Evaluation of disease resistance and tolerance to elevated temperature stress of the selected tissue-cultured *Kappaphycus alvarezii* Doty 1985 under optimized laboratory conditions

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Abstract

Information on the abiotic stress tolerance and ice–ice disease resistance properties of tissue-cultured *Kappaphycus alvarezii* is scarce and can pose a big hurdle to a wider use of tissue-cultured seaweed in the industry. Here, we reported on a study of seaweed-associated bacteria diversity in farmed and tissue-cultured *K. alvarezii*, and ice–ice disease resistance and elevated growth temperature tolerance of tissue-cultured *K. alvarezii* in laboratory conditions. A total of 40 endophytic seaweed-associated bacteria strains were isolated from 4 types of *K. alvarezii* samples based on their colony morphologies, Gram staining properties and 16S rRNA gene sequences. Bacteria strains isolated were found to belong to *Alteromonas* sp., *Aestuariibacter* sp., *Idiomarina* sp., *Jejuia* sp., *Halomonas* sp., *Primorskyibacter* sp., *Pseudoalteromonas* sp., *Ruegeria* sp., *Terasakiella* sp., *Thalassospira* sp. and *Vibrio* sp. *Vibrio alginolyticus* strain ABI-TU15 isolated in this study showed agar-degrading property when analyzed using agar depression assay. Disease resistance assay was performed by infecting healthy *K. alvarezii* with 10^5 cells/mL *Vibrio* sp. ABI-TU15. Severe ice–ice disease symptoms were detected in farmed seaweeds compared to the tissue-cultured *K. alvarezii*. Besides disease resistance, tissue-cultured *K. alvarezii* showed better tolerance to the elevated growth temperatures of 30 and 35 °C. In conclusion, our overall data suggests that tissue-cultured *K. alvarezii* exhibited better growth performance than farmed seaweeds when exposed to elevated growth temperature and ice–ice disease-causing agent.

Keywords *Kappaphycus alvarezii* · Tissue-cultured seaweed · Ice–ice disease · 16S rRNA gene · Carrageenan

Introduction

Seaweed is an important source of food hydrocolloids, such as agar, alginates and carrageenan. Seaweed contains many types of minerals including calcium, magnesium, potassium,

copper and iron, which makes it a good source of nutrients for human consumption. Seaweed is an important member of marine ecosystems as it supports marine organisms individually as a source of food and at marine ecosystem level as habitat formers and primary producers (Egan et al. 2014). *Kappaphycus alvarezii*, a type of seaweed belonging to the red algae species (Rhodophyta) has been intentionally introduced in Africa, South East Asia and America as mariculture for production of carrageenan (Doty 1973; Ask et al. 2003; Seller et al. 2015). *Kappaphycus alvarezii* produces high amounts of kappa-carrageenan, a polysaccharide with alternating units of D-galactose and 3,6-anhydro-galactose, which is found on the outer surface of the seaweed (Sade et al. 2006). Carrageenan is important as it is used as a thickening and stabilizing agent in the food industry. Carrageenan is also used in toothpaste, cosmetics, and medicine and has the potential to be used as a carrier for anti-retroviral drugs for the treatment of HIV (Zacharopoulos and Phillips 1997; Guiry 2015).

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However, the natural stock of seaweed is slowly depleting due to increased incidents in seaweed diseases associated with marine bacteria resulting from an increase in anthropogenic stressors, such as pollutants and climate change (Gachon et al. 2010). One of the common diseases associated with seaweed is the ice–ice disease. Ice–ice disease is characterized by a change in seaweed pigment and softening of the thallus (Doty and Alvarez 1975). This decreases the carrageenan yield and produces inferior quality carrageenan (Mendoza et al. 2002). Ice–ice disease was first reported in 1974 in the Philippines. The severity of this disease was seen in Zanzibar in 2001 where more than 1000 tons of seaweed declined to almost zero in 2008, just over the course of 7 years (Msuya 2013). The pathogenic seaweed-associated bacteria causing ice–ice disease in *K. alvarezii* have been reported by Largo et al. (1995b, 1997, 1998, 1999), Burtado et al. (2003), and Achmad et al. (2016). Largo et al. (1995a) have also investigated the abiotic stresses that induce the early onset of the ice–ice disease in *K. alvarezii* under laboratory conditions.

Tissue culture techniques have been applied to mass produce disease-free plantlets of *K. alvarezii* in the laboratory before transferring them to seaweed farming sites (Reddy et al. 2003; Hurtado and Biter 2007; Yong et al. 2014b). Success in in vitro somatic embryogenesis and regeneration of somatic embryos to whole plantlets was reported by Reddy et al. (2003). These tissue-cultured *K. alvarezii* was shown to have growth rate as high as 1.5–1.8 times over farmed plants (Reddy et al. 2003) and shown to have better carrageenan properties (yield, viscosity, gel strength and sulfate content) than farmed samples (Yong et al. 2014a). Several research groups also claimed to have obtained disease-free *K. alvarezii* through somatic embryogenesis and micropropagation using axenic farmed *Kappaphycus* as parental plants (Reddy et al. 2003; Hurtado and Biter 2007; Yong et al. 2014b). However, tissue-cultured *K. alvarezii* showing the ice–ice disease symptoms were detected in our laboratory-maintained tissue-cultured seaweeds. Information on abiotic stress and biotic agents that induce ice–ice disease in tissue-cultured *K. alvarezii* is limited. Moreover, no study on ice–ice disease resistance of tissue-cultured *K. alvarezii* has been reported. Thus, we aim to investigate the ice–ice disease resistance and elevated growth temperature tolerance of tissue-cultured *K. alvarezii*.

Materials and methods

Plant materials

Four types of *K. alvarezii* samples were used in this study: (1) healthy farmed *K. alvarezii* without disease symptoms (HFC); (2) diseased farmed *K. alvarezii* with apparent

ice–ice disease symptoms (DFC); (3) healthy tissue-cultured *K. alvarezii* without disease symptoms (HTC) and (4) diseased tissue-cultured *K. alvarezii* with apparent ice–ice disease symptoms (DTC).

The farmed seaweed were originally collected from Selaikan Island (4°34'21.28"N, 118°41'41.44"E), Semporna, Sabah and maintained in temperature-controlled lab conditions for at least 1 month before used as explant materials for the tissue culture process. The term 'tissue cultured' seaweed is used for *K. alvarezii* regenerated from the same farmed samples through somatic embryogenesis.

Surface sterilization of *Kappaphycus alvarezii* and culture conditions

Seaweeds were surface sterilized to obtain the axenic cultures according to Hayashi et al. (2008) with modifications. The surface-sterilized materials were transferred to incubation flasks containing culture medium consisting of sterilized artificial seawater (ASW) and Provasoli's enriched seawater (PES) solution (Provasoli 1968). The salinity of the culture medium was maintained at 30 ppt according to Largo et al. (1995a) with modifications. The flasks were maintained at 25 °C in a temperature-controlled laboratory (Largo et al. 1995a). Controlled lighting (12 h light/12 h dark) was supplied by cool-white fluorescent tubes (Philips TLD36W/54–765) at about 50 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ irradiance. Both diseased and healthy seaweed branches were collected from the same farmed or tissue-cultured *K. alvarezii* seedlings that have been grown in the flasks maintained under the same laboratory condition. Five biological replicates from each seaweed types were pooled and used in this study for isolating seaweed-associated bacteria.

Isolation and morphological characterization of seaweed-associated bacteria

Isolation of endophytic seaweed-associated bacteria was carried out as described by Burtado et al. (2003). To ensure the bacteria species isolated are endophytic seaweed-associated bacteria, the seaweeds were surface-sterilized using 75% ethanol for 30 s to remove bacteria attached on the surface of seaweeds. The surface-sterilized seaweed samples were homogenized using sterile mortar and pestle. The samples were diluted with sterilized artificial seawater (ASW) and plated on agar media consisting of ASW and three different types of agar medium: Bacto Agar (BA), Plant Agar (PA) and Marine Agar (MA). The plates were incubated at room temperature for 2 days. Individual colonies with different forms and colour were streaked on respective agar medium to obtain pure bacteria cultures.

The morphologies were observed with a stereo microscope SMZ800N under 40X magnification (Nikon, Japan)

and recorded. Gram staining of pure cultures was performed on single pure colonies using a Gram-staining kit (R & M Chemicals Technology, Switzerland) according to manufacturer's instructions. Slides were then viewed under a compound light microscope ML5000 (Meiji, Japan) to observe cell morphology under 1000× magnification. The bacteria strains were classified based on the descriptor on colour, form, elevation, opacity and surface types (Holt 1994).

Agarolytic analysis of *Vibrio* sp.

Agarolytic analysis was conducted as described by Faturrahman et al. (2011). Pure cultures of *Vibrio* sp. were plated on modified marine agar medium: Difco™ Marine Broth 2216 (BD, USA) supplemented with 1.5% carrageenan (Sigma-Aldrich, USA). Plates were incubated at 30 °C and agarolytic activity was monitored daily, assessed by shallow depressions appearing around the colonies. After 7 days of incubation period, plates were flooded with Lugol's iodine solution and the appearance of pale-yellow zones around colonies against a brown-violet background was considered an indicator for agar-degrading activity.

PCR amplification of 16S rRNA gene and sequencing

16S rRNA PCR was performed as described in Fierer et al. (2005). The oligonucleotide primers Eub338F 5'-ACTCCTACGGGAGGCAGCAG-3' (Fierer et al. 2005) and Eub518R 5'-ATTACCGCGGCTGCTGG-3' (Fierer et al. 2005) were used for PCR amplification of partial 16S rRNA gene. The PCR amplification was performed in T100™ Thermal Cycler (Bio-rad, USA). The PCR products were examined by electrophoresis on 1% agarose gels, purified and sequencing was done by Integrated DNA Technologies Inc. DNA sequence homology searches were performed against prokaryotic 16S rRNA gene sequences maintained in NCBI genbank database using a Blastn algorithm (Altschul et al. 1990). All the sequences were submitted to NCBI genbank database under submission MG952541-MG952566. The bacteria species that show the highest percentage of similarity (> 95%) and lowest *E* value (< 1E−20) with reference 16S rRNA gene sequences based on Blastn analysis results were chosen as the potential bacteria species homologs to seaweed-associated bacteria isolated in this study.

Phylogenetic analysis of *Vibrio* species based on 16S rRNA gene

The 16S rRNA gene sequences of isolated *Vibrio alginolyticus* strain ABI-TU15, *Vibrio xuii* strain ABI-TU21, *Vibrio xuii* strain ABI-TU23 and *Vibrio xuii* strain ABI-TU50 were aligned together with 4 agarolytic *Vibrio* species (*V. agarivorans*, *V. astriarenae*, *V. alginolyticus* and

V. parahaemolyticus), 16 non-agarolytic *Vibrio* species (*V. atypicus*, *V. diazotrophicus*, *V. harveyi*, *V. azureus*, *V. jasicida*, *V. owebsii*, *V. diabolicus*, *V. hyugaensis*, *V. vulnificus*, *V. mediterranei*, *V. shilonii*, *V. nereis*, *V. thalassae*, *V. tritonius*, *V. zhanjiangensis*, *V. xuii* and *V. brasiliensis*) and 2 outgroup (*Photobacterium phosphoreum* and *Aliivibrio fischeri*) using ClustalW embedded in MEGA program version 7.0 (Kumar et al. 2016). The phylogenetic analysis was performed by applying the neighbor-joining method with bootstrap values of 1000 replicates. Evolutionary distances were computed using the Jukes–Cantor method.

Evaluation of growth temperature stress on *Kappaphycus alvarezii*

The evaluation of growth temperature stress was performed as described in Largo et al. (1995a) with modifications. Approximately 2 g (wet weight) of surface-sterilized seaweeds collected from different propagules of healthy farmed (4–5 biological replicates) and tissue-cultured (7–11 biological replicates) *K. alvarezii* were maintained at different temperatures (25, 30 and 35 °C) with agitation at 100 rpm. Two technical replicates were used for farmed and tissue-cultured *K. alvarezii*. Observations were made daily for up to 3 weeks for any sign of stress symptoms. The seaweed samples were then cultured in ASW with PES and maintained at their optimum growth temperature after growth temperature stress tolerance test to access the viability of the stressed seaweeds. The survival rate (%) of stressed seaweed samples was calculated, according to Nohrman (1953) with modification, as follows: Survival rate (%) = (no. of survived branches/no. of death branches + no. of survived branches) × 100%. SPSS version 24 program were used for statistical analysis of the survival rate by considering *P* < 0.05.

Disease resistance assay of *Kappaphycus alvarezii*

Disease resistance assay was performed as described in Largo et al. (1995a). Approximately 2 g (wet weight) of surface-sterilized seaweeds collected from different propagules of healthy farmed (4–5 biological replicates) and tissue cultured (7–15 biological replicates) *K. alvarezii* were inoculated with 10⁵ cells/mL *Vibrio* sp. ABI-TU15 and incubated at different temperatures (25, 30 and 35 °C) with agitation at 100 rpm. Seaweed samples without bacteria inoculation were used as control. Two technical replicates were used for farmed and tissue-cultured *K. alvarezii*. All the cultures were maintained at the respective temperatures described for maximum of 3 weeks for any sign of ice–ice disease symptoms. The culture media were collected after 3 weeks incubation period to examine the presence of *Vibrio* sp. ABI-TU15 using 16S rRNA gene sequencing method as described above. The seaweed samples were then cultured

in ASW with PES and maintained at their optimum growth temperature after disease challenge assay. The survival rate of stressed seaweed samples was calculated as described above.

Results

Diversity of seaweed-associated bacteria in *Kappaphycus alvarezii*

A total of 40 seaweed-associated bacteria strains were isolated from 4 types of *K. alvarezii* samples (Tables 1, 2). These bacteria strains can be classified into 11 bacteria genera: *Alteromonas* sp., *Aestuariibacter* sp., *Idiomarina* sp., *Jejuia* sp., *Halomonas* sp., *Primorskyibacter* sp., *Pseudoalteromonas* sp., *Ruegeria* sp., *Terasakiella* sp., *Thalassospira* sp. and *Vibrio* sp. Interestingly, all bacteria strains isolated in this study are Gram-negative bacteria and most of the bacteria are rod-shaped bacteria except for *Ruegeria* sp. and *Terasakiella* sp. that were ovoid and spiral in shape, respectively (Tables 1, 2).

Twelve bacteria strains such as *Terasakiella* sp., *Thalassospira* sp., *Idiomarina* sp., *Vibrio* sp., *Alteromonas* sp., *Jejuia* sp. and *Aestuariibacter* sp. were isolated from healthy farmed *K. alvarezii*, whereas six strains such as *Vibrio* sp., *Halomonas* sp., *Aestuariibacter* sp. and *Alteromonas* sp. were detected in diseased farmed seaweed (Table 1). For tissue-cultured seaweed samples, *Halomonas* sp. were only found in healthy sample, whereas *Primorskyibacter* sp., *Pseudoalteromonas* sp. and *Thalassospira* sp. were only found in diseased tissue-cultured samples (Table 2). Other bacteria strains found in tissue-cultured seaweed samples, regardless of their health condition, were *Jejuia* sp., *Ruegeria* sp. and *Alteromonas* sp. (Table 2). *Vibrio* species (*V. alginolyticus* and *V. xuii*) were only detected in farmed seaweeds, regardless of their health condition, and absent from all tissue cultured seaweeds (Tables 1, 2).

Tissue cultured *K. alvarezii* shows better tolerance to growth temperature stress

Healthy tissue-cultured and farmed *K. alvarezii* were incubated at different temperatures to evaluate their tolerance toward elevated growth temperature. Only slight colour pigment change was observed for tissue-cultured samples incubated at 30 °C (Table 3; Supplementary Fig. 3b, e). The change in pigmentation was more pronounced for tissue-cultured samples incubated at 35 °C. In addition, cortical cell sediments were observed for all the samples incubated at 35 °C (Table 3; Supplementary Fig. 3c, f, i, l). Bleaching was observed for farmed *K. alvarezii* incubated at 30 and 35 °C (Table 3; Supplementary Fig. 3h, i, k, l). Tissue

fragmentation was observed for farmed seaweeds incubated at 30 and 35 °C (Supplementary Fig. 3h, i, k, l). All the tissue-cultured samples were able to recover from the stress condition upon transfer to fresh culture medium and maintained at 25 °C, whereas all the farmed samples did not survive after 3 weeks of stress treatment at 30 and 35 °C except for samples at 25 °C (Table 3; Supplementary Table 1).

Tissue-cultured *Kappaphycus alvarezii* shows resistance to ice-ice disease

Agarolytic analysis using basal salt media containing 1.5% carrageenan showed that *V. alginolyticus* strain ABI-TU15, when stained with Lugol's iodine solution, able to digest carrageenan to form a pale-yellow zone around colonies (Table 1; Supplementary Fig. 1). This strain was selected for disease resistance assay. Phylogenetic analysis of *Vibrio* species based on 16S rRNA gene sequences showed that *Vibrio xuii* strain ABI-TU21, ABI-TU23 and ABI-TU50 were grouped together with non-agarolytic *V. xuii* and *V. brasiliensis* (Supplementary Fig. 1) whereas ABI-TU15 formed a cluster with two agarolytic *Vibrio* species (*V. alginolyticus* and *V. parahaemolyticus*; Supplementary Fig. 2).

No sign of colour pigment change was observed on the infected tissue-cultured *K. alvarezii* that was incubated at 25 °C (Table 3; Supplementary Fig. 4b, f). However, severe colour pigment change and tissue fragmentation was observed on the infected farmed seaweeds incubated at 25 °C (Table 3; Supplementary Fig. 4j, n). The farmed seaweed in control culture that incubated at 25 °C only showed slight colour pigment change (Table 3; Supplementary Fig. 4i, m). When infected tissue-cultured seaweeds were incubated at 30 and 35 °C, only slight colour pigment changes of seaweed branches were observed on the seaweeds (Table 3; Supplementary Fig. 4c, d, g, h). Sediments were observed for all the samples incubated at elevated growth temperatures (Table 3; Supplementary Fig. 4c, d, g, h, k, l, o, p) with more sediments observed in cultures maintained at 35 °C (Table 3; Supplementary Fig. 4d, h, l, p). The sediments (arrows; Supplementary Fig. 3) were observed under light microscope and appeared to be cortical cells detached from seaweed branches. Although the detachment of cortical cells from seaweed branches was observed on infected tissue-cultured samples the branches remained intact after 3 weeks of disease resistance assay. Bleaching was observed on the branches of infected farmed *K. alvarezii* incubated at 25, 30 and 35 °C (Table 3; Supplementary Fig. 4j–l, n–p). Tissue fragmentation was also observed on the branches of infected farmed samples where samples became soft and fragile when attempts were made to remove them from the culture flasks. All infected tissue-cultured *K. alvarezii* was observed to have recovered from the infection upon transfer

Table 1 Seaweed-associated bacteria found in farmed *Kappaphycus alvarezii*

Seaweed samples	Agar medium	Bacteria ID	Phenotypic characterization					Species identification (16S rDNA; BlastN)				Aga- rolytic analysis
			Pigment	Opacity	Form	Elevation	Surface	Gram staining	Shape	Sequence identity (%)	Description of bacteria	
HFC	MA	ABI-TU16	Cream	Clear	Irregular	Raised	Smooth	Gram –	Spiral	85	<i>Terasakiella</i> sp.	ND
		ABI-TU18	Cream	Clear	Circular	Flat	Smooth	Gram –	Rod	98	<i>Thalassospira indica</i>	ND
		ABI-TU20	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Idiomarina fontislapidosi</i>	ND
		ABI-TU21	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	99	<i>Vibrio xuii</i>	ND
		ABI-TU22	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Idiomarina fontislapidosi</i>	ND
		ABI-TU23	Cream	Clear	Circular	Raised	Smooth	Gram –	Rod	100	<i>Vibrio xuii</i>	ND
		ABI-TU35	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	99	<i>Alteromonas lipolytica</i>	ND
		ABI-TU36	Yellow	Opaque	Circular	Raised	Smooth	Gram –	Rod	96	<i>Jejuia pallidilutea</i>	ND
		ABI-TU37	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	96	<i>Aestuariibacter aggregatus</i>	ND
		ABI-TU38	Yellow	Opaque	Circular	Raised	Smooth	Gram –	Rod	95	<i>Jejuia pallidilutea</i>	ND
PA	PA	ABI-TU48	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Alteromonas lipolytica</i>	ND
		ABI-TU49	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Alteromonas lipolytica</i>	ND
		ABI-TU15	Cream	Translucent	Circular	Raised	Smooth	Gram –	Rod	99	<i>Vibrio alginolyticus</i>	+
		ABI-TU34	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	95	<i>Thalassospira mesophila</i>	ND
		ABI-TU39	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Alteromonas lipolytica</i>	ND
DFC	MA	ABI-TU40	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Alteromonas lipolytica</i>	ND
		ABI-TU50	Cream	Opaque	Irregular	Raised	Smooth	Gram –	Rod	99	<i>Vibrio xuii</i>	ND
		ABI-TU51	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100	<i>Aestuariibacter aggregatus</i>	ND

MA marine agar medium, BA bacto agar medium, PA plant agar medium, HFC healthy farmed *K. alvarezii* without disease symptoms, DFC diseased farmed *K. alvarezii* with apparent ice-ice disease symptoms. ND not determined

+Depression of agar observed on basal salt agar medium

-No agar depression observed on basal salt agar media

Table 2 Seaweed-associated bacteria found in tissue cultured *Kappaphycus alvarezii*

Seaweed samples	Agar medium	Bacteria ID	Phenotypic characterization				Species identification (16S rDNA; BlastN)			
			Pigment	Opacity	Form	Elevation	Surface	Gram staining	Shape	Sequence identity (%)
HTC	MA	ABI-TU25	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	92
		ABI-TU27	Cream	Opaque	Circular	Raised	Smooth	Gram –	Ovoid	95
	BA	ABI-TU41	Yellow	Opaque	ND	ND	Smooth	Gram –	Rod	97
		ABI-TU42	Cream	Clear	Circular	Raised	Smooth	Gram –	Rod	96
		ABI-TU43	Cream	Clear	Circular	Raised	Smooth	Gram –	Rod	96
		ABI-TU44	Cream	Clear	Circular	Raised	Smooth	Gram –	Rod	97
	PA	ABI-TU45	Yellow	Opaque	ND	ND	Smooth	Gram –	Rod	97
		ABI-TU52	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	99
		ABI-TU53	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	95
		ABI-TU54	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100
DTC	MA	ABI-TU55	Yellow	Opaque	Circular	Raised	Smooth	Gram –	Rod	94
		ABI-TU56	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	97
		ABI-TU28	Cream	Opaque	Irregular	Raised	Smooth	Gram –	Rod	99
		ABI-TU30	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	88
		ABI-TU31	Orange	Opaque	Circular	Raised	Smooth	Gram –	Rod	98
		ABI-TU33	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	87
		ABI-TU46	Yellow	Opaque	ND	ND	Smooth	Gram –	Rod	95
		ABI-TU47	Yellow	Opaque	ND	ND	Smooth	Gram –	Rod	95
		ABI-TU57	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	100
		ABI-TU58	Cream	Opaque	Circular	Raised	Smooth	Gram –	Rod	96

MA marine agar medium, BA bacto agar medium, PA plant agar medium, HTC healthy tissue cultured *K. alvarezii* without disease symptoms, DTC diseased tissue cultured *K. alvarezii* with apparent ice-ice disease symptoms, ND not determined due to lack of single colony, NA not available due to no PCR amplification

Table 3 Disease resistance assay of *Kappaphycus alvarezii*

Sample	Temperature (°C)	Control				Treatment			
		Culture medium	Sample pigmentation	Survival rate (%)	Dominant bacteria	Culture medium	Sample pigmentation	Survival rate (%)	Dominant bacteria
Tissue cultured, replicate 1	25	Slightly turbid	Deep brown	100	<i>Halomonas</i> sp.	Turbid	Reddish brown	100	<i>Vibrio</i> sp.
	30	Slightly turbid; sediment	Reddish brown	100	<i>Idiomarina</i> sp.	Turbid	Reddish brown	100	<i>Vibrio</i> sp.
	35	Slightly turbid; sediment	Brown	100	<i>Marinobacter</i> sp.	Turbid; sediment	Deep brown	100	<i>Vibrio</i> sp.
Tissue cultured, replicate 2	25	Slightly turbid	Deep brown	100	<i>Halomonas</i> sp.	Clear	Reddish brown	100	<i>Halomonas</i> sp.
	30	Slightly turbid	Brown	100	Unknown	Clear, sediment	Brown	100	Unknown
	35	Clear; sediment	Reddish brown	100	<i>Alteromonas</i> sp.	Slightly turbid, sediment	Brown	100	<i>Vibrio</i> sp.
Farmed, replicate 1	25	Turbid	Light brown	100	Unknown	Clear, sediment	Bleaching	0	<i>Vibrio</i> sp.
	30	Turbid	Bleaching	0	Unknown	Slightly turbid	Bleaching	0	<i>Vibrio</i> sp.
	35	Turbid	Bleaching	0	<i>Bacillus</i> sp.	Slightly turbid	Bleaching	0	<i>Vibrio</i> sp.
Farmed, replicate 2	25	Slightly turbid	Light brown	100	Unknown	Turbid; sediment	Light brown; bleaching	0	<i>Vibrio</i> sp.
	30	Slightly turbid; sediment	Light brown; bleaching	0	Unknown	Turbid; sediment	Light brown; bleaching	0	<i>Vibrio</i> sp.
	35	Clear; sediment	Light brown; bleaching	0	Unknown	Slightly turbid; sediment	Light brown; bleaching	0	<i>Vibrio</i> sp.

Unknown bacteria species can not be determined due to high sequencing noise

to a fresh culture medium and incubated at 25 °C (data not shown).

Table 3 shows the dominant bacteria species found in all the liquid media cultures after 3 weeks of the disease challenge period. *Vibrio* sp. ABI-TU15 was not detected in the treated flask of replicate 2 of tissue-cultured seaweed incubated at 25 °C and absent in all control cultures (Table 3). On the other hand, *Vibrio* sp. ABI-TU15 remained dominant in the liquid culture media of all treatment cultures for replicate 1 of tissue-cultured seaweeds and treatment flasks of farmed seaweeds (Table 3). In some of the cultures we were unable to identify the dominant bacteria and marked as unknown in Table 3 due to high sequencing noise of 16S rRNA gene sequencing.

Discussion

Tissue-cultured *K. alvarezii* materials were reported to show a higher growth rate (Reddy et al. 2003) and carrageenan properties (yield, viscosity, gel strength and sulfate content)

(Yong et al. 2014a) compared to farmed materials. The production of clonal planting materials through tissue culture is not only important in increasing the seedling stock for seaweed cultivation but can also serve as a consistent supply of selected breeding materials with desired traits such as disease resistance. In addition, tissue culture techniques allow the production of disease-free seaweed stock materials. However, limited information is available on the ice-ice disease resistance properties, abiotic stress tolerance and bacteria diversity of tissue cultured *K. alvarezii*.

In the current study, we isolated 40 endophytic seaweed-associated bacteria strains from four types of seaweed samples. All the bacteria strains isolated in this study belong to Gram-negative bacteria. This is in contradict with seaweed-associated bacteria strains reported by Largo et al. (1995b) where mixed populations of Gram-positive and -negative bacteria were isolated from farmed seaweeds. The difference in bacteria populations found in our study and Largo et al. (1995b) might be due to several factors. For instance, the sample collection location is different between our study and Largo et al. (1995b). We collected farmed seaweed samples

from Selakan Island, Semporna, Sabah, whereas seaweed samples of Largo et al. (1995b) were collected from Dana-jon Reefs, northeastern Bohol, the Philippines. Furthermore, Largo et al. (1995b) isolated the seaweed-associated bacteria from healthy and sickly branches collected from the farm but our samples were maintained in the optimized laboratory conditions before bacteria isolation. Thus, these factors contribute to the difference in bacteria strains isolated in our study compared to those bacteria strains reported in Largo et al. (1995b).

The ice–ice disease was successfully induced under elevated growth temperatures and the severity of the symptoms was enhanced with the introduction of agarolytic *Vibrio* sp. ABI-TU15. This is in line with results obtained by Largo et al. (1995a, b, 1999). Largo et al. (1995a) reported that ice–ice disease can be induced in the laboratory by unfavorable environmental factors, such as high temperature (33–35 °C), low light intensity and low salinity (Largo et al. 1995a) and also could be due to presence of some opportunistic bacterial pathogens (Largo et al. 1995b). Moreover, Largo et al. (1999) showed that primary factors of the ice–ice disease in *K. alvarezii* are the combined effects of abiotic stresses, such as high temperature (33–35 °C), low light intensity and low salinity and biotic agents, such as opportunistic pathogenic bacteria. The authors reported that *Vibrio* sp. P11 build up on stressed thalli of *K. alvarezii* caused early onset of ice–ice disease in stressed thalli, whereas build-up of P11 cell density in non-stressed thalli was not immediately followed by development of ice–ice disease.

In the current study, we showed that tissue-cultured *K. alvarezii* exhibits higher disease resistance against ice–ice disease than farmed samples. The higher disease resistance observed in tissue-cultured seaweeds might be due to the presence of other seaweed-associated bacteria in tissue-cultured seaweeds that are able to inhibit the growth of *Vibrio* species. Largo et al. (1995b) reported that although *Vibrio* sp. can trigger faster ice–ice whitening compared to *Cytophaga* sp., however, it was a weak competitor against Gram-positive bacteria that are found on the seaweed. In the current study, *Alteromonas* sp., *Halomonas* sp., *Jejuia* sp. and *Ruegeria* sp. are the dominant seaweed-associated bacteria found in tissue-cultured samples. Velmurugan et al. (2013) reported that secondary metabolites extracted from *Halomonas salifodinae* MPM-TC showed antibacterial activity against aquatic bacterial pathogens such as *V. harveyi*, *V. parahaemolyticus*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* isolated from infected fish/shrimp. *Ruegeria* sp. has been isolated from seashore sand around a seaweed farm (Park and Yoon 2012). In vitro studies have shown that *Ruegeria* sp. has an antagonistic effect against fish pathogens such as *Vibrio anguillarum*. This reduces the mortality of the fish larvae infected by *Vibrio anguillarum*

(Porsby 2010). Largo et al. (1999) reported that when secondary infection of ice–ice disease by other seaweed-associated bacteria starts, the ability of *Vibrio* sp. P11 to compete with these bacteria gradually diminishes.

We showed that the growth of *Vibrio* sp. ABI-TU15 is inhibited by the presence of *Halomonas* species in some of the tissue-cultured seaweeds tested. In addition, ice–ice disease symptoms were more mild in tissue-cultured samples that harbour *Halomonas* species. Thus, *Halomonas* species might acts as probiotic bacterium that improve the immunity of *K. alvarezii* against opportunistic bacteria pathogens of ice–ice disease. Probiotic *Halomonas* sp. B12 was successfully isolated from the intestine of *Fenneropenaeus chinensis* (Zhang et al. 2008). Oral administration of probiotic *Halomonas* sp. B12 to *F. chinensis* was beneficial to protect the integrity of shrimp intestinal mucosal layer and decreased the total bacterial count of *Vibrio* sp (Zhang et al. 2009). Suantika et al. (2013) reported that the application of indigenous probiotic *Halomonas aquamarina* and *Shewanella algae* in white shrimp (*Litopenaeus vannamei* Boone) cultures was able to inhibit the *Vibrio harveyi* population in which the highest survival rate of white shrimp of ~94% was achieved by the addition of *H. aquamarina* into the culture system. Further analysis is needed to confirm the probiotic property of *Halomonas* species found in the current study.

Vibrio species was not detected in the tissue-cultured *K. alvarezii* in the current study suggesting that the ice–ice disease symptoms observed in our tissue-cultured *K. alvarezii* might be caused by other pathogenic bacteria species. *Ruegeria mobilis* was only found in tissue-cultured *K. alvarezii* samples and absent from farmed *K. alvarezii* samples. *Ruegeria* sp. has been reported to cause bleaching on red seaweed *Delisea pulchra* under laboratory conditions (Campbell 2011) and in the field (Campbell 2011; Fernandes et al. 2011). Besides macroalgae, *Ruegeria* sp. isolated from *Gracilaria* sp. was reported to show algalytic activity on unicellular green algae, *Chattonella antiqua* (Azanza et al. 2013). *Pseudoalteromonas* sp. was detected only in diseased tissue cultured *K. alvarezii*. *Pseudoalteromonas* sp. found naturally on the surface of several seaweeds possesses an inhibitory activity against biofouling organisms. An overpopulation of *Pseudoalteromonas* sp. would cause damage to the seaweed as seen in the rotting disease of kelp (Gachon et al. 2010) and in *Laminaria japonica* thalli (Wang et al. 2008). According to Syafitri et al. (2017), *Alteromonas macleodii* and *Pseudoalteromonas issachenkonii* are the causative agents for ice–ice disease symptoms in farmed *K. alvarezii* collected from Karimunjawa island, North Java Sea, Indonesia and *A. macleodii* showed the highest pathogenicity compared to other causative agents tested. Besides bacteria strains mentioned above, other seaweed-associated bacteria have been reported to cause ice–ice disease in *K. alvarezii* (Achmad et al. 2016). Eight species of ice–ice

disease-causing bacteria (*Shewanella haliotis* strain DW01, *V. alginolyticus* strain ATCC 17749, *Stenotrophomonas maltophilia* strain IAM 12323, *Arthrobacter nicotianae* strain DSM 20123, *P. aeruginosa* strain SNP0614, *Ochrobactrum anthropic* strain ATCC 49188, *Catenococcus thio-cycli* strain TG 5-3 and *Bacillus subtilis* subsp. *spizizenii* strain ATCC 6633) were reported by Achmad et al. (2016) with *S. maltophilia* causing the fastest onset of ice–ice disease symptoms. Further analysis on other seaweed-associated bacteria isolated in the current study is needed to investigate the disease-causing properties of these bacteria.

Conclusion

We showed that tissue-cultured *K. alvarezii* showed resistance against the ice–ice disease than the farmed samples tested. Furthermore, tissue-cultured *K. alvarezii* showed higher tolerance to temperature stress compared to farmed samples. This suggests that tissue-cultured *K. alvarezii* is a better seedling material for seaweed farming. Together with the knowledge on seaweed-associated bacteria found in both tissue cultured and farmed *K. alvarezii* in the current study, will help us in the control and prevention of ice–ice disease in *Kappaphycus*. The finding of disease resistance in tissue-cultured *K. alvarezii* against ice–ice disease allows us to produce improved seaweed stock for seaweed farming. This is important not only for the sustainability of seaweed farming industries but also ensures that the quality of the seaweed material produced meets the safety guidelines for human consumption.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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