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Review Article

What we really know about the dormancy, reproduction, germination and cultivation of charophytes (Characeae)

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Abstract

This expanded review aims to provide information on previous basic research by charophyte experts in the areas of reproduction, dormancy, germination of oospores and cultivation. Therefore, published information, the author's PhD thesis and further results have been combined to summarise the current state of knowledge for Characeae of permanent and temporary waterbodies. The understanding of evolutionary, systematic and molecular pathways require multidimensional approaches using combined results of morphology, anatomy, physiology, genetics, geobotanics and ecology. The application of zonobioms from geobotanies for performed germination studies resulted in a linkage between environmental conditions and dormancy breakage treatment. Besides this, the combination of environmental logger data and species-specific cultivation knowledge resulted in an optimised *in-vitro* system that allow the successful gametangia initiation and maturation under artificial laboratory conditions in monoecious diplostichous *Chara* species.

Keywords

charophytes, reproduction, germination, cultivation, zonobiomes, ecosystem stability

Introduction

Reproduction, one of the most fundamental processes of life, is also one of the most critical steps for aquatic non-seeded plants or algae, such as the submerged living, multicellular charophytes (Characeae), which have evolved by sexual or asexual modes of reproduction. While sexual reproduction depends not only on optimal environmental conditions for the formation and fusion of male spermatozoids with a female oogonium, asexual reproduction relies on fragmentation and specialised vegetative algae parts such as reserve accumulation rhizoid cells or nodal cells (Schubert et al. 2016, Holzhausen et al. 2022). The latter one is assumed as a short-term mechanism and is usually characterised by a lower level of resistance to overlying sediments that causes decomposition and a limited survival time (Skurzyński and Bociąg 2011) Nevertheless, asexual reproduction is common in charophytes. It has been demonstrated for several species in limnic and brackish habitats (e.g. Steinhardt and Selig (2007), Skurzyński and Bociąg (2011), Holzhausen et al. (2017)). Recent studies from North America suggest that vegetative parts of *Nitellopsis obtusa* may also form a perennial reservoir (Pokrzywinski et al. 2020). In contrast, oospores produced by sexual reproduction show a long-term reservoir in aquatic sediments. They are predicted to have a longer and higher survival rate combined with an increased resistance to drought and mechanical stress (Casanova 1994, Rodrigo et al. 2010, Stobbe et al. 2014). However, essential factors, such as: (I) vital material (oospores), (II) suitable conditions for dormancy breakage or (III) optimal germination conditions, are required for colonisation after long- and short-distance transport, recolonisation after disturbance and maintenance of populations. Besides their use in applied science, Characeae are of current evolutionary interest as being part of the sister group of land plants. In order to understand the emergence/disappearance and reproduction of Characeae in our aquatic systems, the limited knowledge of the fundamental molecular processes, the underlying gene regulation and phytohormones, as well as the transcription factors involved, needs to be extended. For this, it would be essential to maintain long-term cultures without massive biofilms and a decay of oospore fertilisation over time. In contrast to *Chara braunii* (Holzhausen et al. 2022), however, no generative propagation could be initiated in monoecious, diplosticious species using existing mineral media (Forsberg 1965b, Andrews et al. 1984a, Wüstenberg et al. 2011). As a solution, a combination of ecological environmental data from outdoor mesocosms (Holzhausen 2019) with proven *in-vitro* methods was found for *Chara hispida*, here referred to as Eco-*in-vitro*-cultivation.

The integration of fields, such as phenology cytology, molecular genetics and physiology, will allow the understanding of these fundamental processes, as well as underlying phytohormone pathways. Therefore, the aim of the manuscript is to summarise existing studies in the fields of:

(I) gametangia formation and fertilisation,

(II) oospore dormancy,

(III) germination induction of vegetative and generative units and

(IV) biomolecular studies.

Formation of gametangia and fertilisation of charophytes

Sexual reproduction

The fusion of male and female gametes and the subsequent maturation of the zygote is called sexual reproduction. This mode of reproduction is described for mostly all charophyte species, even if the frequency of gametangia and oospore formation varies as can be seen in the enormous amount of available literature. Both gametangia, the male antheridia and the female oogonia were produced by the formation of short shoots from the nodal cell of branchlets (Schubert et al. 2016, Schubert et al. In press). Over the last 150 years, various authors published descriptions of cell development and formation of both gametangia. Due to the amount of considered aspects, not all of them can be considered here in detail. More than that, the plethora of techniques for gametangial studies range from simple descriptions over schematic drawings to high-resolution figures of microtome sections. Table 1 summarises available microtomic/microscopic studies of antheridia, oogonia and rhizoids. The full plethora of studies on internodal cells were excluded (Sundaralingam 1954, Fetzmann 1957, Barton 1965a, Barton 1965b, Nagai and Rebhuhn 1966, Pickett-Heaps 1966, Shen 1967c, Barton 1968, Vouilloud et al. 2015).

Male gametangium

The majority of detailed gametangial studies have dealt with male spermatozoids and have considered the morphology, cell division or phylogenetic relationship of charophyte spermatozoids to bryophytes or pteridophytes (Belajeff 1894, Mottier 1904, Karling 1926, Karling 1927, Sasaki 1935a, Sasaki 1935b, Satô 1954, Eggmann 1966, Pickett-Heaps 1967a, Pickett-Heaps 1967b). However, almost all comparisons are based on similarities in structure and form. In contrast to numerous evolutionary studies on the genetic regulations of male and female traits in bryophytes or pteridophytes, only a few studies are available for charophytes, such as the presence of the MIKC-type MADS box gene in oogonia and antheridia of *Chara globularis* (*CgMADS1*), suggesting the presence of these genes before diversification (Tanabe et al. 2004). These genes are thought to be involved in the control of sex-specific haploid cell differentiation and stress response (Qiu et al. 2023).

The development of the two flagellated spermatozoids is well known within this group of algae (Varley 1834, Thuret 1840). A brief historical overview of investigations is given by Vouilloud et al. (2012). In the 19th century, different authors studied the cell development of all three cell types of antheridia (scutum, manubrium, capitulum) in detail (e.g. Varley (1834), Braun (1852), Pickett-Heaps (1967a)), a corresponding summary can be found in

Schubert et al. (2016), Schubert et al. (In press). Intercellular streaming was only observed in the manubrium and capitulum, but never in external scutum cells (Braun 1852). In taxonomy, antheridia size is part of almost all species descriptions and has been used for species delimitation, for example for *Tolypella* species (Holzhausen et al. 2023). However, the developmental stage, environmental conditions, the reproduction mode and the position at the whorl can influence antheridia sizes (Ernst 1918, Kwiatkowska et al. 1996, Calero and Rodrigo 2022), which make it necessary to find a standardised method for reliable regional considerations or species delimitations by antheridia. Fig. 1 shows the typical development of antheridia which changed from green to light yellowish-green in younger stages to an orange antheridium in maturing stages, followed by the release of thousands of spermatozoids. In 1967, Pickett-Heaps suggested a correlation between the orange colour and the accumulation of plastoglobuli as a source of the increasing carotenoid content (Pickett-Heaps 1967a).

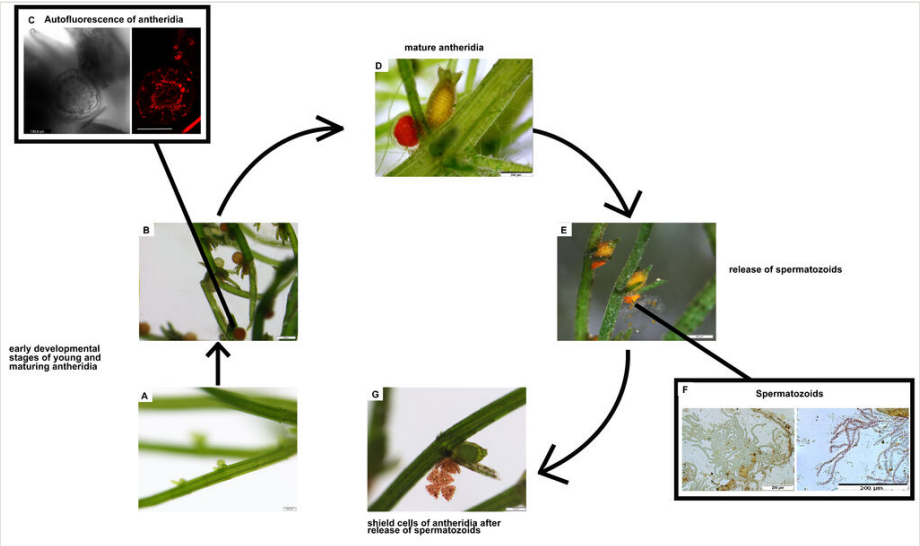

Figure 1.

Developmental stages of charophyte antheridia in accordance with Shepherd and Goodwin (1992). First developmental stage of young (A, B) and maturing (B) antheridia indicated by a green/light yellow colour or the orange colour (B) after shield cell developing (C) Autofluorescence of antheridia detected with a fluorescence microscope, scale = 139.8 µm. (D) Mature antheridia (E-F) release of spermatozoids by opening of shield cells (E) and a detailed view of antheridial filaments (F) (G) shield cells of antheridia after the release of spermatozoids containing plastids with plastoglobuli (photographs: A. Holzhausen).

In addition to morphology, there are several studies, mainly by Kwiatkowska and co-workers, on the cell-to-cell connection via plasmodesmata and the process of endoreplication in *Chara vulgaris* and/or *Chara tomentosa* (Kwiatkowska et al. 1990). The type of plasmodesmata (plugged, unplugged) correlates with the developmental stage and differs in the cell types connected and their internal substances (Kwiatkowska and Maszewski 1985, Kwiatkowska et al. 2003, Kwiatkowska 2003). Accordingly, Shepherd and

Goodwin found out that the symplastic linkage in lateral branches between internodes or internodes and associated nodes is significantly higher in spring and summer (stage of harbouring young and maturing antheridia) than in the post-fertile stage (no new development of antheridia), the mature stage (antheridia reached at least a 32-celled-filament stage) or vegetative branches during winter (Shepherd and Goodwin 1992). In all these three stages, a restricted cell-to cell-communication was found by investigating the transport of 6COOHF in lateral branches. Broken plasmodesmata, therefore, determine the most mature state of antheridia and should be considered in further studies. Furthermore, autoradiographic studies have shown the circadian translational activity within the different cell types of antheridia (scutum, manubrium, capitulum, spermatozoids), including the protein synthesis activity of ^{3}H-leucine (Kwiatkowska 1991, Kwiatkowska et al. 1995, Kwiatkowska et al. 1999, Kwiatkowska and Papiernik 1999). Studies on DNA methylation were performed by Olszewska et al. (1997)

Cell walls of *Chara corallina* antheridia show the presence of cellulose and pectin homogalacturon, whereas arabinogalactan proteins have only been indicated in epidermal shields and anti-xyloglucan in the capitulum (Domozych et al. 2009). The expression of PIN2-like auxin transporters in antheridial filaments was shown by immunofluorescence studies (Zabka et al. 2016). Studies on the effects of gibberellic acid are presented in subsection V, biomolecular studies.

Female gametangium and fertilisation

Compared to male antheridia, there are significantly fewer published studies on female unfertilised gametangia, so-called oogonia. While the male gametangium of charophytes (antheridia) is comparable to the antheridia of bryophytes, the two female gametangia differ. Charophyte oogonia are ovoid and the fertilisation leads directly to the zygote. In bryophytes, however, archegonia are multicellular and lageniform and fertilisation leads to sporophyte development, with the formation of spores (Braun 1852). Previous literature has concentrated on descriptions within general cryptogam overviews, on developmental stages of cell divisions by microtome sections and on size variations (e.g. De Bary 1871 Covich and Tsukada (1969), Maier (1973), Sawa and Frame (1974), Leitch (1986), Leitch (1989), John et al. (1990), Leitch et al. (1990), Kwiatkowska et al. (1996)). The cell development of the female gametangium will not be discussed in detail here. Descriptions can be found in Braun (1852), Sundaralingam (1954), Schubert et al. (2016), Schubert et al. (In press). Microtome studies of female gametangia and included cell compounds are listed in Table 1. Intercellular streaming was observed only in the multicellular basal node, the unicellular internodal cell and the five envelope cells. However, the intercellular streaming within the oogonium could not be observed in detail (Braun 1852).

In 1871, Anton de Bary described the development of the female gametangium up to fertilisation by male spermatozoids (De Bary 1871). Using a Brückean magnifying glass, he was able to observe in detail not only the cell development of the surrounding envelope cells, but also the formation of a tube-like channel, which results from a difference between the convergence of spiral cell tips and cells. Masses of spermatozoids rest near the area of

the five tube-like channels, fixed by the gelatinous intercellular compound of the five narrowed channels, allowing the intrusion of spermatozoids as a dense bundle formation to move to the intercellular part above the oocyte. In contrast, the intercellular part is only traversed by a narrowed channel, which ends in a widened funnel shape at the apex of the oocyte. A loose membrane structure allows individual spermatozoids to pass through the apex. Fertilisation is followed by the formation of a thickened cellulose layer and a thickening and change in the colour of the spiral cells. It is thought that the absence of this layer is associated with infertility, although simultaneously the same amount of amylum is present.

Table 1.

List of cytological studies on gametangia, rhizoids and vegetative cell material of charophytes. The respective object and cell type, method, staining and identified organelles are recorded. If synonyms were used by the authors, common species name is listed first followed by the synonym in brackets.

Reference	Species	Method	Staining	Organelles
Johow (1881)	***Chara vulgaris* (=*C. foetida*)** branchlet, gametangia, rhizoids, nodal cells	-	haematoxylin	nucleus division
Zacharias (1885), Zacharias (1888), Zacharias (1890)	***C. vulgaris* (=*C. foetida*)** male and female gametangia, rhizoids	microscopy	-	• nucleolus • protein • nuclei • rhizoids • geotropism
Overton (1890)	***Nitella syncarpa*, *C. hispida*** female gametangium, branchlets and internodal cells	• staining • microscopy • reaction against concentrated acids	• chloralhydrate • haematoxylin-eosin	nuclei of: • stalk cells • gametangial node cell • “Wendezelle” • spiral cells of oogonia • coronula • “Stachelkugeln”
Kaiser (1896)	***C. vulgaris* (=*C. foetida*), *C. hispida*, *C. canescens* (=*C. crinita*), *N. syncarpa*, *N. flexilis*** apical cell, node cell, branchlets, oogonia/ antheridia, cortex cells	sectioning (3-5 µm)	• Alauncochenille • Haidenhain’s Iron-haematoxylin • haematoxylin-eosin in glycerine • fuchsin • methylene blue	• karyokinesis • nucleus • nucleoli • Altmann’s granula • centrosomes
Goetz (1899)	***N. flexilis*, *N. opaca*, *C. vulgaris* (=*C. foetida*)** internodal cells, oogonia/ oospores	• sectioning • oogonia 10 µm sections • zygotes 20 µm sections	• fuchsin iodine green • fuchsin methylene blue • iron-haematoxylin • maemalaun	• chromatin • nucleolus • membrane
Mottier (1904)	***C. globularis* (=*C. fragilis*)** spermatozoids	sectioning (3-5 µm)	• aniline-safranin • gentian-violet • orange G.	• nucleus • cytoplasm • blepharoplast • cilia

Reference	Species	Method	Staining	Organelles
Oelkers (1916)	***C. globularis* (=*C. fragilis*), *C. vulgaris* (=*C. foetida*), *N. syncarpa*** generative/vegetative tissues	• sectioning (30 µm) • degradation of sugars	Haidenhain's iron-haematoxylin	• „Stachelkugeln" • Aleuron • nuclei of zygotes
Riker (1921)	***C. globularis* (=*C. fragilis*), *C. virgata* (=*C. verrucosa*)**	sectioning (5 – 7 µm)	• Haidenhain's iron-haematoxylin • Flemming's triple stain	mitochondria
Karling (1926)	***C. braunii* (=*C. coronata*), *C. globularis* (=*C. fragilis*), *N. gracilis*** antheridia/oogonia, apical cells, nodal cells	microscopy	• Heidenhain's iron-alum haematoxylin • Flemming's triple stain	• shield cells • manubria • capitula • filaments • nuclei
Linsbauer (1927)	***C. subspinosa* (=*C. rudis*), *C. vulgaris* (=*C. foetida*), *C. globularis* (=*C. fragilis*)** rhizoids	microscopy	• methyl green acetic acid • haematoxylin • borax carmine	nucleolus rhizoidal cells
Schmuckler (1927)	***C. contraria* var. *hispidula*** oogonia	microscopy	-	spiral cells of oogonia
Walther (1929)	***N. syncarpa*, *N. hyalina*, *N. batrachosperma*** oogonia/antheridia, oospores, node cells, branchlets, internodal cells	sectioning (6-16 µm)	• borax carmine • acetic carmine • methyl green • Feulgen reagent	• nuclei • lipids • starch
Sasaki (1935a), Sasaki (1935b)	***N.* sp., *C.* sp.** spermatozoids	-	• gentian-violet • carbol-fuchsin	blepharoplast
Sundaralingam (1954)	***C. zeylanica***	sectioning (5-10 µm)	Haidenhain's Iron-haematoxylin	• antheridium • oogonium • oospores • branchlets • stem (cortex) • internodal cells
Satô (1954)	***C. braunii*** spermatozoids	• electron microscopy • phase contrast microscopy	• carbol-fuchsin • gentian-violet • Heidenhain`s iron-haematoxylin • Feulgen reaction • aniline acidic fuchsin • Janus green • silver nitrate • iodine iodide potassium solution	• mitochondria • chloroplast • starch • blepharoplast • flagella • flagella-fibrils • spermatozoid-nucleus • spherical bodies with granules

Reference	Species	Method	Staining	Organelles
Barton (1965a), Barton (1965b)	***C. vulgaris*** mature lateral cells	sectioning	-	• plasmodesmata • charasome • mitochondria • Golgi apparatus • endoplasmic reticulum • lipid bodies • nucleus • spherosomes
Hollenstein (1966)	***C.* sp.** spermatozoids	X-ray diffraction	-	nuclein content
Shen (1967a), Shen (1967c)	***C. contraria*, *C. zeylanica*** older internodes, rhizoids, protonema, leaves, shoot apices	• sectioning (25 µm) • smear • whole plant part	• fast Green • Schiff's reagent iron haematoxylin	• nuclear DNA contents in mg • sperm (22.5 ± 0.78) • apical cell (45.7 ± 1.6) • nodal cell (48.9 ± 1.59) • nodal cell, metaphase (49 ± 1.23 mg) • nodal cell, half-anaphase (21.9 ± 2.1 mg) • 1-4 internode (124.2 ± 10.3 – 655.2 ± 45.3) • crescentic nuclei, older internodes (174.2 ± 12.2) • spherical nuclei (25.4 ± 1.3 / 46.6 ± 1.5)
Shen (1967b)	***Chara zeylanica*** sperms	Feulgen spectral absorption	Schiff's reagent	sperm nuclear DNA (9.46 ± 0.25 mg)
Pickett-Heaps (1967a), Pickett-Heaps (1967b), Pickett-Heaps (1968)	***C. australis*, *C. fibrosa*, *C.* sp., *N.* sp.** antheridia oogonia	sectioning	• uranium and lead • toluidine blue	• basal cell • chloroplasts • chromosomes • endoplasmic reticulum • cytoplasmic fibrils • Golgi bodies • nucleus • mitotic spindle • polysomes • spermatogenous cell • microtubules
Sawa and Frame (1974)	***Tolypella nidifica*** plant apices with oogonia	sectioning (1-5 µm)	0.5% toluidine blue	sterile oogonial cell
Leitch (1986), Leitch (1989), Leitch et al. (1990)	***C. aspera* (=*C. delicatula*), *C. hispida*, *Lamprothamnium papulosum*** oogonia	sectioning (80-100 nm; > 200 nm)	• Reynold's lead citrate • 4% toluidine blue and 4% malachite green • 4% basic fuchsin	• basal position of nucleolus • chemical composition and development of wall compounds/ layers • sphaerosomes

Reference	Species	Method	Staining	Organelles
Moestrup (1970)	***C. corallina*** spermatozoids	• sectioning • formvar/ carbon-coated grids for electron microscopy	• uranyl acetate • Reynold's lead citrate	different chromosome numbers in single antheridia filaments
Stabenau et al. (2003)	***C. globularis* (=*C. fragilis*)** homogenates of all cells	sectioning	Reynold's lead citrate and uranyl acetate	• peroxisomes • mitochondrium • chloroplast
Hodick (1993)	***C. globularis* (=*C. fragilis*)** protonema from nodes	microscopy	-	• cell division • nuclei
Sievers and Schröter (1971), Braun and Sievers (1993), Buchen et al. (1993), Braun (1996a), Braun (1996b), Braun and Wasteneys (1998)	***C. globularis* (=*C. fragilis*), *N. pseudoflabellata*** protonema, rhizoids	• immune-fluorescence labelling • sectioning (2-3 µm) • slow-rotating-centrifuge-microscope	• fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM • fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG • rhodamine-phalloidin in sodium-potassium-phosphate-buffer • 2% uranyl acetate and 2% lead citrate • acid fuchsin ethanol	• stratoliths • nucleus • microfibrils • actin-cytoskeleton • myosin-related proteins

The molecular signalling pathways involved during fertilisation or meiosis are still unknown. The exact time of meiosis is still unknown. For a summary, see Guerlesquin and Noor (1982), Schubert et al. (2016), Schubert et al. (In press). Five different opposing hypotheses have been proposed for the exact time point of meiosis in charophytes: (I) just before the germination of the oospore (Oelkers 1916), (II) in the early cell stages of the antheridia and oogonia (Tuttle 1924, Tuttle 1926), (III) during the development of the protonema filament (Gonçalves da Cunha 1936, Gonçalves da Cunha 1942) and (IV) immediately after the fertilisation (Shen 1967b, Shen 1967c). Although hypotheses (I) and (IV) are almost identical, both have been listed because of gaps in the descriptions of cell movement and the resting phase before germination. In 1899, Goertz described the observation of a possible delayed fertilisation by the protrusion of starch into the neck of the female gametophyte (V, Goetz 1899). Despite a comparison with *Vaucheria*, no further evidence is found in literature (Oltmanns 1895). The myth of meiosis is not well known, mainly due to complications in microtome sectioning. The high starch content of oogonia and oospores makes the procedure difficult. The establishment of methods for ultrathin sectioning of all developmental stages (from oogonia formation to the released oospore) is one of the most important prerequisites for solving this mysterious process.

Table 2.

Methods for **oospore dormancy breakage** for charophyte species from permanent (p) and temporary (t) habitats from different zonobioms after Pott (2005). These are indicated with Roman numbers as given by the literature, superscript labels indicate the zonobiome and are listed below.

Species	Habitat	Zonobiome	Most effective method to break dormancy (storage conditions)	Reference
Chara aculeolata	p	VIII[1]	+4°C for about 2 months	Forsberg (1965c)
C. aspera	p	VIII	+4°C for about 2 months	Forsberg (1965c)
C. australis	p p	V[2] V	wet under semi-natural conditions	Casanova and Brock (1996) de Winton et al. (2004)
C. braunii	p	I[3]/V	wet storage 22°C	Holzhausen et al. (2022)
C. canescens	p/t p p/t	VI[4] VI VI	dry oospores, low temperatures 12 weeks at 10°C in the dark dessication at 5°C	Ernst (1921) Steinhardt and Selig (2011) Holzhausen et al. (2017)
C. contraria	p/t p p	N/A III[5,*] VII[6]	drying of oospores wet oospores at cold temperatures (3°C) fresh oospores from mother plant	Shen (1966) Proctor (1967) Sabbatini et al. (1987)
C. corallina	p	V	15°C and darkness for up to 4 days	de Winton et al. (2000)
C. globularis	p p	III* V	dry oospores at cold temperatures (3°C) 15°C and darkness for up to 4 days	Proctor (1967) de Winton et al. (2000)
C. hispida	t	VI	drying of oospores, cold treatments of 1-3 months at 4°C	Holzhausen, unpublished data
	p	VIII	+4°C for about 2 months	Forsberg (1965c)
C. muelleri	p/t	V	dry storage in either dark or ambient light conditions	Casanova and Brock (1996)
C. papillosa	p	VI	dessication at 20°C	Holzhausen et al. (2017)
C. rusbyana	p	III*	dry oospores at cold temperatures (3°C)	Proctor (1967)
C. vulgaris	p p p p t	VIII VIII VIII VIII VI	36 days at 18-22°C wet storage 60 days at 4°C and red light dark at 4°C drying of oospores, cold treatments of 1-3 months at 4°C	Kalin and Smith (2007) Sederias (2003) Sederias and Colman (2007) Sederias and Colman (2009) Holzhausen, unpublished data

Species	Habitat	Zonobiome	Most effective method to break dormancy (storage conditions)	Reference
C. zeylanica	p p/t p	VIII N/A III*	4°C for about 2 months cold temperatures (5-7°C) for 10 days wet oospores at cold temperatures (3°C)	Forsberg (1965c) Shen (1966) Proctor (1967)
Lychnothamnus barbatus	p	VI	dessication at 5°C	Holzhausen et al. (2017)
Nitella cristata var. *ambigua*	p/t	V	cold treatment (4-5°C)	Casanova and Brock (1996)
N. furcata	p	VI	4°C and darkness	Sokol and Stross (1986)
	p	V	15°C and darkness for up to 4 days	de Winton et al. (2000)
N. flexilis	p	VIII	247 days sediment storage at 18-22°C, decline of redox conditions in medium	Kalin and Smith (2007)
N. sonderi/*N. subtilissima*	p/t	V	drying of oospores	Casanova and Brock (1996)

- * sample sites are not given for all strains of the collection
- [1] coniferous forest land and taiga
- [2] warm temperate area with indeciduous forests (laurel forests)
- [3] zone of humid tropical rain forests
- [4] temperate, winter cold areas with deciduous forests (= nemoral zonobiom)
- [5] zone of the subtropical hot arid semi-deserts and deserts
- [6] steppes, semi-deserts and deserts

Cultivation and field studies have shown that the process of fertilisation may be dependent on environmental conditions, such as temperature, light or salinity. Cultivation or germination conditions, as well as species-specific growth behaviour, should be considered when comparing existing literature. For example, Karling (1924) compared the maturation of oospores under different light conditions. Whereas, in greenhouses (high temperature during the day, low temperature at night), the oospores matured, in climatic chambers (high temperature during the day and night), the oogonia remained in an unfertilised state. The same has been proposed for *Chara braunii* (Sato et al. 2014, Holzhausen et al. 2022). The same is true for *Chara aspera* with respect to light and salinity. The highest rate of gametangiogenesis was found under natural ecological conditions. Greater variation in conditions compared to the natural ecological status resulted in stress responses such as reduced gametangia numbers or cell death (Blindow et al. 2003). In this context, tracer studies on the polarity and concentration of photoassimilates in the vacuolar sap of different regions of the *Chara* thalli (sugar-phosphates, carbohydrates (sucrose), malate, amino acids) of *C. vulgaris*, *C. hispida* and *C. corallina* showed an assumed correlation between the accumulation of sucrose and reproduction. For a summary, see Schulte et al. (1994). Briefly, they found out that:

1. differences in sap composition are caused by seasonal changes and reproduction (MacRobbie 1962, Barr 1965, Winter et al. 1987),

2. lateral branches as the source of gametangia have the highest photosynthetic activity (Andrews et al. 1984b, Ding et al. 1991a),
3. gametangia are sinks of photosynthates during reproductive periods (Schulte et al. 1994),
4. a higher activity of 1,5-bisphosphate carboxylase (RuBPCase) and a fourfold higher ferricyanide-dependent oxygen evolution in branchlets than in internodes (Ding et al. 1991b),
5. differences between growing and overwintering periods (Shepherd and Goodwin 1992),
6. intercellular transport occurs via plasmodesmata (e.g. Leitch et al. (1990)) and
7. the intercellular transport is determined by the rate and concentration of cytoplasmic streaming and a possibly unclear “intrinsic regulatory process” (Ding et al. 1991a).

Assuming that the rate of cytoplasmic streaming depends not only on general seasonal changes, but also on the exact environmental conditions, the differences in oospore sizes, as reported by different authors, could probably be explained by the number of cell-cell-connections between branchlets and oogonia and the associated rate of sucrose uptake and starch storage, respectively (Holzhausen 2016). Comprehensive analyses, combining phenological and cytological studies, molecular mechanisms and environmental data over several years including the open question of chloride uptake by oogonia, could resolve this open question.

Asexual reproduction of charophytes

Asexual reproduction by vegetative parts is common in aquatic plants and algae. For reviews about this, see for example Lovett Doust and Lovett Doust (1988), Barrat-Segretain (1996), Barrat-Segretain et al. (1998), Barrat-Segretain and Bornette (2000), Cecere et al. (2011), Agrawal (2012). For most Characeae, vegetative reproduction is a widespread feature, except of species with meteoric appearance. However, there is one exception, *Chara canescens*, which was never seen to reproduce vegetatively by formation of modified node or rhizoid cells and is, therefore, dependent on the formation of oospores for reproduction (Krause 1997). In addition to its unique position, this species can reproduce parthenogenetically or sexually through dioecious populations and it has been shown that females of parthenogenetic and sexually reproducing populations are genetically distinct (Schaible et al. 2009a, Schaible et al. 2009b, Nowak et al. 2019) and differ in chromosome numbers (Ernst 1917).

In contrast to generative reproduction, there is a limited amount of literature on asexual reproduction. However, only a few scientists (Braun 1852, Giesenhagen 1897, Migula 1898 , Bharatan 1987, Sederias 2003, Skurzyński and Bociąg 2011, Pokrzywinski et al. 2020) have studied this type of propagation in detail for species of the genera *Nitellopsis*, *Chara*, *Lamprothamnium* and *Paleonitella*. According to the time, cell division in nodal cells and the emergence of new shoots were mainly visualised by schematic representations, as studies and visualisation with modern microscopy techniques does not exist, except for the

rhizoids themselves (e.g. Braun et al. (1996), Braun et al. (1999)). In 1897, Giesenhagen summarised the differences between bulbils and modified rhizoid nodules, based mainly on the work of Migula (Migula 1898) and his own results (Fig. 2A/B). However, Giesenhagen's findings are not entirely consistent with Migula's, as *C. aspera* rhizoidal bulbils are not unicellular and Giesenhagen gives a detailed description of their formation and cell division (Giesenhagen 1897). The different provisioning of individual rhizoidal cells, reflected in the different sizes within the complex, has not been fully elucidated. The number of modified rhizoid cells is species-specific, with more than four being described as unusual in *C. aspera*, whereas complexes of three to five or more than 12 have been described for *Lamprothamnium macropogon* (Braun and Nordstedt 1882, referred as *Lychnothamnus macropogon*). Fig. 2C shows rhizoidal bulbils of *C. aspera* with associated microorganisms. The nearest rhizoidal node or the apical part of the cell is assumed to be responsible for the development of new shoots. In his study, Bharathan describes the same type of 'bulbils' for *Chara hornemannii*, *C. aspera*, *L. papulosum* and *Lamprothamnium succinctum*, but in contrast to earlier studies, they are also referred to as bulbils and not as rhizoid nodules (Bharatan 1987). Interestingly, the author's own results have shown that sterilisation and severance of the connecting rhizoid filaments do not lead to the development of new shoots. So far, no signalling pathways are known for these connecting rhizoid filaments. The study by Bonnot and co-workers showed that the *Chara braunii* ROOT HAIR DEFECTIVE SIX-LIKE genes do not complement the rhizoid or root-hair development in mosses and land plants, initiating a completely different function (Bonnot et al. 2019). In contrast to this, the induction of germination by the rhizoid/shoot nodule complex is simple and occurs within a few days under laboratory conditions (Giesenhagen 1897, Bharatan 1987, Blindow et al. 2009). Vegetative reproduction can occur via modified shoot nodal cells (nodal bulbils) in addition to modified rhizoid nodes. The ability of these cells to produce new shoots after periods of drought, fragmentation or death of apical cells is well documented (Fig. 2D). This ensures the growth of new populations under unfavourable conditions for oospores (Casanova 1994, Casanova 1999, Blindow et al. 2009). Regeneration of fragmented nodal cells requires the formation of lateral branches or cone-like outgrowths (Giesenhagen 1897) as described for the strawberry-shaped nodal bulbils of *Chara baltica*, a limited number of new thalli can be developed by a modified node. These are the same as lateral branches, but greatly reduced. Various studies have been published on the structuring of shoot node cells (Braun and Nordstedt 1882, Giesenhagen 1897, Schubert et al. 2016), including cell division (Fig. 2E-G). Interestingly, different forms of overwintering structures can be observed: star-shaped modified shoot nodes in *Nitellopsis obtusa* (Fig. 2A/B), strawberry-shaped modified nodal cells in *C. baltica* or cardioid-shaped modified rhizoid cells in *Chara fragifera*. The existence of star-shaped bulbils in *Chara connivens*, as mentioned by Clavaud (Clavaud 1863, Giesenhagen 1897), is not fully confirmed. The phenomenon of reduced, starch-filled bulbils acting as reserves can also be found in other species, such as *C. baueri*, *C. vulgaris* (= *C. foetida)*, *C. globularis* (= *C. fragilis)*, *C. hispida* or *C. subspinosa* (= *C. rudis*). Bulbils have also been described in *Nitella* species from the Southern Hemisphere (De Maisonneuve 1859, Casanova 1994, Casanova 1999). However, the formation and gene expression in these vegetative cells is not fully understood, although a correlation with distribution patterns caused by landmass separation and environmental

changes has been hypothesised (Croy 1979). This is supported by barcoding studies that found adaptive mutations of chloroplast and mitochondrial genes in different habitats (Kato et al. 2008, Schneider et al. 2015). The lack of multidimensional approaches, including field, laboratory, microscopic and molecular studies, to address open questions about vegetative reproduction should be addressed in future studies.

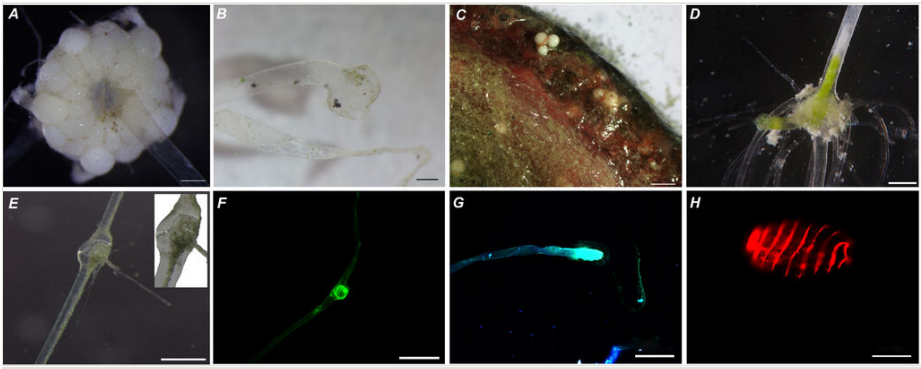

Figure 2.

Reproductive or growth units and rhizoid cells of charophytes. (A) fully developed star-shaped bulbil of *Nitellopsis obtusa*, scale 1 mm. (B) Cell at the beginning of bulbil development, scale 200 µm. (C) rhizoidal bulbil with associated microorganisms of *Chara aspera*, scale 2 mm. (D) growth unit of *Chara filiformis* (stem cell), scale 500 µm. (E) characteristic S-shaped division plane of rhizoid cells (*) of *Chara vulgaris* with additional cells for secondary rhizoids (arrows), scale 1 mm. (F) rhizoid cell of *Chara braunii* S276, stained with DAPI, scale 200 µm. (G) rhizoid tip of *C. braunii* S276, stained with DAPI, 200 µm. (H) oospore of *C. braunii* S276, stained with PI, scale 200 µm (photographs: A. Holzhausen).

Rhizoids of charophytes

Charophyte rhizoids function similarly to terrestrial plants, mainly for anchoring, but also for absorbing nutrients, which are often associated with microorganisms. The cell development of rhizoids has been studied by different authors. Detailed descriptions of cell development, including cell thickening can be found in literature (e.g. Braun (1852), Zacharias (1890), Linsbauer (1927), Shen (1967a), Sievers et al. (1991), Braun and Sievers (1993)).

In general, rhizoids consist of two cell walls, a single-layered outer cell wall and a multi-layered inner cell wall. The components pectin and cellulose are described for both. The outer layer of *Tolypella intricata* f. *humilior* also contains mucilage (Fridvalszky 1958). This exudate and polysaccharide- and glycoprotein-rich coating is also known from ecophysiological studies of *L. papulosum*. A correlation between salinity and the thickness of the mucilage secretion was observed (Shepherd et al. 1999). There are no further studies on rhizoid mucilage in charophytes. Functional characteristics of mucilage on charophyte rhizoids and oospores, especially as hydraulic bridges related to nutrient supply and desiccation tolerance, are not known. In land plants and hydroterrestrial algae,

mucilage has been shown to act as a barrier to pathogens and harmful metals, to protect root tips and to penetrate the soil (Herburger et al. 2022). In addition to mucilage, vesicles containing barium sulphate ($BaSO_4$), which act as gravity-sensing statoliths, are located on the outer cell wall (Hejnowicz and Sievers 1981, Braun and Sievers 1993). The apical part of the rhizoids contains the stationary cytoplasm and the nucleus, whereas the basal part contains the mobile cytoplasm, including the cytoplasmic streaming.

The main focus of research using charophyte rhizoids as model cells is gravitropism. Positive gravitropism, or downward growth, is observed in charophyte rhizoids. The first study was done by Zacharias (Zacharias 1890). He showed that the rhizoid tip cells also grew downwards when the rhizoid position was changed. One hundred years later, it was clear that the actin cytoskeleton of *C. globularis* and *C. vulgaris* (= *Chara foetida)*, which is controlled by actin-binding proteins, plays an important role in the mechanism of gravitropism and in the polarised perception of charophytes (Sievers 1967, Braun and Limbach 2006). A central role in the apical rhizoid zone is supported by immunolocalisation studies of the actin polymerisation factor and profilin. Negative gravitropism is described for protonematal cells in contrast to rhizoids.

Further studies on gene expression as described above are not available for charophytes. However, Sandan's studies of *C. braunii* (= *C. coronata*) and *Nitella flexilis* showed that rhizoid development was enhanced by IAA-K solution as opposed to pure tap water or Knop's solution (Sandan 1955).

Dormancy and germination induction of charophytes

In both permanent and temporary waterbodies, the time between fertilisation and germination is extended by a period of dormancy to increase the possibility of a period of low competition and successful population establishment, although subsequent reproductive capacity is not guaranteed (Casanova and Brock 1990, Vleeshouwers et al. 1995). The adaptive seed trait of temporal loss of germination capacity represents a period of physiological processes, such as mobilising resources and activating/deactivating genes leading to morphological changes and subsequently germination. According to Baskin and Baskin (1985), who described it for land plant seeds, the physiological, morphological and physical state at the time of maturation and germination must be considered and combined with environmental habitat conditions at the time of seed/oospore state changes and during the maturation and germination process.

However, many classification systems have been established because there is no single definition of dormancy (Bewley and Black 1982). The distinction between developmental time, i.e. primary and secondary dormancy, is the simplest and most convenient. While primary dormancy is induced during development and maturation (e.g. Crocker (1916), Hilhorst (1995), Hilhorst (1998), Finch-Savage and Leubner-Metzger (2006)), secondary dormancy is induced only after release from the mother plant and requires the loss of primary dormancy. Interestingly, although not yet confirmed by molecular studies, the hypothetical physiological dormancy model for terrestrial seeds (Hilhorst 1998) also seems

to apply to charophytes (Fig. 3). This combined model describes the regulation of secondary dormancy by temperature and the subsequent triggering of germination by the availability of nitrate, light and gibberellic acids. Figure 3 shows the modified model of Hilhorst (1998) and implies that dormancy and germination are directly correlated with the amount of phytochrome receptors present. These red (R) and far-red (FR) receptors act as light sensors in several plants/algae (Inoue et al. 2019), which are mainly located in the nucleolus. Several germination studies have identified that germination is triggered by the supply of gibberellic acid (Sederias and Colman 2007, Holzhausen et al. 2022), although evolutionary studies have shown that the gibberellic acid pathway has evolved in mosses (Nishiyama et al. 2018). Further studies are needed to determine whether chemical precursors could explain this phenomenon. In addition, endogenous gibberellic acid has been measured in studies on *C. tomentosa* and *C. vulgaris* (Kaźmierczak and Rosiak 2000 Kaźmierczak 2001, Chowdary 2014). Fertilisation was not found to be related to endogenous gibberellic acid content, but to sex. While the concentration in sterile shoots increases towards the apex, the concentration in fertile shoots declines from the apex downwards. In contrast, the levels in male plants exceed those in female plants.

The induction of germination itself is a signal for the end of the resting phase (dormancy) and for the presence of favourable environmental conditions for growth. Sensors are used to provide signals on temperature, light, nutrient availability and hydration status. Competitive conditions, on the other hand, are undetectable and represent a 'trial and error' approach. Individual factors, although not always fully distinguishable, are considered in the following subsection.

Biogeography

The origin of the material (spores/plants/sediments), although neither an abiotic nor a biotic factor, is of crucial importance. This concerns both the biogeographical origin and the habitat type (permanent or temporary biotopes). In addition to morphological differences between regions or water depths (Brzozowski and Pełechaty 2020, Holzhausen et al. 2023) and phenological differences, there could be also programmed physiological differences for germination initiation and dormancy breakage caused by climates.

Most studies with oospores have been carried out on *Nitella* and *Chara* species from permanent waterbodies belonging to the zonobiomes III (sub-tropical arid climates of semi-deserts and deserts; Pott (2005)) and VIII (boreal zone, Table 3). In contrast, most studies on germination use sediments from zonobiome VI, especially from the Baltic Sea (Table 4), while studies on oospore germination are rare.

Studies of *Chara* species from permanent waters in the Northern Hemisphere are less common. Additionally, most studies to date have focused on secondary dormancy and only a few acquire primary dormancy by leaving the mother plant (Forsberg 1965a, Shen 1966) and on differences in the degree of dormancy (Sederias and Colman 2009), see Table 2. Collectively, these studies have shown that species-specific differences exist due to different temporal and spatial offsets of charophytes (Casanova and Brock 1990, Bonis and Grillas 2002) and that freshly extracted oospores have a higher dormancy level than

those extracted from sediments, which may be explained by the inhibitory effect of abscisic acid (e.g. Takatori and Imahori (1971), Casanova and Brock (1996)), which is gradually reduced during sediment storage (Sabbatini et al. 1987, Sederias and Colman 2007, Penfield and King 2009). The most effective method for breaking the dormancy of different species of *Nitella* and *Chara* is listed in Table 2.

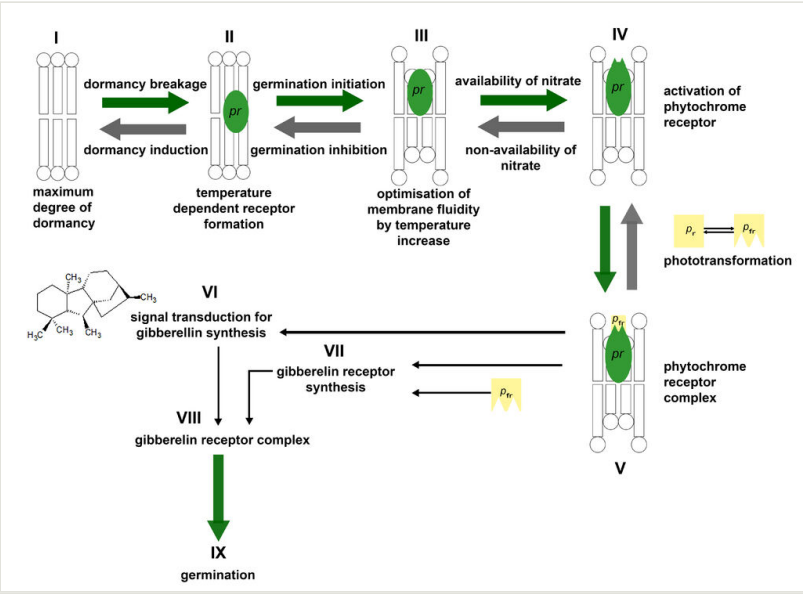

Figure 3.

Modified hypothetical physiological dormancy model of Hilhorst (1998). Originally this model was developed for seeds of magnoliophytes in soil seed banks, but could be particularly applied on charophytes. I. the stage of maximum dormancy is characterised by lacking phytochrome receptors at the plasma membrane. II. The formation of phytochrome receptors (pr) is temperature-dependent. III. Optimisation of membrane fluidity by increased temperatures. IV. activation of phytochrome receptors by nitrate. V. Formation of photoreceptor complexes by binding of active phytochrome (P_{fr}). VI. signal transduction for gibberellin synthesis. VII. gibberellin receptor synthesis VIII. gibberellin receptor complex by binding of gibberellin or gibberellin precursors. IX. germination initiation.

Field studies have shown that charophytes of permanent waters emerge mainly in spring or summer, in their so called “temporal window” (Stross 1989). However, some species prefer autumn or biennial germination periods. The German Charophyte Monograph “Armleuchteralgen. Die Characeen Deutschlands” (Arbeitsgruppe Characeen Deutschlands) and the forthcoming European Monograph (International Research Group of Charopytes, https://www.niva.no/en/featured-pages/international-research-group-on-charophytes) provide a good overview of species-specific ecological niches. In contrast to this, the germination of oospores in temporary waterbodies is dependent on fluctuations in the water level. Adaptions to these unpredictable variations include short or annual life cycles for *C. braunii*, *C. canescens*, *Tolypella* or *Nitella* species (Casanova and Brock 1990, Brock and Casanova 1991). Although interannual differences cause a high variability of initial

environmental conditions on germination and composition of aquatic plant assemblages, simulation studies have shown the effects of the human influence (Rodríguez-Merino et al. 2017). Therefore, it will be necessary to harmonise economic, agricultural and recreational use in order to protect endangered charophyte species as done for *T. salina* from Mediterranean salt marshes (Lambert et al. 2013).

Table 3.

Oospore germination approaches including light and temperature conditions. Listed are the species name, the usual habit (occurrence in temporary/permanent waterbodies), the zonobiome (in correspondence with the published material), experimental temperature(s) in °C (T), the light cycle (LC, intensity in µmol photon/(s*m^2) and light:dark cycle in hours, method of germination approach and reference. Missing information is marked with -. Zonobiomes are abbreviated after Pott (2005) in Roman numbers, zonobiomes are listed below Table 2.

Species	Habitat	Zonobiome	T	LC	Method	Reference
Chara aculeolata	p	VIII	20-25	N/A | N/A	soil-water	Forsberg (1965c)
C. aspera	p	IV	21-29	298 ± 20 | 14:10	Petri dishes with autoclaved sediments, plastic tanks	Calero and Rodrigo (2019)
		VI	10-16	300 | 12:12 and 16:8	glass cylinder with sediment and lake water	van den Berg et al. (1998)
		VIII	20-25	~ 40 | continuous 95 | 16:8	soil-water	Forsberg (1965c)
		VI	20	N/A | natural light	sediment-lake water in 30 l aquarium	van den Berg et al. (2001)
		VIII	room temp.		400ml beakers, sterilised sediment, GF/C-filtered water	Blindow et al. (2009)
C. australis	p/t	V	14.4 – 27.8	N/A | N/A	plastic dishes	Casanova and Brock (1996)
	p	V	14-16	0.1-147 | 14:10	sediments in 30 l tanks with sieved oospores	de Winton et al. (2004)
C. braunii	p/t	I/V/VI	22	< 30 | 16:8	compost-agar	Holzhausen et al. (2022)
		III	25 (50)	N/A | N/A	soil-water	Henderson (1961)
		III*	24	N/A | continuous	soil-water	Proctor (1962)
		III**	25	~ 10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
C. canescens	p/t	IV	21-29	206 ± 26 | 14:10	Petri dishes with sterilised sediments, plastic tanks	Calero and Rodrigo (2019)

Species	Habitat	Zonobiome	T	LC	Method	Reference
		III*	24	N/A | continuous	soil-water	Proctor (1962)
C. contraria	p/t	N/A	22	~ 50 | 12:12	sterilised oospores on agar	Shen (1966)
		III	25	N/A | N/A	soil-water	Henderson (1961)
		III*	24	N/A | continuous	soil-water	Proctor (1962)
		III**	25	~10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
		VII	23	90-100 | 16:8	sterilised oospores in agar with soil-water extract	Kiss and Staehelin (1993)
C. cf. *contraria*	p	VI	20	400 | 12:12	PVC cylinders, rewetted riverine clay + loamy mud or fine sand, tap water	van Zuidama et al. (2014)
C. corallina	p/t	III**	25	~10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
C. delicatula	p	III	20	0-10 | continuous bright light, continuous dim light, dark	sterile test tubes with 1.2% agar	Takatori and Imahori (1971)
C. globularis	p	V	14-16	0.1-147 | 14:10	sediments in 30 l tanks with sieved oospores	de Winton et al. (2004)
		III	25	N/A | N/A	soil-water	Henderson (1961)
		III*	24	N/A | continuous light	soil-water	Proctor (1962)
		VIII	20-25	~40 | continuous	soil-water	Forsberg (1965c)
		III**	25	~10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
		V	N/A	N/A | 6% ambient light	pots with sediments	Matheson et al. (2005)
C. gymnopitys	p	V	-	N/A | continuous	sterilised oospores in Thunberg tubes	Ross (1958)
		III**	25	~ 10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
C. hispida	p	IV	21-29	298 ± 20 | 14:10	Petri dishes with autoclaved sediments, plastic tanks	Calero and Rodrigo (2019)
		VIII	20-25	~ 40 | continuous	soil-water	Forsberg (1965c)

Species	Habitat	Zonobiome	T	LC	Method	Reference
C. hornemannii	p	III*	24	N/A | continuous light	soil-water	Proctor (1962)
C. hydropithys	p	III**	25	~ 10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
C. intermedia	p/t	VI	10	N/A | dark conditions	water	Budnyk et al. (2016)
C. muelleri	t	V	14.4 – 27.8	N/A | N/A	plastic dishes	Casanova and Brock (1996)
C. sejuncta	p	III	20-25	~ 40 | continuous	alcohol + calcium hypochlorite sterilised oospores in test tubes	Forsberg (1965c)
C. vulgaris	p	VIII	20-25	~40 | continuous	soil-water	Forsberg (1965c)
		III	20-30	N/A | dim light	glass jars with sandy loam + tap water	Grant and Proctor (1972)
		VIII	20	N/A | 12:12 (wet/dry)	Petri dishes with 0.2% agar and degassed tap water	Sederias (2003)
		VIII	22	N/A | natural daylight, north-directed window	Petri dishes with BBM, sterilisation with 10% sodium hypochlorite	Kalin and Smith (2007), Sederias and Colman (2007), Sederias and Colman (2009)
C. zeylanica	p/t	N/A	22	~ 50 | 12:12	sterilised oospores on agar	Shen (1966)
		III	25 (5-37)	N/A | N/A	soil-water	Henderson (1961)
		III	24~40	N/A | continuous light	soil-water	Proctor (1962)
		VIII	20-25	N/A | continuous	alcohol + calcium hypochlorite sterilised oospores, soil-water test tubes	Forsberg (1965c)
		III**	25	~ 10 | 12:12	Kanuma clay or 2% agar	Imahori and Iwasa (1965)
		III	10-12 (night), 18-20 (day	50 | outdoor	DEPS in polyethylene trays	Stross (1989)
Nitella cristata var. *ambigua*	p/t	V	14.4 – 27.8	N/A | N/A	plastic dishes	Casanova and Brock (1996)

Species	Habitat	Zonobiome	T	LC	Method	Reference
N. flexilis	p	VIII	20 (wet/dry)	N/A | 12:12	Petri dishes with 0.2% agar and degassed tap water	Kalin and Smith (2007)
N. furcata subsp. *megacarpa*	p	VI	18	monochromatic light | 4, followed by darkness and 18 or broad-band light	112-ml glass jars or shell vials on Bactoagar + membrane filtered lake water	Sokol and Stross (1992)
N. hyalina	p	IV	21-29	298 ± 20 | 14:10	Petri dishes with autoclaved sediments, plastic tanks	Calero and Rodrigo (2019)
N. macrocarpa	p	III**	25	~ 10 | 12:12 |	Kanuma clay or 2% agar	Imahori and Iwasa (1965)

- * Oospores in Proctor (1962) were obtained from the faeces of waterfowls. Only *Chara braunii* and *Chara zeylanica* are present at the study site in Texas. The origin of the oospores is therefore unknown.
- ** Imahori and Iwasa (1965) mentioned the use of material from Proctor's experiments (oospores from Texas).

Table 4.

Sediment oospore germination approaches. Listed are the species name, the usual habit (occurrence in temporary/permanent waterbodies), the zonobiome (in correspondence with the published material), experimental temperature(s) in °C (T), the light cycle (LC, intensity in µmol photon/(s*m^2) and light:dark cycle in hours, method of germination approach and reference. Missing information is marked with -. Zonobiomes are abbreviated after Pott (2005) in Roman numbers, zonobiomes are listed below Table 2.

Species	Habitat	Zonobiome	T	LC	Method	Reference
Chara australis, *C. globularis*	p	V	14-16	0.1-147 | 14:10	sediments in 30 l tanks	de Winton et al. (2004)
C. australis, *C. muelleri*, *Nitella sonderi*, *N. stuartii*, *N. subtillissima*, *N. tasmanica*	p/t	V	14.4 - 27.8	treatments: (I) wet winter (II) dry winter (III) dry summer germination in glass houses	plastic dishes	Casanova and Brock (1996)
C. baltica var. *liljebladii*, *C. contraria*, *C. globularis*, *C. canescens*, *Nitellopsis obtusa*	p	VI	15	15-130 | 16:8	sediments with filtered habitat water	Holzhausen (2016), Holzhausen et al. (2017)

Species	Habitat	Zonobiome	T	LC	Method	Reference
C. canescens	p	VI	15	~ 2 | 12:12	glass beakers, sediment, site waters	Blindow et al. (2016)
C. canescens	t	V	N/A	N/A | N/A	sediments, plastic containers (4 l), fresh water	Casanova and Nichols (2009)
C. canescens, *C. contraria*, *C. vulgaris*, *Tolypella nidifica*	p	VI	15	100 ± 20 | 12:12	glass beakers with sediment and lake waters	Steinhardt and Selig (2011)
C. contraria, *C. globularis*, *C.* sp., *N. obtusa*	p	VI	15	100 ± 20 | 12:12	glass beakers with sediment and lake waters	Steinhardt and Schubert (2012)
C. connivens, *C. aspera* *T. nidifica*, *Lamprothamnium papulosum*	p	VI	15	80-90 | 12:12	glass beakers with sediment and site waters	Nowak et al. (2017)
C. filiformis	p	VI	15	15-20 | 16:8	sediments with filtered habitat water	Holzhausen (2016), Holzhausen et al. (2017)
C. corallina, *C. fibrosa*, *C. globularis*, *N. hookeri / cristata*, *N. leptostachys*, *N. pseudoflabellata*	p	V	N/A	N/A | natural light cycle	outdoor (1.3 m wide×7m long×1 m deep) with ~ 0.5 m water depth, covered with 92% shade mesh	de Winton et al. (2000)
C. fibrosa, *C. zeylanica*	p	II	N/A	N/A | direct sunlight	pots	Guha (1996)
C. hispida, *C. vulgaris*	p	IV	N/A 20	N/A | natural light cycle N/A | natural light cycle 45 | 12:12	outdoor, plastic tank with dechlorinated tap water, covered with metal sheets indoor	Rodrigo et al. (2010) Rodrigo and Alonso-Guillén (2013)
C. spp.	p	V	20	5 | 14:10	plastic pots (130 ml) in containers (115 l) with sediment-water	Kelly et al. (2012)

Species	Habitat	Zonobiome	T	LC	Method	Reference
L. macropogon	t	V	N/A	N/A | partial sunlight with 15% shade by light fibreglass screen mesh; salt treatment	outdoor, polyethylene plastic tanks (1,500 l) with tap water	Porter (2007)
L. sp. aff. *macropogon*	t	V	N/A	N/A	plastic trays (500 ml) in 20 l containers with saline waters	Casanova et al. (2011)
L. papulosum, *N. flexilis*/*opaca*	p	VI	15	30-130 | 16:8	sediments with filtered habitat water	Holzhausen (2016), Holzhausen et al. (2017)
Lychnothamnus barbatus	p	VI	15	30-40 | 16:8	sediments with filtered habitat water	Holzhausen (2016), Holzhausen et al. (2017)
N. micklei, *N. parooensis*	t	II	N/A	N/A	plastic trays with tap or rain waters	Casanova and Porter (2013)
N. mucronata	p	VIII	20	N/A | daylight, south-directed window	glass beakers with sediment and dechlorinated tap water	Stobbe et al. (2014)
N. mucronata, *T. glomerata*	p	VI	15	110-130 | 16:8	sediments with filtered habitat water	Holzhausen (2016), Holzhausen et al. (2017)
N. sp., *C.* sp.	p	IV	N/A	N/A | greenhouse	tanks with sediment and rain water	Casanova (2015)
T. nidifica/*salina*	(p)/t	VI	15-20 salt treatment	110-130 | 16:8	glass beakers with sediment and salt waters	own unpublished results
C. contraria, *C. globularis*, *C. hispida*	p	VI	15-18	110-130 | 16:8	glass beakers with sediment, Wüstenberg medium	Holzhausen (2018)
C. aspera, *C. contraria*, *C. globularis*, *C.* sp.	p	VI	15-18	110-130 | 16:8	glass beakers with sediment, Wüstenberg medium	Holzhausen (2019)

Temperature

The influence of temperature on oospores has been considered by several authors, both on dormancy breakage (Table 2) and on germination of oospores. The temperatures used for germination approaches with oospores or sediments are summarised in Table 3 and Table 4. In most cases, oospores or sediments were pre-treated for days or weeks using cold temperatures between 4°C and 10°C to mimic natural vernalisation. Despite conflicting results, the most effective method for breaking dormancy and subsequently inducing germination appears to be cold treatment of oospores followed by irradiation with long-wave light. There are, however, species-specific and biogeographical differences. For example, the dormancy of freshly-extracted oospores (primary dormancy) of *C. vulgaris* was broken by low temperatures, whereas, for the germination of *Nitella furcata* from New Zealand, storage at room temperatures is preferred (de Winton et al. 2000). The origin of the samples and the associated climatic conditions appear to be of critical importance. The evaluation of the available data shows that a correlation between conditions for dormancy breakage and zonobiome can be made (Table 2). For example, almost all species in zonobiome V (warm temperate zonobiome) can be broken out of dormancy by pre-treatment at 15°C, whereas species in zonobiome VI require a cold storage phase.

Light availability

The growth, the preferred reproduction modus and depth distribution of charophytes is determined by the availability of light and the underwater light climate (Barko and Filbin 1983, Canfield et al. 1985, Küster 1997, Middelboe and Markager 1997, Sagert and Schubert 1999), but also by the germination of oospores. However, multiple interactions with ecological factors such as temperature (e.g. van den Berg et al. (1998)) or phytoplankton biomass (Brunet et al. 2011) hamper the consideration of all light levels seperately. Increasing eutrophication, characterised by an increase in nutrient concentration and associated phytoplankton growth, can also rapidly terminate the existence of charophytes. A number of studies have shown that the reduced availability of light, caused by a high density of phytoplankton, can be a decisive factor in the decline of charophytes (Blindow 1992, Arthaud et al. 2012). In addition, light acts in a variety of ways. Photon flux density, spectral composition or the duration of light exposure (periodicity) can all have important signalling effects. For seeds, studies have also shown that the genetic basis, the position in the sediment, the absorptive capacity of the coat and the state of dormancy play a crucial role (Grime 1979, Grime et al. 1981, Pons 2000). Results are partly contradictory despite the large number of studies on light-induced oospore germination. While several authors have attributed a positive effect to the intensity and quality of the light, others have not observed any effect of these factors on the germination of oospores (Shen 1966, de Winton et al. 2004). This inconsistency may be due to biogeography, species specificity, the type of sample material used (oospores versus sediment sample, fresh oospores versus oospores from diaspore banks) and the synergy thereof. The results of existing literature are summarised Table 3 and Table 4, divided into experiments with sediments or only oospores separated from sediments. For example, Shen (1966) did not observe any effect of light on the germination of *C. contraria* oospores,

whereas Sabbatini et al. (1987) identified this factor as the breaking of dormancy. In this case, however, it is difficult to derive a biogeographical response, because Shen does not specify the origin of the oospores of *C. contraria*. Sabbatini et al. (1987) use oospores from zonobiome III, which come from the dry areas of the temperate zone, characterised by strong annual rainfall variations. These inter-annual rainfall variations, combined with high summer temperatures, initiate the positive trigger for light-induced germination. Similar contradictions could be found for *C. globularis*. Proctor (1967) described a positive effect of light, while de Winton et al. (2004) and Holzhausen et al. (2017) could not confirm this. Furthermore, de Winton et al. (2000) demonstrated the germination capacity of *C. australis* and *C. globularis* under dark conditions (< 0.5 µmol photons m^{-2} s^{-1})). However, there are differences in methodology, particularly in the origin which may explain the wide range of response behaviour (Table 3). In any case, such inconsistencies highlight the need for further basic research on charophyte dormancy and germination to develop reliable, species-specific protocols, including habitat and geographical differences (permanent waters vs. temporary waters, zonobiomes) for continuous cultivation allowing the full life cycle.

Nutrients and phytohormons

The chemical and physical properties of the substrate and medium, including invertebrates and microbes that could change properties and nutrient/phytohormone supply, determine the success or failure of charophyte germination and growth, in addition to the main cues of temperature and light. This includes aspects such as:

- **oospore sterilisation agent and concentration** (Forsberg 1965c, Sederias and Colman 2007, Sederias and Colman 2009, Holzhausen 2019, Holzhausen et al. 2022);
- **inorganic media** (Forsberg 1965a, Forsberg 1965b, Forsberg 1965c, Proctor 1967 , Andrews et al. 1984a, Wüstenberg et al. 2011);
- **content of organic material** (Buljan 1949, Gumiński 1983, Smart and Barko 1984, Kalin and Smith 2007, Pörs and Steinberg 2012, Holzhausen 2016, Holzhausen et al. 2017, Holzhausen et al. 2022);
- **cyanobacteria/phytoplankton** (Casanova et al. 1998, Rojo et al. 2013a, Rojo et al. 2013b, Fukushima and Arai 2015, Pełechata et al. 2016);
- **nutrient concentration** , for example, phosphorus, sulphides or nitrates (Reid et al. 2000, Sederias and Colman 2009);
- **pH value** (Shen 1966, Kim and Mun 1997, Quanter 2020);
- **phytohormones** (Sederias and Colman 2007, Tarakhovskaya et al. 2007, Holzhausen et al. 2022);
- **salt concentration** (Winter and Kirst 1990, Winter et al. 1996, Shepherd et al. 1999);
- **substrate density, structure and water content** (Boedeltje et al. 2002, Matheson et al. 2005, Porter 2007);
- **antibiotics** (Christian 2004);

- activity of **benthic invertebrates** (Kuczewski 1906, Fukuhara and Sakamoto 1987, Kotta et al. 2004, Hansen et al. 2011);
- **epiphytic associated microorganisms** (Hempel et al. 2008, Kataržytė et al. 2017, Rodrigo et al. 2021);
- beneficial **growth promoting substances and phytopathogens** (Wajih and Sinha 1980, Ghazala et al. 2004, Lusweti and Pili 2021).

Interestingly, studies have shown that pure inorganic medium inhibits oospore germination (Imahori and Iwasa 1965). This was strongly supported by a high percentage of germination experiments (see Table 3). In most cases, the addition of organic compounds/ organic material was critical to induce charophyte germination. However, this also increased the risk of unwanted contamination. However, there is a lack of studies on the interaction of germination-promoting micro-organisms. For terrestrial plant seeds and macroalgae, promoting seed germination and growth development was confirmed by producing phytohormones providing water and/or minerals, nitrogen fixation or pathogen defence (Tsavkelova et al. 2007, Spoerner et al. 2012, Wichard 2015). For Characeae, information on associated bacteria is rare (Hempel et al. 2008, Kataržytė et al. 2017) and information on soil microbes, especially in the rhizosphere of *Chara* meadows, is completely lacking. Staining the outer wall of oospores with DAPI showed nucleic acid-rich parts along striae (Fig. 2H).

For most of the above aspects, there is a consensus in the currently available literature. Discrepancies and partly contradictory results exist only for the use and influence of sterilising agents. These range from combined alcohol and calcium hypochlorite to sodium hypochlorite and hydrogen peroxide. The same applies to the concentration (1% 30%) of substance used. While some authors have not been able to detect any effect on the germination of oospores, others have reported an inhibition of germination after the use of high concentrations of hydrogen peroxide (Forsberg 1965c, Holzhausen et al. 2022).

Desiccation/ redox potential

Seeds stored in sediments show long-term viability, whereas seeds stored in moist conditions may lose viability over time (Villiers 1974). Changes in desiccation/redox potential manipulation have also been observed to have species-specific treatment responses. Desiccation of *Nitella cristata* var. *ambigua* oospores increases germination, whereas no effect was observed for *Nitella sonderi* (Casanova and Brock 1996). Dried oospores of *C. canescens*, *C. contraria*, *C. evoluta*, *C. hydropithys*, *C. globularis*, *C. rusbyana*, *C. sejuncta* and *C. zeylanica* germinated after freezing (-20°C), cold (3°C) and warm temperatures (24°C) over a period of up to four years in studies by Proctor (1967). A high percentage of germination of *C. canescens* after six years of wet storage (room temperature, dark) has been shown in our own experiments (Holzhausen, unpublished).

Furthermore, a decisive effect of desiccation and salt concentration on germination has been shown in studies of the germination capacity of *Tolypella salina* oospores from different French salt-marsh sediments (unpublished data). A significant increase in the number of seedlings after desiccation was observed when comparing germination rates of

wet and pre-dried sediments. These results suggest that targeted temporary desiccation and reduction of organic matter layer could allow for a continuous recolonisation of *Tolypella salina* in France.

Seed size and burial depth

In addition to physiological triggers, seed size and burial depth could have an influence on germination. Only a small number of studies on this subject can be found in literature. The seed size of oospores and spores allows the availability of energy reserves in the form of starch grains as a resource for the growth of the transparent seedling part (Venable and Brown 1988, Casanova and Brock 1990, Casanova and Brock 1996), using New Zealand oospores. Their results of highest germination rates up to 50 mm sediment depth were confirmed by most germination studies of sediment samples. Interestingly, they found a relationship between seed size and burial depth: Heavier *C. globularis* oospores germinated from deeper sediment zones than lighter *Nitella* oospores. Those oospores from deeper sediment layers can be considered as a potential internal oospore bank as shown by restoration studies of so-called “ghost-ponds” (Alderton et al. 2017, Sayer et al. 2023). Mediation by soil invertebrates is only known for the seeds of, for example, *Zostera marina* (Blackburn and Orth 2013), but not for charophytes.

Growth and *in-vitro* cultivation

Charophytes have been cultivated for more than 100 years. In most cases, charophytes were cultivated using laboratory-based or greenhouse-based techniques. Problematically, epiphyte overgrowth occurs regardless of the oxygen level in the vessels or the duration of cultivation. Only a few authors (Kuczewski 1906, Karling 1924) have attempted to overcome these problems by adding grazers such as the water snail *Limax paludosa* or daphnia. A drastically reduced selection of cultivation experiments and conditions can be found in Table 5. Due to a lack of information on cultivation and propagation methods, studies involving short-term experiments on photosynthesis are not listed. Nevertheless, they may provide information on light intensities applicable to cultivation methods. In recent decades, aquaculture and outdoor mesocosms have been additionally used to grow and reproduce algal material, for example, for (electro-) physiological studies or regeneration of aquatic systems (e.g. Tazawa et al. (1979), Tazawa et al. (1987), de Winton et al. (2000), Beilby et al. (2006), Rodrigo et al. (2010), Holzhausen (2019), Blindow et al. (2021)). In contrast to constant indoor cultivation conditions, outdoor experiments or greenhouse cultivation require the documentation of environmental conditions to determine influencing factors such as growth, expression of morphological traits or gametangia production. Standardisation of these methods is often lacking. In addition to the extreme growth of microorganisms in culture vessels, the development and maturation of gametangia is often suppressed completely or over time. According to the existing literature, this does not only depend on the periodicity or preference for vegetative growth, but also occurs with repeated reactivation of the material, as can be seen in Karling (1924). The influence of various abiotic factors such as temperature, light regime or media composition, including

amino acids and vitamins, has been the subject of a large number of studies over the last 100 years, but only a few have considered the development of gametangia during experiments. Effects of amino acids and vitamins, added individually or in mixtures, on the growth of protonemata and adult thalli of *Chara zeylanica* were studied by Imahori and Iwasa (1965). Here, they identified casein hydrolysate and polypeptone as effective for promoting growth in protonemata and adult plants, whereas yeast extract and individual amino acids were only marginally effective. In particular, the growth of adult plants was promoted by the vitamins and phytohormones cobalamin, nicotinamide, GA and kinetin (6-furfurylamino-purine). However, thiamine (B1) and pyridoxal (B6) only promoted the growth of the protonemata. Further studies were carried out by Libbert and Jahnke (1965) who found an antagonistic effect of indoleacetic acid/auxin (IAA) and antiauxin (PCIB) in *C. vulgaris* (= *Chara foetida*), *Chara hispida* and *Chara subspinosa* (= *Chara rudis*).

Table 5.

List of references for ***in-vitro*** **cultivation approaches.** Listed are the species name, the cultivation conditions, the observation as well as the respective reference.

Species	Cultivation conditions	Observations	Reference
Chara braunii	aquarian cultures in greenhouse, tap water, sandy loam soil, with snails	• crosses for *C. braunii* failed only for one strain; all other produce oogonia with subsequent darkening by self-fertilisation or crossing • crosses between *C. braunii* and *C. australis* and *C. corallina* were unsuccessful	Proctor (1970)
C. braunii	2000-ml glass vessel or 900-ml glass jar, soil–water medium (SWC-1), 20-25°C, fluorescent lamps (L:D = 12:12), 10–40 µmol m^{-2} s^{-1}	• genetic analysis: Japanese *C. braunii*, formed a monophyletic group, Hawaiian samples represented the sister taxon; two haplotypes (RBCL-1 and RBCL-2) in Japanese *C. braunii* group • morphological analysis: not correlated with genetic results (oospores, stipulodes and bract cells)	Kato et al. (2008)
C. canescens		oospore length differences by temperature and light, substrate and culture medium	Ernst (1918)
C. corallina	Soil-water medium, windowsill with natural light/dark cycle	Aneuploidy and polyploidy in charophytes; cytogenetic species	Chaudhary and Dash (1991)
C. corallina	aquarium with deionised water, fluorescent lamps (L:D = 14:10), soil/sterilised forest soil	anion channels, *in-vivo* caspase-3-like proteinase activity	Berecki et al. (1999), Korthout et al. (2000)

Species	Cultivation conditions	Observations	Reference
C. corallina	tap water with soil extract and rotten leaves, plastic buckets, 25 ± 1°C, fluorescent lamps with 50-60 µmol/m^2s^{-1}), L:D = 15:9	intracellular transport of photoassimilates	Ding et al. (1991b)
C. foetida	dark half-dark full daylight	loss or abnormal cortication in limited light conditions	Müller (1907)
C. fragilis	windowsill (all cardinal directions)/greenhouse/electric illumination	gametangia development independent of collection date and continuous illumination	Karling (1924)
C. fragilis, *C. foetida*	Glass aquarium 4 l; sludge, garden soil, quartz sand or dolomite sand as substrate and/ or floating cultures; destilled water, spring water or mineral media (Detmer, Crone, Beyerinck, Artari, Pringsheim and Benecké); addition of "Purissimum" and "Pro Analysi" salt.	modified mineral medium of Crohn; bicarbonate/CO_2 experiment; effects of chemical compounds on charophyte cultivation	Buljan (1949)
C. fragilis, *C. foetida*, *C. contraria*	Munich tap water, thalli w/wo rhizoids	Effect of $CuSO_4$, alkaloids, strychnine, nicotine and caffeine on the morphology of gametangia	Wallner (1932)
C. fragilis, *C. foetida*, *C. coronata*, *Nitella mucronata*	Vessels with sludge, garden soil, clay, sand or quatz; sunlight, shade and deeper shade	effects on different soils and light conditions on charophyte cultivation	Vouk and Benzinger (1929)
C. globularis	aquarian cultures in greenhouse, tap water, sandy loam soil, with snails	no morphological differences between clones in stipulode development, degree of branchlet cortication, spine cell configuration, length to width ratio of oospores and coronulae shape	Proctor (1980)
C. hispida	windowsill	loss of cortication	Richter (1894)
C. strigosa	cultivation in sodium chloride solution under full or reduced light conditions	high light conditions + full light leads to reduced length growth	Nonweiler (1907)
C. vulgaris	buffered lake water (HEPES); 22°C, 14:10	15W lumilux tubes sterile plants: 10 h light:14 h dark	Schulte et al. (1994)
C. vulgaris	Forsberg mineral medium, Flora white lamps (6.2W*m^{-2}), L:D = 14:10 L:D = 24:0 L:D = 1:23	continuous illumination: exceeding mitotic activity (new nodes), shortens internodal cells, increased rhizoid formation, initiate antheridia, reduced oogonia formation; prolonged darkness: halves the mitotic activity, prolongs internodal cells, blocking of rhizoid formation, reduction of antheridia and oogonia formation	Maszewski (1980)
N. flexilis	outdoor conditions	high temperature and sunlight influenced oospore production	Hodgetts (1921), Hodgetts (1922)

Species	Cultivation conditions	Observations	Reference
N. sp.	autoclaved 2000 ml glass vessel or 900 ml glass jar, autoclaved soil-water medium (SWC- I or SWC-2), fluorescent lamps (L:D = 16:8), 10–40 µmol m^{-2} s^{-1}	combination of scanning electron microscopy and *atp*B and *rbc*L gene sequences resulted in five oospore clades: reticulate or papillate (RP), finely granulate (FG), fibrous (FIB), very finely granulate (YFG) and tuberculate	Sakayama et al. (2004)

Biomolecular studies on charophytes

The number of biomolecular studies on the Characeae is lower than on other plant organisms. However, over the last 100 years, various cell-wall studies, phytohormone studies and cytological studies have been carried out that give first insights into cell composition and cytology of charophytes and are currently investigated in more detail. Especially, phytohormones are known as regulators for development and reproduction in, for example, mosses or land plants. However, only few studies exist for charophytes which describe the regulation of germination and gametangia development by use of phytohormones. The knowlege on biosynthesis and molecular pathways is lacking, but is under investigation.

In addition to the cell walls of antheridia, the **chemical composition** of charophytes has also been studied in internodal cell walls of various species, mainly to elucidate the ion exchange in the cell walls. Overall, charophytes contain the same proteins, pectates, lignin, hemicellulose and cellulose as land plants (Anderson and King 1961a, Anderson and King 1961b, Anderson and King 1961c, Anderson and King 1961d, Foissner et al. 1996, Proseus and Boyer 2005, Proseus and Boyer 2006, Proseus and Boyer 2012). Only the percentages of these differ from those of land plants, for example, the values for lignin in roots/rhizoids. The highest proportions of uronic acid anhydrides and proteins were found in dried cells of *C. australis*, *C. vulgaris* (= *C. foetida*) and *N. translucens* (5-50% of ash). Sugars such as glucose, uronic acid, galactose, arabinose, xylose, mannose and rhamnose were detected in descending order of abundance. Recent studies have shown that *Nitellopsis obtusa*, *C. aspera* and *C. subspinosa* lack hydroxyproline and arabinogalactan proteins, which are common in land plants (Pfeifer et al. 2022, Pfeifer et al. 2023).

Over the last 30 years, Foissner and co-workers have published a vast amount of **cytological and molecular** work on *C. australis*, *C. braunii* and *Nitella* internodal cells. This includes wound healing, exocytosis (Foissner et al. 1996), nuclei fragmentation (Foissner and Wasteneys 2000), the detection of sterol-rich domains (Klima and Foissner 2008), the formation of lipid droplets near the endoplasmatic reticulum (Foissner 2009), the involvement of charasomes in pH banding (Schmolzer et al. 2011, Foissner et al. 2015, Sommer et al. 2015, Eremin et al. 2019) and the inhibition of vesicle transport by Bredfield A (Bulychev and Foissner 2020). The majority of these studies have been carried out using

FM labelling. In addition, molecular and biochemical work was done on vesicular transport and plasma membrane repair by identifying a CaARA6-like protein with GTPase activity and a CaVAMP72 protein (Hoepflinger et al. 2013, Hoepflinger et al. 2014), as well as studies on OH-transporters including the identification of the Slc4-like gene CaSLOT (Quade et al. 2022). Genomic data and protocols available for *C. braunii*, *C. australis* and *C. corallina* have accelerated the ongoing molecular work on charophytes using *in-vitro* cultures (Tsutsui et al. 1987,Nakanishi et al. 1999, Nishiyama et al. 2018, Bonnot et al. 2019, Phipps et al. 2021, Haraguchi et al. 2022, Quade et al. 2022, Heß et al. 2023).

Phytohormone signalling is known to be essential for seed maturation, dormancy, germination and senescence. In land plants and bryophytes, the roles of abscisic acid (ABA), gibberellic acid (GA_3) and auxin are well studied. Their associated enzymes are key players in plant signalling processes, germination and stress response (von Schwartzenberg 2006, Anterola et al. 2009, Thelander et al. 2018). Within Characeae, little is known about the detailed gene regulatory network and the proteins involved in transporting phytohormones and performing specific functions. In the past centuries, the focus on physiological studies has dominated this research, which, for example, demonstrated the auxin accumulation in charophytes. Here, the first studies on auxin in charophytes were carried out by the group of Libbert and co-workers. The synthesis of IAA via a tryptophan-independent pathway was suggested by cut-off studies of apices (Libbert and Jahnke 1965). The presence of polar auxin transport (PAT) in internodal cells and membrane-associated PIN2-like proteins in male antheridia during proliferation, but not during spermiogenesis is confirmed by immunofluorescence labelling studies in *C. corallina* and *C. vulgaris* (Boot et al. 2012). Interestingly, PIN2-like protein expression and auxin accumulation were found to be associated with callose and plasmodesmata connectivity between cells, suggesting that these functional features evolved long before land plants. The highest expression and accumulation were found in capitula cells. In addition, the use of exogenous IAA had been shown to: (i) shorten the proliferative period while PCIB reduces mitotic activity (Godlewski 1980, Zabka et al. 2016) and (ii) ensure the presence of rhizoids during the development of gametangia (Sievers and Schröter 1971) However, recent phylogenetic analyses, based on genomic data, could confirm proteins involved in phytohormone biosynthesis and signalling (Feng et al. 2023).

Within the charophytes, hormone extraction was performed for *C. braunii* and *C. australis* including salicylic acid (SA), ABA, jasmonic acid (JA), indole-3-acetic acid (IAA), jasmonate-isoleucine conjugate (JA-Ile), indole-3-acetyl-aspartate (IAA-Asp), strigolactones and the JA precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA) using LC-MS/MS methods and deuterium-labelled standards (e.g. Delaux et al. (2012), Hackenberg and Pandey (2014), Beilby et al. (2015), Waters et al. (2017), Schmidt (2021)). After detecting low levels of ABA in *C. foetida* cells (Tietz et al. 1989), Hackenberg and Pandey identified the associated G-proteins (Hackenberg and Pandey 2014). Like land plants, they could potentially be involved in phytohormone signalling pathways, as suggested by the findings of a synchronous seasonal and circadian change in ABA, serotonin and melatonin concentrations (Beilby et al. 2015). Furthermore, melatonin findings in *C. australis* have been suggested to be protective against reactive oxygen species. This compound is known

for the freshwater and brackish water species *C. tomentosa* with its reddish tips (Beilby 2016). Detailed interdisciplinary studies to elucidate this evolutionary feature, which has not yet been found in Zygnematophyceae or Coleochaetophyceae, are essential to elucidate its function, molecular signalling pathway and microorganism-associated association (non-sulphur bacteria and cyanobacteria). Evidence for the presence of the serine-threonine phosphatase (PP2C), which acts as an ABA signalling sensor (Ma et al. 2009) has been identified in the *Chara* genome (Nishiyama et al. 2018). Gibberellin-like substances have been ascertained in extracts of *C. braunii* (*C. coronata*) by paper chromatography (Murakami 1966). Additionally, different studies have shown a concentration-depending promoting effect of exogenous GA_3 concerning male gametangia development, germination initiation or RNA and protein biosynthesis in charophytes (Godlewski and Kwiatkowska 1980, Sederias and Colman 2007, Holzhausen et al. 2022). Concentrations of 10^{-7} and 10^{-8} M GA_3 shorten the duration of mitotic divisions in antheridial filaments, increases the number of spermatids per filament by about 200 and increases the lengths of spermatoid cells (Godlewski and Kwiatkowska 1980). Interestingly, Kwiatkowska and Maszewski found a stimulating effect of GA_3 on the incorporation of ^3H adenine into DNA and RNA independent of the cell cycle stage of antheridia as well as an increased capability of the antibiotics actinomycin D binding that indicates at least an indirect participation at the transcription level (Kwiatkowska and Maszewski 1979). The chemical compound responsible for the gibberellin activity could not be identified so far and it needs to be investigated in further studies. One possible explanation for land-plant-like reaction of exogenous gibberellins in charophytes could be the presence of uniport-transporters with activities for different nutrients and simultaneously phytohormone conjugates. Those transporters are well known for plants, especially within the nitrate/peptide transporter family (NRT/PTR) or the SWEET sugar transporters. Recently, in *Arabidopsis thaliana*, the uniporter SWEET13 (AtSWEET13) was identified to transport sugars and gibberellin.

Conclusions

In the previous sections, the wealth of studies on reproduction, germination including oospore dormancy and cultivation that can be used on an *ad hoc* basis and as fundamental resource has been presented. Both the low rates of germination and the difficulty of controlling epiphytic overgrowth have been described in the previous sections. Furthermore, the induction of gametangiogenesis in monoecious and diplostichous *Chara* species, such as *C. hispida*, is not successful under artificial *in-vitro* conditions, as shown by cultures of different populations in different common media (e.g. Forsberg (1965b), Andrews et al. (1984a), Wüstenberg et al. (2011)) and under continuous light cycles of 16 h light to 8 h dark or 12 h:12 h (Fig. 4).

In contrast, cultivation of *C. hispida* in outdoor mesocosms at the Rostock University showed that cultivation under natural conditions including temperature, light and continuous oxygenation induced germination, gametangia initiation and maturation (Holzhausen 2019). Logger data of temperature and light intensities including the light-dark

cycle were used to identify temperature and light ranges in which the processes of dormancy breakage and germination initiation, gametangia development and maturation take place. Although the decisive factor could not be identified, the transfer of the recorded logger data (temperature, light-dark cycle, intensity, Holzhausen (2019)) to *in-vitro* cultures showed that the induction of gametangia and maturation of oospores is possible (Fig. 4) using nutritional medium (Wüstenberg et al. 2011).

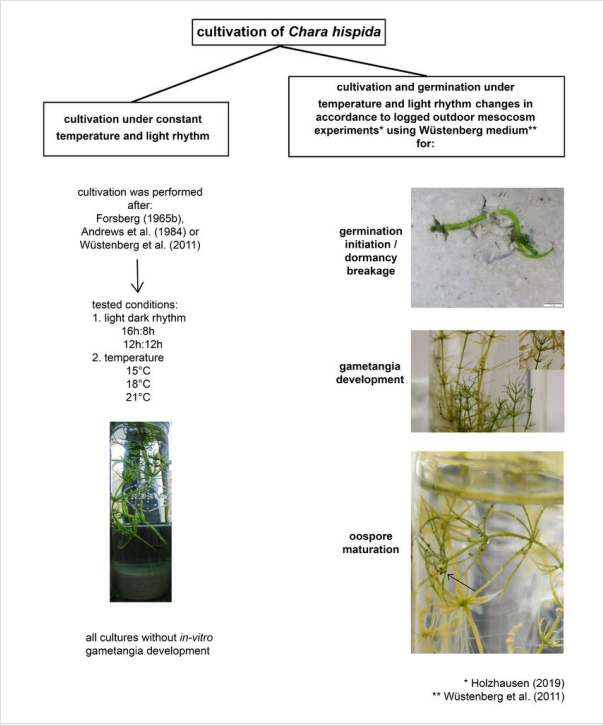

Figure 4.

Cultivation of *Chara hispida*. While no gametangia could be developed under conditions of common temperature or light regimes and nutritional media, the use of Eco-*in-vitro* cultivation including the use of logged environmental data from outdoor mesocosm experiments (Holzhausen 2019) is successfully proven for *in-vitro* gametangia initiation/maturation (photographs: A. Holzhausen).

This Eco-*in-vitro*-culture method could be particularly useful for species with low in vitro gametangiogenic potential such as *C. hispida*. In contrast to environmental alga material, these cultures have a drastically reduced microbiome which facilitates the identification of contaminant-specific and bacterial-specific gene sequences. The further understanding and integration of evolutionary, systematic and ecological knowledge will enable the establishent of various *in-vitro* (and axenic) cultures (axenic strain *C. braunii* S276, Holzhausen, unpublished) that will allow the identification of reproduction-related gene networks at different reproduction states and of different reproduction modes.

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Conflicts of interest

The authors have declared that no competing interests exist.

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