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# High monospore-producing mutants obtained by treatment with MNNG in *Porphyra yezoensis* Ueda (Bangiales, Rhodophyta)

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## Abstract

Two high monospore-producing pigmentation mutants were obtained by treatment with MNNG in *Porphyra yezoensis* Ueda. The mutants produced many monospores in young gametophytic blades (1 month old) and old large blades (3 months old). Monospore production of the mutants was affected by the culture conditions. The higher the temperature and/or the light intensity, the more the number of monospores released. When the conchospores and monospores of the mutants were in a monoculture at 15 and 20 °C, they did not develop into large blades because their germlings repeatedly released many monospores. However, they developed into large blades when they were in a co-culture with the large gametophytic blades of the wild-type or other pigmentation mutants. One high monospore-producing red mutant (*rm-1*) was genetically characterized by crossing with the wild-type which does not easily release monospores. In the $F_1$ gametophytic blades from heterozygous conchocelis produced in the crosses, there were unsectored blades (2 types) and sectored blades (6 types) consisting of 2, 3 or 4 sectors having both parental color phenotypes. Sectors of both parental colors appeared in the $F_1$ sectored blades in the proportion 1W:1.03R, indicating that the mutant *rm-1* has a mutation for its color phenotype. In the 4-week-old $F_1$ blades, high monospore production occurred only in the unsectored red blades and the red sectors of the sectored blades. These results indicate that the tendency of high monospore production is associated with the color phenotype of the mutant, suggesting that high monospore production of the mutant *rm-1* is controlled by gene(s), which is closely linked with the gene for the mutant color.

## Introduction

*Porphyra yezoensis* Ueda is a commercially important red alga, which has been extensively cultivated in Japan, China and South Korea. *P. yezoensis* has a biphasic life history that alternates between macroscopic, foliose, gametophytic blades and microscopic, shell-boring, sporophytic filaments referred to as the 'conchocelis' phase. Reproduction in *P. yezoensis* involves both sexual and vegetative reproduction cycles. Vegetative reproduction occurs by production of monospores (equal to archeospores) from young gametophytic blades. Monospores play an important role as secondary 'seeds" in large-scale cultivation of *P. yezoensis*. Monospore germlings grow faster than conchospore germlings and the monospores can probably be used as a primary seed source (Li & Cui, 1980; Li, 1984; Chen et al., 1985). The primary source of spores for seeding nets in *P. yezoensis* has been conchospores. This requires culturing of conchocelis filaments on shells, a process that is relatively expensive and labor-intensive. In addition, unwanted genetic diversity is introduced at the time of meiosis in the first four cell divisions of the germinating conchospore. Vegetative propagation would solve these problems. By eliminating the conchocelis phase, production costs would be lowered and genetic diversity eliminated. However, the major difficulty in

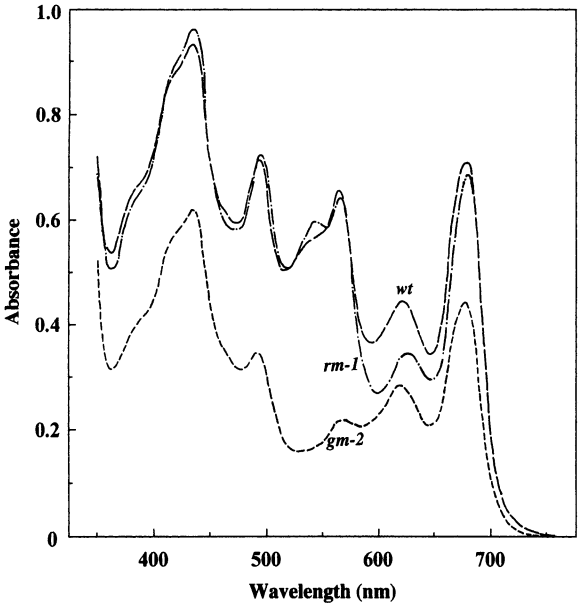


*Figure 1. In vivo* absorption spectra of gametophytic blades of mutants (*rm-1* and *gm-2*) and the wild-type (*wt*) in *Porphyra yezoensis* after 3 months in culture. Absorption maxima ($\lambda_{max}$) are numbered from left to right.

using *P. yezoensis* monospores as the primary seed source is obtaining sufficient quantities of them from the cultivated strains. Selection of high monospore-producing mutant strains will probably make a great progress in this problem.

Recently, we obtained a large number of *P. yezoensis* pigmentation mutants by treatment with a chemical mutagen (Yan & Aruga, 1996, 1997a,b, 1998). Simultaneously, several high monospore-producing mutants which released many monospores from the gametophytic blades as compared with the wild-type and other mutants, were selected. This study will primarily report monospore production of two high monospore-producing mutants in *P. yezoensis*, and the genetic transmission characteristics of one of the mutants.

## Materials and methods

In the present study, a wild-type strain of *Porphyra yezoensis* Ueda (U-511, Ohme et al., 1986), whose free-living conchocelis has been maintained in the laboratory, was used. Stock culture of its free-living conchocelis was performed as described by Kato & Aruga (1984).

A chemical mutagen, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Wako Pure Chemical Industries, Ltd., Japan), was used to induce mutations by dissolving it in the culture medium in a concentration of 30 ppm. Culture medium and methods for gametophytic blade mutagenesis with MNNG and mutant isolation were the same as those described in Yan & Aruga (1997a).

Two high monospore-producing mutants, red (*rm-1*) and green (*gm-2*) mutants obtained by treatment with MNNG, were used. The large gametophytic blades of the mutants were obtained by co-culturing their small conchospore germlings with the large gametophytic blades of the wild-type strain (5–15 cm long) at 15 °C under 90 $\mu$mol photons $m^{-2}$ $s^{-1}$ (10L:14D). After ca. 3 months in culture, when the blades of the mutant *rm-1* and the wild-type had grown to 10–15 cm in length, blade discs (9 mm in diameter) were cut out with a borer and cultured separately at different temperatures (10, 15, 20 and 23 °C) under 40 $\mu$mol photons $m^{-2}$ $s^{-1}$ (10L:14D), or at 15 °C under different light intensities (6, 12, 36, 72 and 98 $\mu$mol photons $m^{-2}$ $s^{-1}$) with a 10L:14D photocycle, or co-cultured in different combinations of these two types at 15 °C under 80 $\mu$mol photons $m^{-2}$ $s^{-1}$ for examining monospore production. Ten vinylon monofilments (3 cm long) were added in each of the culture flasks. After 3 weeks in aerated culture, the blade discs were removed from the flasks but the cultures were kept up. After 3 d, the culture medium was refreshed and monospores attached on the flask wall and the vinylon monofilments were cultured for another 10 d. The monospore germlings were then detached and counted microscopically.

Crossing experiments between the mutant (*rm-1*) and the wild-type (*wt*) were done in a similar method as described by Yan & Aruga (2000). However, conchospores released from heterozygous conchocelis were cultured to obtain $F_1$ gametophytic blades at 20 °C under 40 $\mu$mol photons $m^{-2}$ $s^{-1}$ (10L:14D), because 20 °C is more favorable to the early development of *P. yezoensis* conchospores than 15 °C (Yan et al., 1999). After 4 weeks in culture, the color phenotypes and monospore production of the $F_1$ gametophytic blades from heterozygous conchocelis were examined microscopically and classified for genetic analysis.

Examinations of *in vivo* absorption spectra of gametophytic blades of the mutants and the wild-type were done as in Yan & Aruga (1997a).

## Results

### In vivo *absorption spectra of mutant gametophytic blades*

After 3 months in culture, conchospores of the mutants (*rm-1* and *gm-2*) and the wild-type (*wt*) of *P. yezoensis* developed into large gametophytic blades. The *in vivo* absorption spectra of the gametophytic blades, which were grown under the same culture conditions and at the same age, are illustrated in Figure 1. Both mutants showed 5 absorption peaks similar to those of the wild-type. The red mutant *rm-1* showed distinct differences in $_3\lambda_{max}$ and $_4\lambda_{max}$, the former being double-peaked and the latter being lower and shifting towards longer wavelengths by 3–4 nm as compared with the wild-type. The green mutant *gm-2* showed significantly lower $_2\lambda_{max}$ and $_3\lambda_{max}$ as compared with the wild-type.

### *Monospore production of mutants*

Two-week-old gametophytic blades of both mutants (*rm-1* and *gm-2*) and the wild-type (*wt*) in *P. yezoensis* did not release any monospores (Fig. 2A–C). Four-week-old wild-type blades did not release monospores (Fig. 2D). However, the blades of the mutants *rm-1* and *gm-2* usually produced many monospores after 3 weeks in aerated culture (Fig. 2E,F). When the conchospore germlings of the mutants were cultured at 15 °C under 80 $\mu$mol photons $m^{-2}$ $s^{-1}$ (10L:14D), only the basal portion of the blades remained after monospore release (Fig. 3G,I). In monoculture, the mutant germlings did not develop into large blades because they repeatedly released many monospores (Fig. 3D,E). However, if the 2-week-old mutant germlings were co-cultured with the large wild-type blade (5–15 cm long), they developed into large blades (Fig. 3A–C). When the 4-week-old conchospore germlings, which were releasing monospores in monoculture, were transferred into a flask containing several large wild-type blades to co-culture, they stopped producing monospores in a week and finally developed into large blades. The mutant germlings also developed into large blades by co-culturing with large blades of other pigmentation mutants. The monospores released from the mutant blades developed into blades (Fig. 3F,H), which again produced many monospores in 3–4 weeks in monoculture.

Not only did the young mutant blades produce many monospores, but the large mutant blades (10–15 cm long, 3 months old) grown by co-culturing with the wild-type blades, also produced many monospores when they were in a monoculture. When the blade discs taken from the large blades of the mutant *rm-1* were cultured at different temperatures from 10 to 20 °C, the higher the temperature, the more the number of monospores released. However, almost no monospores were obtained when they were cultured at 23 °C (Fig. 4).

Monospore production of the mutant blade discs was also affected by light intensity. From 6 to 72 $\mu$mol photons $m^{-2}$ $s^{-1}$ (10L:14D), the higher the light intensity, the more the number of monospores released. When the blade discs were cultured under 6 $\mu$mol photons $m^{-2}$ $s^{-1}$, very few monospores formed. At 98 $\mu$mol photons $m^{-2}$ $s^{-1}$, the monospores production decreased (Fig. 5). Meanwhile, the wild-type blade discs in the same size were also examined under the same conditions, but almost no monospores were obtained. When the mutant blade discs were co-cultured with the wild-type blade discs of different numbers, the number of monospores released from the mutant discs decreased with increase in the numbers of the wild-type discs (Fig. 6). When the number of the wild-type discs was 2–3 times the mutant discs, the number of monospores released from the mutant significantly decreased. The mutant discs produced fewer monospores (approx. 1/100 time that produced in monoculture) when they were co-cultured with more than 4 times of the wild-type discs. However, almost no monospores were produced from the wild-type blade discs in either monoculture or mixed cultures (Fig. 6).

### *Coincidence of color and high monospore production*

Reciprocal crosses between the mutant (*rm-1*) and the wild-type (*wt*) were performed to ascertain whether or not mutations of the mutant *rm-1* are nuclear mutations and whether the high monospore production coincides with the mutant color phenotype. Because the *P. yezoensis* gametophytic blade is monoecious, self- and cross-fertilization will possibly occur simultaneously in each blade in a co-culture. In crosses of *rm-1*×*wt*, carpospores produced by the mutant blade developed into two types of conchocelis, the red and the wild-type. Conchospores from the red conchocelis developed only into the red unsectored $F_1$ gametophytic blades, indicating that the red conchocelis was homozygous due to self-fertilization. On the other hand, conchospores from the wild-type conchocelis developed into both unsectored and sectored

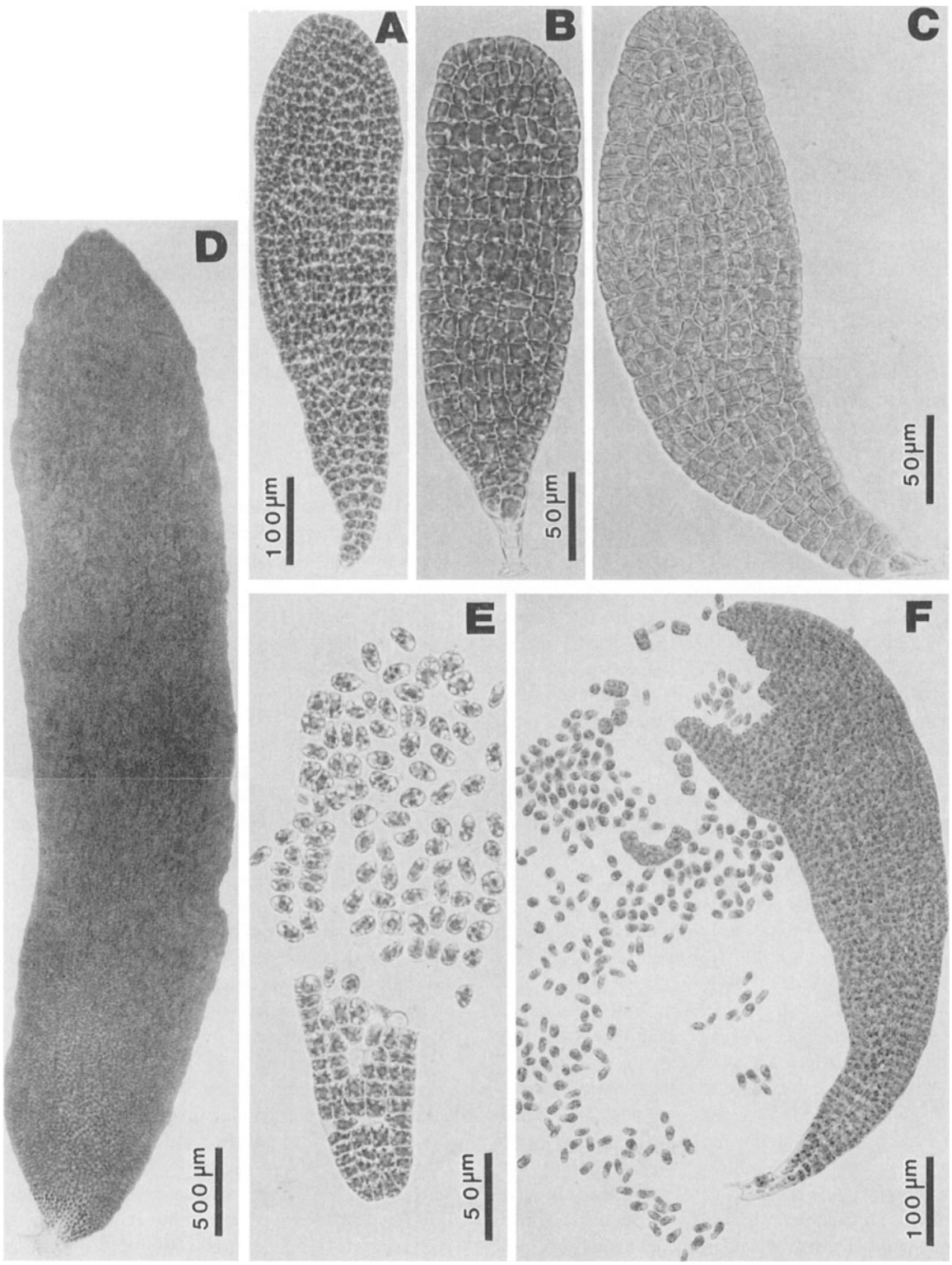


*Figure 2.* Monospore production of young blades in mutants (*rm-1* and *gm-2*) and the wild-type (*wt*). (A) *wt*; (B) *rm-1*; (C) *gm-2*; (D) *wt*; (E) *rm-1*; (F) *gm-2*. Age: 16 d old in A–C; 25 d old in D and 22 d old in E–F.

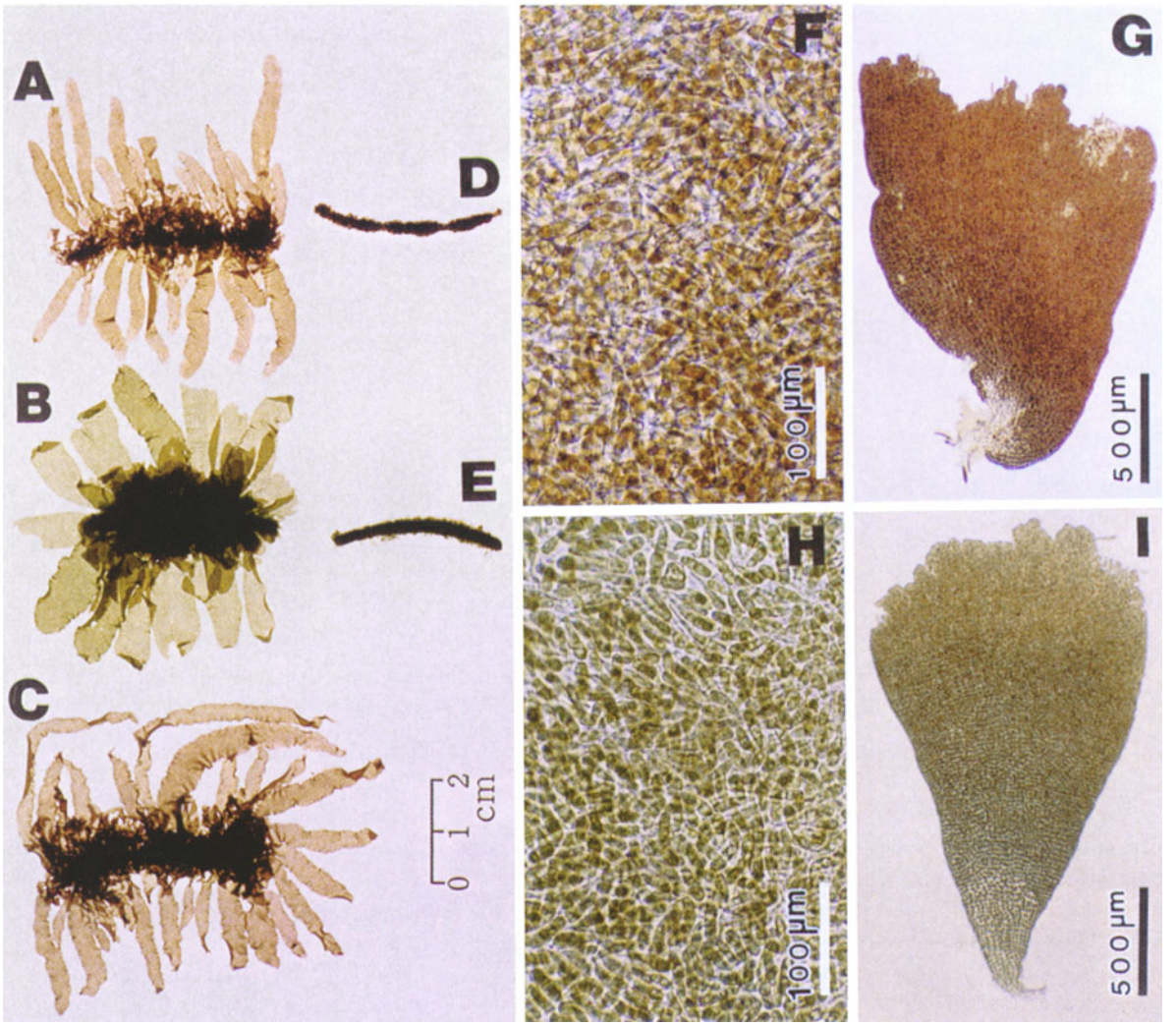


*Figure 3.* Growth and monospore production of mutant blades (*rm-1* and *gm-2*) in both monoculture and co-culture with the wild-type (*wt*). (A–C). Blades of *rm-1*, *gm-2* and *wt* grown in co-culture; (D) and (E) Blades of *rm-1* and *gm-2* grown in monoculture; (F) Monospore germlings of *rm-1* attached on the surface of blades shown in D; (G) Blade shown in D after monospore release; (H) Monospore germlings of *gm-2* attached on the surface of blades shown in E; (I) Blade shown in E after monospore release; A–E, at the same magnification. Age: 42 d old in A–E, G and I, and 3–5 d old in F and H.

$F_1$ gametophytic blades having both parental color phenotypes, indicating that the wild-type conchocelis was heterozygous due to cross-fertilization. However, in these crosses, carpospores from the wild-type blade only yielded wild-type conchocelis. Conchospores from some conchocelis colonies developed only into the wild-type unsectored $F_1$ gametophytic blades, indicating that these conchocelis were homozygous. On the other hand, conchospores from other conchocelis colonies developed into both unsectored and sectored $F_1$ gametophytic blades having both parental color phenotypes, indicating that these conchocelis were heterozygous.

Color phenotypes and blade types of $F_1$ gametophytic blades from the heterozygous conchocelis produced in the cross *rm-1*♀×*wt*♂ are shown in Table 1. Only both the parental color phenotypes appeared in $F_1$ gametophytic blades, indicating that the mutant *rm-1* has a single mutation with respect to color. There were two types of unsectored blades and six types of sectored blades consisting of 2, 3 or 4 sectors. The sectored $F_1$ blades, reflecting the color segregation at meiosis, represented 98.9% of the total, giving a

*Table 1.* Color phenotypes, blade types and color segregation of $F_1$ gametophytic blades from heterozygous conchocelis in the cross *rm-1*♀×*wt*♂ in *Porphyra yezoensis*. W. wild-type; R. red

| Blade types | Number of blades *rm-1*♀×*wt*♂ |
|---|---|
| Unsectored | |
| W | 9 |
| R | 3 |
| Sectored[a] | |
| W+ R | 472 |
| R +W | 392 |
| W+ R +W | 78 |
| R +W+ R | 100 |
| W+ R +W+ R | 7 |
| R +W+ R +W | 4 |
| Unsectored | 1.1% |
| Sectored | 98.9% |
| Segregation ratio of colors in sectored blades | 1152W : 1184 R (1W : 1.03R) |

[a] Color sectors are shown in the order from the base to the apex.

nearly perfect 1:1 segregation ratio (1152W:1184R) in the sectored blades.

After 4 weeks in aerated culture, monospore production of the $F_1$ blades was examined microscopically. Many monospores released only from the unsectored red blades and the red sectors of the sectored blades. High monospore production was observed not only in the upper but also in middle and lower red sectors of the sectored blades when they were cultured with aeration. In the absence of large wild-type blades, the young red unsectored $F_1$ blades and the red sectors of the sectored $F_1$ blades often vanished after releasing many monospores, leaving only unsectored wild-type blades or wild-type sectors. However, when the $F_1$ blades were cultured without aeration, fewer monospores were usually released from the red unsectored blades and the upper red sectors of the sectored blades. Most (98.8%) of the monospore germlings developed from the monospores of the $F_1$ blades were the red one, which released monospores again in 3-4 weeks.

## Discussion

Several high monospore-producing mutants were obtained by treatment with MNNG (Yan, 1997). Most of them were different in color compared to the wild-type. Out of seven such mutants, six were red, red

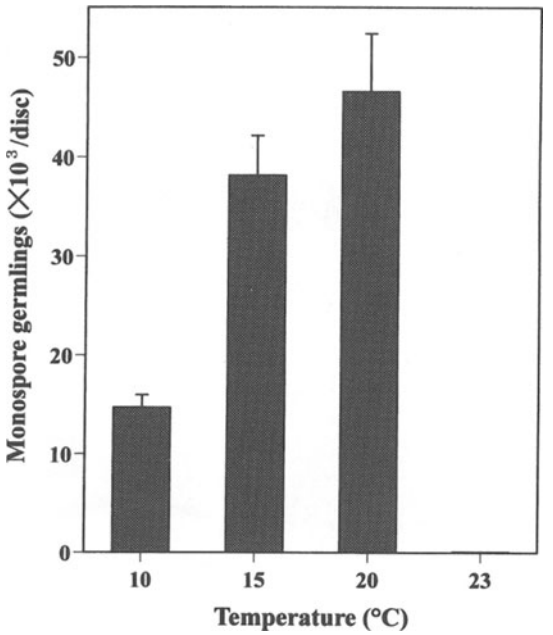


*Figure 4.* Effects of temperature on monospore production of the mutant (*rm-1*) blade discs. Monospore germlings developed from the monospores released from the mutant blade discs. Results represent the means of three independent experiments ± SE.

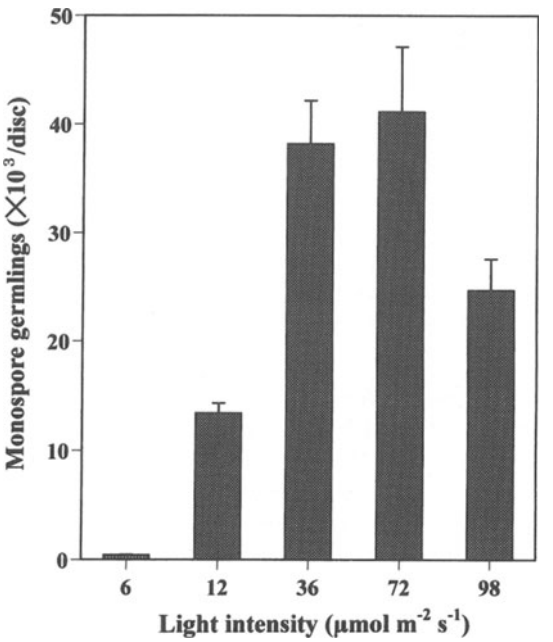


*Figure 5.* Effects of light intensity on monospore production of the mutant (*rm-1*) blade discs. Monospore germlings developed from the monospores released from the mutant blade discs. Results represent the means of three independent experiments ± SE.

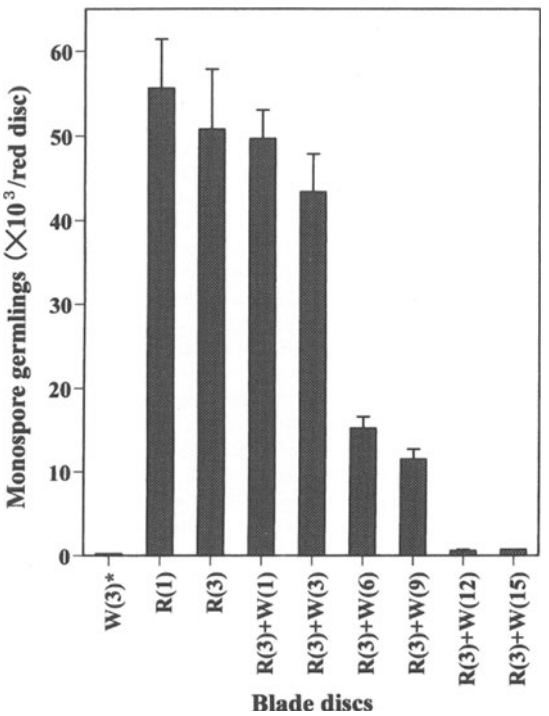


*Figure 6.* Monospore production of the mutant (*rm-1*) blade discs (R) in monoculture and co-culture with the wild-type blade discs (W). Monospore germlings developed from the monospores released from the mutant blade discs. *Monospore germlings of the wild-type blade discs. The number of the blade discs is shown in parentheses. Results represent the means of three independent experiments ± SE.

orange, yellow orange and green, and one was like the wild-type in color. In general, the greenish mutants obtained with MNNG treatment tend to produce fewer monospores compared to the wild-type and other pigmentation mutants. Therefore, the high monospore-producing green mutant *gm-2* is particularly interesting. The high monospore-producing mutants *rm-1* and *gm-2* matured much later than the wild-type. Blade discs taken from the 3-month-old blades of both the mutant *rm-1* and the wild-type, which were cultured under the same conditions and at the same age, were cultured alone under the same conditions for growth test. After 20 d in culture, the mutant blade discs and the wild-type discs grew respectively to 11.6 and 2.2 times the original size in diameter, suggesting that the old mutant blade discs continued to exhibit fast growth rate.

Gametophytic blades of the high monospore-producing mutants *rm-1* and *gm-2* were thinner (their blade thickness was 69–74% of the wild-type) and lacked elasticity as compared with the wild-type and other pigmentation mutants. In the sectored $F_1$ gametophytic blades from heterozygous conchocelis produced in the cross *rm-1*♀×*wt*♂, blade thickness of the red sector was only 72% of the wild-type sector. These results suggest that components and construction of both cell wall and cell wall matrix in these mutants might have been changed. When cultured under the same culture conditions, the gametophytic blades of the spontaneous red mutant (*C-22*) (Aruga & Miura, 1984), which showed very similar color and *in vivo* absorption spectra to the mutant *rm-1*, did not release many monospores, indicating that these two mutants are different.

Culture conditions, e.g., temperature, light intensity and aeration, significantly affected monospore production of the mutants. The young mutant blades, which developed from either conchospores or monospores, often began to release many monospores in the fourth week in aerated culture but released fewer monospores even in the sixth week of culture without aeration. When culture conditions were quickly changed, e.g., by changing all the culture media, applying aeration and increasing temperature and/or light intensity, the mutant blades usually released so many monospores that the upper portions of the blades quickly vanished. When the mutant blade discs were cultured at too high a temperature (23 °C) or under too low a light intensity (6 $\mu$mol photons $m^{-2}$ $s^{-1}$), no substantive growth was observed and very few monospores were obtained. The mutant discs (*rm-1*) taken from the large blades (10–15 cm long, 3 months old) usually began to release monospores after the discs had grown to 1.5–2.5 times the original size in diameter after about 2 weeks of aerated culture. However, the mutant discs taken from the young blades (3–5 cm long, 5 weeks old) often released many monospores within a week under the same culture conditions. These results suggest that monospore production of the mutants varied according to variations in their physiological environment and the age of their blades.

The young blades and the large blade discs of the mutant *rm-1* often began to release monospores simultaneously in the periphery and the central part of the blade or blade disc. However, the wild-type blade usually released only very few monospores in the periphery of the blade. During the formation of monospores, most parts of the mutant blades faded 2–3 days before monospore release. After releasing many monospores, most parts of the blade vanished and the mucous substances remained.

When the blades or blade discs of the mutant *rm-1* were co-cultured with large blades or blade discs of the wild-type, they stopped producing monospores and developed into large blades. The results suggest that the mutant probably needs some substance(s) released by the large wild-type blades to prevent monospore production. However, there is another possibility that the nutrients in the culture medium will be more quickly exhausted by adding the large wild-type blades or its blade discs into cultures of the mutant blades or blade discs, and let monospore production of the mutant blades be prevented.

In $F_1$ gametophytic blades from heterozygous conchocelis in the crosses of *rm-1*×*wt*, the high monospore production of the blades was associated with the mutant color. This result suggests that the high monospore production of the mutant is controlled by gene(s) that is closely linked with the gene for the mutant color. However, the number of genes involved in this high monospore production of the mutant was unclear.

The high monospore-producing mutants reported in the present study will be useful for studying the mechanism of formation and release of monospores in *P. yezoensis*. The mutant *rm-1* will also be directly used in obtaining large number of monospores as a primary source of spores for seeding nets in *P. yezoensis*. It will also be used in further breeding because it has several other desirable characters, including fast growth and late maturation relative to the wild-type.

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