentration was needed to attain the effect of

Table 2. Chemical composition of test media 43 and 54–62, and the number and weight of zygospores formed on each medium.

Medium[a]	Glu (mM)	NH_4Cl (mM)	NH_4NO_3 (mM)	$(NH_4)_2SO_4$ (mM)	$CaCl_2$ (mM)	$Fe(NO_3)_3$ (μM)	$ZnSO_4$ (μM)	$MnSO_4$ (μM)	$NaMoO_4$ (μM)	Number of Zygospores[b]	Weight of Zygospores[c]
43	5	5	0	0	0.25	3.7	3.5	2	0.2	183	A
54	5	5	0	0	**0.5**	3.7	**7**	2	**0.4**	218	A
55	**10**	5	0	0	**0.5**	3.7	**7**	2	**0.4**	180	A′
56	**10**	**10**	0	0	**0.5**	3.7	**7**	2	**0.4**	136	A′
57	5	5	0	0	**0.5**	**7.4**	**7**	**4**	**0.4**	200	A
58	5	5	0	0	**1**	3.7	**14**	2	**0.8**	142	A⁺
59	**0**	**0**	**5**	0	0.25	3.7	3.5	2	0.2	0	nd
60	5	**0**	**5**	0	0.25	3.7	3.5	2	0.2	154	A′
61	**0**	**0**	0	**5**	0.25	3.7	3.5	2	0.2	0	nd
62	5	**0**	0	**5**	0.25	3.7	3.5	2	0.2	104	A′
SI	5.3	0	0	0	0.25	3.7	3.5	2	0.2	98	A
SI+MSX	2 mM MSX in SI medium									0	nd
PDAYC	40g Potato Dextrose Agar (Difco), 1g Yeast Extract, 1g Casitone in 1L of distilled water									140	B

a) Amount of nitrogen sources and/or inorganic elements was changed in each medium as compared with medium 43 as shown by bold, underlined figures.

b) The number is expressed per unit area (10 mm^2).

c) The weight (mg per a zygospore) is expressed by the following indexes: $0.05<A^+$, $0.04<A<0.05$, $0.025<A'<0.04$, $0.015<B<0.025$. nd: not determined.

Glu. Replacement of Glu with an inorganic nitrogen source, NH_4Cl (Am), greatly decreased the number of zygospores at a concentration of 2.5 mM, the same concentration at which Glu resulted in threefold stimulation, and completely suppressed zygospore formation at 5 and 10 mM. The same was true for the addition of NH_4NO_3 (medium 59) or $(NH_4)_2SO_4$ (medium 61) instead of Glu (Table 2). Certain combinations of Glu and Am had a synergistic effect on zygospore production (Fig. 3). The effect was typically observed at the combination of 5 mM Glu and 5 mM Am, and of 10 mM Glu and 5 mM Am. An amount of Am greater than the optimum in every combination caused a reduction of production, which was sharp at 2.5 and 5 mM Glu. The greater the increase in the amount of Glu, the less Am addition was inhibitive, from the view of the Glu/Am ratio. Another ammonium compound, NH_4NO_3 (medium 60), also increased zygospore production in co-application with Glu, but its effectiveness was less than that of NH_4Cl (Table 2). MSX, known as a glutamine synthetase inhibitor, strongly inhibited the progress of mating without significantly inhibiting growth (Table 2).

Development of a new medium for high production of zygospores On the basis of the above results, we developed a new medium (mSI+Am) for high production of zygospores (Fig. 4). Its chemical composition is shown as medium 54 (Table 2). It includes the combination of 5 mM Glu and 5 mM Am as nitrogen sources and doubles the amount of $CaCl_2$, $ZnSO_4$ and $NaMoO_4$ as inorganic nutrients as compared with SI (Table 2). In fact, the number of zygospores formed on the new medium was far greater than those on other media, 2.2- and 1.6-fold more than SI and PDAYC, respectively. Note that zygospore weight on PDAYC was half that on SI and mSI+Am (Table 2).

Mating of β-carotene mutants on the new medium When two β-carotene-deficient mutants, C2 (−) and C3 (+), whose combination produced a small number of zygospores on PDAYC or SI, were mated on mSI+Am medium, zygospore production was greatly accelerated

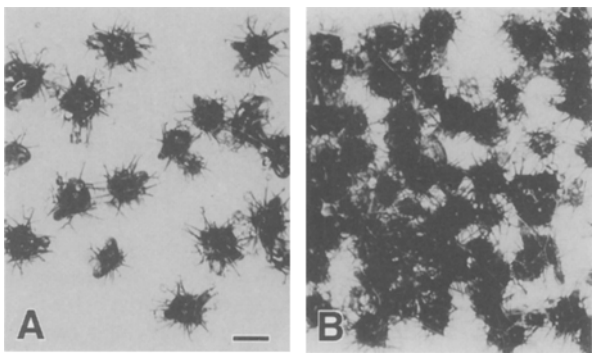

Fig. 4. Zygospores formed on SI (A) and mSI+Am (B) media. IGE1101 (−) and IGE1103 (+) were mated for 20 d at 16°C. Medium 54 was designated mSI+Am. Bar=1 mm.

Table 3. The number of sexual organs at each developmental stage in the mating between C2 (−) and C3 (+) strains, which are β-carotene-deficient mutants.

Medium	Developmental stages							
	S2	S3	S4	S5	S6	S7	S8	Total
PDAYC	310[b]	76	6	0	0	0	0	392
SI	572	78	8	0	2	0	8	668
mSI+Am[a]	990	194	46	4	12	8	42	1296

a) Medium 54 was designated mSI+Am.
b) The number of sexual organs is expressed per unit area (10 mm^2).

(Table 3). mSI+Am was recognized to stimulate sexual development itself and its progression, suggesting that the medium may be useful for genetic analysis of weak mating reactions such as in this combination.

Effect of chemical composition on zygospore weight Zygospore weight was 0.045 mg (index A) on SI and 0.022 mg (index B) on PDAYC. Even with a change in inorganic chemicals (medium 05–21), the index was A or A′ ($0.025<A'<0.04$) (Fig. 1). As shown in Figs. 2 and 3 (medium 22–53), although Glu alone and in combination with Am maintained an index of A′ to A^+ ($0.05<A^+$), except in the case of 100 mM Glu alone, nitrogen sources other than Glu were less effective in developing heavy (i.e., large) zygospores. In fact, 20 mM Asp alone had an effect equivalent to 20 mM Glu alone in production of zygospores, and showed an index of B′ ($B'<0.015$) (Fig. 2). With medium 54, mSI+Am, zygospore development was characterized by not only high production but also index A (Table 2).

Discussion

The effects on zygospore production of glucose (C-source), thiamin, elemental nutrients, and N-compounds were investigated with 62 test media. By modifying SI described by Sutter (1975), we developed a synthetic medium, mSI+Am, which can produce twice as many zygospores as was previously possible. Essential to this medium are the simultaneous addition of Glu and Am. Glu was the most effective as a single nitrogen source among the seven N-compounds tested, and its effectiveness was enhanced synergistically by Am, although this N-source was inhibitive when used alone. For vegetative growth, Asn is known to be the best nitrogen source in *Phycomyces* (Burkholder and McVeigh, 1940; Ødegård, 1952; Hilgenberg et al., 1987), leading Sutter to propose SIV. Asn given as a single N-source liberates Am by the action of asparaginase, which is more active in cultures grown in the dark than in the light (Hilgenberg et al., 1987). As the progress of mating continues well in the dark (Hocking, 1967; Yamazaki et al., 1996), in the case of SIV, the accumulation of Am may be sufficient to inhibit mating. This implies that the nitrogen metabolic pathway is essentially involved in the mating responses of this fungus, especially in zygospore development.

Nitrogen assimilation involves Am as a key intermediate in fungi: (1) Am is assimilated into Glu by biosynthetic glutamate dehydrogenase; (2) Am and Glu are joined to yield Gln by glutamine synthetase; and (3) Glu and Gln formed are used to yield other amino acids by transamination (Griffin, 1994; Moore-Landecker, 1996). Therefore, although Glu alone is still effective to synthesize amino acids in anabolic pathways in the dark (a good condition for mating), the simultaneous addition of Am would strongly promote the pathway, leading to the development of large zygospores with high density. The fact that no zygospores were formed on SI containing MSX, an inhibitor of glutamine synthetase, whose effect on vegetative growth is only a slight reduction of mycelial growth and sporangiophore formation, supports the hypothesis that mating processes may be more sensitive than vegetative processes to free Am over a certain level.

In an early study on factors affecting in gametic reproduction of *Phycomyces*, the benefical effect of Glu was concluded to be primarily due to its buffering action (Robbins and Schmitt, 1945). The pH of SI, SIV, and mSI+Am was initially around 5.5, and this may shift to a lower pH during culture (see Burkholder and McVeigh, 1940; Robbins and Schmitt, 1945; Ødegård, 1952). It is well known that mycelial elongation is strictly reduced at a pH around 3 in *Phycomyces*, resulting in compact colony formation (Cerdá-Olmedo, 1987). The vegetative growth was, however, almost the same in the three media in this study (data not shown), confirming that Glu, not Asn in SIV, plays an important role in the mating response of this fungus through nitrogen metabolism together with Am.

A similar bridge involving Asp may be expected. It was shown in an early study that Asp as the sole nitrogen was greatly effective in production of zygospores in *Phycomyces* (Leonian and Lilly, 1940; note that Glu was not used in the study.) The simultaneous addition of Am, however, did not promote production; on the contrary, no zygospores were formed, while growth was greatly stimulated. They mention that this is possibly ascribable to a balance of Asp/Am ratio. In the present work, the Glu/Am ratio was found to be very important for such promotion. It has been pointed out that the C/N ratio is also important to zygospore production in *Phycomyces* (Leonian and Lilly, 1940; Benjamin and Hesseltine, 1959). However, the addition of two times more glucose did not influence the development in our case.

As for inorganic elements, the addtion of four times greater amounts of these elements as those in SI was stimulative in the case of $CaCl_2$, $ZnSO_4$, $NaMoO_4$; the effect of $CaCl_2$ was particularly remarkable owing to the non-use of agar. Both Zn and Mo are essential microelements and play diverse roles in the cell, but they are mainly associated with enzymes (Moore-Landecker, 1996). Zn normally activates enzymes of glutamate dehydrogenase and the TCA cycle, accelerating carbon and nitrogen metabolisms. Mo acts as an electron carrier in the enzymatic reduction of nitrates. This involvement is, however, unlike that of *Phycomyces*, which is deficient in nitrate utilization (Garcés et al., 1985; Fukui et al., 2000). It is well known that Ca is involved in many essential cell functions in eukaryotic cells: maintenance of the structural integrity of the cell membranes, enzyme activity, and the operation of microfilaments and microtubules (Moore-Landecker, 1996). However, in an early study, it was reported that microelements Fe, Zn, Mn, Mo, and Ga had no specific effect on zygospore formation (Leonian and Lilly, 1940)

It should be kept in mind that responsiveness may be influenced by genetic and physiological factors: Different wild strains used have genetic backgrounds that affect their responses; starvation for a particular nutrient during culture dependent on the medium selected often has complex effects (Griffin, 1994). A simple combinational sum of individual nutrients at concentrations promoting zygospore production is not always best, as shown in media 54 (mSI+Am) and 58.

The new medium was recognized to stimulate zygospore production in the mating of C2 and C3 mutants characterized by a low level of mating. The genus *Phycomyces* includes at least two species, *P. blakesleeanus* and *P. nitens* Kunze (Benjamin and Hesseltine, 1959). In some Mucoralean fungi including *Phycomyces*, progametangia or gametangia are producible by interspecific or intergeneric mating, but in general they never produce complete zygospores (Satina and Blakeslee, 1930). Factors such as a cell-to-cell recognition system may be involved in the later stages of mating (Ootaki et al., 1996). However, the statement that a few black zygospores were seen when a *P. blakesleeanus* strain was mated with *P. nitens* strains suggests the possibility of mating between them until the final stage (Benjamin and Hesseltine, 1959). This implies that the medium may be very useful for genetic anlysis and for elucidating mechanisms of the mating reaction involved in mutant strains whose mating is often or sometimes poor, depending on the kind of mutants, in strains of the genus *Phycomyces*, and in Mucorales as well.

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