Effects of extrinsic abiotic factors on induction of gametogenesis and efficacy of a device for the segregation of non-fused gametes and zygotes in the green alga Ulva lactuca

Nikunj Balar, Santlal Jaiswar and Vaibhav A. Mantri

ABSTRACT
Seaweed aquaculture is one of the fastest growing marine sectors with 31.2 million tonnes year−1 of harvest worth over 11.7 billion USD. Commercial operations still rely on traditional techniques but innovative strategies are essential for future expansion. The present paper reports on a simple device that could effectively segregate non-fused haploid gametes from diploid zygotes in the green alga Ulva lactuca. The differential phototactic behaviour of gametes and zygotes was used for their separation. Approximately 56.2% non-fused haploid gametes and 5.3% diploid zygotes were separated after 5 min and after 1 hr and 2 hr, the composition of gametes was 26.6 and 8.8%, respectively while that of zygotes 32.7 and 57.4%, respectively. The effects of extrinsic abiotic factors, including light, temperature, salinity, photoperiod, dehydration and thallus fragmentation, on the induction of gametogenesis was found to be limited at 35°C (13.33 ± 5.77%) and a short day 9:15 h light:dark photoperiod (20 ± 4.08%), while dehydration treatments enhanced spore induction. This study demonstrated the efficient and cost-effective utilisation of locally available materials in producing a simple, small, precise, durable and easy to use system, which also saved on energy. The effective segregation of non-fused gametes from zygotes aids in cytological preparation, bio-chemical characterisation investigations as well as breeding and hybridisation all of which have direct implications on the future farming of Ulva and other species of seaweeds with similar life phases.

Introduction
Seaweeds are a diverse assemblage of marine macroalgae which are economically, as well as ecologically, important. This renewable resource is commercially utilised for food and animal feed as well as for the extraction of phycocolloids having varied applications (Barbier et al., 2019). Global seaweed production currently stands at 31.2 million tonnes year−1 (95% accounted to farming) worth 11.7 billion USD per annum with a 6.8% annual increase (Food and Agriculture Organization of the United Nations (FAO), 2018). To keep pace with ever-increasing seaweed feed-stock requirements, it is essential to develop innovative and sustainable technologies to bridge the supply and demand gap. Species of the green seaweed Ulva, commonly known as “ao-nori”, have been cultivated due to their high protein content, robust nature, exceptionally high growth-rate and broad range of tolerance to environmental fluctuations (Balar and Mantri, 2019). Existing commercial production is focused on niche, high-value, edible products in Japan with approximately 1,500 tonnes dry weight output per annum (Carl, 2014). However, unlike other commercially farmed seaweeds where clonal propagation is practiced, this genus relies solely on motile reproductive cells, i.e. zoospores or gametes, for large-scale farming. Thus, information pertaining to mechanisms of induction of reproduction in these economically important taxa is essential. Ulva lactuca has been utilised in aquaculture as an efficient bio-filter for mariculture effluents in IMTA system (Ben-Ari et al., 2014). Utility of biomass in human and animal nutrition is well established (Ortiz et al., 2006; Tabarsa, Rezaei, Ramezanpour, & Waaland, 2012). Species determination in Ulva requires examination of gametes as well as zoospores (Hiraoka et al., 2017) and determination of life cycle pattern (Liu, Yu, Yan, Zhang, & Zhou, 2015). Such studies have led to advances in laboratory culture (Vesty, Kessler, Wichard, & Coates, 2015) as well as out-door tank culture (Ashkenazi, Israel, & Abelson, 2019) and artificial seeding for commercial farming (Ohno, 2006).
The gametophytic and sporophytic thalli of *Ulva* spp. are isomorphic in the wild which makes them difficult to differentiate. Understanding the effects of physiological stimuli on the induction of reproduction is critical to improve mariculture programmes, as well as natural resource management. This whole process goes through three distinct phases, namely: determination, differentiation and swarming or gamete release (Richard & Oertel, 2010). These processes can be triggered by various physiological stimuli in the form of extrinsic abiotic factors, i.e. light, temperature, salinity, photoperiod, dehydration, thallus fragmentation, etc. Balar and Mantri (2019) listed abiotic stimuli responsible for the induction of reproduction in 27 different case studies of *Ulva*. Additionally, hypoxia and sulphide concentrations and extended dark periods were found to influence the production of gametes in *Ulva* spp. (Corradi, Gori, & Zanni, 2006). Salinity, photon fluence rate and fragmentation affected the induction of sporulation in *Ulva* (*Enteromorpha*) prolifera (Dan, Hiraoka, Ohno, & Critchley, 2002). Temperature was found to be critical for sporulation in *U. lactuca* (Niesenbaum, 1988), whereas both temperature and photoperiod were required for sporulation in *Ulva* sp. (Carl et al., 2014).

Further isolation and segregation of reproductive stages of *Ulva* sp. is necessary for several studies (Balar and Mantri, 2020). In a simple technique, a flask with a slender side-arm was used for the segregation of gametes in *Ulva* (Supplementary fig. S1a). This technique was originally developed for concentrating phototactic flagellates from debris (Meeuse, 1963). The flagellates would be concentrated at a point representing the “focal” line of the outer convex surface of a container using a point-source of light. However, this technique is limited for routine and larger scale operations due to the disturbance occurring during decanting of medium. Gamete purification has been achieved using capillary pipettes with uni-lateral illumination (Supplementary fig. S1b). The gametes were found to be aggregated at the tip of the capillaries after 20–30 min (Vest et al., 2015). Liu et al. (2015) reported that separation of zoospores and gamete from a mixture required 5–7 days (Supplementary fig. S1c).

Determination of pre-zygotic and post-zygotic isolation is essential for understanding reproductive relationships, confirming sexual boundaries and identifying species delineation in *Ulva* (Hiraoka et al. 2017). Testing self-compatibility and interspecific copulation are essential steps in breeding. The current protocol follows: (i) synchronised gamete induction and release; (ii) concentration of gametes into small droplets; (iii) mixing desired gametes; (iv) microscopically ascertaining conjugation and (v) isolation of zygotes from the opposite side of droplets. Furthermore, precise separation and confirmation of non-fused gametes from zygotes is difficult on a real-time basis. The procedure is time consuming and requires a great degree of precision.

An apparatus was also developed using a plastic centrifuge tube and two pipettes, with a light source from the bottom (Supplementary fig. S1d). The gamete solution was applied from the top and zygotes were collected again from top (Nordby, 1976). The efficiency of this device was confirmed by identifying heterozygotes and slender and wild type partheno-sporophytes after two weeks in culture. Therefore, it is evident that existing methods were not entirely satisfactory due to being cumbersome and limited success. Similarly, the scale on which they can be operated is limited and further confirmation of separation success requires long incubation periods making them practically unusable for the needs of a large-scale operation.

Nevertheless, there exists subtle yet important differences between the motile stages of gametes and zygotes, such as ploidy and their eco-physiological responses, which can be used for the effective segregation for scaled-up operations. Phototaxis has been used for separating flagellates, gametes and zoospores in seaweeds. This study describes a device to segregate zygotes and non-fused gametes in an efficient manner using these principles. The investigation demonstrates technical advancement in terms of simplicity of design, operation, reduced time between gamete mixing and acquisition of zygotes along with accuracy of segregation. We believe that the device has implications for basic (e.g. cytological, bio-chemical) and applied (e.g. breeding, hybridization and farming) investigations.

### Materials and methods

**Collection of materials and molecular identification**

Healthy and mature fronds of an *Ulva* species were collected from a single site at Veraval (20.55°N and 70.20° E), on the west coast of India. The fronds were immediately transported to the laboratory under cool conditions, cleaned and only gametophytic thalli were used for induction of reproduction. To confirm the presence of gametophytic thalli, fronds were chopped to induce reproduction and supplied with enriched seawater. Gametes were released three days after replacing the medium. Observation of released biflagellate gametes under microscope (100x magnification) confirmed the gametophytic nature of fronds. To overcome the issue of phenotypic plasticity and to identify the *Ulva* species, the plastid molecular marker, *rbcL* was...
used. The cetyl trimethylammonium bromide (CTAB) method was used for genomic DNA extraction following the methods of Varela-Álvarez et al. (2006). Quantification, as well as quality, of extracted DNA was determined using absorption of samples at wavelengths of 260 and 280 nm and additionally by 0.8% agarose gel electrophoresis. The detailed protocol for DNA extraction is provided in supplementary material. The *rbcL* gene was amplified using a 5 ng template DNA concentration in a reaction tube. PCR was performed in 50 µl reaction volumes, containing 1 µl of template DNA, 200 pM dNTP’s, 0.5 PM of each primer, 1.5 mM MgCl₂, 5 µl 10x reaction buffer and 1.25 units of Taq DNA polymerase (molecular biology grade chemicals from M/s. Sigma Aldrich). The PCR was performed in a Biorad thermocycler (M/s. Biorad MyCycler, USA) using the following protocol: initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C. The amplified product was checked on a 1.5% agarose gel containing EtBr. The *rbcL*-forward and *rbcL*-reverse green seaweed, specific primers sequences were 5'-GCTGGWGTAAAAGATTAYCG-3' and 5'- TCACGCAACGCATRAASGGG-3' respectively. Amplified products were purified using a gel extraction kit (Genelix Biotech Asia Pvt. Ltd.). Sequencing of the amplified product was obtained using the same primers that were used for amplification at M/s. Macrogen Inc., South Korea. The sequence obtained was submitted to the National Center for Biotechnology information (NCBI) and a number for the deposited sequence was obtained. A phylogenetic study was undertaken using software MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). DNA sequence alignment of the *Ulva* species was created and additional sequences were as downloaded from the Genbank database (selected from Hughey et al., 2019). A phylogenetic tree was constructed using the Maximum Likelihood method and Tamura-Nei model. 1000 replicates were performed to compare the relative support of branches (Tamura & Nei, 1993).

**Identification of life-phase and induction of gametogenesis**

Cleaned *Ulva* sp. fronds were used to identify the life-cycle phase and only gametophytic thalli were used for the induction of reproduction. The conventional approach which investigates one factor at a time to optimise induction of gametogenesis was followed (Yong et al., 2014). Induction of gametogenesis was tested using 5 mm diameter discs excised from a healthy thallus (basal portion excluded) for each set of experiments. The experiments were designed to standardise a single parameter in the following sequences of salinity (i.e. 30, 25, 20, 15 ppt), temperature (i.e. 35, 30, 25, 20, 15°C) and photoperiod (i.e. 15:9, 12:12, 9:15 h), followed by dehydration (i.e. 0, 15, 30, 60 min). The discs were incubated in autoclaved seawater (ASW) as a medium in 90 mm diameter, disposable sterile Petri dishes. The dishes were kept in a Multi-thermo incubator (MTI-202, Eyela, Japan) at 50 µmol photons m⁻² s⁻¹ provided by white, fluorescent light. All of the discs were observed individually under an inverted microscope (Olympus BX60, Japan) on the first, second and third days for formation and release of gametes and the data presented as percentage induction of gametogenesis on the third day. Results are expressed in terms of mean ± standard deviation. One-way ANOVA was used to test the effects of different salinities, temperatures, photoperiod and dehydration on gamete induction, the significant differences were determined at p ≤ 0.05.

**Device for segregating non-fused gametes and zygotes**

An image of the device used for segregating non-fused gametes and zygotes, along with detailed engineering drawings are provided (Figure 1a, Figure 1b). The design consisted of a square wooden box, 32 x 32 x 32 cm made with stiff sides and weighing ~4.4 kg. It was partitioned with a wooden separator ~2 cm thick. The upper 16 cm portion was painted white while the lower part was painted black. The wooden separator was painted likewise. A 5-watt LED, was fixed at the centre of the upper lid making leaving approximately 8 cm from the sample tubes. The box lid hinged about an axis located on one end of the box. The holes with 1 and 1.7 cm diameter were made in the wooden separator to hold 2 ml Eppendorf tube and 15 ml test tube, respectively. The gametes were induced from pieces of mature thallus (5 g) by following the described method (Supplementary fig. S2). This modular device was tested for its efficiency in segregating gametes. The gametes released and segregated using the device were collected by pipetting out the concentrated mass from the upper part of the test tubes. These were observed under a microscope (EVOS Auto FL, Invitrogen, USA) to confirm their biflagellate status and size. The gametes released from two different thalli were checked for compatibility. The compatible gametes were then mixed in equal proportion into 15 ml test tube to enable fusion. They were further studied using a flow cytometer (BD Accuri™ C6 Flow Cytometer, USA) equipped with blue (448 nm) and red (635 nm) lasers and run in the linear mode, with a flow rate of 40 µl min⁻¹, a total of 10 000 events were recorded. Forward-scatter (FSC) and side-scatter (SSC) were recorded for individual particles as they passed through the laser. The data were then processed using BD ACCURI C6 software. The
readings were taken at 5 min, 1 hr and 2 hr intervals after the gametes were mixed.

Results

Molecular analysis

Sequence data was obtained for approximately 750 bp amplified rbcL. The obtained sequence was submitted to the NCBI database (MN509806). The phylogenetic tree constructed with the Maximum Likelihood method and Tamura-Nei model gave comparable tree-topologies with distinct clades. The analysis involved 36 nucleotide sequences. There were total of 1428 positions in the final dataset. The collected species in the present study formed a separate entity within Ulva lactuca (Supplementary fig. S3). A well-supported monophyletic clade was formed with the collected sample which was a sister group to all others. Although not directly relevant to the present study, but it is worth reporting that the outer group taxa (i.e. Ulvaria obscura, Umbraulva olivacens and Percursaria percura) formed a well-supported, but separate clade. The large Ulva clade in the phylogenetic tree was resolved in seven different lineages. The U. spinulosa, U. taeniata and reticulata clades were the closest to the collected Ulva lactuca sample. Morphological and anatomical observations (data not shown), coupled with strong evidence of molecular phylogeny, confirmed the identity of the species collected from Veraval, India, in the present investigation as U. lactuca.

Identification of life-phases and induction of gametogenesis

The small (< 5 μm), bi-flagellate nature and positive phototaxis confirmed the identity of gametophytes which were further used for the experiments related to induction of reproduction and gamete fusion. On the first day of incubation discs showed a normal organisation of cells (Supplementary fig. S4a); gametogenesis started from day two onwards. Induction was indicated by a change in colour from light green to hyaline. The process of gametogenesis was found to be continuous, first developing at the periphery of the discs then rapidly progressing towards their centres. Microscopic examination confirmed the multiplication of the intra-cellular plastids from few to many which then formed granulated masses. After the first dark period, the content of the cells concentrated at one end, towards the direction of illumination. This phenomenon was found to be coordinated whereby several neighbouring cells quickly transformed from vegetative to a gamete-mother-cell. The second dark period gave rise to the formation of gametes (Supplementary fig. S4b) and their subsequent release. Induction of gametogenesis improved with increase in salinity up to 25 ppt and then decrease with further increase; similarly, increase in temperature, enhanced induction up to 25°C and after that registered a decrease (Figure 2(a–c)). It was found to be limited by higher temperature of 35°C (13.33 ± 5.77%) and by short days of 9:15 dark:light photoperiod (20 ± 4.08%). Dehydration period (> 15 min) was found to be a critical factor which achieved the successful induction of gametogenesis. Therefore, optimum salinity was found to be 25 ppt followed by 25°C temperature and 12:12 h photoperiod, while 100% induction was achieved at 15 as well as 60 min dehydration (Figure 3). Significant variations in gamete induction were recorded at different salinities, temperature and photoperiod, however no significant differences were observed in gamete induction at different dehydration times (Table 1).
Device for segregating non-fused gametes and zygotes

The experimental assessment of the constructed device confirmed its utility for the segregation of zygotes from non-fused gametes in a simple manner. The six positions available to hold tubes of different sizes (i.e. three for 2 ml Eppendorf tubes and three for 15 ml centrifuge tubes) facilitated the handling of 6–45 ml liquid samples in a single run. The light source was active only in one chamber, with the simultaneous provision of a dark environment in the other. This unique set up achieved effective segregation within a single enclosure. Segregation (> 50%) occurred within 2 h, with manual intervention alone. Its utility was confirmed by ‘Flow cytometer” analysis based on size variation of two fundamentally different reproductive cells. The mean size of non-fused gametes was 3.8 ± 0.5 μm with that of the zygotes being 10.48 ± 1.75 μm (Supplementary figs 5a–c). The mean FSC-A for non-fused gametes was 42 475; that of the zygotes was 594 263. At 5 min interval 56.2% of population represented non-fused haploid gametes while 5.3% were diploid zygotes. Composition of the non-fused gamete population decreased over time as syngamy progressed. At the 1 hr and 2 hr intervals it was 26.59 and 8.8% respectively, the diploid zygote population was 32 and 57.4% respectively (Figure 4). The segregation process did not require any regulation, constant monitoring and data acquisition and was operated continuously for longer periods thereby improving through-put. In addition, the closed assembly circumvented the possibility of evaporation of media from the tubes during the segregation process.

Discussion

Our results are in accordance with studies in Ulva pertusa (Hiraoka & Enomoto, 1998), U. prolifera (Cui et al., 2018) and U. fasciata (Mantri et al., 2011). However, most of the earlier studies were conducted using sporophytic fronds and gametogenesis was seldom studied. It was found that a combination of 25°C, 25 ppt salinity, 12:12 L:D photoperiod with 50 μmol photons m−2 s−1 radiation was conducive to gamete induction on the third day of incubation using U. lactuca in the present study. This is in contrast to Ulva sp. collected in the Sacca di Goro, Italy where,
20 ± 1°C, 152 µmol photons m⁻² s⁻¹ radiation, 16 h photoperiod and 30 ppt salinity were effective in inducing gametogenesis after three days in (Corradi et al., 2006). A photoperiod of 12:12 L:D and 20–75 µmol m⁻² s⁻¹ irradiation at 20°C were required for gamete formation in U. prolifera (Lin, Shen, Wang, & Yan, 2008). A temperature of 20°C, 17.7 h L:D, photoperiod and 60–120 µmol photon m⁻² s⁻¹ radiation successfully induced gamete formation in U. mutabilis (Spoerner, Wichard, Bachhuber, Stratmann, & Oertel, 2012) and in U. linza (Vesty et al., 2015). Besides these extrinsic factors, dehydration (Corradi et al., 2006); addition of nutrients (Lin et al., 2008) and fragmentation (Spoerner et al., 2012; Vesty et al., 2015) was also reported as triggers for gametogenesis. Similarly, in the present investigation, dehydration was required to obtain 100% gametogenesis in U. lactuca. There are interspecies differences in extrinsic physiological drivers for inducing reproduction and such studies are crucial not only for developing successful mariculture programs but also natural resource management (Hiraoka, Ichihara, Zhu, Ma, & Shimada, 2011). Further, mechanisms of artificial induction of gametogenesis ensures consistent production as the donor clone remains the same, contrary to natural seeding where spores/gametes comes from genetically different clones. To the best of our knowledge this is the first report pertaining to the effects of extrinsic abiotic factors on the induction of gametogenesis in U. lactuca.

Breeding techniques have considerably improved the prospects of commercial seaweed farming and during the past two decades, 47 certified seaweed cultivars have been developed in eastern Asian countries (Hwang, Yotsukura, Pang, Su, & Shan, 2019). “Improved varieties” in the brown algea Saccharina (Laminaria) and Undaria and the red alage Gracilaria and Porphyra sensu lato are now available for commercial operations. However, selective breeding in the green alga Ulva has lagged behind, partly due to sexual incompatibility barriers and largely due to the absence of protocols for segregation of zygotes from non-fused gametes. Hybridisation experiments were originally attempted by Foy (1934) in U. mutabilis by mixing dense gamete suspensions of different parental strains at the end of a droplet on a glass slide from where strong light has
been provided to facilitate fusion. After successful copulation of the gametes the hybrid zygotes were isolated via negative phototaxis. The method has not changed after nine decades and was employed to achieve hybridisation in *U. spinulosa*, *U. prolifera* and *U. linza* (Hiraoka et al., 2011; Hiraoka & Shimada, 2004; Hiraoka, Shimada, Ohno, & Serisawa, 2003).

The currently practised techniques for evaluating reproductive phases and their motile stages (i.e. gametes and spores) are really only suitable for academic research. They are inadequate in scaled-up operations aimed at developing applied phycology protocols and field testing. Furthermore, it is argued that a high concentration of gamete cells in a small volume potentially influences the negative phototaxis. The current protocol only handles 0.1 ml solution in single operation. Contrary, a single device presented here can handle 6–45 ml samples in a single operation, thereby increasing the efficiency of output several folds.

The present device utilises completely opposite behaviour exhibited by non-fused gametes and zygote towards light simultaneously in two separate chambers. Effective segregation was achieved in the separate light and dark chambers, thus gametes remain in the top portion with the light source (due to positive phototaxis), while fertilised zygotes descend into the dark zone – (largely due to negative phototaxis and density). This is the innovative feature of the investigation. Additionally, accuracy of this procedure was also found to be high. The confirmation of efficient segregation (i.e. 5.3% at 5 min to 57.4% at 2 h of mixing the gametes) through flow cytometer, confirmed the advantage of this technique over the conventional methods in use.

The only other apparatus described in the literature was developed for performing hybridisation experiments in *Ulva* (Nordby, 1976). However, the utility of this apparatus was not convincingly verified. Besides, it was suggested to examine the reliability by microscopically counter checking the zygotes for presence of non-fused gametes (Phillips, 1990). Nevertheless, to confirm the segregation culture test (2 weeks) was recommended wherein heterozygotes and slender and wild type partheno-sporophytes could be visually identified. This technique also required a great degree of expertise to carry out the task of segregating the zygotes. On the contrary, the device described in this study could be operated reliably by local fishermen in order to perform relatively basic procedures aimed at breeding such as conjugation and zygote separation without specialised training. We also believe that routine use of this device will augment selective breeding efforts resulting in new varieties with improved products, advanced genetic make-up, faster environmental adaptability and increased disease resistance. This further saves the cost involved in microscopy and reduces time and drudgery associated with the current droplet practice. We also demonstrated the economical use of locally available materials in the construction of durable and easy-to-use, energy saving system requiring only modest investment thereby making it affordable to several applied phycology applications. The slight heat generated by lamp needs to be dissipated by opening the lids of this device to allow suitable cooling, after every cycle of operation. Further developments are desired to make the process more mechanised. We also are in the process of introducing high quality, but cost-effective fibre-based materials for construction and an external battery. This would make it durable, light in weight and maximise its utility in remote coastal villages where availability of electricity is scare.

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