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Influence of five organic antifouling candidates on spore attachment and germination of a fouling alga *Ulva pertusa*

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Abstract: *Screening of test chemicals or formulations for antifouling (AF) activity is important to get first hand information on their nontoxic repelling activities. Especially spores of a fouling alga, Ulva pertusa were used in this study to test the AF efficiency of five organic chemicals. Coatings made with 100 $\mu g\ cm^{-2}$ of citral and eugenol significantly inhibited the spore attachment. A low concentration (1 $\mu g\ cm^{-2}$) of solanesol exhibited effective AF activity against spore attachment. Spore germination was sensitive to different AF candidates screened in this study. Based on the attachment and germination response of Ulva pertusa spores, AF efficiency of five organic AF candidates is discussed*

Key words: *Organic antifouling (AF) candidates, Spore attachment and germination, Ulva pertusa, Nontoxic AF activity*

Introduction

Marine surface exposed to seawater is rapidly colonized by microorganisms, along with various organic molecules (dissolved organic molecules such as polysaccharides, proteins and proteoglycans), which together develops to a bioorganic film (Callow and Fletcher, 1994; Abarzua and Jakubowsky, 1995; Keough and Raimondi, 1995). The physical and chemical characteristics of this bioorganic film greatly influence the settlement of algal spores and animal larvae. There have been many studies about various species being attracted or repelled by these surface properties e.g. phototaxis, thigmotaxis and chemotaxis (Fletcher and Callow, 1992; Callow and Callow, 1998, 2000; Callow *et al.*, 2000). Wide range of variation in settlement dynamics across the species clearly showed specificity of individuals to select their substrate for the settlement. In particular chemical cues present in the biofilm play an important role in the successful settlement of fouling organisms. To identify effective antifouling (AF) agent, tests on initial stages of biofouling e.g. algal spore or larval attachment is necessary. Moreover, studies on attachment of initial stages of fouling algae on AF test surfaces can be very useful to assess the nontoxic nature of the AF agent. Especially, surface specificity in algal spore attachment has been well investigated (Callow and Callow, 2000; Finlay *et al.*, 2002). Spore attachment bioassays are proved to be an important measure of both AF efficiency and toxicity, and have long been used to assess the natural and synthetic AF agents (Fletcher, 1991; Leitch and Puzzuoli, 1992; Miyauti, 1993; Hattori and Shizuri 1996; Shin and Smith, 2001).

Several natural products originating from plants possess bioactive properties. In earlier studies, essential oils from plants such as *Citrus limon*, *Zingiber officinale*, *Syzygium aromaticum, Jasminum officinale* and *Nicotiana tabacum* (with one of the active components citral, β-myrcene, eugenol and solanesol) were reported to influence on microbial growth (Bhawasar *et al.*, 1965; Baratta *et al.*, 1998; Dorman and Deans, 2000; Palic *et al.*, 2002). Similarly, marine natural products containing terpenes and phenolic compounds were screened for AF activities (Faulkner, 1997; Kamenarska *et al.*, 2002; Tsoukatou *et al.*, 2002). Considering these proven bioactivities, citral, β-myrcene, eugenol, cis-3 hexenyl acetate and solanesol were chosen for AF screening.

A screening method for AF substances using spores of the fouling macroalga *Ulva conglobata* was reported by Hattori and Shizuri (1996). In which prevention of spore attachment or germination on AF candidate-coated surface was considered as an AF function. In order to find out the nontoxic AF activity of five selected organic AF candidates, their effects on attachment and subsequent germination of spores of a fouling alga *Ulva pertusa* were studied.

Materials and Methods

Ulva pertusa is a green tide alga densely occurring along the Korean coast and recognized as monospecific fouling alga (Sidharthan *et al.*, 2004). Because of a high reproductive potential of *Ulva pertusa,* it has been used as a test alga. Matured *Ulva pertusa* plants were collected from the east coast and transported to laboratory in an ice box. Individual *Ulva* plants were separated and thrice washed with distilled water to remove the epiphytes and debris. Finally once washed with filtered seawater (2 μm). To facilitate spore liberation, *Ulva* fronds were placed on a tray and carefully blotted dry with paper towels and kept under fluorescent lamp (50 $\mu m^{-2}\ s^{-1}$) for 10 hr. In between, after 6 hr filtered seawater (2 μm) sprayed on *Ulva* plants to make it partially wet. At the end of this pretreatment *Ulva* plants were kept in one liter beaker and about 500 ml of filtered seawater (2 μm) was added. After 5 min *Ulva* plants were removed from beaker and

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Table - 1: Chemical characteristic of antifouling (AF) candidates

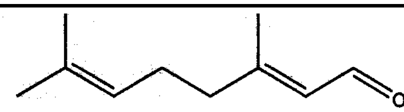
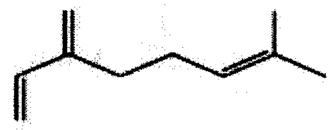
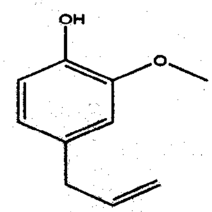
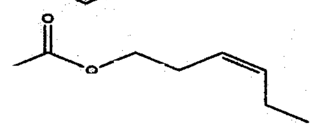
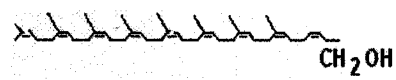
AF candidate	Chemical name (IUPAC)	Chemical group	Source	Structure
Citral	3,7-dimethyl 2,6-octadienal	Monoterpene	*Citrus limon*	
β- myrcene	7-methyl-3-methyleneocta-1, 6-diene	Monoterpene	*Zingiber officinale*	
Eugenol	2-methoxy-4-(2-propenyl) phenol	Phenol	*Syzygium aromaticum*	
Cis-3 hexenyl acetate	Hex-3-enyl acetate	Aliphatic ester	*Jasminum officinale*	
Solanesol	3,7,11,15,19,23,27,31,35-nonamethyl hexatriaconta-2,6,10,14,18,22,26,30,34-nonaen-1-ol	Polyprenol	*Nicotiana tabacum*	

Table - 2: Regression analysis of total spore attachment and germination on coatings of five AF candidates

AF candidates	Spore attachment		Spore germination	
	r- value	*t*- value	r- value	*t*- value
Citral	0.647	3.013*	0.677	3.292^{NS}
β -myrcene	0.898	7.646**	0.785	4.628*
Eugenol	0.715	3.661*	0.701	3.514*
Cis-3 hexenyl acetate	0.636	13.269**	0.730	8.725**
Solanesol	0.685	3.418*	0.601	2.697^{NS}

Significant at *p<0.05; ** p<0.01; NS: Not significant

spore released into seawater (~ 10^{5-7} spores ml^{-1}) was used for further experiments. Initial spore density in this spore suspension was determined using haemocytometer.

Spore attachment and germination experiments were carried out as outlined by Hattori and Shizuri (1996). The chemical characteristics of AF candidates are given in Table 1. AF candidates [citral (monoterpene), β-myrcene (monoterpene), eugenol (phenol), cis-3 hexenyl acetate (aliphatic ester) and solanesol (alcohol)] were dissolved in MeOH. From these stock solutions dilutions were made to make 1, 10, 100 & 1000 µg cm^{-2} concentrations. Two ml aliquot of each AF candidate was then poured into polystyrene petridishes (Corning: 60 x 15 mm) and swirled repeatedly to spread the AF aliquots uniformly all over the petridish surface. AF coated petridishes were dried at 30 ºC for 24 hr. Series of AF concentrations were used to give a final concentration of µg cm^{-2} of the surface. For the spore culture, in each AF coated petridish 10 ml of provosoli enriched seawater (PES) medium (Provosoli, 1968) was added. Each petridish was inoculated with $6x10^3$ spores and incubated in culture chamber under 14:8 LD cycle at 24±1 ºC for five days. At the end of experiment spore suspension was carefully discarded and to remove the unattached or loosely attached spores, gently washed with filtered seawater (2 µm). Total number of spores attached and number of germinated spores were examined in 10-20 fields of view in triplicates under microscope (400 x). Results expressed in percentage increase/ decrease over control. Data collected were subjected to linear regression analysis and *t*-test (p<0.05; p<0.01).

Results and Discussion

Among the five organic AF candidates tested, solanesol exhibited effective AF activity against *Ulva* spore attachment as well as germination rate. On 100 µg cm^{-2} coatings of citral and eugenol more than 90% reduction in spore attachment were seen (Fig. 1 and 3). On the highest concentration of 1000 µg cm^{-2} coatings made with citral, eugenol, solanesol and β-myrcene no spore attachment was observed as a result of their nontoxic repellent activity. In the present study, results showed promising

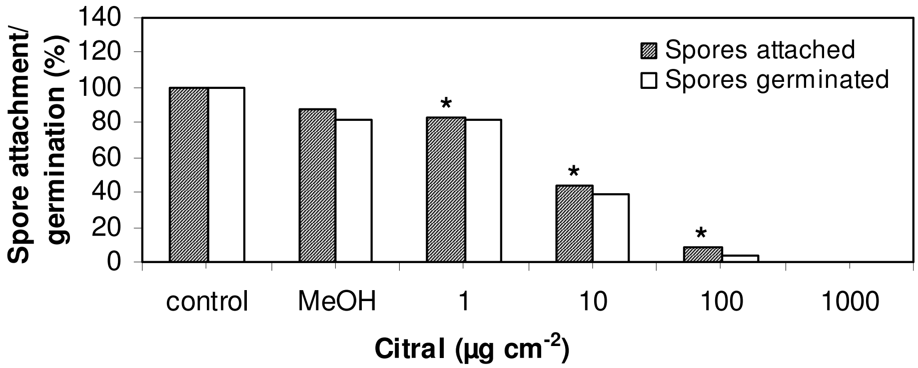


Fig. 1: Antifouling activity of citral on spore attachment and germination of a fouling alga *Ulva pertusa*. Percentage of reduction when compared to control (significant at *p<0.05; ** p<0.01)

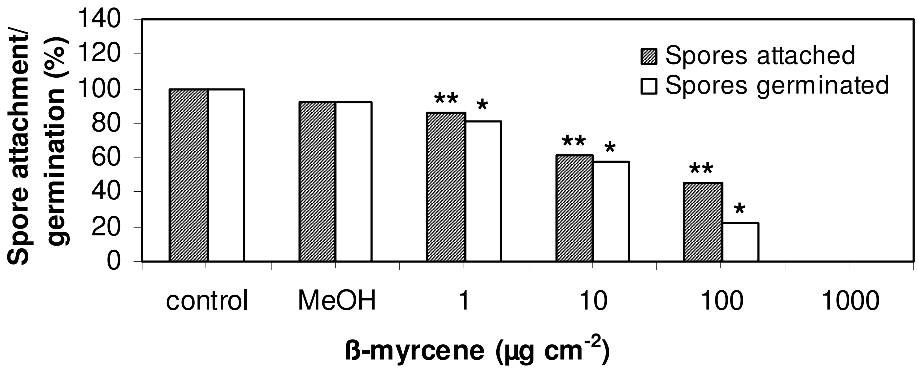


Fig. 2: Antifouling activity of ß-myrcene on spore attachment and germination of a fouling alga *Ulva pertusa*. Percentage of reduction when compared to control (significant at *p<0.05; **p<0.01)

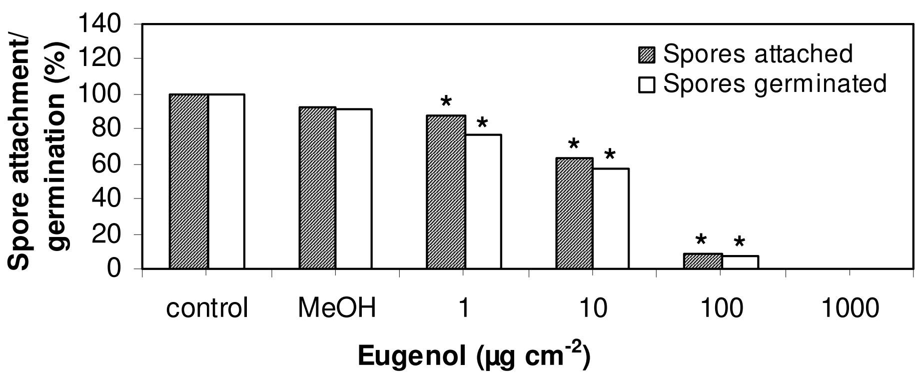


Fig. 3: Antifouling activity of eugenol on spore attachment and germination of a fouling alga *Ulva pertusa*. Percentage of reduction when compared to control (significant at *p<0.05; ** p<0.01)

AF efficiency of aromatic AF candidates. An aliphatic ester, cis-3-hexenyl acetate significantly reduced the spore attachment as well as spore germination to 66 and 40% respectively, at 1000 $\mu g\ cm^{-2}$ ($p<0.05$; $p<0.01$) (Fig. 4).

Solanesol had significant AF action against *Ulva* spores with more than 80% reduction in spore attachment ($p<0.05$) (Fig. 5). When compared to eugenol, citral and solanesol, the AF action of β-myrcene was found to be less (Fig. 2). Burridge *et al.* (1995) reported that TBT concentrations 10^{-6} mg l^{-1} and higher completely inhibit the germination of spores of a fucoid alga *Phyllospora cosmosa*. At 3 mg l^{-1} of copper sulfate, 80 to 60% decreases in *Ulva* spore attachment and 60 to 20% decreases in *Ulva* spore germination rates were reported (Hattori and Shizuri, 1996). Whereas a toxic reference AF chemical TBTO which was banned to use in AF paints showed over 75% decrease in spore

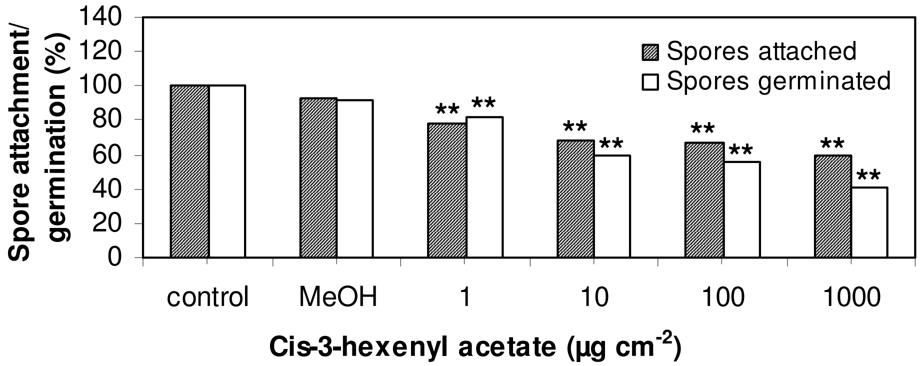


Fig. 4: Antifouling activity of cis-3-hexenyl acetate on spore attachment and germination of a fouling alga *Ulva pertusa*. Percentage of reduction when compared to control (significant at *p<0.05; ** p<0.01)

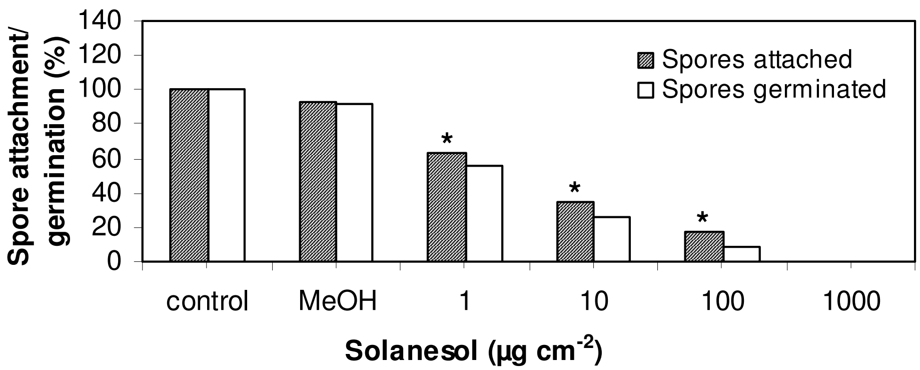


Fig. 5: Antifouling activity of solanesol on spore attachment and germination of a fouling alga *Ulva pertusa*. Percentage of reduction when compared to control (significant at *p<0.05; ** p<0.01)

attachment of *Ulva conglobata* at 0.10 mg l^{-1}. They suggested that inhibition of *Ulva* spore attachment by copper sulfate was mainly due to the toxicity, whereas a repellent effect was found in response to toxic tributyltin oxide (TBTO).

The differential response of *Ulva* spore attachment as well as germination to five AF candidates was analyzed for the Linear Regression (LR) coefficient of determinations (r^2); *t*-test revealed significant differences among the AF concentrations administered (Table 2).

A conventional antifouling paint includes broad spectrum biocides which kills the propagules during the initial attachment process. Inorganic copper and zinc; and organometallic tin compounds are widely used in conventional AF paints. The basic AF mechanism involved is, toxic effects of metal ions loaded or uncoupling of oxidative phosphorylation and electron transport. In such AF paints there is no measure to control the release of metal ions. So fouling organisms approaching the coating are to be disrupted by metal toxicity; their normal enzymatic and metabolic activities impacted which leads to death (Rittschof, 2000). Whereas the organometallic tin compounds interfere with membrane systems of the organisms and disrupt essential cellular energy functions (Boyer, 1989). The concept of nontoxic antifoulants emerged from the unfouled marine organisms due to their self protecting deterrent chemical production (Targett *et al.*, 1983; Targett, 1988; Rittschof, 1999). Similarly, allelochemicals released by some marine algae, on the other organism which always inhibits the growth or other metabolic activities of later was reported by Harlin (1996). Therefore it is considered that the functional ecology can provide effective AF principles. One of the most basic and precarious process of life-cycle of benthic marine organism is colonization onto new substratum. Attachment and settlement of fouling organisms, animals and algae and their larvae and spores, respectively, considered as most important stage of their life-cycle to establish and grow. Parallel to this prevention of attachment of juvenile stages (spores or larvae) of fouling organism is important process in nontoxic AF strategies (Finlay *et al.*, 2002). Both positive and negative response of spore adhesion of a green alga *Enteromorpha* increased on increasing number of carbon atoms in the surface (Callow and Callow, 1998, 2000). Some of the negative chemical cues (bacterial origin) present in the biofilm found to inhibit the settlement of *Ulva* spores (Holmstrom *et al.*, 1996). These studies clearly indicated the influence of chemical cues in the attachment of fouling organisms. Such negative chemical cues can be used in the nontoxic AF technologies. In the present study also AF candidates, citral, eugenol and β-myrcene exhibited such nontoxic AF activity against the attachment of *Ulva* spores at low concentrations. The AF repelling mechanism involved in these organic chemicals to be studied further.

Acknowledgements

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