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Table: 1, Figs : 5

Anti-cyanobacterial allelopathic effects of plants used for artificial floating islands

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ABSTRACT

This study aimed to identify the plants suitable for artificial floating islands. We screened the 7 plants spp. [Canna (*Canna generalis* L.H.Bailey), grassy-leaned sweet flag (*Acorus gramineus* Sol. ex Aiton), lesser bullrush (*Typha angustifolia* L.), purple loosestrife (*Lythrum salicaria* L.), reed (*Phragmites australis* (Cav.) Trin. ex Steud.), softstem bulrush (*Scirpus tabernaemontani* Gmel.) and yellow iris (*Iris pseudacorus* L.)] showing the anti-cyanobacterial allelopathic effects through release of anti-cyanobacterial compounds from their roots. To determine their potential anti-cyanobacterial allelopathic effects, we prepared methanolic extracts from the roots and investigated their effects on growth of cyanobacterium *Microcystis aeruginosa*. We found that *I. pseudacorus, A. gramineus, T. angustifolia, S. tabernaemontani* and *P. australis* had anti-cyanobacterial compounds in their roots. The culture solutions from these plants were used in cyanobacterial assays, which showed that only *T. angustifolia, S. tabernaemontani* and *P. australis* released anti-cyanobacterial compounds from their roots. Thus *T. angustifolia, S. tabernaemontani* and *P. australis* are potential beneficial species for vegetation on artificial floating islands. These species may enhance the water purification owing to anti-cyanobacterial allelopathic effects.

Keywords: *Acorus gramineus,* Aquatic and terrestrial plants, artificial floating island, *Canna generalis,* cyanobacterium, growth inhibition, *Iris pseudacorus, Lythrum salicaria, Scirpus tabernaemontani, Typha angustifolia*

INTRODUCTION

In eutrophicated ponds and lakes, cyanobacterial blooms cause serious problems in use of water (for fisheries and water-supply). Improving the water quality by reducing the nutrients load is a practical method to control cyanobacterial blooms. However its effectiveness is limited, because it is difficult to control non-point sources and/or direct nutrient loading, such as fertilizer application to soils to maintain vegetation in closely located areas and fisheries. Therefore, vegetated artificial floating islands have been proposed to remove nutrients and restore aquatic ecosystems. Artificial floating islands with vegetation have been used to remediate many lakes and ponds (8,9,12,13). Such floating islands can effectively remove nutrients and reduce cyanobacterial biomass (8,13),

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though management of vegetation is inevitable due of nutrients removal. Light interception and/or removal of nutrients can suppress the cyanobacterial growth (12), positive effects of floating islands may also include improving the zooplankton community (8) and allelopathic effects of vegetation (12).

Some macrophytes cause allelopathic growth inhibition of cyanobacteria (7,8,10,14,16,21,23-25), it can be expected that vegetation on artificial floating islands may also cause allelopathic growth inhibition of cyanobacteria. Some studies have reported that various plants contain anti-cyanobacterial compounds and are therefore, suitable for growth on artificial floating islands. These plants include *Acorus gramineus* (5)*, Typha latifolia* (1), *Phragmites australis* (*communis*) (11,14) and *Cyperus rotundus* (2).

The release of anti-cyanobacterial compounds from vegetation could improve the water purification ability of artificial floating islands, as these compounds may prevent or lessen the severity of cyanobacterial blooms. It is therefore necessary to determine whether anti-cyanobacterial compounds are in fact released. It is important to recognize that anti-cyanobacterial allelopathic compounds (allelochemicals) should be released from the roots, not from non-submerged plant organs. However, previous studies have concentrated on anti-cyanobacterial compounds in leaves or stems (1,14,19). In some cases, the part of plant tested was not reported (5,11,18). Furthermore, the environmental conditions (light intensity and nutrients availability), may influence the allelopathic effects of macrophytes (6). Therefore, the influences of such factors on allelopathic effects should be determined to ensure the optimum allelopathic activity.

In this study, we screened several aquatic and terrestrial plants with potential anti-cyanobacterial allelopathic effects by cyanobacterial assays using extracts of root tissues and then the anti-cyanobacterial allelopathic effect was demonstrated by confirming cyanobacterial growth inhibition by culture solutions. Furthermore we investigated, whether plant cultivation temperature affects its anti-cyanobacterial allelopathic effects.

MATERIALS AND METHODS

Plants and cyanobacterium

Plants used in these experiments were obtained from Tojaku Engei Co., Ltd., Kyoto, Japan. We used 7 test plants species : canna (*Canna generalis* L.H.Bailey), grassy-leaned sweet flag (*Acorus gramineus* Sol. ex Aiton), lesser bullrush (*Typha angustifolia* L.), purple loosestrife (*Lythrum salicaria* L.), reed (*Phragmites australis* (Cav.) Trin. ex Steud.), softstem bulrush (*Scirpus tabernaemontani* Gmel.) and yellow iris (*Iris pseudacorus* L.) Plants roots were carefully washed with tap water to remove soil and debris, then plants were cultivated at 20-30°C in a greenhouse under 0-100 μmol m^{-2} s^{-1} light intensity. Plants roots were immersed in 4 L growth medium [containing 0.4 $mg{\cdot}L^{-1}$ $(NH_4)H_2PO_4$, 0.06 $mg{\cdot}L^{-1}$ KNO_3, and 0.15 $mg{\cdot}L^{-1}$ $MgSO_4$]. Temperature was maintained at 15°C or 25°C in experiments investigating the effects of plant cultivation temperature on the anti-cyanobacterial allelopathic effect, and light conditions were as follows: 12 h-dark:12 h-light photoperiod, 100 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ light intensity. We used the cyanobacterium *Microcystis aeruginosa* (NIES-87), obtained from Microbial Culture Collection of the National Institute for Environmental Studies (NIES). *M. aeruginosa* was

cultured in a modified C (CB) medium (20) with an exception that pH of medium was adjusted to 7. *M. aeruginosa* was grown at 25°C under 50 μmol·m^{-2}·s^{-1} light intensity, and growth was monitored by measuring turbidity (ANA-108, Tokyo Koden, Japan).

Potential anti-cyanobacterial allelopathic effects

Root extracts from the test plants were assayed for anti-cyanobacterial activity using *M. aeruginosa* as the target species. Roots were first cleaned with MillQ water, then cut into 1-cm pieces and extracted with methanol [1:1 (w/v) roots: methanol)] for 24 h at 25°C with shaking at 70 rpm on a rotary shaker (6). After 24 h, the methanol was replaced with fresh solvent. In total, the methanol extraction was repeated three times. Finally, the methanol extracts were combined and concentrated *in vacuo* at 30°C. The concentrated extract was filtered through a grass fiber filter (GF/F, Whatman, UK) and then a portion of filtrate (<1000 μl) was added to 100 ml autoclaved CB medium in 500 ml Erlenmeyer flask, before inoculation with *M. aeruginosa.* The same volume of methanol was added to the cyanobacterial medium in control. The experiments were carried out in tripricate.

Demonstration of anti-cyanobacterial allelopathic effect

As mentioned later, the methanol extracts of *I. pseudacorus, S. tabernaemontani, T. angustifolia, A. gramineus,* and *P. australis* showed strong growth inhibition of *M. aeruginosa,* therefore the culture solutions from these plants were assayed to determine whether they inhibit the growth of *M. aeruginosa.* Since the biomass of roots per stock depends on the plant species and cutting roots seriously damaged the plants, the concentration of roots was not adjusted during the preparation of culture solutions (Table 1).

Table 1. Roots concentrations in culture solutions

Plant	Root concentration [kg-wet·m^{-3}]
Scirpus tabernaemontani	100
Acorus gramineus	25
Iris pseudacorus	25
Typha angustifolia	25
Phragmites australis	9.4

Two stocks of plants were cultivated for 10 days and then nutrients equivalent to CB medium (20) were added to the culture solution to ensure sufficient nutrient availability in the cyanobacterial assay. The enriched culture solution was filtered through an autoclaved membrane filter (0.45 μm, Advantec, Japan). A 100 ml filtrate was added to 500 ml Erlenmeyer flask and the flask was inoculated with *M. aeruginosa.* The experiments were done in tripricate. For the plants which inhibited the cyanobacterial growth, the experiments were repeated twice for *S. tabernaemontani* and *P. australis* or 4-times for *T. angustifolia*. Finally, *t*-test was done for a statistical comparison of *M. aeruginosa* growth in control and culture solution of each plant.

Effect of temperature on anti-cyanobacterial allelopathic effect

To determine if the plant cultivation temperature affected the anti-cyanobacterial allelopathic activity, we cultivated *T. angustifolia* (a plant showing anti-cyanobacterial

allelopathic effects), at 15°C and 25°C for 10 d. The culture solutions from plants grown in these conditions were used in a cyanobacterial assay. We measured the dissolved organic carbon (DOC) concentration of culture solution with a total organic carbon analyzer (TOC5000, Shimadzu, Japan). The resultant culture solutions were assayed with *M. aeruginosa* in triplicate as described above. To compare the anti-cyanobacterial allelopathic effect, results of cyanobacterial assays were statistically analyzed with one-way ANOVA, followed by a *post hoc* Scheffé's test.

RESULTS AND DISCUSSION

Potential anti-cyanobacterial allelopathic effects

Methanol extracts inhibited the growth of *M. aeruginosa* (Fig. 1). This result confirmed that anti-cyanobacterial compounds exist in the roots of *S. tabernaemontani*, which have a potential anti-cyanobacterial allelopathic effect.

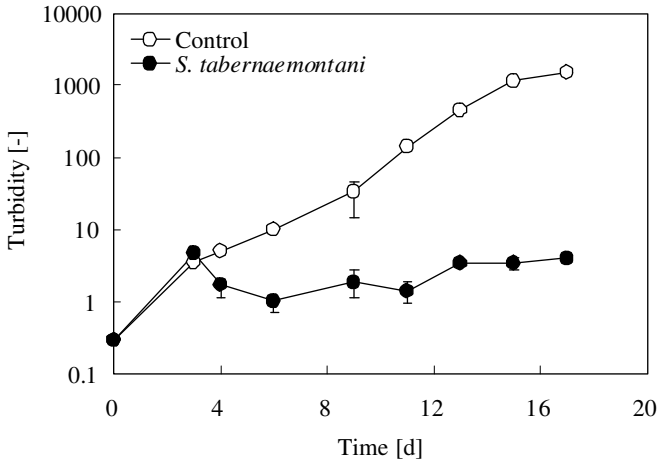

Figure 1. Effects of *S. tabernaemontani* extract (3 kg-wet·m^{-3}) on the growth of *M. aeruginosa*. Bars indicate standard deviation (n=3).

The methanolic extracts from roots of all plants, except *L. salicaria* inhibited growth of *M. aeruginosa* (Fig. 2). The *C. generalis, I. pseudacorus, S. tabernaemontani*, *T. angustifolia, A. gramineus* and *P. australis* showed potential for allelopathic growth inhibition in *M. aeruginosa.* The anti-cyanobacterial allelopathic effects of *I. pseudacorus, S. tabernaemontani, T. angustifolia, A. gramineus* and *P. australis* were comparatively strong, hence, these five plants were used for further testing.

In a previous study, anti-cyanobacterial compounds were found in *A. gramineus* plants (5), but that study did not report the dose-response relationship. Thus, we cannot compare the potential anti-cyanobacterial allelopathic effects in this study to the previous study.

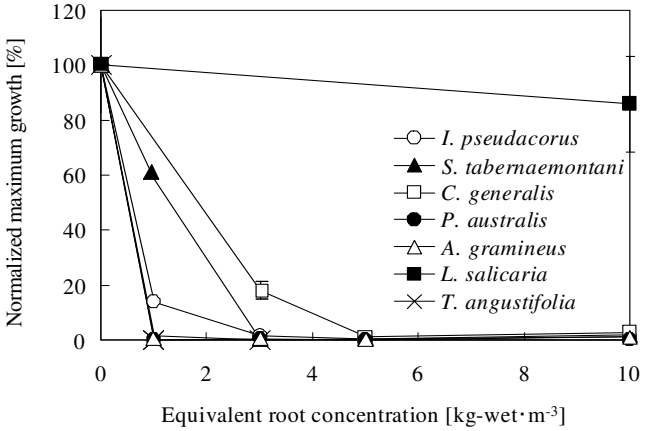

Figure 2**.** Dose-response relationship between the equivalent root concentration of methanol extract and inhibition of *M. aeruginosa* growth. The normalized maximum growth is percentage of maximum growth of *M. aeruginosa* as affected by the methanol extract to that in the control. Bars indicate standard deviation (*n*=3).

Occurrence of anti-cyanobacterial allelopathic effect

The culture solutions of *T. angustifolia* and *S. tabernaemontani* inhibited the growth of *M. aeruginosa* (Fig. 3). This effect was consistently observed when assays were repeated. These results confirmed that anti-cyanobacterial allelochemicals were present in culture solutions of these plants. As the methanolic root extracts also inhibited the growth of *M. aeruginosa*, these results suggested that *T. angustifolia* and *S. tabernaemontani* release anti-cyanobacterial allelochemicals through their roots.

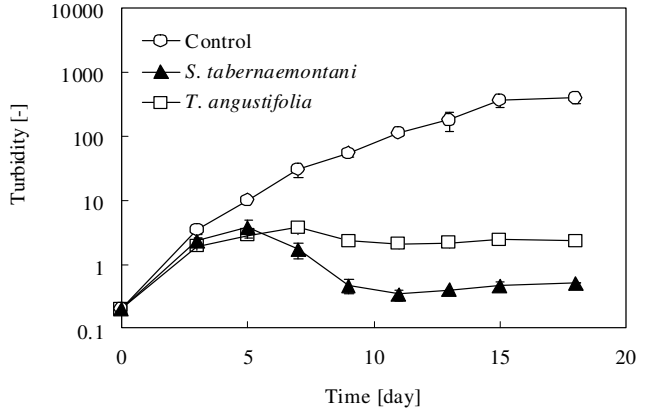

Figure 3. Effects of culture solutions of *T. angustifolia* and *S. tabernaemontani* on the growth of *M. aeruginosa.* Bars indicate standard deviation (*n*=3).

P. australis was also found to release anti-cyanobacterial allelochemicals through its roots (Fig. 4). In our previous study, anti-cyanobacterial allelochemicals (such as gallic and protocatechuic acids) were found in leaves and stems of *P. australis* (14), while ethyl 2-methylacetoacetate was identified in ethanolic extract of its roots (11). *T. angustifolia* contains flavonoids (22) and certain flavonoids (catechin and quercetin) have anti-cyanobacterial activity (15,19). To search for allelochemicals, the compounds in culture solutions and methanol extracts of *T. angustifolia, S. tabernaemontani,* and *P. australis* should be analyzed in detail in future research.

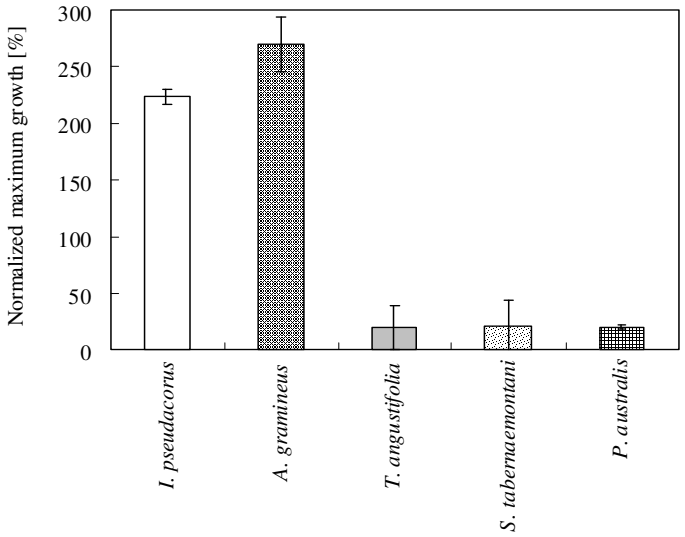

Figure 4. Effects of plant culture solutions of 5 test plant spp. on the *M. aeruginosa* growth. The normalized maximum growth is percentage of maximum growth of *M. aeruginosa* in the culture solution to that in the control. Bars indicate standard deviation (n=3 for *I. pseudacorus* and *A. gramineus*; n=6 for *S. tabernaemontani* and *P. australis*; n=12 for *T. angustifolia*).

Roots exudates of *I. pseudacorus* and *A. gramineus* stimulated ($p < 0.01$) also inhibited the growth of *M. aeruginosa* (Fig. 4). The root concentration was 25 kg-wet$\cdot$m^{-3} in culture solutions (Table 1). However, the corresponding methanolic extracts were prepared using the same quantity of root tissue and these extracts strongly inhibited the growth. These results indicate that anti-cyanobacterial compounds in roots of *I. pseudacorus* and *A. gramineus* might not be released, or that the amounts released were insufficient to inhibit the growth of *M. aeruginosa.* The reasons underlying the growth stimulation are unknown, but it may be that compounds accelerating the growth of *M. aeruginosa* were released. In allelopathy, positive effects on target organisms are also known (6) and the extracts of certain aquatic plants stimulates the algal growth (17).

We did not quantitatively determine the anti-cyanobacterial allelopathic effect in the culture solution assays. However, the results demonstrated that *T. angustifolia,*

S. tabernaemontani and *P. australis* release anti-cyanobacterial allelochemicals through their roots. This indicates that the water purification abilities of artificial floating islands could be increased using such allelopathic plants, which could control cyanobacteria growth.

In a further experiment, we investigated the effects of cultivation temperature on the anti-cyanobacterial allelopathic effects. *T. angustifolia* was selected, as it showed a strong allelopathic effect in previous experiments. A *post hoc* Scheffé's test showed the significant differences in the growth of *M. aeruginosa* between the control and *T. angustifolia* culture solution prepared at 25°C after 7 d ($p < 0.01$) (Fig. 5). The culture solution prepared at 15°C after 13 d significantly inhibited the growth ($p < 0.05$). Furthermore, after 5 d, the growth of *M. aeruginosa* in culture solution of *T. angustifolia* prepared at 25°C was significantly decreased than that prepared at 15°C ($p < 0.01$), thus confirming that the allelopathic effects of plants grown at 25°C were stronger than plants grown at 15°C. Although the 15°C temperature is not optimum for cyanobacterial growth, but this result showed that plant cultivation temperature affects the allelopathic effects of *T. angustifolia*. Thus, variation in cultivation temperature might be one reason for the observed variations in the inhibitory effect of *T. angustifolia* culture solution, because the culture solutions in that assay were prepared without controlling temperature.

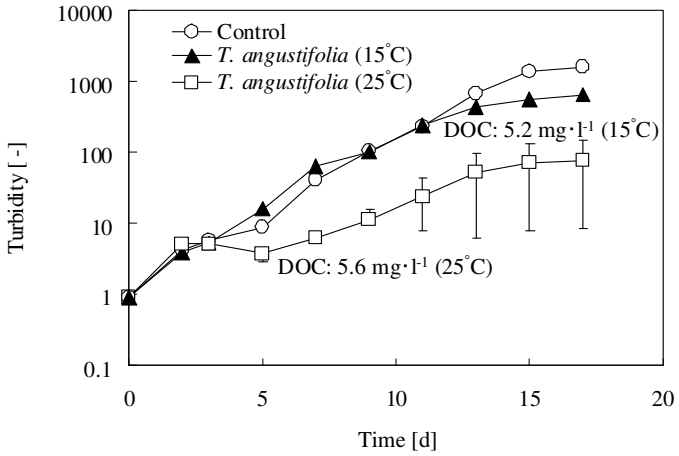

Figure 5. Effects of culture solutions of *T. angustifolia* grown at 15°C and 25°C on the growth of *M. aeruginosa.* Bars indicate standard deviation (n=3).

It is not surprising that temperature affects the activities of plants. The inhibitory effects of *T. angustifolia* culture solution became higher when plants were grown at 25°C, thus we speculated that this was due to increased exudation of organic compounds at higher temperatures. However, the difference in DOC concentration of the culture solution was less than 10%; 5.2 mg·l^{-1} at 15°C and 5.6 mg·l^{-1} at 25°C. Another possible explanation is that the composition of organic compounds in culture solution changed at higher temperatures. Seasonal changes in carbohydrate translocation and synthesis of structural

carbon components has been reported in *T. angustifolia* (3), though these may also be affected by the plant growth phase. Since we did not characterize the organic compounds dissolved in the culture solutions of *T. angustifolia*, we cannot explain the observed differences in the inhibitory effect. However, our results demonstrate that there may be an optimum temperature at which anti-cyanobacterial allelopathic plants release the most effective types and/or amounts of allelochemicals.

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