

The Effect of Cytoplasmic Factors on the Variability of *Aspergillus* strains

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Received February 14, 1962

Vliv cytoplasmatických faktorů na variabilitu kmenů rodu *Aspergillus*

Kmeny *Aspergillus amstelodami* No. 5, *A. chevalieri* A 24 a *A. ruber* No. 71 byly pasážovány po 21 generací pomocí monokonidiové izolace na sladidinové půdě a na Czapekově půdě s 20% sacharosou. Bylo pozorováno postupné zvyšování tvorby konidií, různě na obou půdách. Nedošlo ke snížení tvorby perithecií nebo jejich vymizení. U kmenů *A. amstelodami* No. 5 na sladidinové půdě a *A. ruber* No. 71 na Czapekově půdě se sacharosou vznikly celkem tři morfologické změny, charakterisované především zpomaleným růstem a zpožděnou diferenciací v prvních dnech inkubace a reversibilitou k původnímu fenotypu u některých kolonií v dalších generacích. U kmene *A. chevalieri* A 24 se na sladidinové půdě objevily dvě rozdílné fenotypové změny, jež jsou charakteristické a stálé. Vyznačují se, mimo jiné morfologické znaky, též zpomaleným růstem a zpožděnou diferenciací v prvních dnech inkubace.

Tyto výsledky jsou srovnávány s pokusy JINKSE (1954, 1956) a diskutován původ pozorovaných změn a vliv půd. U reversibilních změn lze usuzovat, že jsou mimojaderného původu, neboť je možno s velkou pravděpodobností vyloučit změny podmíněné mutacemi genomu a segregací jader následkem heterokaryose. U změn, u nichž k reversi nedochází, není jejich původ jasný. Zvýšená sporulace je připisována změnám v cytoplasmě.

Summary

Strains *Aspergillus amstelodami* No. 5, *A. chevalieri* A 24 and *A. ruber* No. 71 were maintained for 21 generations on wort and on Czapek's medium with 20% sucrose by means of monoconidial isolation. A gradual increase in conidium formation was observed which was different on the two media.

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No decrease in the formation of perithecia or their disappearance could be observed. In strains *A. amstelodami* No. 5 on wort and *A. ruber* No. 71 on Czapek's medium with sucrose, three morphological changes could be detected, characterized first of all by retarded growth and delayed differentiation during the first days of incubation and by reversibility to the original phenotype in some colonies during subsequent generations. *A. chevalieri* A 21 displayed two different phenotypic changes on wort medium, the changes appearing to be characteristic and permanent. They are accompanied, in addition to other morphological features, by retarded growth and delayed differentiation during the first days of incubation.

The present results are compared with those of JINKS (1954, 1956) and the origin of the changes observed and the effect of media is discussed. As concerns the reversible changes it can be assumed that they are of extranuclear origin since it appears to be justified to rule out changes caused by the mutation of the genome and by segregation of nuclei due to heterokaryosis. With changes where no reversion can be observed the origin is not clear. Increased sporulation is ascribed to changes in the cytoplasm.

Introduction

The problem of cytoplasmic heredity has recently become the centre of attention of investigators. It is especially to microbial genetics and some new inherent properties of microorganisms that our thanks are due for new information about the mutual relationship between the nucleus and the cytoplasm and their role in heredity. The problems and the present state of knowledge about cytoplasmic heredity have been discussed by EPHRUSSI (1953), RHOADES (1955), NANNEY (1957) and others.

The variability of colonies of monosporic origin in *Aspergillus fischeri* was taken up by GREENE (1933). His results, however, do not permit any conclusions to be drawn as to the variability of colonies formed by monosporic transfers.

JINKS (1954, 1956, 1957, 1958) investigated the changes occurring in colonies of strains of the genera *Aspergillus* and *Penicillium* during monosporic transfers through sexual and asexual spores and during propagation with the aid of hyphal fragments. His work has produced the following results: In *Aspergillus nidulans* (JINKS 1954, 1956) there is a pronounced decrease in the formation of conidia in the sexual line but the decrease does not last. In the asexual line there is a decrease in perithecia which disappear completely in the 14th generation. As long as some perithecia persist the situation is not irreversible and through transfers via sexual spores the original state can be restituted. With *Penicillium cyclopium* which forms no sexual spores, asexual spores and hyphal fragments were used during transfers (JINKS 1954, 1956). In the asexual line the formation of conidia increased. In the line propagated only by vegetative hyphae the formation of conidia decreased and after 10 generations spores disappeared completely. JINKS arrives at the conclusion that "the normal nucleus is capable of determining and responding to the full range of differentiating cytoplasm so that a full range of organs is produced.

The failure of differentiation must reside in the cytoplasm". The very probable theory can be deduced from this, viz. that the variability of colonies of the asexual line is due to the irregular distribution of cytoplasmic particles while the uniformity of the sexual line colonies is caused by a reorganization of cytoplasm during the formation of ascospores.

MATHER and JINKS (1958) are of the opinion that in *Aspergillus* the cytoplasm during sexual reproduction is stabilized and standardized so that each new individual is equipped with balanced cytoplasm. Without it the smooth development and differentiation of the new individual could never take place. "Sexual reproduction is the time of nuclear variation, springing from recombination of the genes. Somatic development, on the other hand, is relatively a period of nuclear stability, for although nuclei may show changes during development, these are secondary and not the prime cause of differentiation. With the cytoplasm the situation is reversed. Somatic development and differentiation depends characteristically on cytoplasmic change, while sexual reproduction is the time of cytoplasmic stabilization and standardization" (MATHER, JINKS 1958).

It was the aim of the present work to verify whether the results obtained by Jinks during propagation through asexual spores are also valid for other species of *Aspergillus* and whether the result can be affected by the cultivation medium.

Material and Methods

Three strains of the *Aspergillus glaucus* group were used: *A. amstelodami* No. 5, *A. chevalieri* A 24 and *A. ruber* No. 71 which were obtained from the collection of the Microbiological Institute of the Czechoslovak Academy of Sciences in Prague. The strains conform to the description given by THOM and RAPER (1945). Czapek's agar medium with 20% sucrose (THOM and RAPER 1945) and a 5% wort medium with 2.5% agar were used. Czapek's medium with 20% sucrose was also used for the isolation of conidia and for the evaluation of the number of conidia in all series. The cultures were grown in a thermostat at 30° C.

Conidia for isolation were taken from each colony after a six-day incubation when the ascospores were not yet developed. For isolating, the monosporic isolator Meopta (NEČASEK, PALEČKOVÁ and TESAŘ 1954) was used. Germinated spores were isolated.

The isolation of ascospores was not carried out as no criterion was known according to which the differentiation between ascospores and conidia could be performed.

For examining the changes in the number of conidia Czapek's agar medium with sucrose was inoculated in a Petri dish with a suspension of spores using a single test-tube every time, containing a 21—28-day-old culture. The dishes were then incubated for 72 hrs. at 30° C. After this period a cork-borer was used to punch out 12 mm. disks from the heavily overgrown agar, all spores were washed down with lactophenol and the spore suspension diluted as required. Counting was carried out in a Büchner chamber. The values obtained were expressed as the number of spores per mm³ if 1 ml. lactophenol was used for washing.

Results

The morphology of colonies and the number of conidia produced per unit area over 21 generations were examined using *A. amstelodami* No. 5, *A. chevalieri* A 24 and *A. ruber* No. 71 on wort medium and on Czapek's agar with

20% sucrose, i.e. in a total of six series. All the strains formed a large amount of conidia and perithecia. From each series a total of 10 germinating spores were isolated from the original culture inoculated by injection and from each colony thus formed further generations were independently isolated, using one colony every time. The isolation was carried out after 6 days. Beginning with the 9th generation less lines were left for technical reasons but from each line a greater number of colonies was isolated so that the number of colonies (10) in the individual generations was maintained. When any change took place the number of colonies isolated was increased so that the change observed could be examined.

Table 1. Altered and original phenotypes in subsequent generations of an altered colony of *Aspergillus amstelodami* no. 5. The changes occurred once during the 10th (I), once during the 17th (II) generation

	Generation	Total number of colonies	Number of altered colonies	Number of colonies of unchanged phenotype
I	11	7	4	3
	12	24	20	3
	13	10	3	7
	14	15	12	3
	15	15	9	6
	16	20	1	19
	17	15	11	4
	18	20	2	18
II	18	15	4	11
	19	15	4	11
	20	19	1	18

In the series of *A. amstelodami* No. 5 and *A. chevalieri* A 24 on Czapek's medium with 20% sucrose and in *A. ruber* No 71 on wort medium no morphological changes of colonies could be observed during the experiment.

In the series of *A. amstelodami* No. 5 on wort two morphological changes appeared during the experiment, in the 10th and in the 17th generations. Both changes were of the same type and were reversible. The altered colonies were characterized by retarded growth and delayed differentiation (retarded formation of perithecia) during the first phases of colony growth but later the differences were compensated and after 16 days of incubation the altered colonies differed from the normal ones only in the formation of isolated white sterile hyphae in the colony but the colour and size of the colonies were the same as in normal colonies. After 28 days of incubation the centre of the unaltered colonies falls through to form a "crater" while in the altered ones this phenomenon does not appear and the centre displays a larger number of sterile white hyphae. A greater number of colonies was always isolated from colonies of altered character. Part of these preserved the changed phenotype while a part reverted to the original unchanged character. The same conditions existed in the colony where the change occurred during the 17th generation. The frequencies are shown in Table 1. which shows that the frequency of the two phenotypes (changed and unchanged) in the individual generations are very varied and no regularity can be detected.

A change of a very similar character was observed in one case during the 5th generation in the *A. ruber* No. 71 series in Czapek's medium with 20% sucrose. The altered colonies differed from normal ones in retarded growth and delayed differentiation during the initial phase of growth of the colony, later on, however, the colonies did not differ from normal ones. This change, too, was reversible and occurred with the following frequency: during the 6th generation the character persisted in all 3 isolated colonies, during the 7th generation 4 out of 10 colonies reverted to the original unchanged character of the colony. During the 8th generation none of the 10 colonies displayed the altered character, just as during the 9th and 10th generations.

In the series of *A. chevalieri* A 24 on wort agar two types of change were observed. Both these types differed very strikingly in their morphology from the original type of the colony and no reversion was observed here. The type A change appeared once during the 12th and once during the 16th generation. Colonies of this type differed from the original unchanged colonies in their irregular shape and in the fact that in their centre there was a white bulge formed by sterile mycelium with a dark middle in which rich conidiophores were formed. The type B change occurred during the 16th generation from the type A colony. Colonies of this type are somewhat smaller than the original unchanged ones, are regular in shape and have a definite greenish colour. Both these types of changes are permanent, no reversion having been observed. The changes are characterized by retarded growth and a delay in differentiation in young colonies just as with colonies of changed character in strains *A. amstelodami* No. 5 and *A. ruber* No. 71. Colonies of type A and B transferred by inoculation to Czapek's medium with 20% sucrose differ from normal colonies in retarded growth but no delay in the differentiation of perithecia takes place. No other morphological differences were observed. On inoculation back to the wort medium they again yielded colonies of type A and B.

The formation of perithecia was investigated only macroscopically without numerical evaluation. No great decrease in their number was observed. This is of importance in view of the fact that JINKS (1954, 1956) found that their formation gradually decreased to disappear completely during the 14th generation.

The method of evaluation of conidia formation is rough and inaccurate and allows the assessing of only the general trend in intensity of conidia formation. It shows, however, that on grouping the results from 4 generations there appears an increasing tendency in all the series (Fig. 1, 2, 3). In all three strains incubated on wort agar the formation of conidia increases substantially between the 4th and 10th generation while in the series propagated on Czapek's medium with sucrose the formation of conidia rises especially between the 12th and 16th generation, the absolute values being less. The increased formation of conidia persisted over 5 subsequent generations propagated by loop inoculation.

Discussion

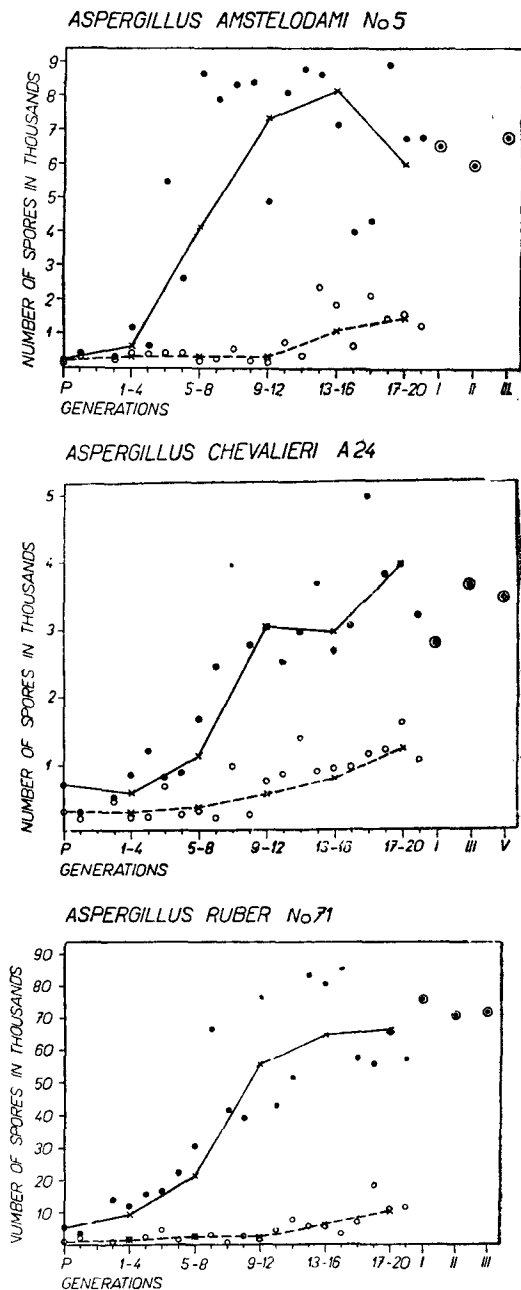
The results obtained by JINKS (1954, 1956) for *Aspergillus nidulans* do not appear to be valid for all strains of this genus. It should be noted here that

JINKS does not mention the cultivation medium with which he worked so that the results are not strictly comparable. It cannot be assumed, however, that the results would be so much affected by the cultivation medium. It appears that, in contrast to *Aspergillus nidulans*, in the three strains used here (of the *Aspergillus glaucus* group), when monosporic propagation through conidia over 21 generations is used no visible decrease in the number of perithecia or their complete disappearance takes place. In JINKS' paper (1954) no observation of individual morphological changes, either reversible or irreversible, are mentioned that would differ from the other colonies; similarly, no case of growth retardation and of differentiation delay in young colonies is mentioned. JINKS does not state the amount of material used in his work so that it is hard to assess the probability with which the contingent tendency toward similar changes would be displayed in his set.

Fig. 1, 2, 3. Increase in the formation of conidia during monosporic propagation over 21 generations in strains of *Aspergillus amstelodami* No. 5, *Aspergillus chevalieri* A 24 and *Aspergillus ruber* No. 71.

● values for individual generations on wort medium; ○ values for individual generations on Czapek's medium with 20% sucrose; ◊ values for loop inoculation after terminated monosporic propagation on wort medium during the 1st, 3rd and 5th generations; — curve of increase of conidia formation on wort medium, the class interval being represented by an average value from 4 generations; - - - - - curve of increase of conidia formation on Czapek's medium with 20% sucrose, the class interval being represented by the average value from 4 generations.

The figures indicate the number of spores per mm², if 1 ml. lactophenol was used for washing off the spores from unit area.



The present results are also at variance with those of GREENE (1933) working with *Aspergillus fischeri*.

The question is whether the individual changes observed in the three strains are of cytoplasmic or nuclear origin and whether these changes are caused by the monosporic propagation or by random mutations.

Especially in those cases where the reversion to the original phenotype appeared it cannot be assumed that the change is due to mutation. Back mutations occur at a frequency of 10^{-5} — 10^{-10} . In the strain *A. amstelodami*, however, 104 out of 175 colonies reverted to the original character, in *A. ruber* 14 cases out of 23. In no case did the colonies of the altered phenotype exhibit morphologically different sectors which must be assumed to exist during back mutations of traits localized in the nucleus.

It is known about the *Fungi imperfecti* that sporulation is affected by heterokaryosis and by segregation of nuclei during heterokaryosis (HANSEN 1938). It is impossible with *Aspergillus*, however, that during monoconidial propagation heterokaryosis would occur since the nuclei of all conidia in one conidial chain are formed by the division of a single original nucleus in the sterigma (THOM, RAPER 1945). If thus any of the conidia should be polynucleate the nuclei must be identical genetically. Since no heterokaryosis is possible here the changes cannot be due to recombination processes, such as mitotic recombination.

In those cases when reversions toward the original phenotype were observed the only remaining explanation lies in the conditioning of the corresponding changes by the cytoplasm. In the way of a model it can be envisaged that a certain component of the cytoplasm becomes diluted during repeated conidial isolation and that the decrease in its concentration brought about the observed change. The reversion was then caused by restitution of this cytoplasmic component to its normal state.

Changes in *A. chevalieri* A 24 which were not generally reversible can be considered as selection of nuclear mutants. Since, however, these changes have the property in common with the reversible changes in that their young colonies exhibit a retardation of growth and delayed differentiation and since, on transfer to Czapek's medium with 20% sucrose, the type A and B colonies differ only in the intensity of growth rather than in any morphological feature, it cannot be ruled out that the changes might be caused by a cytoplasmic factor.

In spite of objections that can be levelled against the technique of counting the conidia the results obtained clearly show that we are dealing here with a gradual increase in the formation of conidia. The most likely and simple explanation of this lies in cytoplasmic changes caused by monosporic propagation.

The effect of the medium was interestingly reflected in *A. chevalieri* A 24. Colonies of type A and B formed on the wort medium and displaying, in addition to morphological changes, a retarded growth and delayed differentiation, were monosporically propagated to Czapek's medium with 20% sucrose. Here a retardation of growth takes place in young colonies but differentiation is not delayed. No other morphological changes were observed. These colonies, re-inoculated to the wort medium again gave rise to colonies of type A

and B. It is somewhat surprising that the retarded differentiation takes place on the wort medium which is rich in nutrients and growth factors and that no delay of differentiation can be observed in Czapek's medium with 20% sucrose, which, with the exception of the high sugar content, is poor in nutrients and growth factors. THOM and RAPER (1945) state that in species of the *Aspergillus glaucus* group typical perithecia and conidiophores are formed only on media with a high osmotic pressure. Since the osmotic pressure of the substrate apparently plays an important role in the differentiation of perithecia and conidiophores it can be concluded that the differences between the two media might be due to the big difference between their osmotic pressures.

It can thus be generally said that differentiation is delayed on a wort medium more easily than on Czapek's sucrose medium. The same holds for the rate of increase in the number of conidia. On Czapek's medium there is either something preventing the ready cytoplasmic changes (this could explain the greater stability during conidia formation in series propagated on Czapek's medium with 20% sucrose) or else the altered type is seen to a much smaller degree (is masked). In this sense it would also be possible to interpret the greater stability during conidia formation in series propagated on Czapek's medium with 20% sucrose and perhaps even the complete lack of manifestation of morphological changes of type A and B in *A. chevalieri* A 24 when incubated on Czapek's medium. We do not know, however, whether the changes of type A and B are of cytoplasmic or of nuclear origin. This case shows, however, that such a masking effect could be due to the greater osmotic pressure of the substrate which apparently contributes to a ready differentiation of perithecia.

The frequency of phenotype changes in the experiment, five cases on wort and a single one on Czapek's medium with 20% sucrose, suggests that on the wort medium the strains are less stable and more liable to morphological changes than on Czapek's medium with 20% sucrose.

Acknowledgement: The author wishes to thank Dr. J. Nečásek for many helpful discussions and suggestions.

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Влияние цитоплазматических факторов на изменчивость штаммов рода *Aspergillus*

BIOLOGIA PLANTARUM (PRAHA) 5 (2) : 100—108, 1963

Штаммы *Aspergillus amstelodami* № 5, *A. chevalieri* A 24 и *A. ruber* № 7 пассажиrowали в течение 21 поколения при помощи моноконидиевой изоляции на среде с суслом и на среде Чапека с 20% сахарозой. Наблюдалось постепенное повышение образования конидий, по-разному на обеих средах. Снижения образования перитециев или их исчезновения не наблюдалось. У штаммов *A. amstelodami* № 5 на среде с суслом и *A. ruber* № 7 на среде Чапека с сахарозой возникли в общем три морфологических изменения, характеризующихся прежде всего замедленным ростом и задержкой дифференциации в первые дни инкубации и обратимостью к исходному фенотипу у некоторых колоний в последующих поколениях. У штамма *A. chevalieri* A 24 на среде с суслом появились два разных фенотипических изменения, которые характеристичны и постоянны. Они обладают, кроме других морфологических признаков, также замедленным ростом и задержкой дифференциации в первые дни инкубации.

Данные результаты сопоставлены с опытами Джинкса (1954, 1956) и дискутировано происхождение наблюдаемых изменений и влияние среды. В случае обратимых изменений возможно судить о их внеядерном происхождении, так как с большой вероятностью возможно исключить изменения обусловленные мутациями генома и сегрегацией ядер вследствие гетерокариоза. В случае изменений, у которых обратимости не наблюдается, их происхождение не ясно. Повышенная споруляция объясняется изменениями в цитоплазме.