ISSN 1021-4437, Russian Journal of Plant Physiology, 2015, Vol. 62, No. 2, pp. 153–160. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © N.A. Davidovich, O.I. Davidovich, Yu.A. Podunai, K.I. Shorenko, M.S. Kulikovskii, 2015, published in Fiziologiya Rastenii, 2015, Vol. 62, No. 2, pp. 167–175.

REVIEWS

Reproductive Properties of Diatoms Significant for their Cultivation and Biotechnology

N. A. Davidovich[a], O. I. Davidovich[a], Yu. A. Podunai[a], K. I. Shorenko[a], and M. S. Kulikovskii[b]
[a] *Karadag Nature Reserve, Feodosiya, Crimea, 298188 Russia;*
e-mail: karadag-algae@yandex.ru; nickolaid@yandex.ru
[b] *Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, Borok, Yaroslavl oblast, 152742 Russia*

Received July 01, 2014

Abstract—Unicellular algae and diatoms (Bacillariophyta) in particular, have attracted increasing attention as the objects of biotechnology. Diatoms are known to produce mucopolysaccharides, fats, and fat-like substances suitable for production of biodiesel, unusual pigments (e. g., marennine), and nanosized siliceous structures. It should be noted that only few diatom species out of great number living on the Earth are used in biotechnology. About 100000 species of diatoms occur in nature. The use of diatoms in biotechnology is restricted by little-studied life cycle and biology of reproduction. This review summarizes data on biological properties of diatoms, which should be taken into account when they are used as the objects of culturing and particularly as clones.

Keywords: Bacillariophyta, cultivation, clone, biology of reproduction

DOI: 10.1134/S1021443715020041

INTRODUCTION

Several recent decades witnessed active search for new objects (and first of all, microorganisms) for industrial cultivation with the purpose of production of useful substances. In this relation, diatoms (Bacillariophyta) capable of producing substances of various nature (fats, fatty acids, polysaccharides, liposoluble and water-soluble pigments, nanosized siliceous structures, etc. [1–3]) become increasingly attractive. Diatoms form an evolutionary young group of algae comprising more than 100000 diverse species [4, 5]. In respect to evolution, diatoms are unique. They are believed to originate as a result of several consecutive acts of symbiogenesis of an initially heterotrophic organism with algae [6–8]. As a result, in addition to the genes of the initial heterotrophic eukaryote, the genome of diatoms incorporates genes of symbiont organisms (i.e., green [9] and red [10] algae); at the expense of the horizontal gene transfer, they acquired genes of bacteria and protozoa. Diatoms carry numerous genes borrowed at different stages (also by symbiont organisms) from various prokaryotes: cyanobacteria, proteobacteria, and Archaea [10]. Such a polytypic gene assembly accounts for unusual properties and capabilities of diatoms including uncommon pathways of biosynthesis. Diatoms have such photosynthetic pigments as chlorophylls *a* and *b*, fucoxanthin, diadinoxanthin, and diatoxanthin.

In spite of enormous species diversity, only several diatoms are currently used for mass cultivation. One of the main barriers hindering progress in introduction and maintaining new objects of cultivation is a weak knowledge of reproductive biology of diatoms including their life cycles, crossing systems, and the process of sexual reproduction. Clonal cultures (strains) seem to be most efficient and have the greatest technological and commercial potential. This implies two tasks: (1) acquiring (isolation from natural environment, selection, and genetic engineering) the most efficient genetic lines and (2) long-term maintenance of strains in culture without deteriorating their genetic purity.

Based on the long-term experience in laboratory cultivation, we aimed to summarize and systematize theoretical information on the biology of diatoms, which may be useful in solving the problem of their practical maintaining as clones (genetically uniform lines).

LIFE CYCLE

The data about life cycle of diatoms are presented quite extensively in the literature; they deal with the properties of certain species and general patterns [11–19]. The life cycle of diatoms is very specific, and first of all one should pay attention to characteristic changes in their dimensions (Fig. 1). Initial cells are the largest. They arise as a result of sexual reproduction, and each of them gives rise to a new clone of vegetatively dividing cells with a unique gene assembly. During the vegetative phase of the life cycle (Fig. 2), the cells of diatoms divide into two parts, and because they carry a protective siliceous frustule made of two

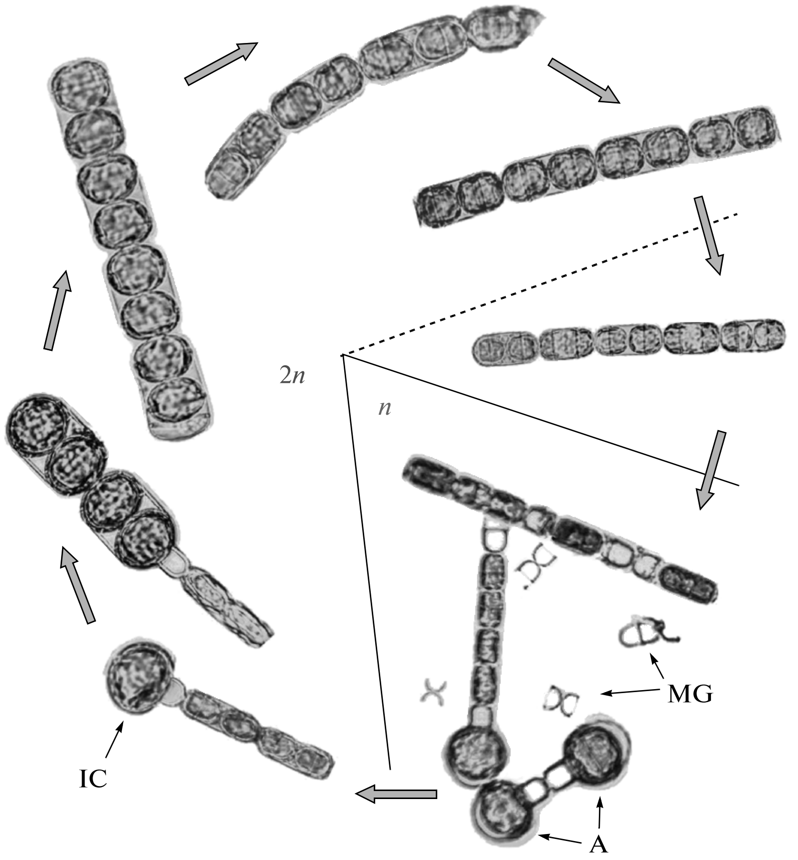

Fig. 1. Life cycle of centric diatom *Melosira moniliformis* (O.F.Müller) C.Agardh.
IC—initial cell obtained as a result of sexual reproduction giving rise to a new clone and notable for the largest species-specific size; A—auxospore (rapidly growing cell arising from zygote); MG—hollow frustules of male gametangia. Dotted line shows critical size above which the cells become sexually inducible. During the greater part of the life cycle lasting for 3–6 months in this species the cells are in the diploid state (2*n*); only gametes are haploid (*n*).

halves (thecae) one of which covers the other as the lid of box (box pattern), each daughter cell inherits one half. The second lacking part is built up within the existent theca. As a result, after division one cell has the same size as the parental cell and the other cell is a little smaller. Such a phenomenon is characteristic of the majority of diatom species with few exceptions [20]. Size reduction approximately corresponds to double thickness of the thecae in the girdle zone (where the thecae overlap), and in respect to individual division it is comparatively small and equal to fractions of micrometer. However, after numerous divisions (taking from several months to several years depending on species and growth conditions) the cells become critically small and their further existence is doubtful.

A new cycle of the initial cell formation prevents further decrease in cell dimensions; they restitute their initial size typical of the species in the beginning of the life cycle. This occurs by means of production of auxospores (Fig. 2). Auxospores or germinating spores are the cells produced from a zygote that results from gamete syngamy. Auxospores and auxosporulation are known only in diatoms. Auxospores expand reaching maximal species-specific sizes during several hours. When the growth of auxospore is completed, the frustule of future initial cell is deposited inside. It is important that auxospore is not just a cell capable of expanding. It is a product of sexual reproduction carrying a new gene assembly.

This pattern of decrease and restoration of dimensions in the course of the life cycle was described at the end of the 19 century [21, 22] and is known as MacDonald–Pfitzer rule [17, 23]. Several considerations important for cultivation follow from this pattern.

(1) It is impossible to cultivate endlessly the strains (clones) of alga obeying the MacDonald–Pfitzer rule, because the duration of strain existence is determined by the length of its life cycle. Progression of life cycle depends on the rate of cell divisions, and this feature can be used for conservation and subsequent use of a clone portion, for instance by incubating this portion at low illuminance and low temperature.

(2) The cultured clone loses its genetic homogeneity as soon as the process of sexual reproduction takes

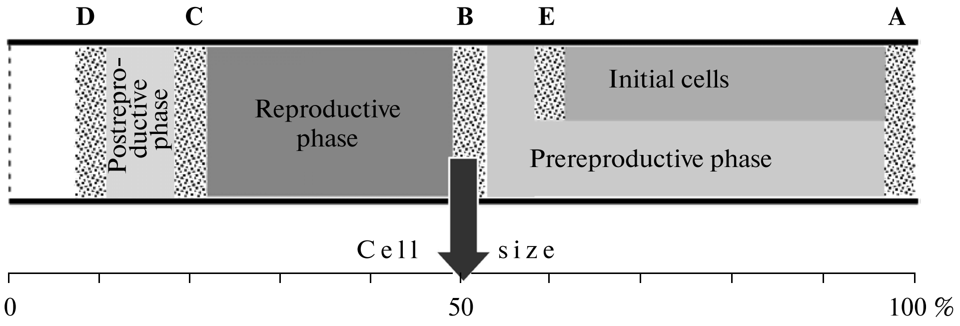

Fig. 2. Phases of ontogenesis of diatoms and the size of cells in respective phases.
A–E crucial points (phase bounds) in ontogenesis.

place; as a result, numerous new genetic lines arise. Sexual reproduction within a clone (homothallic reproduction) is possible but it is only characteristic of centric and some pennate diatoms (see below), not typical for all diatoms. Formation of initial cells may serve as a simple indicator that intraclonal reproduction occurred. Initial cells are notable for their dimensions: the apical length in pennate and the diameter in centric diatoms are several times greater than respective dimensions of parental clone.

(3) On the contrary, when sexual reproduction is desirable, for instance in an effort to select certain strains, it is important to consider the fact that life cycle in diatoms comprises two phases: prereproductive and reproductive (generative) (Fig. 2). In prereproductive phase, the cells starting from initials divide only vegetatively. One of necessary conditions for progression towards the generative phase is an achievement by cells of a certain critical size, which is a cardinal point in the life cycle in accordance with Geitler [11, 24]. For the majority of diatoms, the critical size is close to 50% of the highest species-specific size [16]. Having reached the critical upper border of the size range allowing reproduction, the cell can proceed to meiosis provided that all other necessary conditions (availability of a sexual partner in the case of dioecism as well as favorable temperature and light conditions) are satisfied. Transition to reproduction is not obligatory: some cells continue dividing mitotically, and their sizes decrease. In the ontogeny, some species reach the lower threshold size after which the cells lose the ability to enter the sexual process but can for some time divide mitotically. In this relation, the open and closed size ranges allowing sexual reproduction [14] are distinguished.

In practice, the critical upper border is rapidly attained owing to acceleration of cell division upon changes in medium composition, illumination, and temperature or in some species by means of artificial decrease in cell size due to mechanical breaking the tips or reisolating deformed short-cut cells sometimes arising in old cultures in steady or degenerative phases. It is impossible to achieve sexual reproduction in overgrown culture; it only occurs in healthy culture in the exponential phase of growth.

(4) It was repeatedly shown [11, 12] that no stimuli can initiate sexual process in the cells if they did not achieve proper sizes; therefore, cultivated clones will remain genetically homogeneous provided they remain in prereproductive phase. Cultivation in postreproductive phase is also possible but it is not promising because by this time cells in the clone become very small, often acquire deformities and abnormalities of the frustule, and their growth rate markedly decreases.

DIPLOIDY AND DIOECY

Diatoms are diplobionts and the number of chromosomes reduces at the stage of gametogenesis; therefore, only their gametes are haploid while during the most part of the life cycle cells are diploid. Diatoms reveal sex that in centric and pennate diatoms is determined and realized differently. This is one of their major differences.

In centric diatoms, the same clone can produce both female and male gametes; in other words, the sex of specific clone is not genetically predetermined. Manifestation of the sex depends on cell size and environmental conditions [12].

In pennate diatoms, the sex is genetically determined and, judging from the available data, pennates follow MF/FF inheritance pattern, where male sex is determined by MF combination of sexual factors and female sex is defined by FF combination; in other words, male sex is heterogametic [17, 25–28]. Heterogametic nature of the male sex was for the first time proved in the course of investigation of the breeding system in *Nitzschia longissima* (Brébisson) Grunov [25]. Judging from gamete morphology, some clones of this species (solely male) were capable of limited intraclonal allogamous reproduction. This phenomenon was described as facultative andromixis [29]. Progeny of male clones included both male and female initial cells, which is only possible in case of heterogamety of the parental clone. Subsequently, similar phenomenon was also described in *Tabularia fasciculata* (C.Agardh) D.M.Williams & Round [30] and corroborated in *Seminavis robusta* D.B.Danielidis & D.G.Mann [31]. Diatoms have no sex chromosomes [32, 33].

Thus, centric diatoms are genetically monoecious and pennate diatoms are genetically dioecious (diclinous). Therefore, for centric diatoms maintained in culture, intraclonal reproduction is typical although in nature allogamy is probably more frequent. Normal reproduction of pennate diatoms requires sexual partners from two different clones (male and female).

Centric and pennate diatoms differ also by the types of sexual process: in centric diatoms oogamy occurs, whereas in pennate diatoms oogamy is not known. Gametes produced by them are about the same size but may differ in morphology and behavior in some pennate species [13, 17, 18, 34].

Dioecy of pennates to which the majority of currently existent diatom species belong makes it possible to draw some generalizations useful for applied works.

(1) It seems possible to cultivate clones of only one sex, that exclude spontaneous sexual reproduction and the loss of pure genetic line.

(2) The opportunity of directional crossing and classical selection opens up.

(3) When genetic material of pennate diatoms is maintained in collections of living cultures or under deep freezing, one should bear in mind their genetic dioecy and preserve the clones of opposite sexes.

CROSSING

The system of crossing implies possible pathways of sexual reproduction and involved types of sexual process. There are two pathways of sexual reproduction (homothallic and heterothallic) involving only one clone or two clones of opposite sex. Homothallic pathway of reproduction is characteristic of centric diatoms; however, in pennate diatoms the cases of homothallism are also known [17, 24, 35]. In some species, homothallism is characteristic of only one sex [17, 35], which apparently depends on gamete mobility rather than on genetic constraints. There exist diclinous species where distinct dominance of heterothallism is associated with rare cases of intraclonal reproduction in each sex. In such cases, male sex revealed greater efficiency of homothallic reproduction [27].

The types of sexual process are much more numerous than the pathways of reproduction. Oogamy is usually opposed to nonoogamous types [12, 34]. The most comprehensive classification proposed by Geitler [36] suggests the following types in pennate diatoms, depending on the number of parental cells (gametangia) participating in the process and the number of gametes produced by each of them: (1) normal type (each of two gametangia participating in the process produce two gametes); (2) reduced type (each of two gametangia produces one gamete with the other degenerating); (3) automixis (the process occurs within a single gametangium producing two gametes that subsequently fuse in case of paedogamy; otherwise, the process involves only nuclear transformations without protoplast division in the case of autogamy); (4) apomixis is actually asexual reproduction however leading to the formation of auxospore.

The patterns of oogenesis and spermatogenesis in centric diatoms are diverse [12, 37, 38]. In centric diatoms, oogenesis of three types occurs: type 1, when two egg cells are formed, each having one functional and one pycnotic nucleus; type 2, when an egg having functional and pycnotic nuclei and a residual body are formed; type 3, with only one egg with one functional and two pycnotic nuclei. Spermatogenesis starts with a series of differentiating mitoses that transform male cells into spermatogonia. After final mitosis, spermatogonium becomes spermatocyte and then proper spermatogenesis begins (it may be hologenic or merogenic) [12, 13].

Homothallic and heterothallic pathways of reproduction may be associated with different types of sexual process. For instance, in *Ulnaria ulna* (Nitzsch) Compére heterothallism is associated with a normal type of sexual process, whereas homothallic reproduction occurs by means of paedogamy (a sort of automixis) [28]. Most often, the system of crossing combines both pathways with greater or lesser dominance of one of them [39]. In centric diatoms homothallic by nature, interclonal reproduction is maintained by production of predominantly female or male sexual products in the cells of different sizes in the beginning and the end of the generative phase accordingly [12] and by existence of clones where only one sex predominantly develops [40]. In the latter case, the mechanism of sex determination requires in-depth study. Interclonal crossing in centric diatoms could result in inbreeding depression as it was for example in the second generation of monoeciously reproduced *Cyclotella meneghiniana* Kützing [31].

GENETIC ENGINEERING AND CLASSICAL BREEDING

Culturing of microorganisms embraces the following stages: (1) isolation of individual strains from the environment; (2) determination of conditions optimizing the growth of organisms and production of desired substances; (3) changes in the properties of cultured organisms by modification of their genetic characteristics; and (4) growing the culture of modified organisms without the loss of acquired properties.

The properties of cultured organisms may be modified in different ways, primarily, by means of classical selection with crossing as the main mechanism altering genetic diversity. Earlier, mutagenesis induced by chemical and radiation agents was considered efficient. Recently, considerable progress was made in manipulations at the molecular genetic level (genetic engineering). In diatoms, this concerns genetic transformations aiming at acquiring resistance to antibiotics [41], modifying structure of siliceous cell wall [42, 43], etc.

In nature, genetic purity of the species is preserved. First of all, this is ensured by reproductive barrier preventing interbreeding of unrelated forms and operating all over from the molecular level to the population scale. Secondly, at intracellular and molecular levels there operates a mechanism of repairing defective molecules that carry hereditary information and become injured for various reasons. This is the most efficient in the case of sexual reproduction.

Genetic engineering makes it possible to negotiate the reproductive barrier between species by means of transfer of some genetic material from donor to recipient species. However, any genetically modified organism (clone) may lose its useful properties after crossing with other clones or intraclonal reproduction; therefore, information about the life cycle and the role of sexual reproduction therein is crucial for culturing commercially efficient objects, particularly those produced by the methods of genetic engineering.

FACTORS INITIATING SEXUAL REPRODUCTION

Irrespective of the mode of production of new gene assemblies providing the exploited organisms with new properties desirable for humans, preservation and transfer of these properties in generations remains a crucial goal. It this relation, it is necessary to get to know principles, rules, and properties characterizing sexual reproduction of cultivated organisms. Initiation of sexual reproduction in specific clones becomes a foreground problem, the same as understanding the way of transfer of essential genetic traits through the generations.

As it was mentioned above, the cell size (apical length in pennate and frustule diameter in centric diatoms) is an internal nongenetic factor regulating the onset of sexual reproduction.

When the appropriate size is reached, the next crucial point is an availability of sexual partner in the species that to a greater or lesser extent need it in the cases when mating system combines different ways of reproduction (homo- and heterothallic). Homothallic reproduction does not require sexual partner, but in the majority of species where homo- and heterothallism occur together this pathway is not dominant. In many cases, homothallic reproduction is spontaneous and hard to control. For instance, in the alga *Haslea ostrearia* (Gaillon) Simonsen well known for its specific blue pigment, homothallic reproduction was found first [44, 45]. Over three subsequent decades, in spite of active investigations no new data about reproduction were obtained. We have discovered heterothallic reproduction in this species [46] by means of numerous successful crossing the clones and repeating the experiments in the course of several years. Only after expiration of two years, intraclonal reproduction was confirmed. Thus, it can be argued that heterothallism is basic in this species.

Finally, a group of key factors are represented by environmental conditions, such as temperature, irradiance, photoperiod, light spectral quality, and salinity [12, 13, 47–50]. Sexual reproduction can be stimulated or inhibited under the influence of one or several factors; therefore, each algal species requires optimal conditions. As it was noted above, sexual reproduction can be obtained only in the cultures at exponential phase of growth. If the culture overgrows (when even vegetative divisions cease probably because of self-inhibition by metabolic products), sexual reproduction is unlikely. Bright light usually suppresses sexual reproduction. Some algae need dark periods and they cannot reproduce under continuous illumination; in other species, this process does not depend on dark periods and their duration [12, 14, 48, 50]. Thus, continuous high-intensity illumination can suppress sexuality in certain species, ensuring at the same time high rate of vegetative division.

Some researchers believe that the sexual cycle of most diatoms cannot be controlled in the laboratory [51]. However, our experience has shown that, when certain conditions are satisfied, sexual reproduction may be initiated in most species of diatoms. Sometimes it is necessary to carefully search for appropriate conditions and agents capable of initiating sexual reproduction but in many cases this is quite feasible. At the same time, there exist difficult species actually lacking sexuality, for instance, the centric diatom *Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal or the pennate diatom *Phaeodactylum tricornutum* Bohlin. Although *T. pseudonana* and *P. tricornutum* are the first and so far the only diatoms which genomes were sequenced in full [10, 52], they are insufficiently investigated in respect to sex expression and sexual reproduction. In this relation, the choice of these objects for sequence analysis cannot be considered as a proper one. It is also appropriate to refer to a short paper [53] describing the nuclear transfer between the cells of *P. tricornutum*, which was not associated with formation of auxospores and initial cells and could not be regarded as a normal sexual process. Sexual reproduction was not found in those few species, which are notable for permanent cell size throughout the life cycle [20, 23]. It is interesting that *T. pseudonana* and *P. tricornutum* are exactly such atypical species [8].

SELECTION OF SPECIES, SPECIES FRAMEWORK, AND BIOGEOGRAPHY

In spite of the progress in genetic engineering and resources of genetic modification and transgenesis, any biotechnological process deals with cultivation of individual representatives of a specific species. A biological species carries a certain gene assembly distributed among all its representatives. Sampling of one or several representatives from one or several populations does not guarantee the choice of most appropriate object of cultivation. In respect to classical genetics based on crossing and selection of the organisms, it is advisable to use several genetic lines of a specific species. In this relation, determination of the species framework becomes important.

In diatoms, because of difficult differentiation by morphological or genetic characteristics, the sexual compatibility of clones from different populations is an important evidence of their affiliation to the species and can be directly confirmed only by the experiment. In the majority of diatoms, transfer of traits through the generations also depends on reproductive compatibility of clones. When the samples are collected in natural habitats, especially from distant populations, it is impossible to be sure of authenticity of particular species. Biogeographic investigations of the recent decade have shown that, concurrently with cosmopolitan species [53–56], there exist species with the restricted geographical area [57–62]. In this relation and taking into consideration the existence of cryptic complexes, isolation of individual species from natural samples is sometimes difficult [63, 64]. Therefore, for subsequent use in biotechnology it is advisable to collect samples and introduce into culture several clones (genetically uniform lines) from different ecotopes. Later on, this will make it possible to select the most productive, resistant, and reproductively compatible clones, which ensures further selection.

Maintenance of living species in the laboratory or special collections and continuous work with cultures create the history of clone generations (genealogy), which may be strengthened by the data about their molecular genetic sequences, cultivation experience, and norms of physiological and adaptation reactions. Biology and reproduction of species are always the issues of high priority.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project no. 14-04-90427_Ukr_a.

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Translated by N. Balakshina