Factors influencing the induction of adventitious bud and callus in the brown alga *Sargassum horneri* (Turner) C. Agardh

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Running title: Adventitious bud and callus induction from *Sargassum* explants

Abstract

A high frequency of callus induction and propagation from leaf explants of the brown alga *Sargassum horneri* was achieved when grown in medium supplemented with 5 μM uniconazole, which is a triazole-type inhibitor of cytochrome P450 enzymes, within two months of culture. Adventitious buds were efficiently formed from the pigmented callus after transfer to medium without uniconazole, indicating that treatment with uniconazole was more beneficial for the regeneration of the alga. The results showed that favorable culture conditions for induction of adventitious buds and calli included temperatures of 15°C to 25°C and light levels of 20 to 200 μmol photons m^{-2} s^{-1}. Blue light promoted the production of adventitious buds and calli. The frequency of formation of adventitious buds and calli in explants from thalli with one leaf was more than 90%, while it was 10% only when explants sourced from thalli with nine to eleven leaves. These findings will be useful for clonal propagation and storage of seed materials for mariculture of *Sargassum* species.

Key index words: brown alga; clonal propagation; explant culture; seed material; plant growth regulators; uniconazole

Introduction

Species of *Sargassum*, a genus of brown algae (Phaeophyceae), forms luxuriant forests on rocky coasts around the world and play an important role in the coastal ecosystems that provide spawning and nursery habitats for a variety of marine organisms (Choi et al. 2008; Komatsu et al. 2008). *Sargassum* is also an effective algal biofilter for the removal of nutrients from effluent water of fish farms (Pang et al. 2009). In addition, the genus has been recently receiving attention as a valuable source of health improvement because these algae contain a number of useful bioactive compounds including polyunsaturated fatty acids, fucoxanthin, polysaccharide, and polyphenols, which exhibit diverse biological activities such as anti-obesity, anti-inflammatory, antioxidant, antitumor, and antiviral activity (Miyashita et al. 2011; Liu et al. 2012; Yende et al. 2014). Despite their value as resources, the area of natural *Sargassum* beds has been decreasing due to anthropogenic causes including land reclamation (Terawaki et al., 2003; Okuda 2008). In addition, elevated sea temperature caused by global warming will change distribution of *Sargassum* species, leading to changes in the habitats of marine organisms (Komatsu et al. 2014). Thus, effective methods for the maintenance and restoration of populations of *Sargassum* species are urgently required, and are also of paramount importance.

Tissue culture techniques have potential for application to the micropropagation of desired strains in seaweeds. To date, there are many studies wherein successful induction and regeneration of calli have been reported from a diverse group of macroalgae (Reddy et al. 2008; Baweja et al. 2009). Two types of callus, colorless callus and pigmented callus, have been developed from sectioned tissues of *Sargassum*

species (Polne-Fuller and Gibor 1987; Hwang et al. 1994). There have also been reports of the development of adventitious tissues from undamaged or injured tissue of some *Sargassum* species under laboratory culture. For example, regenerated shoots were directly produced from excised tissues of *S. heterophyllum* and *S. confusum* (Mooney and Van Staden 1985; Kirihara et al. 1997) and the formation of adventitious buds from excised leaves was also found in *S. horneri* and *S. thunbergii* (Nanba 1993; Li et al. 2010). In addition, the spontaneous formation of adventitious embryos from intact leaves was observed in young thalli of *S. macrocarpum* (Yoshida et al. 1999). However, little is known about factors influencing the induction of callus and adventitious tissues of *Sargassum*.

Sargassum horneri (Turner) C. Agardh is one of the *Sargassum* species chosen for the construction of artificial *Sargassum* beds in Japan and Korea because of their fast growth rate, with seedlings growing up to 2-3 m long within 10 months after transplantation (Yamauchi 1984; Choi et al. 2008). In the present study, we examined the factors influencing the formation of adventitious buds and calli of *S. horneri* toward the development of efficient methods for the production of artificial seedlings.

Materials and methods

Algal materials

The mature female thalli bearing fertilized eggs of *S. horneri* were collected from the coast of Hakodate (Hokkaido prefecture, Japan) in July 2013. Fertilized eggs were shed on plastic Petri dishes containing autoclaved artificial seawater (ASW) from the mature

receptacles by coverslips. Microscopic contaminants were removed by manual brushing under a stereo microscope. After brushing, fertilized eggs were washed three times by pipettes in new ASW and were cultured at 20°C under photoperiod regimes: 12 h light:12 h dark with cool-white fluorescent lamps at 80 μmol photons m^{-2} s^{-1}. Embryos were cultured in plastic Petri dishes containing 40 mL sterile ESL medium (Kitade et al. 2002) to which 2.0 mg germanium dioxide (GeO_2) per 1 L ESL had been added to inhibit the growth of diatoms. Culture medium was exchanged every week. Juvenile thalli that grew to ca. 10 mm in blade length with three to four leaves were used for the experiments of the effect of environmental factors on development of adventitious buds and callus. Juvenile thalli with one leaf, two leaves, three to four leaves, or nine to eleven leaves were used for the experiments of the effect of different developmental stages on formation of adventitious buds and callus.

Induction conditions for development of adventitious buds and callus

Tissues from undulate leaves of juvenile thalli with three to four leaves were cut into segments (ca. 2 mm x 2 mm squares) using a dissecting knife. The 20 explants were transferred into each Petri dish with 40 mL of sterile ESL medium. The effect of environmental factors on development of adventitious buds was examined through the following experiments. Twenty explants per condition were cultured in a cultivation chamber set at various temperatures (from 5 to 30°C) and photon flux densities (from 0 to 200 μmol photons m^{-2} s^{-1}), while the other experimental conditions were the same as described above for embryo cultures. Another 20 explants each were cultured at 20°C and 80 μmol photons m^{-2} s^{-1} under blue, red and white light conditions. White

fluorescence (PA-LOOK fluorescent lamp; Panasonic Co., Ltd., Osaka, Japan) was used as a source of white light (peaks at 450, 540 and 610 nm). Blue light (400-530 nm, peak at 470 nm) and red light (600-700 nm, peak at 660 nm) were supplied by light-emitting diodes (MIL-B18, MILR18; Sanyo Electric Biomedical Co., Ltd., Japan). Leaves of juvenile thalli at different developmental stages – thalli with one leaf (first leaf), two leaves (first leaf), three to four leaves (undulate leaf), or nine to eleven leaves (serrate leaf) – or undulate leaves of regenerated juvenile thalli with three to four leaves from adventitious buds were used for explant cultured at 20°C and 80 μmol photons m^{-2} s^{-1} condition. The number of adventitious buds and callus from the explants was measured under a microscope after one month.

To examine the effects of a plant growth regulator (PGR) on callus induction, 20 explants from undulate leaves of juvenile thalli with three to four leaves were cultured at 20°C and 80 μmol photons m^{-2} s^{-1} with ESL medium containing auxin [indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)], cytokinin [trans-zeatin and 6-benzylaminopurine (BA)], and Gibberellin A3 (GA) (Wako Pure Chemical Industries, Osaka, Japan) at concentrations of 0.1, 1.0, 10.0, and 100 μM for one month. To study callus induction, another 20 leaf explants were also cultured at 20°C and 80 μmol photons m^{-2} s^{-1} with Uniconazole P (Wako Pure Chemical Industries), a gibberellin biosynthesis inhibitor, at concentrations of 1.0, 3.0, 5.0, 7.0, and 10.0 μM. The efficiency of callus induction was examined in the same way as the induction of adventitious buds. To examine regeneration of callus, induced calluses were transferred from ESL medium supplemented with uniconazole to ESL medium without uniconazole and cultured at 20°C and 80 μmol photons m^{-2} s^{-1}.

Statistical analysis

Data are expressed as the means ± standard deviation (S.D.) of two independent experiments with 20 explants each condition. To compare the numbers of adventitious buds and calli formed per leaf explant, we used a non-parametric analysis of variance (Kruskal-Wallis test) followed by the Steel-Dwass multiple comparison test. Pairs of means for which $P<0.05$ were considered significantly different.

Results

Direct regeneration of adventitious buds from leaf explants

Adventitious buds were produced from both the section area and the surface area of leaf explants of *S. horneri* after 2-4 weeks (Fig. 1a), and they subsequently grew into adventitious shoots (Fig. 1b). After 4 months of cultivation, normal juvenile thalli that formed serrated leaves were regenerated from leaf explants via adventitious shoots (Fig. 1c). Various water temperatures and photon flux densities were tested to find out appropriate culture conditions for the induction of adventitious buds from the leaf explants. As shown in Tables 1 and 2, adventitious buds were efficiently induced in explants incubated at temperatures from 15°C to 25°C and light intensities from 20 to 200 μmol photons m^{-2} s^{-1}, while no adventitious bud formation was observed at 5°C or in dark conditions. The frequency of adventitious bud formation from explants incubated at 20°C was 37.5%. The effects of light wavelengths on induction of

adventitious buds are shown in Fig. 2. The number of adventitious buds under the red light condition was less than one-tenth of that under white or blue light. Additionally, elongation of adventitious shoots was repressed under red light (Fig. 2b).

Next, juvenile thalli at different ages and juvenile thalli regenerated from adventitious shoots were used for evaluating the formation of adventitious buds in explants of various origins. The formation rate of adventitious buds in explants from thalli with one leaf was more than 90%, while it was 7% only when explants sourced from thalli with nine to eleven leaves (Fig. 3a). The number of adventitious buds per explant significantly decreased for older leaf tissues (Fig. 3b). In addition, the frequency of adventitious bud formation in explants from regenerated juvenile thalli via adventitious shoots was lower than that in explants from juvenile thalli derived from zygotes as well as the number of adventitious buds per explant (Fig. 3c and d).

Indirect regeneration of adventitious buds via callus

Callus from leaf explants were not induced by treatment of auxins (IAA and 2,4-D), cytokinins (trans-zeatin and BA), and gibberellin (GA) (data not shown). However, uniconazole at concentrations of 5 to 10 µM stimulated formation of callus but not adventitious buds from leaf explants, while at 0 to 3 µM produced adventitious buds but not callus (Fig. 4). Treatment with 5 µM uniconazole induced the highest number of calli per leaf explant and led to more efficient callus propagation when compared with concentrations greater than 7 µM. All calli induced by uniconazole were dark brown pigmented calli but not clear and filamentous calli. Propagation of callus from explants was observed during two months of culture in ESL medium containing 5 µM

uniconazole (Fig. 5a and 5b). To induce plant regeneration, each propagated callus was transferred to ESL medium without uniconazole. After one month, all calli formed adventitious buds, and the number of adventitious buds regenerated from callus was approximately 20-fold greater than those leaf explants not treated with uniconazole (control) (Fig. 5c-5f).

For efficient callus induction, the leaf explants were cultured with 5 μM uniconazole under various conditions. As shown in Table 3, calli were efficiently induced in explants incubated at 20°C. In addition, blue light promoted the production of calli, while red light repressed it. Interestingly, when explants were treated with uniconazole at 30°C, adventitious buds were observed in 20% of explants. As shown in Fig. 6, the number of calli induced per leaf explant was significantly influenced by the age of the explant of origin, similar to the induction of adventitious buds. The frequency of calli in explants from thalli with one leaf was ca. 90%, whereas those explants from thalli with nine to eleven leaves was 2.5% (Fig. 6a). The number of calli per explants significantly decreased in thalli with nine to eleven leaves (Fig. 6b).

Discussion

In this study, the induction and propagation of pigmented callus was observed in leaf explants of *S. horneri* grown medium supplemented with 5 μM uniconazole. Furthermore, induced calli treated with uniconazole produced significantly high number of adventitious buds than did explants not treated with uniconazole.

Calli of brown algae are generally classified into broad two types, clear callus and pigmented callus. Only ca. 10% of tissue sections from *Sargassum* species formed calli,

and the clear calli of *Sargassum* species did not differentiate into plantlets, whereas ca. 30% of the pigmented calli differentiated into young plants (Polne-Fuller and Gibor 1987; Kumar et al. 2007). The frequency of calli in explants from *S. horneri* thalli with one leaf was ca. 90%, and all induced calli regenerated into normal young plants (Fig. 6). These results indicate that uniconazole treatment can efficiently induce calli from *Sargassum* tissue and lead to plantlet regeneration.

Uniconazole has been reported as a triazole-type inhibitor of cytochrome P450 enzymes for t-zeatin biosynthesis (Sasaki et al. 2013), brassinosteroid biosynthesis (Iwasaki and Shibaoka 1991), and abscisic acid (ABA) catabolism (Saito et al. 2006), as well as GA biosynthesis (Izumi et al. 1985). Thus, these PGRs may be involved in callus formation in *S. horneri*, but it has not been reported that they affect callus induction in brown algae. In higher plants, loss-of-function mutations impaired in cell wall synthesis lead to PGR-independent callus formation (Ikeuchi et al. 2013). For example, in *Arabidopsis thaliana*, *tsds*, mutants with impaired in biosynthesis of cellulose, grows as a callus without the otherwise indispensable supply of auxin and cytokinin (Frank et al. 2002; Krupková and Schmülling 2009). Interestingly, ancymidol, known as an azole-type inhibitor of cytochrome P450 enzymes similar to uniconazole, can inhibit cellulose synthesis as well as GA biosynthesis (Hofmannova et al. 2008). Thus, uniconazole may inhibit cellulose synthesis in *S. horneri*, thereby leading to callus formation, although further experiments are required to clarify the underlying mechanisms.

Development of techniques for maintenance of seed stock is important for mariculture of seaweeds. Vegetative microscopic gametophytes are available for stock strains of several useful brown algae, such as *Saccharina japonica* (Kombu) and

Undaria pinnatifida (Wakame), which provides a constant supply of sporophytes. In *Sargassum* species, embryos were stored under low temperature and low light intensity; however, the germination rate of embryos stored for more than two years decreased to 10% (Yoshida et al. 2000). It has been reported that callus cells of macroalgae maintained their viability for over three years on agar medium under low light and that 70-90% of the calli could be regenerated after prolonged storage (Polne-Fuller and Gibor 1987). Thus, uniconazole is a useful reagent for developing seed stocks of *Sargassum* species as well as for efficient production of seedlings.

The effects of environmental factors and explant origins on formation of adventitious buds and calli were also examined. Water temperatures have a greater influence than photon flux densities in the formation of both adventitious buds and calli in *S. horneri*. Optimum water temperatures of formation of adventitious buds and calli are consistent with those for the growth of whole plants in *S. horneri* (Baba 2007). In contrast to water temperatures, the growth rate of *S. horneri* thalli decreased under low photon flux densities (Yoshida et al. 1995), whereas the frequency of callus induction of *S. horneri* did not decrease under even 20 μmol photons m^{-2} s^{-1}, indicating that optimum photon flux densities for the induction of calli are inconsistent with the optimal conditions for the growth of whole plants. Interestingly, callus formation in *S. japonica* was induced even in the dark (Mizuta et al. 2007), while the explants cultured under dark conditions did not produce any calli in *S. horneri*.

Light wavelength has a marked effect on production of adventitious buds and calli in higher plants (Morini et al. 2000; Hunter and Burritt 2004). In contrast to higher plants, little is known concerning the effect of light wavelength on both adventitious buds and calli in macroalgae, but in the Laminariaceae, it has been demonstrated that

cultivation under blue light favored sporophyte regeneration from callus-like cell suspension (Asensi et al. 2001), whereas callus formation was repressed by blue light (Mizuta et al. 2007). In contrast, *S. horneri* explants cultured under red light exhibited a decrease in the induction rate of adventitious buds and calli compared with those cultured under white or blue light, indicating that adventitious bud and callus formation was stimulated by blue light. These results suggests the differences of callus formation in responsive to light wavelengths between Laminariaceae and Sargassaceae. In this study, formation of adventitious buds was also promoted by blue light. In Fucales, blue light have the effect on induction of the photopolarization of the zygote and stimulate cytoplasmic ion gradients and actin localization that are present during the photopolarization (Bisgrove and Kropf, 2007). Localization of actin filaments was observed in the tip of adventitious buds of *S. horneri* by staining Alexa Fluor 488 phalloidin (data not shown). These findings suggested that the mechanism of adventitious buds formation might have a similar that of photopolarization of the zygote. Some previous studies have suggested that photoreceptors are involved in the responses to different light wavelengths in the development of adventitious tissue and callus in higher plants (Huan and Tanaka 2004; Saitou et al. 2004). Aureochromes, the new type of blue light receptor, have been identified in the brown algae Fucales and Laminariales (Takahashi et al. 2007; Deng et al. 2014). Thus, Aureochromes may serve as photoreceptors for the formation of adventitious buds and calli in brown algae.

The younger explants of *S. horneri* showed a much higher capacity for forming calli and adventitious buds than did the older explants or regenerated plantlets. Several studies have shown that younger explants exhibit greater morphogenic potential than older explants in higher plants (Nikam and Shitole 1999; Mok and Norzulaani 2007).

Collection or cultivation of young tissue is also a critical step for successful micropropagation in macroalgae (Baweja et al. 2009). Loss of morphogenic ability during ageing has been related to changes in gene expression caused by DNA methylation (Fraga et al. 2002), and DNA methylase inhibitors repressed adventitious bud induction in *Petunia* leaf explants (Prakash et al. 2003). In addition, alterations of DNA methylation levels were observed in calli and regenerated plantlet (Gao et al. 2014; Stelpflug et al. 2014). These findings suggest that changes in DNA methylation may cause the low frequency of callus and bud induction in older explants and regenerated plantlets from *S. horneri*.

In conclusion, we have developed a simple and efficient technique for the formation of adventitious buds via the calli induced by uniconazole in *S. horneri*. This technique is potentially useful for clonal propagation of strains with desired traits such as production of high contents of valuable metabolites. The present method for callus induction is also important for maintenance of elite strains as seed banks for future cultivation.

Acknowledgments

We are grateful to Takashi Tani (Tetsugumi industry Co., Ltd., Japan) for kindly helping to collect mature thalli of *S. horneri* and to Dr. Hajime Yasui (Hokkaido University, Japan) for kindly providing microscopes and helpful discussion. This work was supported by a National Project for the Formation of Tohoku Marine Science Center (Innovation Strategy for the Industrial Seaweed Utilization Supporting Regional Seeds and Local Features of Sanriku Coast) grant from MEXT (Ministry of Education, Culture, Sports, Science, and Technology of Japan).

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Figure legends

Fig. 1 Development of adventitious buds from leaf explants of *S. horneri*

(a) Adventitious bud formation on a leaf explant (scale bar, 1 mm). (b) Regenerated shoots via adventitious buds after one month (scale bar, 1 mm). (c) Regenerated serrated leaves via adventitious buds after four months (scale bar, 2 mm).

Fig. 2 Effect of light wavelength on the adventitious bud formation in the leaf explants of *S. horneri*

Adventitious bud formation was observed in the leaf explants cultured under white light (a), red light (b), and blue light (c). The number of adventitious buds per leaf explant in different light qualities (d). Data represent the result for two independent experiments with 20 explants each condition. The asterisk indicates a significant difference at $P<0.05$ (by the Steel-Dwass test).

Fig. 3 Effect of different explant origin on the adventitious bud formation in the leaf explants of *S. horneri*

(a) The formation rate of adventitious buds in leaf explants from juvenile thalli at different developmental stage. Leaves of juvenile thalli at different developmental stages – thalli with one leaf (first leaf), two leaves (first leaf), three to four leaves (undulate leaf), or nine to eleven leaves (serrate leaf), were cut into explants. (b) The numbers of induced adventitious buds per leaf explant from juvenile thalli at different

developmental stages. Data represent the result for two independent experiments with 20 explants each condition. Different letters indicate significant differences at $P<0.05$ (by the Steel-Dwass test). (c) Comparison of the rate of formation of adventitious buds in explants from juvenile thalli via adventitious shoots with those formed via zygotes (d) Comparison of the number of adventitious buds per leaf explant from juvenile thalli via adventitious shoots with those formed via zygotes. Data represent the result for two independent experiments with 20 explants each condition. The asterisk indicates a significant difference at $P<0.05$ (by the Steel-Dwass test).

Fig. 4 Callus induction from the leaf explants of *S. horneri* by uniconazole treatment
The explant of *S. horneri* treated with 0 μM uniconazole (a), 3 μM (b), 5 μM (c), 10 μM (d), (Scale bar, 1 mm) The number of adventitious buds (white column) or callus (black column) per leaf explant with different concentration of uniconazole (e). Data represent the result for two independent experiments with 20 explants each condition.

Fig. 5 Regeneration of shoots from calli induced by uniconazole treatment
(a) Propagation of pigmented calli from leaf explants during 2 months of culture in ESL medium containing 5 μM uniconazole (scale bar, 1 mm) (b) Magnified view of pigmented callus during 2 months of culture in ESL medium containing 5 μM uniconazole (scale bar, 100 μm) (c) Formation of adventitious buds from propagated callus without uniconazole (scale bar, 1 mm) (d) Magnified view of adventitious buds (arrows) on propagated pigmented callus (arrowhead) without uniconazole (scale bar, 100 μm) (e) The formation rate of regenerated adventitious buds from callus. Callus formation was induced by treatment with 5 μM uniconazole for 2 months. Control

indicates leaf explants not treated with uniconazole. (f) The number of adventitious buds per callus or leaf explant not treated with uniconazole (control).

Fig. 6 Induction of callus in the leaf explants of *S. horneri* at different developmental stages. (a) The rate of callus formation in explants from juvenile thalli at different developmental stages. Leaves of juvenile thalli at different developmental stages – thalli with one leaf (first leaf), two leaves (first leaf), three to four leaves (undulate leaf), or nine to eleven leaves (serrate leaf), were cut into explants. (b) The numbers of calli induced per leaf explant from juvenile thalli at different developmental stage. Data represent the result for two independent experiments with 20 explants each condition. Different letters indicate significant differences at $P<0.05$ (by the Steel-Dwass test).

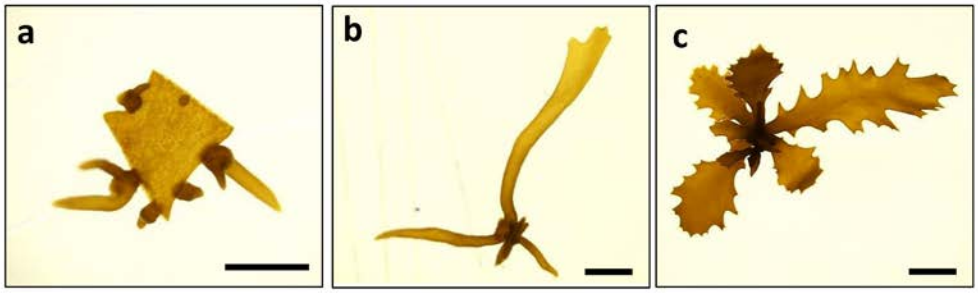

Fig. 1

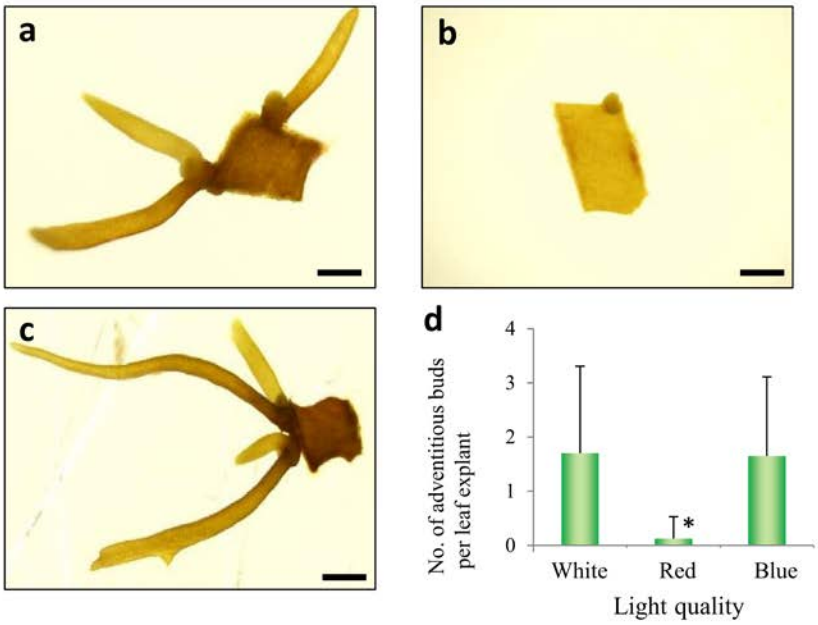
a
b
c
d
No. of adventitious buds per leaf explant
4
3
2
1
0
*
White
Red
Blue
Light quality

Fig. 2

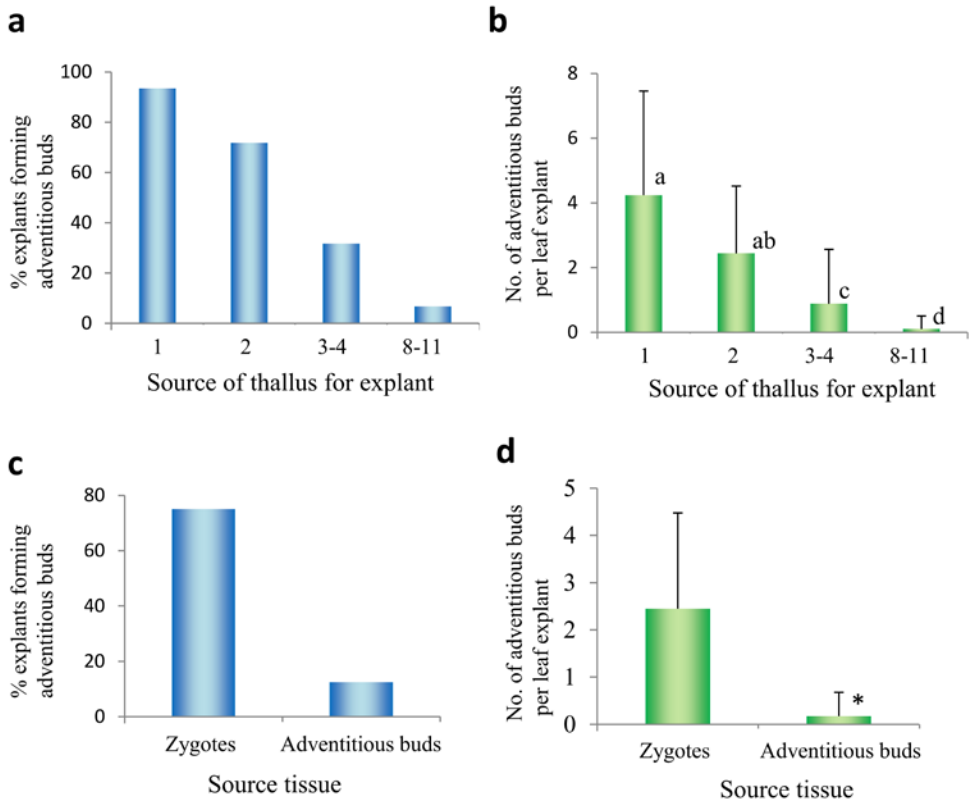
a
% explants forming adventitious buds
100
80
60
40
20
0
1
2
3-4
8-11
Source of thallus for explant
b
No. of adventitious buds per leaf explant
8
6
4
2
0
a
ab
c
d
1
2
3-4
8-11
Source of thallus for explant
c
% explants forming adventitious buds
80
60
40
20
0
Zygotes
Adventitious buds
Source tissue
d
No. of adventitious buds per leaf explant
5
4
3
2
1
0
*
Zygotes
Adventitious buds
Source tissue

Fig. 3

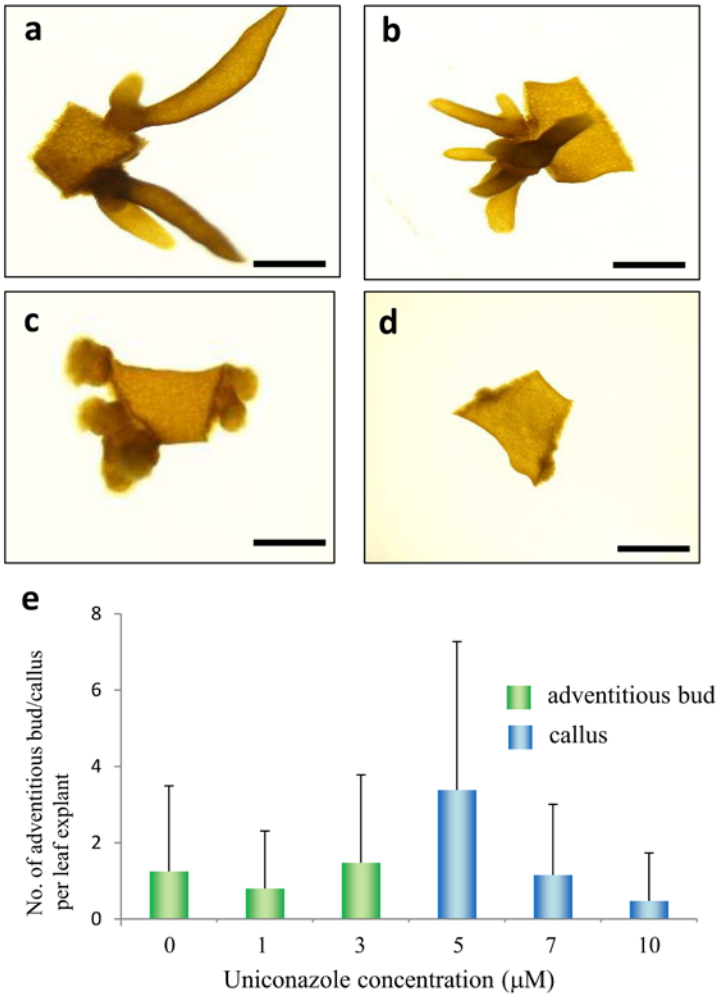
a
b
c
d
e
No. of adventitious bud/callus per leaf explant
0
2
4
6
8
adventitious bud
callus
0
1
3
5
7
10
Uniconazole concentration (μM)

Fig. 4

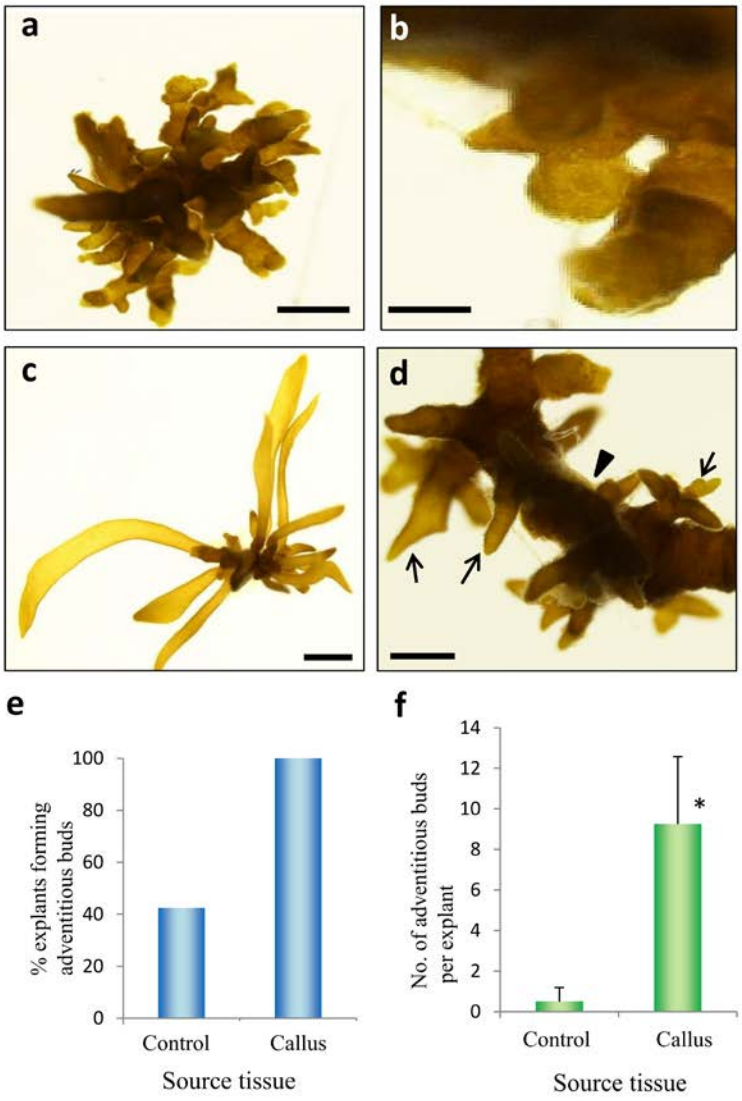
a
b
c
d
e
% explants forming adventitious buds
100
80
60
40
20
0
Control
Callus
Source tissue
f
No. of adventitious buds per explant
14
12
10
8
6
4
2
0
*
Control
Callus
Source tissue

Fig. 5

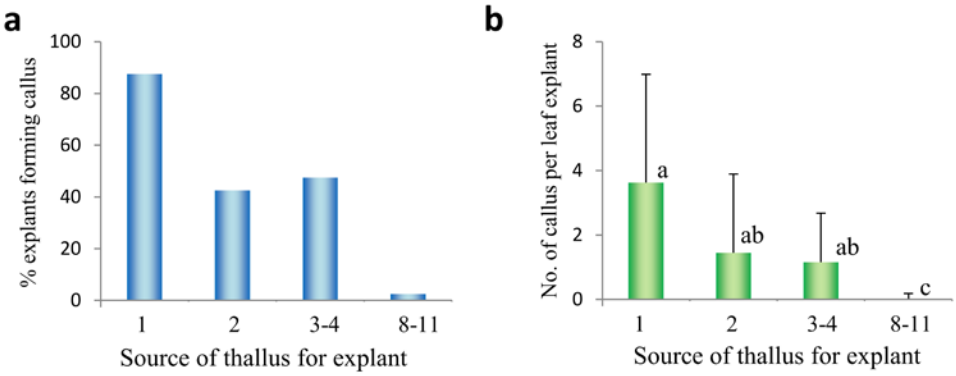
a
% explants forming callus
100
80
60
40
20
0
1
2
3-4
8-11
Source of thallus for explant
b
No. of callus per leaf explant
8
6
4
2
0
a
ab
ab
c
1
2
3-4
8-11
Source of thallus for explant

Fig. 6

Table 1. Effect of water temperatures on adventitious bud formation in the leaf explants of *S. horneri*

Water temperature (°C)	% of explant forming adventitious buds	No. of adventitious bud per explant
5	0.0	0.00
10	7.5	0.13
15	27.5	0.50
20	37.5	0.60
25	32.5	0.40
30	2.5	0.03

Data are expressed as the means of two independent experiments with 20 explants each condition.

Table 2. Effect of photon flux densities on adventitious bud formation in the leaf explants of *S. horneri*

Photon flux densities (μmol photons m^{-2} s^{-1})	% of explant forming adventitious buds	No. of adventitious bud per explant
0	0.0	0.00
20	50.0	0.75
40	45.0	1.08
80	37.5	0.73
100	22.5	0.43
200	35.0	0.88

Data are expressed as the means of two independent experiments with 20 explants each condition.

Table 3 Effect of environmental factors on induction of callus in the leaf explants of *S. horneri*

	Water temperature (°C)			Photon flux densities (μmol photons m^{-2} s^{-1})			Light quality		
	5	20	30	20	80	200	White	Red	Blue
% of explant forming callus	0.0	37.5	2.5	50.0	37.5	30.0	42.5	27.5	45.0
No. of callus per explant	0.00	1.20	0.03	1.05	1.20	1.35	1.45	0.95	3.08
% of explant forming adventitious buds	0.0	0.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0

The explant of *S. horneri* treated with 5 μM uniconazole for induction of callus.
Data are expressed as the means of two independent experiments with 20 explants each condition.