Further studies on the physiology of the sporulation of the myxomycete *Physarum nudum*

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In a previous paper (Rakoczy 1962) it has been stated that in constant conditions the development of the myxomycete *Physarum nudum* is characterized by a remarkable regularity of sporulation. In light this process takes place after 9–10 days following reinoculation; in darkness plasmodia show potential readiness to sporulation after 8 days; in these conditions, however, this process can be induced only by a suitable light impulse. The results of previous studies suggested that reinoculation leads to a kind of rejuvenescence of the plasmodium followed by a renewal of vegetative growth. The phase of readiness to initiate morphogenetic processes by the plasmodium is related to the age of the culture (this age being counted from the moment of transfer of the plasmodium onto fresh medium). During previous experiments care was taken to maintain constant such factors as: temperature, light intensity, the kind of medium and its composition and the pre-treatment of the plasmodium.

It may be expected that a study on the action of the mentioned factors on sporulation will give a detailed insight into the mechanism of this process and presumably will also give an answer to the question whether the phase of sporulation readiness is correlated with the age of the culture or with the parameters the change of which is closely connected with the time factor (for instance exhaustion of nutrient substances, attainment of a certain size by the plasmodium and so on).

MATERIAL AND METHODS

The myxomycete *Physarum nudum* Macbr. provided the experimental material; it was cultured on oats flakes medium in darkness, in previously described conditions (Rakoczy 1962). The fragment of the plasmodium used as inoculum for a new culture was rapidly weighted on a torsion balance. The inocula were allowed to grow on oatmeal agar (Howard 1931) or on nonnutrient agar (agar washed with distilled water) poured on Petri dishes of various diameters, mostly 7.5 cm. The cultures were grown in light or in darkness in a thermostat at 21°C. Fluorescent tubes emitting white so called "day" light constituted the light source. The light intensity on the level of the plates with the plasmodia was 4800 ergs/cm² sec in the range 400–700 nm (= about 1600 lx).

Besides to qualitative observations the results were registered 1) by measuring the mean time of sporulation — this means the time expressed in hours and elap-

sing from the moment of the reinoculation of the plasmodium onto fresh medium to the moment when black sporangia were formed, 2) by estimating the percent of sporulating plasmodia; the estimation was based on 6–10 replications, each replication including 10 Petri dishes, 3) by determining the fresh and dry weights of the plasmodia. The plasmodia were very carefully separated from the surface of the nutrient agar, placed on cover glasses in a humid atmosphere and weighted on a torsion balance with a precision attaining 0.2 mg. The dry weights of the plasmodia were determined in a similar manner after the cover glasses with the plasmodia have been dried at 105°C for 24 hours. The fresh and the dry weights are the averages of 6 replications.

RESULTS

I. Plasmodia of the myxomycete *Physarum nudum* reinoculated on fresh media and allowed to grow in continuous light sporulated after 9–10 days. During this period the plasmodia showed vigorous growth this, however, led to an exhaustion of the nutrients and to a deterioration of the growth conditions.

Preliminary series of the experiments were made in order to establish which of the following 3 factors: chronological age, exhaustion of medium or suitable size of the plasmodium is the factor inducing the sporulation process. In these experiments plasmodia were reinoculated every 7 days on oatmeal agar plates (7.5 cm of diameter and 15 ml of the medium) and were kept in continuous light. The fresh weights of the inocula were 10, 100 and 500 mg. It follows from Fig. 1 (upper columns) that the cultures which developed from small inocula (10 mg) did not sporulate at all in spite of their exposition to continuous light; they died

Fig. 1. Results of the culture of *Physarum nudum* plasmodia in continuous light conditions. Reinoculation every 7 and 5 days. Fresh weights of the inocula used for reinoculations were: 10, 100 and 500 mg. White columns show the % of the nonfruiting, healthy cultures, black columns — % of the dead plasmodia and shaded columns — % of the sporulated cultures. Numbers by columns indicate the time (in hours) for sporulated or dead plasmodia. Numbers under columns indicate the sequence numbers of the reinoculation.

after few reinoculations. For instance, after the third and the fifth reinoculation the death rate of plasmodia amounted to 30 and 100 percent respectively. On the contrary the plasmodia which developed from larger inocula (100 or 500 mg) did not perish and after a few reinoculations they rapidly sporulated often notwithstanding the fact that the nutrients have not been exhausted yet (Fig. 1, upper columns: 100 mg inocula — after the second reinoculation the sporulation took place after 28 hours, and for 500 mg inocula after inoculation — after 170 hours).

Similar results were obtained when the time elapsing between two reinoculations was shortened to 5 days (Fig. 1 — lower columns).

The results of the preliminary experiments did not give a clear picture which factor, besides light, was responsible for sporulation. It is however possible, basing on these results, to advance a few suppositions:

1) Reinoculation is not a factor which wipes out completely the biological past of the plasmodium. The physiological state of the plasmodia used as the inocula is not without influencing the later results of the experiments. This is shown in Fig. 1 for cultures which developed from 500 mg inocula (upper and lower columns).

2) It may be supposed that the size of the plasmodium has a great influence on sporulation. The dependence of this process on the weight of the inoculum and on the moment of reinoculation is shown in Fig. 1. The longer the time elapsing between the reinoculations the greater is the final weight of the plasmodium (upper and lower columns for 100 and 500 mg).

3) Light is a factor decreasing the vegetative growth rate of the plasmodia. This is shown by the high death rate of the plasmodia allowed to grow in continuous light but in conditions inhibiting sporulation (small inocula, frequent reinoculations). On the contrary the plasmodia cultured in the same way but kept in darkness show a high vitality.

II. It follows from the results of preliminary experiments that the size of the plasmodium seems to be an important factor affecting sporulation. Therefore, in further experiments the influence of the size of the inoculum on sporulation made the object of a special study.

Inocula of fresh weight between 2 to 1000 mg cut from stock cultures grown in darkness were used in these experiments. The inocula were transferred onto nutrient agar on Petri dishes (7.5 cm diameter) provided with 15 ml of the medium and exposed to continuous light. The time of sporulation was the criterion adopted for the interpretation of the experimental results. They (Fig. 2, curve a) point to a close correlation between the time of sporulation and the weight of the inoculum. The smaller the weight the longer the time of sporulation. For instance plasmodia grown from 2 mg inocula needed 400 hours to sporulate, whereas for plasmodia which started from 500 mg inocula, the time of sporulation was reduced to 150 hours. If the plasmodia were transferred on nonnutrient agar then the sporulation occurred after a time which was much shorter than in the previous cases; as matter of fact the time of sporulation was constant and showed no relation to the weight

of the inoculum (Fig. 2b). Only when very small fragments were used as inocula (2 mg) the time of sporulation was slightly lessened and exceeded the admissible error; in this case about 40 per cent of the cultures died without sporulation.

Basing on these results it must be supposed that the time of sporulation is rather determined by the nutritive conditions than by the size of the plasmodium.

The above results are easily explained if it is assumed that there exists some kind of antagonism between the vegetative growth and the generative processes (sporulation). Lack of food by stopping growth directs the development towards

Fig. 2. Relation between the time of the sporulation (y — axis = hours) and the fresh weight of the inoculum (x — axis = mg); *a* — plasmodia grown on oats flakes agar medium, *b* — plasmodia on nonnutrient agar medium.

sporulation. This stage is attained after a short time which shows no relation to the weight of the plasmodium. Favorable conditions for vegetative growth (nutrient agar) direct the development towards an increase of the plasmodium size, and this process can continue as long as it is not limited by the exhaustion of the nutrients. The greater the inoculum the more rapid this process.

III. If it is assumed that sporulation depends on the amount disposable nutrients, then it is expected that the time of sporulation will depend on the amount of these nutrients.

The answer to this question may be obtained by culturing plasmodia on Petri dishes of various diameters (from 1.8 to 20 cm). The depth of the agar layer was 1/2 cm and care was taken to keep constant the amount of oatmeal per cm² of agar area.

The relation between the time of sporulation and the area of the medium is presented in Fig. 3 for inocula whose weights were 2 and 500 mg. Unfortunately, the 500 mg inocula placed on the smallest Petri dishes (1.8 cm diameter) died in a short time. It appears from Fig. 3 that there is a certain correlation between the amount of the nutrient compounds and the time of sporulation: the greater the area

of the dishes the longer the time of sporulation; but the correlation is rather slight and this would indicate that the amount of nutrients is not an important factor affecting the sporulation.

IV. The above formulated suggestions concerning the relation between sporulation and the size of the inoculum or the amount of nutrients were confirmed by the results of the third series of experiments. They seem rather to indicate that the ability to sporulate is related to the attainment of a critical size by the growing plasmodium (at least in nutritive conditions).

This critical size is attained the sooner the larger is the inoculum.

If this supposition is correct then the fresh or dry weights of the plasmodia just before sporulation should show close values with no relation to the size of the inoculum.

Table 1

Fresh weights of the plasmodia at the moment of sporulation in dependence on the size of the inoculum

Fresh weight of the inoculum (mg)	Fresh weight of the plasmodium 12 hours before sporulation (mg)	Increase of fresh weight of the plasmodium for 1 mg fresh weight of the inoculum (mg)
2	172	85
10	191	18.1
500	564	0.13

Table 1 presents the fresh weights attained about 12 hours before sporulation by plasmodia developed from inocula of different sizes.

V. It appears from the earlier experiments that the size of the inoculum exercises a certain influence on the time of sporulation but in nutrient conditions only (part of experiments II). This influence can not be interpreted either as a simple relation between the amount of nutrients (III) or attainment of a suitable size of the plasmodium or of the increase of the preliminary amount of the plasmodium (IV). Probably the readiness to stop the vegetative growth and to initiate sporulation process is controlled by some inner correlative system and therefore such factors as the quality of the nutrient medium or the weight of the inoculum can modify the time of sporulation at to a limited extent only, and it is impossible to state the simple relation between the time of sporulation and the mentioned factors.

The last series of experiments was made in order to obtain more information about the character of the growth curve. It was expected to discover some symptoms indicating that the plasmodium is approaching to sporulation, i.e. is acquiring the ability to sporulate. In order to obtain the data necessary for the tracing of

the growth curves (Fig. 4) plasmodia growing on nutrient agar in light conditions were collected at constant time intervals and their fresh and dry weights were determined.

It results from Fig. 4 that the curves representing growth (as dry weights of the plasmodia) present a similar shape, which, is independent of the size of the

Fig. 3. Relation between the time of sporulation (y — axis = hours) and the size of Petri dishes with medium (x — axis = cm^2). Fresh weights of the inocula: a = 2 mg; b = 500 mg.

Fig. 4. Growth curves of the plasmodia: x — axis = the time in days counted from the moment of reinoculation; y — axis = dry weight of the plasmodium in mg. The fresh weight of inocula: 2, 100, 500 mg. The dashes show the mean time of sporulation; ○, ●, □ cultures in light conditions; × — × cultures in darkness.

inocula. As it is shown in Fig. 4 the period of exponential vegetative growth is followed by a period of declining growth rate and a period characterized by a reduction of the dry weight. In all cases the moment of the sporulation coincides with the beginning of the period of the dry weight reduction. Therefore this reduction can be considered as a symptom of readiness to sporulate.

It is interesting to compare the growth curves corresponding to plasmodia start-

ing from the inocula of the same size but grown in light and in darkness. It appears once more from this comparison that light is a factor not favorable for vegetative growth.

Table 2
Dry weight of the plasmodia on nonnutritive agar

Dry weight of the plasmodia (mg)	
Immediately after collecting from nutrient agar (in darkness)	After remaining 48 hrs on nonnutrient agar in light conditions
30	23
32	24
30	22.5
28	22
28	22.5
Average: 29.8	22.8

Another observations which have been made are that growth is also inhibited in dark cultures, i.e. in non sporulating one's. In the last period the decrease of dry weight takes also place. In both cases (in light and in darkness) the period of dry weight reduction coincides with the stated readiness for sporulation, which in turn, may be initiated by a light stimulus. Plasmodia placed on nonnutritive agar, independently of the size of the inoculum enter immediately into the phase of dry weight decrease (Table 2), and this is accompanied by early sporulation on the condition that the plasmodia are at the same time exposed to light.

DISCUSSION

In the literature concerning the relation of the Myxomycetes sporulation on nutrient conditions many discrepancies are found. For instance a series of data indicates that the presence of fresh or at least nonexhausted nutrients is not a factor inhibiting sporulation. Howard (1931) stated that plasmodia of *Physarum polycephalum* often formed sporangia in spite of a supply of fresh nutrient material. According to Seifriz and Russell (1936) the exhaustion of nutrients does not always induce sporulation in *Physarum polycephalum*, whereas plasmodia often sporulated in spite of the presence of nutrients. Such a situation occurs not only in laboratory conditions but also in nature; for instance it may be assumed that a rotten wood on which a plasmodium appears contains a sufficient amount a nutrient substances, this, however, does not prevent sporulation. On the other hand many observations point to a correlation between the lack of nutrients and sporulation. E.g. Lister (1925) maintains that plasmodia of many species of the Myxomycetes, among others *Badhamia utricularis* continue their vegetative growth as long as there is a sufficient amount of nutrients in the environment. When the

nutrients are exhausted the plasmodia begin to sporulate. Camp (1937) also observed that the sporulation of *Physarum polycephalum* is correlated with nutritive conditions. The time of sporulation was lengthened proportionally to the number of feedings and after their cessation sporulation took place.

Our experimental results corroborate both these observations.

Thus, during growth of *Physarum nudum*, a relation between sporulation and the amount or the quality of nutrients were observed. In certain conditions, however, sporulation takes place in spite of the addition of fresh nutrient. It seems that it would be premature to consider the informations in the literature as discrepant and contradictory.

In many cases the influence of nutrients on sporulation was investigated without taking in account the light conditions, which are a deciding factor in inducing sporulation both in *Badhamia utricularis* (Sobels and van der Brugge 1950) and *Physarum polycephalum* (Gray 1953; Daniel and Rusch 1961, 1962).

Besides, sporulation presumably is a complicated developmental process and accordingly is not probable that only one external factor e.g. the amount or the quality of nutrients or the age would exclusively determine the change from the vegetative to the generative (sporulation) phase. The results of the present paper indicate that neither the chronological age nor the amount of the nutrients or the size attained by the plasmodium are solely responsible for inducing sporulation. On the other hand it has been shown that each of these factors influences this process indirectly and can to certain extent lengthen or shorten the period of vegetative development. It seems that the induction of sporulation in the examined species may be explained by a competition between the antagonistic processes of vegetative and generative development.

Lack of nutrition directs the developmental processes immediately to sporulation. In the presence of nutrients causes growth of the plasmodium whose the length of the vegetative phase depends on the amount of nutrients, or the final weight of the plasmodium and most probably on many other factors. But these factors can influence the length of the vegetative phase to a certain extent only. After some time the growth is inhibited by some inner mechanism shifting the balance from vegetative growth to sporulation. In the phase of growth cessation the plasmodium is ready to sporulation and this process can be induced by suitable light conditions.

The growth curves obtained for *Physarum nudum* developing on oats flakes agar are similar to the respective curves found by Daniel and Rusch (1961) for *Physarum polycephalum* cultivated in microbiologically pure liquid media. They give on that the optimum phase of growth which is immediately followed by complete check of this process is one of the factors inducing sporulation. The agreement of the mentioned observations with our results permits to assume that the regulation of growth and sporulation proceeds on a similar way in both examined species.

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SUMMARY

A study was performed on the dependence of the sporulation process in the myxomycete *Physarum nudum*, on such factors as the amount of nutrients, the size of the inocula, the frequency of reinoculations.

It has been found that each of the mentioned factors exercises a certain influence on the length of the vegetative growth period but none of them is the sole and principal factor responsible for the induction of sporulation.

Presumably the principal factor inducing sporulation is an internal mechanism shifting the balance between the antagonistic processes of vegetative and generative development. The cessation of growth is a symptom indicating that the plasmodium has attained the phase of sporulation readiness. The factor which made the object of our study are only to modify (to lengthen or to shorten) the period of the vegetative phase. Suitable light conditions are an indispensable factor for the induction of sporulation.

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Dalsze badania nad fizjologią zarodnikowania śluzowca Physarum nudum

Streszczenie

Zbadano wpływ szeregu czynników takich jak: obecność i ilość substancji pokarmowych, wielkość inoculum, częstość przeszczepiania na wystąpienie procesu zarodnikowania w kulturach śluzowca *Physarum nudum*.

Stwierdzono, że każdy z wymienionych czynników wywiera pewien wpływ na długość okresu wzrostu wegetatywnego, ale żaden z nich nie jest wyłącznym czynnikiem regulującym w sposób prosty proces sporulacji.

Stwierdzone zależności należy tłumaczyć regulowaniem procesu zarodnikowania przez wewnętrzny mechanizm korelacyjny przesuwający w pewnej fazie rozwojowej równowagę między dwoma antagonistycznymi procesami jakimi są wzrost wegetatywny i rozwój generatywny (sporulacja). Wyrazem wystąpienia fazy gotowości do zarodnikowania jest zahamowanie wzrostu.

Warunkiem koniecznym do rozpoczęcia procesu sporulacji w takiej fazie są odpowiednie warunki świetlne.