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Ecology Note

Phytoremediators from abandoned rice field

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Abstract We screened about 50 species of plants collected from local abandoned rice fields for capability to decolorize Remzol Brillinant Blue R (RBBR), an anthraquinone dye. We chose *Rumex crispus* L. subsp. *japonicus* (Houtt.) Kitamura (a curly dock; "Gishi-gishi" in Japanese) and rice, and investigated the capability of these plants to remove bisphenol A (BPA), an endocrine disruptor, from the culture medium. Aseptically-grown curly dock appeared to completely remove BPA added in the culture medium (1000 mg BPA kg^{-1} fresh plants, 40 mg l^{-1}) by 15 days after treatment (DAT). At 7 DAT methanol-extractable BPA was present in curly dock. However, no such BPA was detectable at 14 DAT. Curly dock was found to remove completely BPA at an environmental pollution level (1 μg l^{-1}) by 15 DAT. Rice showed a similar or less capability to clean up BPA from the culture medium.

Key words: Bisphenol A, phytoremediation, remzol brillinant blue R (RBBR), rice, *Rumex crispus* L.

In this study, we collected plants and seeds from local abandoned rice fields in Higashi-Hiroshima city, Hiroshima, Japan in the fall of 1999–2003, and screened for those that bear high capability to decolorize an anthraquinone dye, Remazol Brilliant Blue R (RBBR). The decolorization of this dye has widely been used as an indicator for rapid screening in fungal screening for strains of high dioxin degradation (Sato et al. 2002; Novotny et al. 2001; Vyas and Molitoris 1995).

Among about 30 monocot plant species such as those of Poaceae and Cyperaceae tested, their decolorization capabilities varied as shown in Figure 1A. Because precise identification of young seedlings of monocot species was difficult, and also because rice itself was found to show a high decolorization capability, we chose rice as a monocot plant for further study. About 20 dicot plant species including those of Polygonaceae, Umbelliferae, Compositae were similarly tested, and *Rumex crispus* L. subsp. *japonicus* (Houtt.) Kitamura (a kind of curly dock; "Gishi-gishi" in Japanese), a member of Polygonaceae was found to be the one of the best in RBBR decolorization assay. *Rumex* species are perennial and form thick taproots, which might be suited for cleaning up pollutants. Three other Rumex species (*R. acetosa, R. acetocella* and *R. madaio*) tested, however, showed no appreciable decolorization. Thus, we chose curly dock as a dicot plant for further study. Figure 1B shows decolorization of RBBR by aseptically-grown seedlings of curly dock.

In the observed differences in decolorization among plant individuals (see Figure 1), various factors (separately or in combination) such as those enzymes secreted from roots, bacterial and/or fungal activities associated with roots and uptake of the dye into plants are involved. Laccase and peroxidase enzymes have been shown to participate fungal decolorization of this dye (Sato et al. 2002; Novotny et al. 2001; Vyas and Molitoris 1995). Addition of 200 μM hydrogen peroxide to the culture medium for curly dock (see Figure 1B) greatly stimulated decolorization of RBBR, suggesting involvement of peroxidase in decolorization by this plant (Takahashi et al., unpublished results). In the following part of this study, aseptically-grown curly dock and rice were used to test for their capability to clean up an organic pollutant from the culture medium.

Bisphenol A (4,4′-isopropylidenediphenol; BPA) was chosen as a model pollutant in this study. BPA is one of the pollutants of the hydrosphere and pedosphere that is known to cause defects of the genital system (Vom Saal et al. 1998) and have an estrogenic activity (Scippo et al. 2004; Markey et al. 2005). BPA is detected in fresh water (1 μg l^{-1}), freshwater sediment (1,100 μg kg^{-1} dry weight), saltwater sediment (120 μg kg^{-1} dry weight) and wastewater (0.09–3.9 μg l^{-1}) (http://www.safe.nite.go.jp/english/index.html). BPA is used as the monomer of polycarbonate resins. World (in 2002) and domestic (in 2000) production of BPA is estimated to be 2.8 million tons and 0.4 million tons, respectively (http://www.

Abbreviations: RBBR, Remazo Brilliant Blue R; BPA, Bisphenol A; DAT, day after treatment

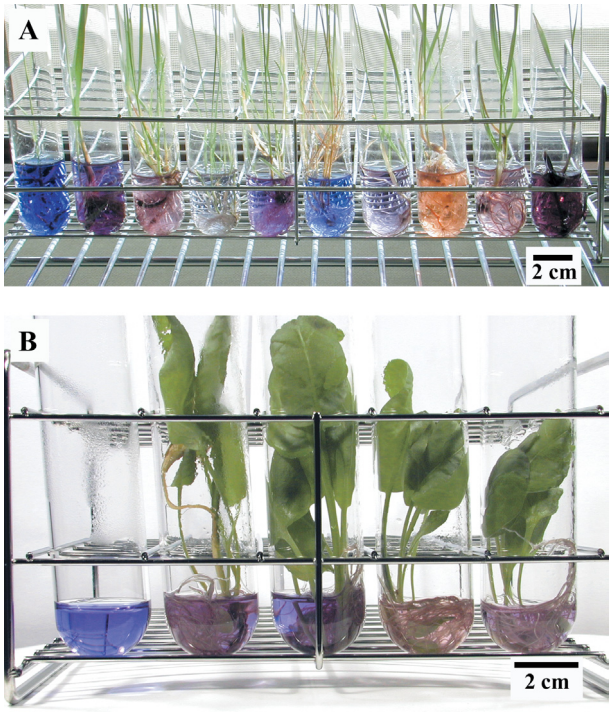

Figure 1. Decolorization of Remazol Brilliant Blue R (RBBR) by aseptically (B) and non-aseptically grown (A) plants. (A) Various monocot plants were collected from the abandoned rice field, washed with wash with tap water, and incubated in 0.1% Hyponex containing 5 mM 2-morpholinoethanesulfonic acid (MES; Sigma, St. Louis, USA) and 0.02% (w/v) RBBR (Sigma, St. Louis, USA) at pH5.6 under the natural light at 22±3°C for 3 days. (B) Seeds of *Rumex crispus* L. subsp. *japonicus* (Houtt.) Kitamura (a curly dock; "Gishi-gishi" in Japanese) collected from the abandoned rice field were surface-sterilized using 2.5% sodium hypochlorite and washed with sterilized water, placed in water at 4°C overnight and sown on MS medium (Murashige and Skoog 1962) containing 1% (w/v) sucrose and 0.3% Gellan Gum (Wako Pure Chemical Ind., Osaka, Japan). They were allowed to germinate at 25±0.3°C under a 16 h light (70 μmol photons m^{-2} s^{-2})/8 h dark cycle for several days. After germination, seedlings were grown for 2 more weeks, after which they were transferred to the same medium without sucrose in plastic containers (Agripot, Kirin, Japan) and grown for 4 more weeks. The seedlings were then transferred to test tubes containing MS medium with 5 mM MES (pH 5.6) and 0.02% (w/v) RBBR and incubated for 3 more days. The leftmost test tube without plants shows the initial color of RBBR.

bisphenol-a.org/index.html). The standard for the specific migration limits into food is 3 ppm (EU) and 2.5 ppm (Japan) (ECB 2003).

Transactions of plants with BPA have been investigated by the previous authors. Glycosylation and other modification of BPA in cultured cells of several plant species (Hamada et al. 2002; Chai et al. 2003; Nakajima et al. 2004) or in plants per se (Nakajima et al. 2002; Noureddin et al. 2004a, b) are known. These modifications reportedly decrease estrogenic activity of BPA (Morohoshi et al. 2003; Tsutsumi et al. 2001). Rice (Noureddin et al. 2004a), water convolvulus (Noureddin et al. 2004b) and tobacco (Nakajima et al. 2004) are reported to remove BPA from liquid media. However, no plants from the abandoned rice field have been studied

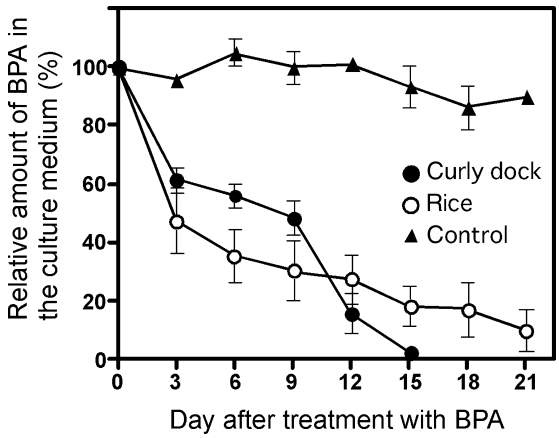

Figure 2. Time course of disappearance of bisphenol A (BPA) from the culture medium by curly dock and rice over 21 days after the start of treatment (DAT).

An aseptically-grown plant of curly dock (ca. 0.2 g fresh weight) or rice (0.1 g fresh weight) of 6 weeks or 10 days after germination respectively was placed in 5 ml 0.1% hyponex (Hyponex, Osaka, Japan) with BPA (Wako Pure Chemical Ind., Osaka, Japan) in a test tube (2.5 cm diameter and 12 cm long) and incubated at 25±0.3°C under a 16 h light (70 μmol photons m^{-2} s^{-1})/8 h dark light cycle. BPA to plant mass ratio was 1000 mg BPA kg^{-1} fresh plants, and BPA concentration was 40 (curly dock) or 20 (rice) mg l^{-1}. Every 3 days, 100 μl culture medium were collected, and analyzed by HPLC (Waters 2690 Separation Module, Milford, MA, USA) under the following conditions: column, XTerra® MS C18 5 μm 4.6×100 mm; mobile phase, 35% acetonitrile; flow rate, 1 ml min^{-1}; detection, absorption at 277 nm; temperature, 25°C; internal standard, 4-isopropyl phenol. One experiment consisted of 4 replicates, and data represent means±SD. Control corresponds to the medium without plants.

on their capability to clean up BPA from liquid media.

Seeds of curly dock collected from local abandoned rice fields were surface-sterilized, germinated and cultured on MS medium (Murashige and Skoog 1962) as described in the legend of Figure 1. Rice (*Oryza sativa* L. cv. Nipponbare) seeds were a gift from Prof. Kunisuke Tanaka, Kyoto Prefectural University. Aseptic growth of rice was done in the same way as for curly dock. Six-week-day old curly dock and ten-day old rice were used.

Figure 2 shows that the time course of disappearance of BPA from the culture medium by curly dock and rice over 21 d after the start of treatment (DAT). The BPA to plant mass ratio was 1000 mg BPA kg^{-1} fresh plants, and the initial concentration of BPA was 40 (curly dock) or 20 (rice) mg l^{-1}. Note that this BPA concentration was more than four orders of magnitude higher than the value in fresh water in Japan (see above).

Curly dock rapidly decreased BPA concentration of the culture medium, and almost no detectable BPA remained by 15 DAT. Very similar results were obtained with rice except that ca. 20% of added BPA remained in the culture medium at 15 DAT. Since the initial concentration of BPA was two times higher in curly dock than rice medium, it was concluded that curly dock had a higher capability to remove BPA than rice. It should be noted that BPA concentration in the culture medium

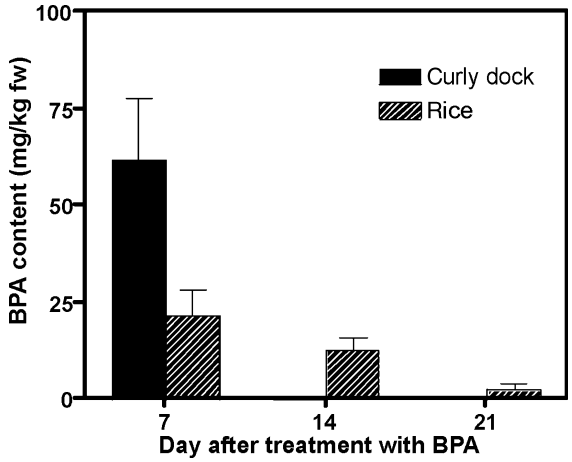

Figure 3. Changes of methanol-extractable BPA in curly dock and rice. At 7, 14 and 21 DAT, plants of curly dock and rice were removed from the culture media, washed with distilled water, ground in liquid N_2 using a mortar and pestle, and further homogenized with 300 μl methanol. After centrifugation at 11,000 *g* for 30 min and evaporation of methanol, the residues were suspended in 200 μl distilled water, and analyzed by the HPLC. One experiment consisted of 4 replicates, and data represent means ± SD.

without plants was decreased by ca. 10% of the initial concentration by 15–20 DAT (see Figure 2) and that when autoclaved (at 121°C for 30 min) plants were added, BPA concentration in the culture medium was decreased by 10–20% more by 15–20 DAT.

Figure 3 shows the quantity of BPA extractable with methanol from curly dock and rice at 7, 14 and 21 DAT. There were an appreciable amount of methanol-extractable BPA both in curly dock and rice at 7 DAT. This accounted for only less than 10% of the BPA that disappeared from the culture medium at 7 DAT. Methanol-extractable BPA completely disappeared by 14 DAT in curly dock or almost completely by 21 DAT in rice. This strongly suggests that BPA taken up into plants was metabolized. It is probable that the BPA absorbed from the culture medium by curly dock and rice was converted to some non-extractable form inside of the plants. Consistent with this is the previous report (Noureddin et al. 2004b) that approx. 50% of the absorbed BPA by water convolvulus were converted to a polymerized and/or tightly bound form in plant residues.

Environmental pollution level of BPA is 20,000 to 40,000 times less than the concentration of BPA in the abovementioned experiments (see above). We next addressed whether curly dock and rice can remove BPA at an environmental pollution level from the culture medium. Curly dock or rice (10 g fresh weight each; BPA to plant mass ratio was 100 μg BPA kg^{-1} fresh plants) were placed in 1-l culture medium containing BPA (1 $\mu g\,l^{-1}$) and incubated in the same way as described above. At 15 DAT, plants were taken out from the culture media, and 100 ml dichloromethane was added to each of the media, and the mixture was shaken vigorously for 5 min. The dichloromethane layer was recovered and evaporated, after which the residues were suspended in 200 μl distilled water and analyzed for BPA by the HPLC (see legend of Figure 2). By this procedure, BPA was concentrated by about 5,000-fold and the recovery rate was estimated to be about 70%. It was found that no BPA was detectable from both culture media of curly dock or rice, indicating that curly dock and rice remove BPA at as low as environmental pollution level from the culture medium.

Our result with rice lines with the previous authors' results (Noureddin et al. 2004a). However, quantitative comparison of data for the capabilities to remove BPA from liquid media is hampered at this time because detailed experimental conditions such as BPA to plant mass ratio are not available. *Rumex crispus*, a closely related species to curly dock, is reported to remove cadmium ions from wastewater (Cha 1992). In conclusion, our present findings show that curly dock, which is one of the major species in the abandoned rice fields and it also inhabitates in riversides in Japan, is a potent phytoremediator (phytoextractor) of BPA in the hydrosphere and pedosphere.

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