



Research Article

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Control of oomycete pathogens during *Pyropia* farming and processing using calcium propionate

Yong Tae Kim¹, Ro-won Kim², Eunyoung Shim², Hana Park¹, Tatyana A. Klochkova³ and Gwang Hoon Kim^{1,*}

¹Department of Biology, Kongju National University, Gongju 32588, Korea

²Biotechnology Research Institute, Kongju National University, Gongju 32588, Korea

³Department of Biology, Kamchatka State Technical University, Petropavlovsk-Kamchatsky 683003, Russia

The oomycete pathogens *Pythium porphyrae*, causing red rot disease, and *Olpidiopsis* spp. causing *Olpidiopsis*-blight, cause serious economic losses to *Pyropia* sea farms in Korea. During the washing step for *Pyropia* processing, these pathogens proliferate rapidly, significantly reducing the quality of the final product. To develop non-acidic treatments for these pathogens, various calcium salts were tested against the infectivity of *P. porphyrae* and *Olpidiopsis pyropiae* on *Pyropia* gametophytes, and calcium propionate was the most effective. When *Pyropia* blades were immersed in 10 mM calcium propionate for 1 h after inoculation with the oomycete pathogen, infection rate of both oomycete pathogens on day 2 was significantly lower (7.1%) than control (>95%). Brief incubation of *Pyropia* blades in calcium propionate also reduced the spread of infection. The infected area of *Pyropia* thallus was reduced to 14.3% of the control in 2 days after treatment with 100 mM calcium propionate for 30 s. In field experiments conducted in actual aquaculture farms, it has been shown that a brief 30 s wash every two weeks with 100 mM calcium propionate can effectively reduce the spread of oomycetes throughout the entire culture period. The above results suggest that calcium propionate can be a useful means for controlling the spread of oomycetes not only during laver processing but also during aquaculture.

Keywords: calcium propionate; *Olpidiopsis pyropiae*; oomycete; *Pyropia*; *Pythium porphyrae*

INTRODUCTION

The global production of cultivated macroalgae more than doubles every decade, and the production of red algae has overtaken brown algae since 2010, dominating the market (Badis et al. 2020b). Production of laver (*Pyropia* spp., formerly known as *Porphyra*) (Zuccarello et al. 2022) has also doubled during the same period, primarily due to a rapid expansion in China and Korea, but this rapid development of aquaculture is followed by a similarly increasing economic burden of disease outbreaks (Cottier-Cook et al. 2016, 2021). Disease management

is a growing concern for *Pyropia* farmers, and now contributes up to half the running cost of a farm (Kim et al. 2014). Infected *Pyropia* are still harvested and used, but crop yield and quality are severely reduced (Klochkova et al. 2017), and market prices may drop by up to one-third of non-infected crops. Infection continues to spread after harvest. The harvested thallus is washed alternately with seawater and fresh water over 2–3 days to remove contaminants, and then goes through a drying process. During this time, the infection spreads further and the dead



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*Corresponding Author

E-mail: ghkim@kongju.ac.kr

Tel: +82-41-850-8504, Fax: +82-41-850-8504

cell debris promotes bacterial growth, further deteriorating the quality of the final product, but there are no effective and environmentally safe countermeasures to date (Kim et al. 2014).

Various species of fungal, viral, and oomycete pathogens infecting *Pyropia* crops have been described worldwide (Sekimoto et al. 2008, Kim et al. 2016, Klochkova et al. 2016, Mo et al. 2016, Badis et al. 2018, 2020a). Among them, oomycete pathogens such as *Pythium porphyrae* and *Olpidiopsis* spp. cause the greatest damage to *Pyropia* aquaculture (Kim et al. 2014, Klochkova et al. 2017). *Pythium porphyrae*, a causative agent of red rot disease, is one of the most serious oomycete pathogen first reported and in *Pyropia* farms in Asia (Arasaki 1947, Takahashi et al. 1977, Park et al. 2001, Ding and Ma 2005). The geographic distribution of this oomycete includes the Southern Hemisphere where *Pyropia plicata* in New Zealand was found to be infected with the same species (Diehl et al. 2017). The oomycetes *Olpidiopsis porphyrae* and *O. pyropiae* are responsible for the destructive outbreaks of *Olpidiopsis*-blight in Asia (Sekimoto et al. 2008, Kim et al. 2014, Klochkova et al. 2016, Kwak et al. 2017). Two novel *Olpidiopsis* species infecting red algae have been reported in Scotland (Badis et al. 2018). A few more species of this genus have been reported to infect other red algae: *O. bostrychia* infecting *Bostrychia moritziana* (West et al. 2006, Sekimoto et al. 2009); *O. feldmanni* infecting *Asparagopsis* sp. (Fletcher et al. 2015); and *O. heterosiphoniae* infecting *Dasyisiphonia japonica* (Klochkova et al. 2017). Investigations into the molecular mechanisms by which these oomycete pathogens infect red algae have only recently begun (Im et al. 2019).

Acid-wash of cultivation nets is the most commonly used measure in *Pyropia* farms to reduce epiphytic green algae, diatoms, and even oomycete pathogens (Park et al. 2001). A boat equipped with a large tub passes underneath the cultivation net and the net gets immersed in the organic acid in the tub while the boat slowly moves forward. Because of huge area to cover and limited tolerance of *Pyropia* cells to acidic solution the immersion time should not exceed 30 s. As *Pyropia* tolerates acidic solutions better than other epiphytic organisms this treatment has been regarded useful to clean the cultivation nets (Park et al. 2001), but this method has little effect on *Olpidiopsis* blight and is a burden on the environment (Kim et al. 2014). As regulations on aquaculture methods are strengthened, interest in non-acidic treatment is increasing.

Calcium salts has been widely used to control oomycete pathogens in land plants because calcium treatment

is known to significantly reduce zoospore production and viability of oomycetes (Brunelli 1995, Nigro et al. 1997, Campanella et al. 2002). It has been reported that the formation of zoosporangia and cleavage of the zoosporangial cytoplasm to zoospores in *P. porphyrae* is dependent on extracellular calcium and the calcium ion could regulate zoospore release and infectivity of *P. porphyrae* (Addepalli and Fujita 2002). However, the effectiveness of calcium salts varies greatly depending on the type of crop and pathogen, so it is important to choose the appropriate calcium salt.

In this study, we developed a non-acidic treatment for oomycete diseases in *Pyropia* farms. We observed the effects of various calcium salts on the progression of oomycete disease and proposed a more effective methods for field practice.

MATERIALS AND METHODS

Isolation and culture of pathogens and host

Pythium porphyrae was isolated from infected blades of *Pyropia yezoensis* cultivated in Jindo, southwestern seashore of Korea in December 2010 and designated as strain KNU-pyp2. *Pythium porphyrae* strain was maintained on Arasaki B medium (Arasaki et al. 1968) at 20°C in the dark on agar plates. To obtain zoospores from *P. porphyrae*, the agar discs of mature fungal hyphae were inoculated into liquid culture medium (Fujita and Zenitani 1977) and grown for 5 days. The zoospores from the liquid culture were induced under axenic conditions following the protocol of Uppalapati and Fujita (2000).

Pyropia yezoensis infected with *O. pyropiae* were collected from a commercial plantation in Seochon, Korea. After being transported to the laboratory, blades were cut into approximately 1 × 1 cm pieces and kept in modified Grund medium (MGM) with constant aeration at 10°C under 30 µmol m⁻² s⁻¹ light intensity (16 L:8 D). Stock solution of MGM consisted of C₁₀H₁₄O₆N₂Na₂·2H₂O (Na₂EDTA) 3.72 g, FeSO₄·7H₂O 0.28 g, Na₂HPO₄·12H₂O 10.74 g, NaNO₃ 42.5 g, MnCl₂·4H₂O 0.019 g, vitamin B₁₂ 1 mg, ddH₂O 1 L, and was diluted to a concentration of 1 mL per 1 L of filtered autoclaved seawater with salinity of 30 psu (Klochkova et al. 2012). Cultures of *Olpidiopsis* sp. were constantly maintained for over 10 years by transferring a piece of infected blades to a solution containing healthy *Pyropia* blades every two weeks.

Pyropia yezoensis was collected from a commercial plantation in Seochon, and kept in PES medium with

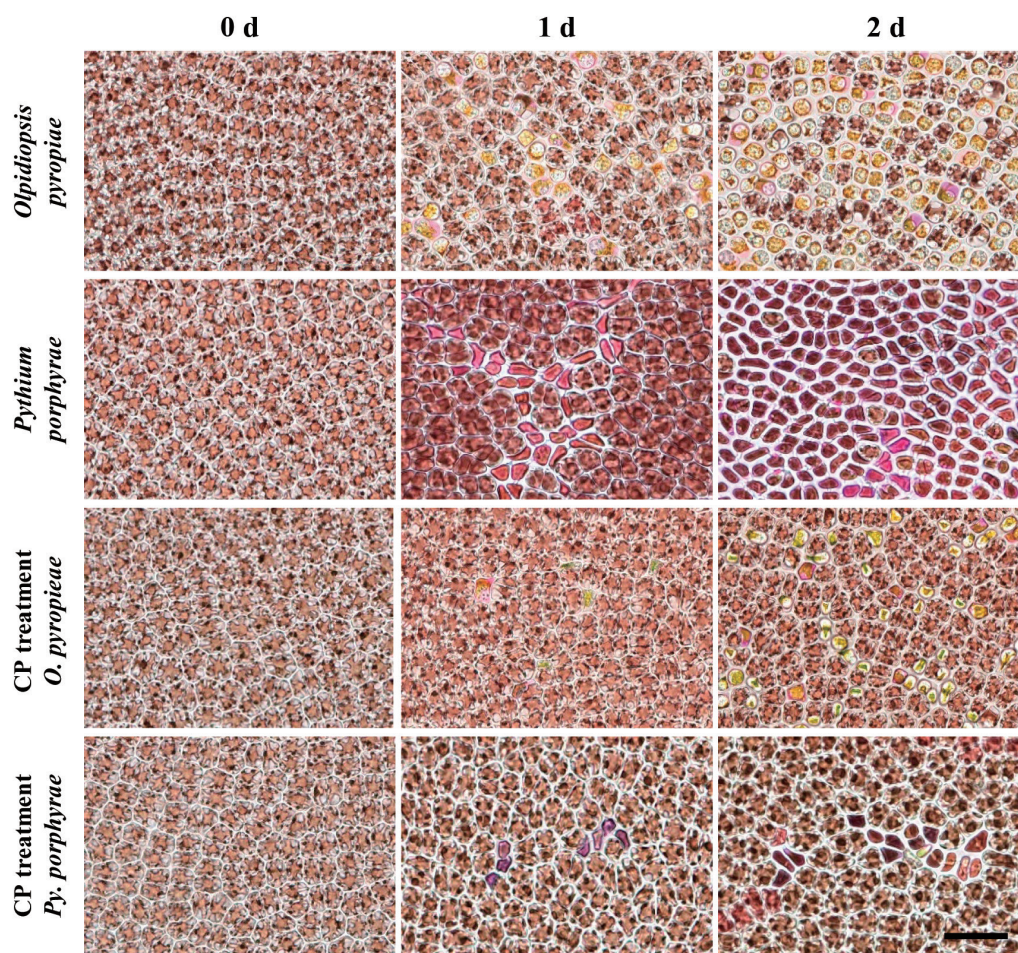


Fig. 1. Spread of oomycete infection in closed tanks over time. Infection with *Olpidiopsis pyropiae* and *Pythium porphyrae* spread to more than 97% of the thallus in 2 days, whereas infection with both oomycetes was limited to 10% of the thallus in tanks treated with 10 mM calcium propionate (CP). Scale bar represents: 50 μ m.

constant aeration at 10°C and 30 μ mol m⁻²s⁻¹ irradiance (16 : 8 h L : D). The medium was replaced every 2 weeks. The cohorts of numerous monospores released from the gametophyte thallus were grown in an environmentally controlled chamber and were used for the infection experiment. Indoor culture strains of *Pyropia* used in this study have been deposited in culture collection of the National Marine Biodiversity Institute of Korea (MABIK) (No. KNU000212, KNU000216).

Effects of calcium salts on infection of *Pythium porphyrae* and *Olpidiopsis*

An inoculum of *P. porphyrae* and *Olpidiopsis* zoospores was prepared using stock cultures described above. Healthy blades of *P. yezoensis* were mixed with oomycete-infected thalli and incubated in seawater containing 10

mM of different calcium salts for 1 h, and the spread of infection was measured after day 2. In order to observe if a brief calcium pretreatment is effective against oomycete infection, *Pyropia* blades were dipped in a solution containing calcium propionate for 30 s, then transferred to seawater containing the zoospore inoculum, and the progress of infection was measured two days later. The infected area of each experimental group was photographed with a camera attached to Olympus BX51 microscope (Tokyo, Japan), measured with an image analysis program (Image-Pro; Media Cybernetics, Silver Spring, MD, USA), and then compared with the control group (no calcium salt treatment) and calculated as a percentage. All the experiments were carried out three times and each in quadruplicate samples and their standard deviations were calculated.

Field test of calcium propionate treatment

A field test was conducted to observe if the treatment with calcium propionate could inhibit oomycete infection in an aquaculture plantation located in Seocheon, west coast of Korea. A boat equipped with a large tub containing 100 mM calcium propionate in seawater used as per standard practice (see above). Because of large area to cover the immersing time was restricted in 30 s. This calcium propionate wash was done once at a time. *Pyropia* blades were collected every two weeks and the oomycete-infected areas were measured and compared with nearby control nets treated with acid-wash using the methods described above.

RESULTS

Spread of oomycete infection during laver processing

After harvesting, a large amount of *Pyropia* is packed into a water tank, transported to a drying facility, and then washed several times with seawater and freshwater for two days. When oomycete-infected thalli are mixed, the infection spreads rapidly during this process which is carried out at room temperature. When we observed the spread of infection over time, oomycetes spread much faster in the tank than in the field (Fig. 1). The infected area, which was initially barely visible, expanded to more than 97% of the thallus within two days in tanks containing thallus infected with *O. pyropiae* or *Pythium prophyrae* (Fig. 1).

Table 1. Calcium salts used in the experiment, their pH in seawater and *Pythium prophyrae* infection area compared to control when treated with 10 mM concentrations

Treatments	Concentration 10 mM		
	g L ⁻¹	pH	Infection rate (%)
Control	-	8.15	100.00 ± 14.86
Calcium gluconate (CG)	22.4	7.36	46.10 ± 3.88
Calcium chloride (CC)	5.5	7.92	45.92 ± 3.75
Calcium lactate (CL)	15.4	7.86	36.98 ± 4.85
Calcium silicate (CS)	6.4	5.24	26.92 ± 2.15
Calcium nitrate (CN)	11.9	7.80	23.60 ± 0.97
Calcium L-ascorbate dihydrate (C.LA)	21.3	7.15	22.30 ± 0.97
Calcium cabonate (C.CA)	5.5	5.36	20.76 ± 1.83
Calcium acetate (CA)	8.1	7.44	15.92 ± 1.50
Calcium propionate (CP)	9.3	7.88	7.07 ± 2.10

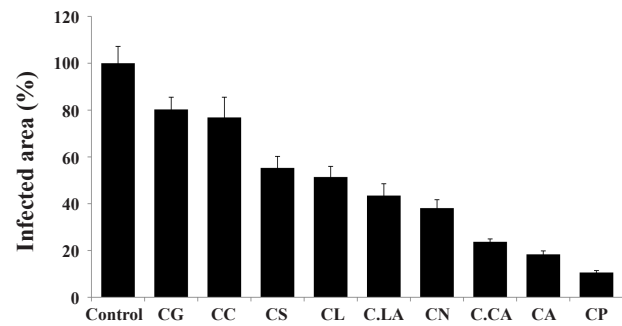


Fig. 2. Effect of treatment with 10 mM calcium salts on the spread of *Olpidiopsis pyropiae* infection compared to control. CG, calcium gluconate; CC, calcium citrate; CS, calcium silicate; CL, calcium lactate; C.LA, calcium L-ascorbate dihydrate; CN, calcium nitrate; C.CA, calcium carbonate; CA, calcium acetate; CP, calcium propionate.

Effects of calcium salts on the spread of infection

When *P. yezoensis* thalli infected with *P. prophyrae* was incubated with 10 mM calcium salt for 1 h in seawater prior to transfer to fresh seawater, the infection area was significantly reduced day 2 after all calcium treatments (Table 1). In particular, in the calcium propionate treatment, it was only 7.07% of the control. The pH of 10 mM calcium propionate dissolved in seawater was 7.88, which was closer to seawater pH than other salts (Table 1). In *O. pyropiae* infection, the infection-spreading effect of calcium salts was slightly lower than in *Pythium prophyrae* infection, but calcium propionate still effectively inhibited the spread, which was 10.5% of the control (Fig. 2).

Effects of brief treatment of calcium propionate on the spread of infection

Brief incubation of infected *P. yezoensis* blades in calcium propionate for 30 s significantly reduced the spread of oomycete infection, but higher concentrations were required compared to 1 h incubation to have a significant effect (Fig. 3). When infected thallus was incubated in 100 mM calcium propionate for 30 s, *Pythium prophyrae* infection was 14.3% of control and *Olpidiopsis pyropiae* infection was 20.17% of control (Fig. 3).

Field test of calcium propionate treatment

An experiment to control spread of oomycetes in the field using calcium propionate was conducted at an aquafarm in Seocheon, the west coast of Korea, and two control farms located next to the experimental site (Fig. 4A). A boat equipped with a large tub containing 100 mM

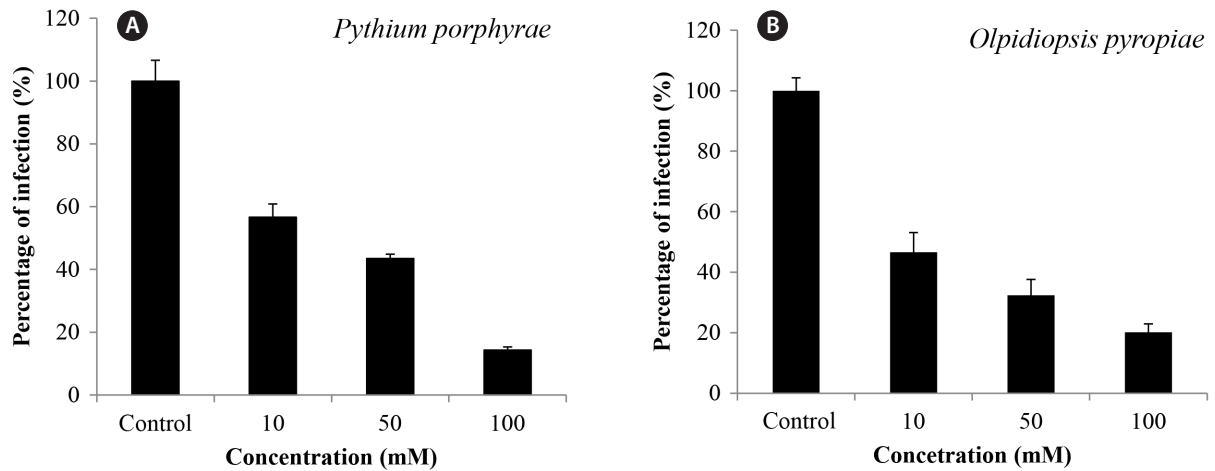


Fig. 3. Effect of treatment with 10, 50, and 100 mM calcium propionate for 30 s on the spread of *Pythium porphyrae* (A) and *Olpidiopsis pyropiae* (B) infection after 2 days.

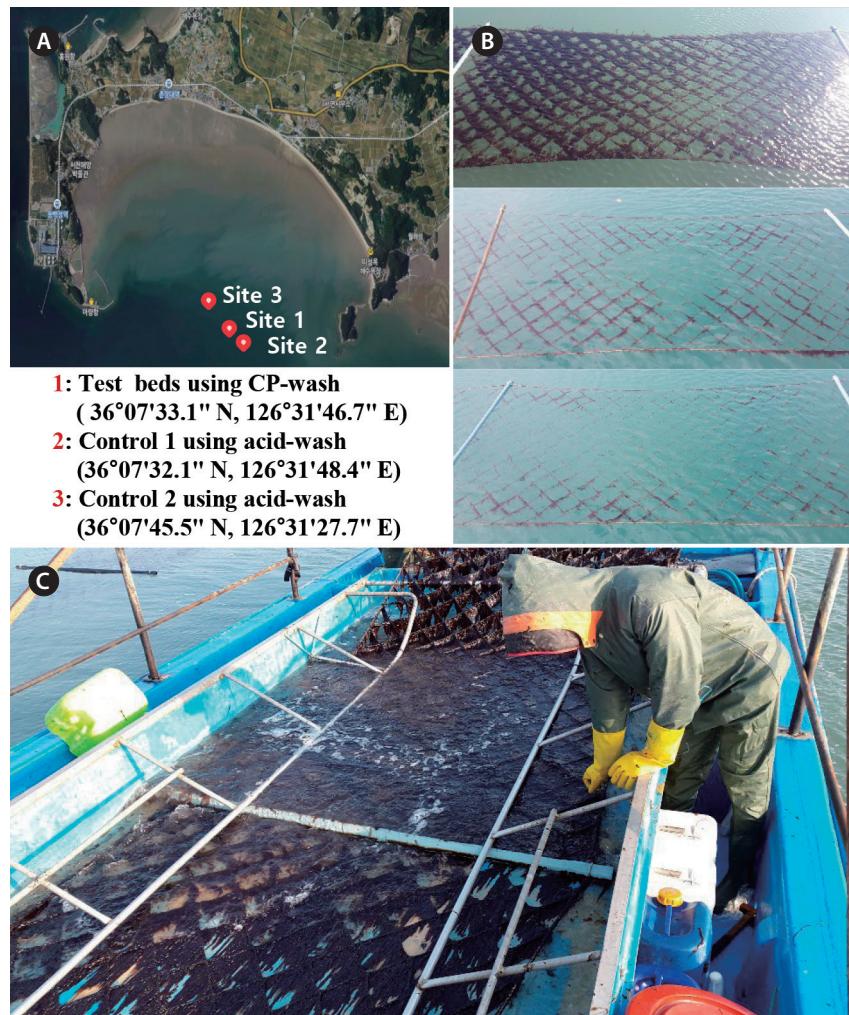


Fig. 4. Field test of calcium propionate (CP) treatment. (A) Satellite image of the experimental and control farms located in Seocheon. (B) Image of culture net of experimental farm treated with calcium propionate (upper). Images of culture nets of control farms using acid wash (middle, lower). (C) Treatment of aquaculture nets with calcium propionate on a boat.

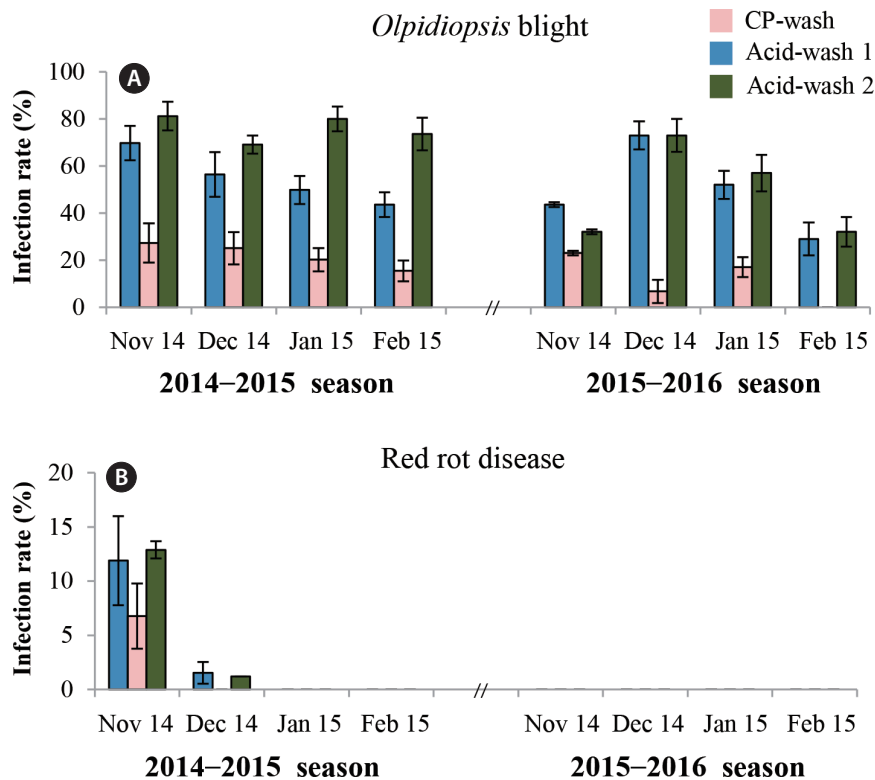


Fig. 5. Incidence rates of *Olpidiopsis* blight (A) and red rot disease (B) in *Pyropia yezoensis* collected from farms in Seocheon during the 2014–2016 seasons. CP, calcium propionate.

calcium propionate in seawater was slowly advanced under the net so that the cultivation net was submerged in the solution for 30 s (Fig. 4C). During two seasons in which the field tests were conducted, *Olpidiopsis*-blight attacked the area and devastated the entire aquaculture farms in the area, but the damage was noticeably less in the cultivation nets treated with calcium propionate (Fig. 4B). During the 2014–2016 seasons *Pyropia* blades were collected to measure oomycete infection whenever possible (Fig. 5). Red rot disease caused by *Pythium pophyrae* hit the farm early in the first season and *Olpidiopsis* blight persisted throughout two seasons, but the infection rates were always lowest in the experimental site treated with calcium propionate (Fig. 5).

Effect of calcium treatment on production volume and market price

The yield and auction prices of *P. yezoensis* produced in the test farms were obtained from local fisheries cooperatives with the permission of the farmers running the experimental area (Table 2). During two farming seasons, the cultivation nets using calcium propionate treatment

earned a minimum of 18.8% and a maximum of 63% more than other nets using acid-wash. Total production decreased by 25–30% in all farms in the second year, but the unit price per kg was higher in the calcium propionate treated areas than in the other two area, so the profit margin was even larger (Table 2).

DISCUSSION

The results of this study show that treatment with calcium propionate can be used as an effective means of controlling the spread of oomycetes during the production and processing stages of *Pyropia* while improving product quality with a relatively low environmental impact compared to the currently used acid treatment. *Olpidiopsis*-blight and red rot disease caused by oomycete pathogens are dominant diseases in *Pyropia* farms in Korea (Cho and Chang 1986, Kim et al. 2014). Oomycete infections not only reduces aquaculture production, but also spread throughout the distribution and processing stages, significantly reducing product competitiveness. Recent estimates from the Korean Ministry of Marine Affairs and

Table 2. Yield and revenue of test farms using calcium propionate (CP-treatment) versus acid-washing during the 2014–2016 seasons

Year	No.	Harvest date	CP-treatment			Control 1 (acid-wash)			Control 2 (acid-wash)		
			Weight (kg)	Unit price (won)	Total revenue (won)	Weight (kg)	Unit price (won)	Total revenue (won)	Weight (kg)	Unit price (won)	Total revenue (won)
2014–2015	1	Dec 13, 2014	225	120,000	225,000	465	115,000	445,625	870	105,000	716,250
	2	Dec 27, 2014	765	116,000	739,500	240	110,000	220,000	420	123,000	430,500
	3	Jan 4, 2015	510	105,000	446,250	810	115,000	708,750	1,060	107,500	845,875
	4	Jan 21, 2015	930	126,000	976,500	720	137,000	822,000	135	115,000	129,375
	5	Feb 16, 2015	900	103,000	772,500	450	113,000	416,250	270	117,000	263,250
	6	Mar 7, 2015	945	69,000	543,375	690	67,000	385,250	600	66,000	330,000
	7	Mar 27, 2015	900	66,000	495,000	1,410	83,000	975,250	930	38,145	295,625
	8	Apr 7, 2015	1,920	66,000	1,056,000	1,035	52,000	448,500	360	70,000	210,000
2015–2016	Total		7,095	-	5,254,125	5,820	-	4,421,625	4,645	-	3,220,875
	1	Nov 23, 2015	465	90,000	348,750	300	85,000	255,000	315	78,000	247,400
	2	Jan 8, 2016	584	116,000	522,000	465	117,000	453,375	405	75,000	253,125
	3	Jan 12, 2016	780	125,000	812,500	795	112,000	742,000	885	124,000	883,500
	4	Jan 27, 2016	645	118,000	634,250	675	123,000	691,875	660	127,000	883,500
	5	Feb 5, 2016	480	150,000	937,500	570	147,000	698,250	765	142,000	905,250
	6	Feb 21, 2016	825	132,000	1,266,250	210	120,000	210,000	240	127,000	254,000
	7	Feb 21, 2016	870	160,000	1,160,000	840	119,000	833,000	562	145,280	681,000
2015–2016	8	Mar 9, 2016	825	160,000	1,100,000	825	117,000	804,375	420	147,000	514,500
	Total		5,474	-	6,781,250	4,680	-	4,687,875	4,252	-	4,622,275

Fisheries show that on average, 10% of the annual *Pyropia* production is lost due to pathogens (Klochkova et al. 2012, Cottier-Cook et al. 2021). Yield and value losses due to *Olpidiopsis*-blight and red rot disease have been reported as high as 15–20 and 20–30%, respectively (Park et al. 2000), and losses due to oomycete diseases have been reported as high as 77.5% in the past (1983–1985) (Cho and Chang 1986). Our three-year investigation in *Pyropia* farms showed that currently used acid-wash methods cannot effectively control the infection and spread of oomycetes, especially *Olpidiopsis* spp. (Kim et al. 2014). Moreover, the transportation and manufacturing processes of *Pyropia* harvested from farms does not allow for the use of acid treatment.

Government regulations for disease control in South Korea's seaweed farms are very strict. Chemical treatments are currently limited to a few organic acids or concentrated salts that have been approved through government review and testing in the field (Kim et al. 2014). Farmers also try to avoid chemical treatments as much as possible to reduce operating costs. *Pyropia* cultivation takes place between October and March, when the water temperature is relatively low in part because the spread of disease is slower during this time. If there is a concern that the water temperature will not drop low enough to prevent the spread of infection other methods can be employed. The freezing net method is sometimes used, in which nets are stored at -20°C and then reinstalled in the field at the appropriate time (e.g., Fujita and Migita 1980, Ding and Ma 2005, Klochkova et al. 2012), but this reduces growth time and is expensive to implement. In some areas, farmers expose aquaculture nets to the air for several hours during the day, taking advantage of the fact that *Pyropia* thalli are more resistant to cold and desiccation than other organisms (Klochkova et al. 2012). The most common method is acid-washing, which is not effective in preventing the spread of infectious disease (Kim et al. 2014). Despite all these attempts, oomycete pathogens are still the biggest problem for *Pyropia* farms. This study shows that calcium propionate salts are very useful in controlling oomycete pathogens, although government regulations need to be modified before this method can be used more broadly. However, calcium propionate treatment can be a useful means of effectively preventing the spread of infection during the transportation and processing step of harvested *Pyropia*, even without legislative changes.

Calcium salt treatments are widely used to eliminate oomycetes that cause root / fruit rot in many important crops including citrus, soybeans, potatoes, onions, and

apples, and have low environmental impact (Kaiser et al. 2011, Türkkan 2013). The inhibitory effects of calcium salts on oomycete pathogens include reduced germ tube growth, reduced mycelial growth *in vitro*, and reduced severity of infection of pretreated host tissues (Biggs et al. 1997, Campanella et al. 2002). While the types of calcium salts applied to different crops vary greatly, calcium propionate is one of the most effective calcium salts against oomycete pathogens on a wide variety of terrestrial crops (Kaiser et al. 2011). Calcium propionate has been used for decades as a food additive to inhibit mold in bakery products (Furia 1973). It also occurs naturally in butter and some types of cheese and is regarded as relatively safer than other food additives by the European Food Safety Authority (EFSA Panel on Food Additives and Nutrient Sources Added to Food 2014).

The regulatory role of external calcium on zoospore release, development, and infection of *P. porphyrae* has been studied, using EGTA to inhibit the influx of external calcium, but the regulatory role of calcium salts has not been studied (Addepalli and Fujita 2002). Calcium (Ca^{2+}) is a universal cell signaling molecule involved in myriad biological responses in a variety of organisms (Zhang et al. 2014, Aldon et al. 2018), but little is known about why calcium salts inhibit oomycete reproduction and infection to a much greater extent than their hosts. To develop more effective treatments for oomycete pathogens, research into calcium signaling during oomycete infection is needed.

This study showed that treatment with 100 mM calcium propionate for 30 s using the same method used by fishermen for acid-washing can effectively control oomycetes in *Pyropia* aquaculture, but aquaculture is regulated by much stricter regulations than land-based crop cultivation, this method cannot be immediately applied in field practice, except in test farms, under the current legal system. In the future, efforts should be made to revise legislation and educate fishermen to ensure more effective and environmentally-friendly control of oomycetes can be utilized.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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