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Assessment Method for Leaf Litters Allelopathic Effect on Cyanobacteria

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ABSTRACT

A new bioassay technique combining leaf disk and soft-agar over-layer methods was developed to investigate the allelopathic effect of deciduous leaf litters on the growth of cyanobacteria (*Microcystis aeruginosa* Kütz.). Bioactive substances exuded from leaf disks caused inhibitory plaques on the agar plate containing cyanobacteria, and the rate of diffusion depended on the specific leaf disk area. Most of the leaf litters collected around reservoirs in Japan showed inhibitory activity to *M. aeruginosa*, with *Rhus trichocarpa* Miq., *Quercus variabilis* Blume and *Mallotus japonicus* (Thunb.) Muell. Arg. being the strongest among the 22 tested species.

Key words: allelopathy, bioassay; leaf disk, *Microcystis aeruginosa*.

INTRODUCTION

Severe outbreaks of cyanobacteria (blue-green algae) in shallow lakes and reservoirs, called "water blooms", cause serious problems with regard to effective utilization of water resources. *Microcystis* is often associated with the formation of hepatotoxic peptides such as microcystin (Carmichael 1992). The presence of high concentrations of microcystin can bring about serious problems in public water supplies. Some techniques for suppressing the occurrence of water blooms have been introduced in the past, such as cultivating plants with high nutrient absorption characteristics (i.e., *Eichhornia crassipes* (Mart.) Solms-Laub.) in a lake (Otsuki et al. 1990), and cutting off the supply of nutrients like nitrogen (N), phosphorus (P), and iron (Fe) which are key factors to the growth of cyanobacteria (Sakamoto 1966, Welch et al. 1992). However, high nutrient concentrations in lakes do not totally explain the occurrence of water blooms (Moss 1998). For example, in some shallow lakes, large macrophyte populations can suppress water blooms in spite of eutrophication (Scheffer et al. 2001, Takamura et al. 2003). Based on this observation, other important factors are inferred to play an active role in the competition between macrophytes and cyanobacteria. One explanation for this phenomenon is the release of chemical compounds by these plants that inhibit algal growth (Morris et al. 2003). These bioactive substances are called allelochemicals, and the mechanism involved is called allelopathy (Molisch 1937).

Allelopathy has been reported in littoral areas (Gross 1999), and in the last decade, many studies have demonstrated the algicidal effect of leaf litter and straw (Newman and Barrett 1993, Ridge et al. 1999). A method to assess allelopathic effects of leaf litters to higher plants has been established (Fujii et al. 2004), however, no method to determine the effect of leaf litters on cyanobacteria has been reported. This study evaluates the allelopathic effect of deciduous leaf litters on bloom-forming cyanobacteria by combining the soft-agar over-layer technique (Uchida et al. 1998, Yamamoto 1978) and leaf disk method.

MATERIALS AND METHODS

The algal strain (NIES-88) *Microcystis aeruginosa* used in this bioassay was obtained from the microbial collection of the National Institute for Environmental Studies (NIES), Japan. It was pre-cultured in sterilized CT medium (Watanabe and Ichimura 1977) for 15 d under the following conditions: a photosynthetic photon flux density of 66.6 µmol photons/m^2/s, 12:12 h light: dark photoperiod, and 30:25 C, respectively. The algae were collected by centrifugation at 3000 g for 30 min.

CT medium (10 ml) with agar (0.75% w/v) was poured onto petri dishes (90 mm in diameter) and left to gelatinize (bottom layer). CT medium (3 ml) with agar (1.0% w/v) at 40 to 50 C was mixed well with 3 ml of the concentrated algae suspension. This algae-mixed medium was then spread onto the gelatinized bottom layer, and left to harden (top layer).

Deciduous leaf litters for 22 species collected around the reservoirs at South Hyogo Prefecture (34N-134E) were drilled with a leaf disk punch (Fujiwara Scientific Co., Tokyo, Japan) and sterilized with ultraviolet irradiation (257.3 nm, 6 h for each faces). Paper disks (ADVANTEC, Japan) sterilized with heat (150 C, 6 h) were used for control. Both leaf and control disks were placed over the top layer of the agar plates; a total of two disks were on a plate. These plates were then incubated under the same pre-culturing conditions for 7 d. A schematic diagram of this method is shown in Figure 1. The degree of algae growth inhibition was determined by both size and characteristics of the plaque zones formed around the leaf disk. All experiments were run in quadruplicate.

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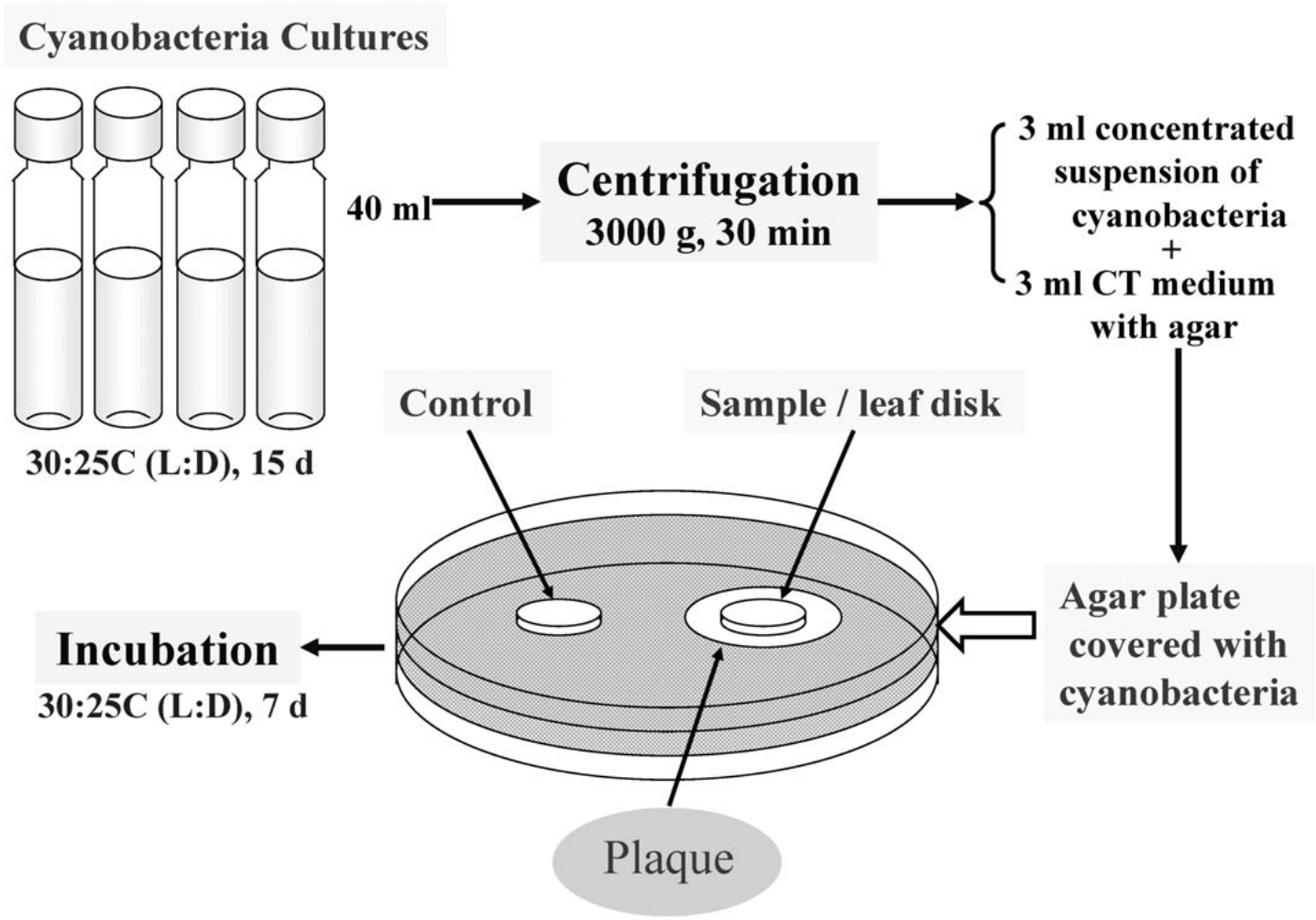

Figure 1. Schematic diagram of the assessment method used in this study. Leaf disks sterilized with ultraviolet irradiation (257.3 nm, 6 h for each faces) were placed on the top layer of cyanobacteria (*M. aeruginosa*). Paper disks sterilized with heat (150 C, 6 h) were used for control. Cell numbers per plate calculated by the growth curve are 4.78×10^7 (assumed 50% recovery rate). After incubation for 7 d, the plaque zone diameter was measured in mm.

RESULTS AND DISCUSSION

After incubating the plates for 7 d, inhibitory plaques (zone plaques) of different sizes and shapes were observed on top of the agar plate (Table 1). Control disks resulted in no plaque formation. *Rhus trichocarpa*, *Quercus variabilis* and *Mallotus japonicus* produced larger diameter plaque zones than the others (Table 1, Figure 2). Since the leaf disk surface was initially sterilized, plaque formation is likely due to its exudates and not of the action of microorganisms. The formation of plaque zones suggests the presence of active allelopathic compounds in the leaf litters of the tested species.

Additionally, the variation in sizes and shape of plaque zones may indicate the activity-dependent nature of the leaf litter species. Evaluation of the relationship in size between leaf disks and plaque zones of *R. trichocarpa* (most active) and *Ficus erecta* Thunb. (least active) showed that both parameters are highly correlated (r = 0.899) in the former, but not (r = 0.252) in the latter (Figure 3), indicating that the rate of diffusion depends on the specific leaf disk area and species. *R. trichocarpa*, *Q. variabilis* and *M. japonicus* showed large, clear plaque zones, suggesting the presence of strongly active allelopathic substances effective against *M. aeruginosa*.

Leaf litters may strongly influence algal communities especially in shallow lakes and reservoirs (Gross 2003). There are numerous reports regarding allelopathic effects from leaf litter leachates. For example, it has been known for many years that poor plant growth under the black walnut trees (*Juglans nigra* L.) and the Japanese red pine trees (*Pinus densiflora* Sieb. et Zucc.) are due to allelopathic effect of their leaf litters (Massey 1925, Lee and Monsi 1963). However, reports that discuss the relationship between leaf litter leachates and aquatic organisms hardly exist. This study provides a potential method for improving investigations regarding the influence of shoreline and littoral zone vegetation on the occurrence of water blooms.

Results of this study suggest that deciduous leaf litters accumulating on littoral zones of lakes and reservoirs contain active compounds which are inhibitory to the growth of *M. aeruginosa*, and that leaf litter from *R. trichocarpa*, *Q. variabilis* and *M. japonicus* can be potential natural inputs in controlling their excessive growth. More studies of this nature

TABLE 1. LIST OF SAMPLES (LEAF DISKS) WITH THEIR CORRESPONDING PLAQUE ZONE DIAMETER AND CHARACTERISTICS. D: THE PLAQUE BOUNDARY IS DISTINCT. ND: THE PLAQUE BOUNDARY IS NOT DISTINCT. P: THE PLAQUE SHAPE IS PROTEAN.

Sample	Plaque zone	
	Diameter (mm)	Characteristics
Rhus trichocarpa Miq.	65	ND, P
Quercus variabilis Blume	64	D
Mallotus japonicus (Thunb.) Muell. Arg.	60	ND, P
Vaccinium oldhami Miq.	49	D
Rhus succedanea L.	46	D
Viburnum erosum	43	D
Prunus lannesiana (Carr.) Wils.	40	D
Vaccinium ciliatum	35	D
Castanea crenata Sieb. et Zucc.	34	D
Vaccinium smallii A. Glay var. *glabrum* Koidz.	29	D
Parabenzoin praecox (Sieb. et Zucc.) Nakai	29	D
Salix caenomeloides Kimura	28	D
Quercus acutissima Carruth.	28	D
Photinia glabra (Thunb.) Maxim.	28	ND
Lyonia neziki Nakai et Hara	27	D
Vitis coignetiae Pulliat	25	D
Rhus javanica L.	23	D
Ilex pedunculosa Mip.	20	D
Quercus glauca Thunb.	20	D
Quercus aliena Blume	14	D
Ficus erecta Thunb.	12	D
Wisteria brachybotrys Sieb. et Zucc.	0	Not measurable
Control	0	Not measurable

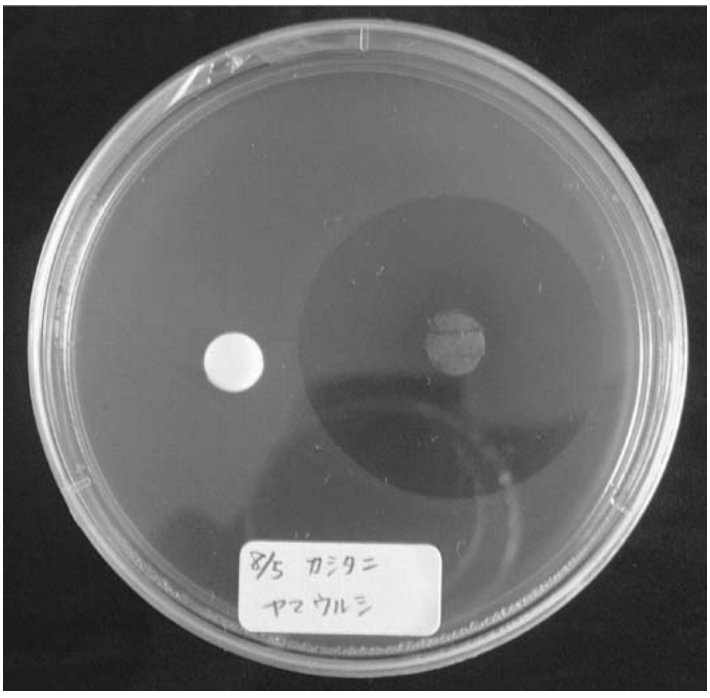

Figure 2. Growth response of cyanobacteria to leaf disks. Disks were placed on the top layer of cyanobacteria (*M. aeruginosa*). After incubation for 7 d, plaque zone formation around the disks was examined. Leaf disk: *Rhus trichocarpa*. Control: Paper disk.

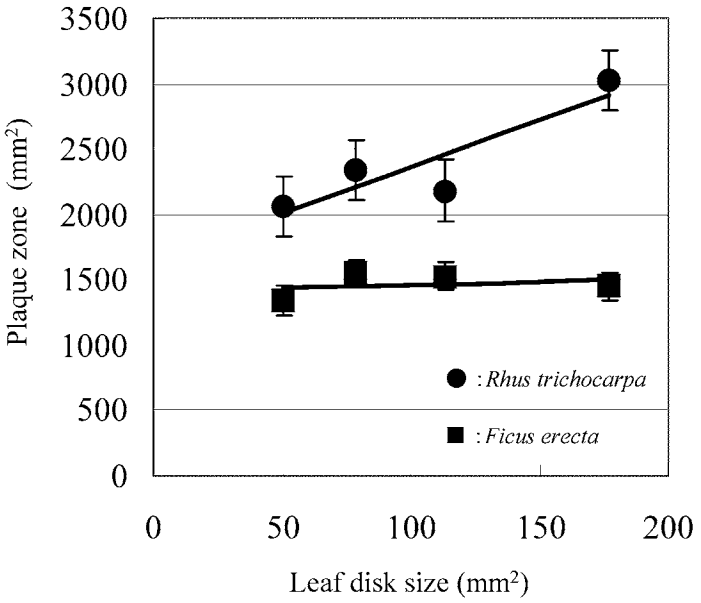

Figure 3. Relationship between the leaf disk (mm^2) and the plaque zone size (mm^2). *Rhus trichocarpa* (●): r = 0.899, n = 16, $p < 0.001$; *Ficus erecta* (■): r = 0.252, n = 14. Error bars show standard errors.

are necessary to elucidate further the extent and nature of the inhibition. Other cyanobacteria species should be tested as well. The inhibitory effect of deciduous leaf litters based on their decomposition rates should also be investigated.

For the suppression of water blooms, the long-term goal remains that of reducing nutrient inputs, but, even if this is achieved, continued management will often be necessary to maintain lakes and reservoirs in the "desired" state. Natural leaf litter input is one factor that may help to minimize the likelihood of excessive algal growth. The bioassay system introduced here could be a useful tool for providing information that will be helpful in determining the role of shoreline vegetation in impacting algal communities in littoral zones of lakes and reservoirs.

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