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The induction of reproductive cell formation of *Ulva pertusa* Kjellman (Ulvales, Ulvophyceae)

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SUMMARY

Synchronous zooid formation in *Ulva pertusa* Kjellman was induced in excised disks maintained in sterilized seawater at 20°C, 12:12 h L : D cycle and fluorescent light at 100 μmol photons m^{-2} s^{-1}. Zooids were released from mature disk tissue on the morning of the second or the third day after excision. The degree of zooid formation was found to be dependent on disk size and the region of the mother thallus from which the disk tissue was excised. Zooid formation was induced in more than 90% of small disks (0.9 mm in diameter) which were taken from the margins of the *Ulva* thalli. When disks were incubated together with a perforated mother thallus, the disks remained sterile. The presence of maturation inhibitors in vegetative thalli is suggested.

Key words: induced reproduction, maturation inhibitor, reproductive cells, *Ulva pertusa*, zooid formation.

INTRODUCTION

In the green algal genus *Ulva*, vegetative cells from the margins of the thallus transform directly into zoosporangia or gametangia, and release numerous zoospores or gametes, respectively. In the field, the periodic maturation of *Ulva* is observed around spring tide (Smith 1947; Chihara 1969; Sawada 1971; Sawada and Watanabe 1974; Okuda 1975). In the laboratory, it has been reported that strains of *Ulva mutabilis* Føyn produced zooids 3 days after they had been placed into fresh growth medium (Føyn 1959). Nordby and Hoxmark (1972) reported that cutting the thallus of *U. mutabilis* into small fragments induced a high degree of synchronous zooid formation. Using this method, many cytological and biochemical reports on mitosis and meiosis in *U. mutabilis* were published in the 1970s (Nordby and Hoxmark 1972; Bråten and Nordby 1973; Hoxmark and Nordby 1974; Nordby 1974; Hoxmark 1975; Nilsen and Nordby 1975; Nordby 1977; Løvlie and Bryhni 1978). Zooid formation and liberation were observed in isolated single protoplast cells of three species of *Ulva* (Reddy *et al.* 1989). Recently, Stratmann *et al.* (1996) reported the extraction of reproductively mature inhibiting materials from the growth medium of several day-old cultures and the suspension of single-layered thallus fragments in *U. mutabilis*. There has been no report of artificial induction of reproductive maturation in other species of *Ulva*, and of the contribution of the tissue size and the region excised from the mother thallus to the degree of zooid formation.

MATERIALS AND METHODS

Well-developed thalli of *U. pertusa* Kjellman were collected from the lower tidal zone at Iwaya, Awaji Island, Hyogo Prefecture, Japan from August 1995 to October 1996. After collection, thalli were maintained in running seawater, at 15–25°C, with a 12:12 h L : D cycle (6:00–18:00-light period) under 50–80 μmol photons m^{-2} s^{-1} irradiance until required for experiments. The sex of the thalli was determined by the types of zooids released from the mature disks. Quadriflagellate zoospores were microscopically distinguished from biflagellate gamete, and the sex of the gametes was determined using the crossing test of Tatewaki (1979).

Variously sized disks were excised from the upper, middle and basal regions of membranous vegetative thalli using leather craft punches. The disks were rinsed with autoclaved seawater (ASW) and transferred into culture vessels (60 mm diameter, 90 mm high) containing ASW. The volume of ASW provided was 5 mL per 1 cm^2 of disks. These were placed under 20°C, with a 12:12 h L : D cycle (6:00–18:00-light period), under cool white fluorescent light (100 μmol photons m^{-2} s^{-1}). All disks were punched at 12:00 noon. Observation of the disks was made at 6:00 and 12:00 every day. When maturation of disks was observed at 6:00, observations for determination of the time of zooid release were made at hourly intervals from 6:00. In some cases, perforated mother thalli were cultured under the same conditions mentioned above.

The mature area of the thallus, which was to form zooids, was recognized due to a color change from green to yellowish or brownish as observed with the na-

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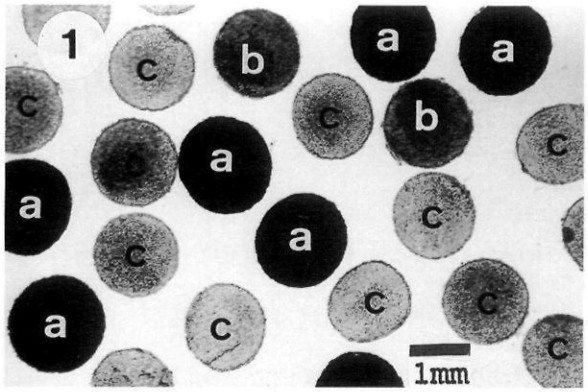

Fig. 1. Sporophytic disks 2 days after excision. Disks that failed to mature (a), partially matured (b) or entirely matured (c) were found to release zoospores.

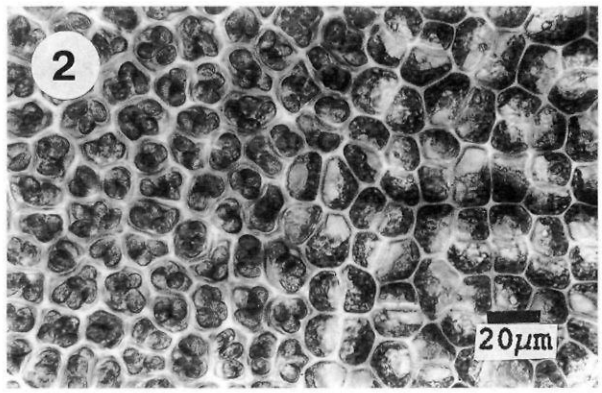

Fig. 2. The boundary area of reproductively mature cells (left) and non-mature cells (right) on a sporophytic disk.

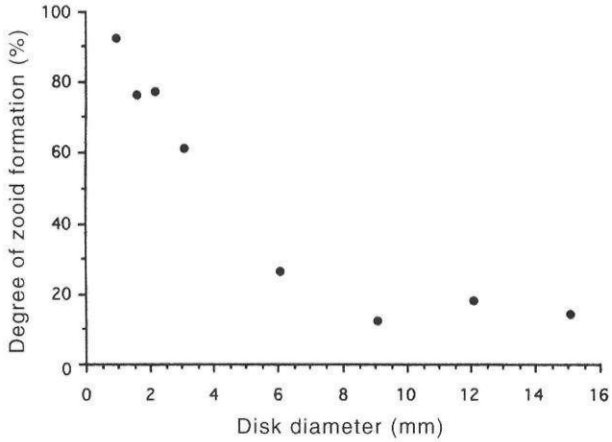

Fig. 3. The degree of zooid formation 3 days after excision of various-sized disks from a sporophyte thallus.

ked eye. The disks with mature areas were ranked into weighted groups according to the table of Nilsen and Nordby (1975). The degree of zooid formation in a region of thallus was assessed according to the number of classified disks.

To compare the zooid formation of disks derived from different portions of a mother thallus, five large disks of 15 mm diameter were obtained from the upper, middle, and basal part of a sporophyte thallus. Furthermore, 80 small disks of 0.9 mm diameter were punched from each of the 15 mm diameter disks. The degree of zooid formation in each portion of the thallus was compared to the degree of zooid formation in the 0.9 mm diameter disks, 4 days after punching.

In order to investigate the effect on zooid formation of retaining excised disks together with mother thallus in the same culture vessel, 200 disks of 1.5 mm diameter were prepared from the upper part of a thallus. One hundred disks were incubated with the mother thallus. Another 100 disks were incubated without the mother thallus as a control. The degree of zooid formation was compared between the two disk groups.

In order to survey the effect on zooid formation in disks maintained in culture medium containing supernatant from the mother thallus, supernatant was prepared by homogenization of a concentration of sporophytic disk at a 15 mm diameter per 2 mL sterilized seawater, which was then pressure filtered through a GS filter (0.22 μm; Millipore, Bedford, MA, USA). A dilution series of the supernatant was made with sterilized seawater. One hundred 1.5 mm diameter disks from the sporophyte were incubated in each diluted supernatant and the degree of zooid formation was assessed over the 3 days following punching.

RESULTS

As shown in Fig. 1, zooid formation occurred partially or entirely in the disk. Partial zooid formation in a disk was expressed in the form of sharply divided areas of matured and non-matured cells as observed in fertile thalli in the field (Fig. 2).

The disks of 0.9, 1.5, 2.1, 3.0, 6.0, 9.0, 12.0 and 15.0 mm diameter were excised from the upper part of the same sporophyte (about 30 cm height), of which the numbers were 200, 100, 100, 50, 20, 10, 5 and 5, respectively. The degree of zooid formation in each size disks was investigated 3 days after excision. As shown in Fig. 3, the degree of zooid formation increased as the disk size decreased, reaching a maximum of 93% of those disks of 0.9 mm diameter. As disk size increased, the degree of zooid formation decreased to a minimum of 12% of those disks of 9.0 mm diameter. The 1.5 mm disks were employed in the following experiments, both for the convenience of experimental handling and because of a high degree of zooid formation.

One hundred disks of 1.5 mm each from the upper parts of 100 mother thalli were incubated. As shown in Table 1, zooid formation occurred in the disks from 93 mother thalli, which contained 42 sporophytes, 27 female gametophytes and 24 male gametophytes. Table 2 shows the daily average degree of zooid formation of the disks from the 93 mother thalli. The zooid formation

Table 1. The degree of zooid formation in disks (1.5 mm diameter) from 100 thalli 4 days after punching

Zooid formation rate in the disks (%)	No. mature thalli		
		Gametophyte	
	Sporophyte	Female	Male
100–81	39	7	3
80–61	2	4	2
60–41	1	6	4
40–21	0	6	6
20–1	0	4	9
0 (no maturation)		7*	

*Disks from seven thalli did not mature, so remained sterile.

Table 2. The percentage frequency of daily zooid formation

Time after punching		Zooid formation (%)	
Day	Hour	Sporophyte	Gametophyte
1	24	0	0
2	48	70	13
3	72	25	24
4	96	0	9
5	120	0	2
6	144	0	0

Disks (1.5 mm diameter) were observed at noon each day. The average zooid formation rate for each day was calculated from 42 sporophytes and 51 gametophytes.

was induced in 70% of the disks from the sporophytes within 2 days, and more than 90% of the disks matured within 3 days, whereas zooid formation was induced in 40% of the disks from the gametophytes within 3 days. The degree of zooid formation had a large variation, depending on individual mother gametophytes (Table 1). The zooids were always released in the morning, 2–5 h after the light period began; no release was observed at any other time. The sterile disks grew up and enlarged over time. Perforated mother thalli still remained sterile for 1 week after punching. In a few cases, when the upper part of a mother thallus was densely perforated by punching, it matured.

The degree of zooid formation was examined in different portions of a mother thallus. As shown in Fig. 4, more than 90% zooid formation was induced in the small disks from the upper parts of the thallus, while almost all disks from near the rhizoid did not mature.

Table 3 shows the degree of zooid formation of disks incubated with or without the mother thallus. The disks incubated without the mother thallus matured, whereas ones incubated with the mother thallus never matured even 1 week after excision of the disks.

Figure 5 shows the effect of supernatant on zooid formation of mature disks. Eighty-two percent of the disks incubated in ASW without supernatant formed zooids,

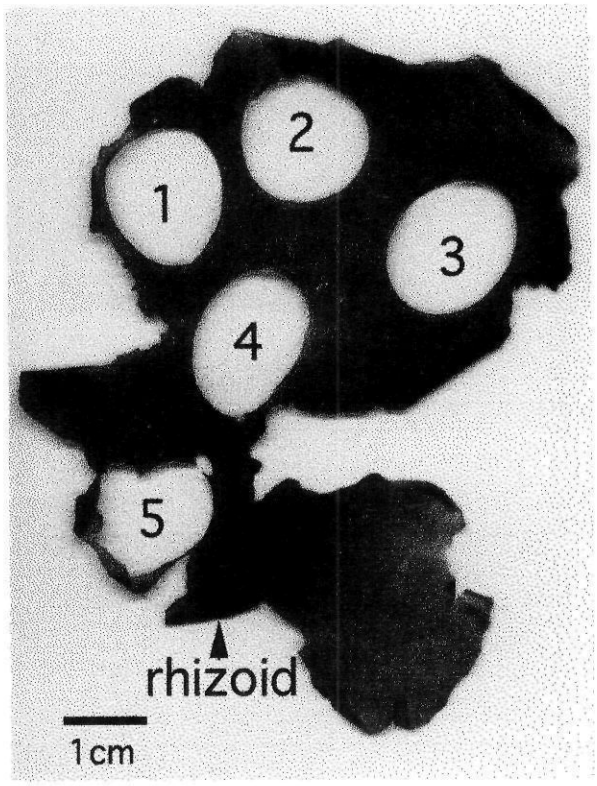

Fig. 4. The degree of zooid formation 3 days after excision in each position (1–5) of the thallus (sporophyte). The small disks from 1, 2, 3, 4 and 5 showed 94, 92, 90, 24 and 2% zooid formation, respectively.

Table 3. Comparison of zooid formation of disk groups on the third day after excision and incubation in the presence (+) and absence (−) of the perforated mother thallus

Mother thallus	Sporophyte	Gametophyte
+	0%	0%
−	58%	16%

while the degree of the zooid formation declined to 5–7% with more than half the concentration of the original supernatant.

DISCUSSION

Nordby and Hoxmark (1972) reported that a high degree of synchronous zooid formation was induced by means of cutting the thallus of *U. mutabilis* into small fragments (about 0.4 mm) with a razor blade and by incubating them in fresh seawater. The present study reveals that the zooid formation is easily induced in *U. pertusa* by excision of small disks (0.9–2.1 mm diameter) and incubating these in sterilized seawater.

Nordby (1977) reported that variation of the fragment size did not influence zooid formation greatly. In the present study, the degree of zooid formation was investigated in various-sized disks cut to exact sizes using punches. All of the excised disks retained the dou-

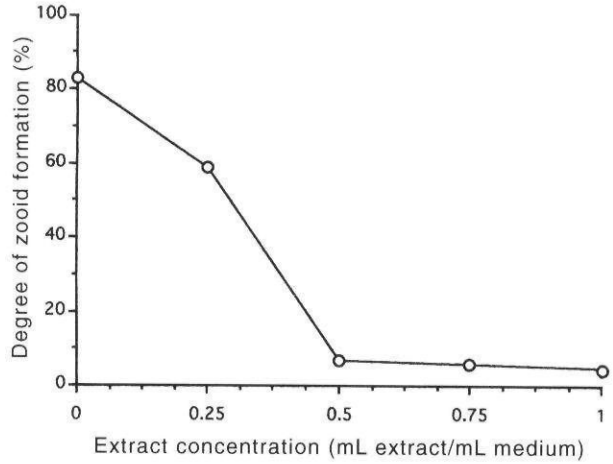

Fig. 5. Dilution effect for extracts prepared by homogenization of sporophyte tissue at a concentration of one disk (15 mm, diameter) per 2 mL seawater. Sporophyte disks of 1.5 mm diameter were used as test disks to determine the degree of zooid formation.

ble-layered cell structure (distromatic) which is characteristic of *Ulva*. As shown in Fig. 3, our results clearly show that the disk size affected the rate of zooid formation.

More than 400 wild thalli of *U. pertusa* were used in these disk perforation experiments; nevertheless, no disk was found to mature and release zooids before 2 days had passed after having been punched. At least 2 days was needed for the completion of the maturation process. In *U. mutabilis*, under optimal culture condition, the maximal sporulation of fragments would also occur after 48 h (Nordby 1977).

The zooids of *U. pertusa* were usually released from mature disks within 2–5 h after commencement of the light period, even if the maturation of the disks was incomplete on the second day and completed on the third or fourth day. When the second dark period was extended for a few hours, the release from the matured disks was postponed (M. Hiraoka, unpubl. data). These results suggest that light probably promotes release of zooids. Stratmann *et al.* (1996) isolated a swarmer inhibitor of low molecular mass from the medium after incubating thallus fragments of *U. mutabilis* for about 20 h. They described that mature gametes became motile and were released from the gametangia when the concentration of the swarmer inhibitor declined below the concentration threshold. The relationship between such a swarming inhibitor and the light promotion of zooid release in the ulvacean thallus remains to be investigated.

Thiadens and Zeuthen (1967) argued that factors in the fresh medium could be responsible for the induction of spore formation in *U. mutabilis*. However, Nordby (1974, 1977) proposed that the vegetative thalli of *U. mutabilis* would produce some substances inhibiting their zooid formation and, when a thallus was fragmented and the inhibitor leaked out from the thallus, zooid formation would occur. This hypothesis is based on two observations: (i) a lower percentage frequency of zooid formation was caused by the higher density of the thallus fragments to the fresh medium; and (ii) the double-layered thallus fragments tended to sporulate later than single-layered thallus fragments torn with the mixer. Furthermore, he pointed out that the double-layered structure of the ulvacean thallus could be responsible for maintaining a sufficient concentration of the sporulation inhibitor during vegetative growth. Actually, Nilsen and Nordby (1975) tried to extract the sporulation inhibitor from the vegetative thalli in *U. mutabilis*. However, although the extracts from fragmented and boiled thalli showed the inhibition of sporulation, the actual inhibitor in the vegetative thallus was not characterized. Stratmann *et al.* (1996) have recently reported that the thallus of *U. mutabilis* produces at least two kinds of sporulation inhibitor, one of which is a glycoprotein and the other is a non-protein of very low molecular mass. In *U. pertusa*, when the disks were incubated with the mother thallus, zooid formation in the disks was never observed (Table 3). The supernatant from the homogenization of vegetative thallus obviously inhibited zooid formation (Fig. 5). The present results suggest that the vegetative thallus of *U. pertusa* also produces such an inhibitor.

The smaller disks showed the highest degree of zooid formation (Fig. 3). If the inhibitor leaked out from the circumference of the disks, the smaller disks would easily leave off the inhibitor and form zooids at a high degree. The densely perforated upper part of the mother thallus sometimes matured reproductively. These observations in *U. pertusa* can be explained by Nordby's inhibitor hypothesis. Reddy *et al.* (1989) reported that each single cell of protoplast isolated from three species of *Ulva* discharged many motile spores after cell wall regeneration. Their observation does not contradict the inhibitor hypothesis.

Ulva pertusa displayed different degrees of zooid formation according to the portion of the thallus used to produce disks. Almost all of the disks from the upper part matured within 3 days, while only a few from the basal part matured (Fig. 4). There is no report that in *U. mutabilis* the portion of the thallus cut into fragments influences the degree of zooid formation. In the serial studies on *U. mutabilis*, the fast growing and undifferentiated mutant 'Slender' (Føyn 1959; Løvlie 1964) was used. 'Slender' thallus lacks rhizoidal cells and consists of homogeneous cell type (blade cell) (Nordby and Hoxmark 1972). Therefore, *U. mutabilis* could have no different degree of the zooid formation depending on the cut portion. In contrast, the thallus of *U. pertusa* has a basal, rhizoidal structure and consists of at least two kinds of cells (blade cell and rhizoid cell). The results presented here suggest that the vegetative thallus of *U. pertusa* may have the concentration

gradient of inhibitor from the rhizoid to the margin of thallus, or there could be more cells with high sensitivity to the relief of the inhibition of zooid formation in the marginal part than in the basal part of a thallus.

The perforation method used for inducing reproductive maturity has a greater effect on sporophytes than on gametophytes (Tables 1,2). There was no significant difference between male and female gametophytes in the results. The sporophyte produces zoospores by meiotic and mitotic cell division, while the gametophyte produces gametes by only mitotic cell division. The difference between sporophyte and gametophyte mentioned above may be attributed to the difference in the systems of the zooid formation.

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