Open-closure trap system modeled in tissue-cultured *Dionaea muscipula* controled by certain chemical substances in culture media

Katsuhiko Kondo[1,*], Sayuri Ichiishi[2], Hiroshi Kajita[2], Tsukasa Iwashina[3], Rie Suzuki[1], Yusuke Kondo[2] and Toshiharu Nagamitsu[2]

[1]Laboratory of Plant Chromosome and Gene Stock, Faculty of Science, Hiroshima University, 1-4-3 Kagamiyama, Higashi-Hiroshima City 739-8526, Japan
[2]Plant Bio-technology Section, Sanmei Electric Co., Ltd., 5-7, Sumiyoshi-Cho, Hannda City 475-0862, Japan
[3]Tsukuba Botanical Garden, National Science Museum, 4-1-1, Amakubo, Tsukuba City 305-0005, Japan

Abstract. Correlationships between certain macro-components of 1/2 strength of MS agar medium supplemented with no growth substance and growth, red-color pigment and closure and re-opening in traps were studied in cultured *Dionaea muscipula in vitro* generated from multiple shoots. The plants cultured in 1/2 strength of MS agar medium modified with 10.31mM NH_4NO_3 and 9.40 mM KNO_3 but no other macro-component and supplemented with 0.75 or 0% sucrose continuously proliferated by multiple shoots and generated large, green-colored plants, while those with dilution of those nitrogen components to 1/2 down to 1/8 and increase of sucrose up to 1.5% under continuous light exposure did not much proliferate but formed better carnivorous condition in relatively inverse proportion to depths of red-color anthocyanin pigmentation spread from glands to the upper surface of the trap, to plant sizes and dry weight and moreover trap healthy movement. If one or two plants, with four to five leaves by removal of old leaves, originated from big masses of multiple-shoot plantlets were placed in low population density on the solid medium, they got produce bigger leaves with trap. Deposit of he anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-0-glucoside and cyanidin 3-0-glucoside (chrysanthemin) was an index of trap movement.

Introduction

Dionaea muscipula Ellis, the Droseraceae is the king of carnivorous plants and scientifically and ornamentally very meritorious resource (Kondo and Kondo, 1983). The species is endemic to North and South Carolinas of the U.S.A. (Kondo and Kondo, 1983). The species has commonly dark red, red, yellowish red, yellowish green to green color in plant body in wild, cultivated and even *in vitro* culture conditions. These differences in color pigmentations may be correlated with differences in amounts of nutrient uptake from prey or soil, soil pH, light intensities or other environmental factors in habitats or laboratory and in genetic diversity within the species according to our experiences and may be furthermore correlated with trap movement (Kondo, unpublished).

Plants of *D. muscipula* which have stable red color are horticulturally more meritorious than those which have green color. Some strains of the species artificially

*Dedicated to Prof. Emeritus C. Ritchie Bell, The University of North Carolina, Chapel Hill, and Tokuyoshi (Masahiro) Kondo, KK's late father.

selected contain stabilized deep red pigment in the whole plant body *in vivo* cultivated condition to be the commercialized cultivars of 'Red Dragon', 'Red Giant', 'Red Purple', and 'Royal Red'. However, these cultivars can be artificially removed red pigment and kept green color by adjusting some medium components and light intensity *in vitro* culture (Kondo unpublished).

Dionaea muscipula, which is eutrophic plants containing chlorophyll in their leaves for photosynthesis, commonly occupy sunny, open, relatively closed ecosystem where the soil is poor in nutrient substances, wet and acid and intake and absorb nutrients directly from insect or small animal resources by insect-eating mechanisms differentiated in leaves (Lloyd, 1942). However, under shade conditions or at low CO_2 availability, the resultant negative photosynthetic benefits in those species as well as the other carnivorous plants are counterbalanced by organic carbon uptake from prey (Adamec, 1997). Darwin (1878, 1899) and some other workers (Kellermann and Raumer, 1878; Thum, 1988, 1989; Gibson, 1991) experimented and stated that the plants of some *Drosera* species fed artificially meat, aphids, or fruit flies increased numbers of flowers, total weight of seeds and winter buds, or dry weights of summer and winter plant, more leaves, and a larger trapping area. Thus, animal food supply is an important limiting factor for the *Drosera* species in the field (Thum, 1988, 1989; Gibson, 1991). However, the plants of *Dionaea muscipula* fed and examined mineral nutrients grew poorly, while the non-fertilizing controls grew prominently (Robert and Oosting, 1958; Juniper *et al.*, 1989). Studies on correlationships between nutrients, anthocyanin color pigmentation and moreover trap movement in carnivorous plants are lacking. The culture of another carnivorous *Utricularia praelonga* St. Hil. (Idei and Kondo, 1998) showed different organogeneses, micropropagation, growth form, and so on by adjusting KNO_3 concentrations between 24.73 and 3 mM as well as BAP (N_6-benzylaminopurine) concentrations in B5 (Gamborg *et al.* medium) liquid medium (Gamborg *et al.*, 1968).

Since these differentiations can be correlated with differences in nutrient substances in habitats, *Dionaea muscipula* as well as many other carnivorous plants can be cultured in a closed, well-controlled microenvironment *in vitro* to study the relationships between growth and micropropagation habits, trap closure and re-opening and pigmentation and specific chemical components of the medium. These data would also contribute to satisfactory ecophysiological treatment of the carnivorous plants.

Materials and Methods

Plant Materials - Individual plantlets of *Dionaea muscipula* both erect and rosette forms used as explants *in vitro* were planted on basal, 1/2, 1/4 and 1/8 strengths of MS (MS, 1/2 MS, 1/4 and 1/8; Murashige and Skoog) medium (Murashige and Skoog, 1962) supplemented with 0, 0.75, and 1.5% sucrose on each 80 ml medium supplemented with no growth regulator at pH 5.6 in each culture vial 58X80X129.5 mm in size and were placed at 25℃under 3500 lux continuous illumination. After five months they propagated average six plants per explant by adventitious buds and multiple shoots.

Each plant with 4-5 leaves and traps ca 1 cm long was used for the present experiment.

Effects of the macro-element of 1/2 MS and its diluents and sucrose concentrations on growth, trap closure and re-opening and color pigmentation - Plants of *D. muscipula in vitro* obtained were utilized to study effects of the macro-element at 1, 1/2, 1/4, 1/8 and 1/16 strengths or 0 in 1/2 MS medium and sucrose at concentrations of 0, 0.75, and 1.5% on growth, trap closure and re-opening and color pigmentations.

Effects of five macro-components in 1/2 MS on growth, trap closure and re-opening and color pigmentation - Plants of *D. muscipula in vitro* were also utilized to study effects of the five macro-components in 1/2 MS, such as NH_4NO_3, KNO_3, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4, by remaining or removing, on growth, trap closure and re-opening and color pigmentation.

Visible color identification in plant bodies - Identification of visible coloration on plant bodies caused followed the Japan Color Standard for Horticultural Plants (Japan Color Research Institute).

Detection of anthocyanin - Every 0.2 g fresh weight of plant bodies easpecially leaves per sample was utilized to extract anthocyanin pigments with 1 ml MeOH-HCl mixture (methanol:hydrochloric acid=1000:1) for 3 h to overnight and filtrated by Toyopak ODS M (Tosoh) and Maisyoridisc H-13-5 0.45 μ m (Tosoh) pre-cartridge. Following Iwashina (1996), the composition of plant extracts was determined by HPLC (high performance liquid chromatography; JASCO HPLC System) with the Model 880-51, two-line degasser and Syringe loading sample injector 25 Model 7125 (Rheodyne Inc.). Multi channel UV-visible detector Multi-330 connected with a computer was used to record the chromatogram data and UV-visible spectra. The dimension of the column was 120 X I.D.4 mm (Tosoh TSK-Gel, ODS-80TM). Samples (10 μ l each) were injected and eluted with H_3PO_4-AcOH-CH3CN-H2O (3:8:6:83) at a flow rate of 1.0 ml/min at a pressure of 99-106 Kg/cm^2. Compounds were detected at 360-660 nm for the presence of anthocyanin.

Identification of the anthocyanins contained in the two species was made by HPLC comparisons with authentic samples. For double identification of the anthocyanin bands separated from the crude extracts with BAW (n-BuOH-HOAc-H2O, 4:1:5, upper layer) by mass-PC (paper chromatography) were redissolved in 0.01% MeOH-HCl. After they were concentrated, they were eluted and separated using Sephadex LH-20 column with 70% MeOH and a drop of HCl, and purified individual anthocyanins. UV (ultra violet) spectra were measured by an UV spectrophotometer. Those anthocyanins were directly hydrolyzed in 12% HCl for 3 min on heating, cooled down, added and shaked with isoamyl alcohol to separate anthocyanidin in alcohol layer and glucose in water layer. Those anthocyanidin and sugars were identified by PC comparisons with their authentic samples. PC and UV spectral data of isolated anthocyanins were listed as follows: Pelargonidin 3-0-glucoside. PC: Rf 0.49 (BAW), 0.12 (1% HCl), 0.60 (FAH=formic acid:HCl:H_2O, 5:1:4), 0.36 (AAH=HOAc:HCl:H_2O, 15:3:82); color (visible)-orange. UV: 0.01% MeOH-HCl max 511 nm; +$AlCl_3$ $\triangle\lambda$ 0 nm; E440/Emax=33.6%. Pelargonidin 3-0-galactoside. PC: Rf 0.36 (BAW), 0.12

(1% HCl), 0.62 (FAH), 0.37 (AAH); color (visible)-orange. UV λ max 0.01% MeOH-HCl 5ll nm; +$AlCl_3$ $\triangle\lambda$ 0 nm; E440/Emax=33.6%. Cyanidin 3-0-glucoside. PC: Rf 0.27 (BAW), 0.06(1% HCl), 0.50 (FAH), 0.26 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 529 nm; +$AlCl_3$ $\triangle\lambda$ 44 nm; E440/Emax=23.8%. Cyanidin 3-0-galactoside: PC: Rf 0.24 (BAW), 0.06 (1% HCl), 0.50 (FAH), 0.26 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 530 nm; +$AlCl_3$ $\triangle\lambda$ 43 nm; E440/Emax=24.8%. Cyanidin 3,5-di-O-glucoside. PC:Rf 0.17 (BAW), 0.16 (1% HCl), 0.67 (FAH), 0.42 (AAH); color (visible)-purplish red. UV: λ max0.01% MeOH-HCl 522 nm. Delphinidin 3-0-glucoside. PC: Rf 0.19 (BAW), 0.03 (1% HCl), 0.39 (FAH), 0.17 (AAH); color (visible)-reddish purple. UV: λ max0.01% MeOH-HCl 540 nm; +$AlCl_3$ $\triangle\lambda$ 47 nm; E440/Emax=22.3%

Results and Discussion

Traps got much larger if one or two plants with four to five leaves each were isolated from a mother mass of plantles generated by multiple shoots or precocious branching and were conditioned in very low population density. Additionally, removal of old leaves from plant body *in vitro* could be another factor to make the traps larger.

Modified 1/2 to 1/8 MS media with less or no macro-element and with more sucrose induced red-color pigmentation in the upper surface of trap lobe and furthermore in the whole leaves in *Dionaea muscipula* after four months culture (Ichiishi *et al.*, 1999). They had trap closed if their trigger hair was touched and stimulated. However, they made plant growth slow down. In contrast, 1/2 MS media with more to complete macro-elements in absolute strength promoted deeper green colored in the whole plant bodies and larger growth and more proliferation of plantlets by multiple shoots or precocious branching.

Moreover, the modified 1/2 MS media with no NH_4NO_3 performed a little red colored glands, glandular tissues and trigger hairs on the upper surface of the trap but green colored in the lower surface of the leaves of the species after four months cultivation and the modified 1/2 MS medium with no NH_4NO_3 and no KNO_3 performed red coloration in the upper surface of the trap lobe and relatively red color in the whole leaves and reduction of dry weight. These red-colored traps could be made themselves closed and reopened if their trigger hairs were artificially touched twice or more. Too large traps as well as too small traps in the plant *in vitro* did not show trap movement although the trigger hair was stimulated; traps average 1 cm long were just right size for active trap movement. A natural habitat of *D. muscipula* in North Carolina, U.S.A. had low contents of NH_4^+ (2 mg/Kg dry weight), PO_4 (less than 2 mg/Kg), K (2 mg/Kg) and Mg (1 mg/Kg) and no content of NO_3^-, Ca and Mn (Robert and Oosting, 1958). The plants in the natural habitat of the species showed commonly red color in the upper surface of the trap lobe and yellowish green in the other lower surface. On the other hand, the lack of NH_4NO_3 and $MgSO_4 \cdot 7H_2O$ among the macro-components of the 1/2 MS medium exhibited healthy-looking plant bodies without any dead leaf but no plant

growth perhaps due to balanced combination of N, P, K, and Ca. In contrast, the lack of $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 among the macro-components of the 1/2 MS medium treated the leaf and shoot tips dead perhaps due to unbalanced combination of less Ca against more N and K.

The anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-0-glucoside and cyanidin 3-0-glucoside (chrysanthemin).

Thus, dilution of NH_4NO_3 and KNO_3 down to 1/2 to 1/16 strengths in volume and increase of sucrose up to 1.5% in the 1/2 strength of MS medium and continuous light illumination mainly promoted and could be in relatively inverse proportion to depths of red-color anthocyanin pigmentation spread from glands to entire leaves of the species and best trap closure and re-opening.

Insects contain total nutrients of N (99-121 g/kg dry weight), P (6-14.7 g/kg), K (1.5-31.8 g/kg), Ca (22.5 g/kg) and Mg (0.94 g/kg) (Reichle *et al.*, 1969; Watson *et al.*, 1982; Dixon *et al.*, 1980) that are somewhat smilar to the medium requirements studied here. In nature, preys would be more atractive and captured to red-colored plants than to green-colored ones in the species. Generally speaking, if plants get heavy stress of temperature, physical damage of organ, lack of nitrogen and phosphoric acid, light, or other environmental factors, they often show anthocyanin color pigmentation (Harborne and Grayer, 1988). *Dionaea muscipula* as well as other carnivorous plants could have adaptation strategies to barren, wet and low pH soil condition by interaction between leaf carnivory and low root nutrient especially both NH_4+ and NO_3^- depended greatly on pH (Adamec, 1997). The present examination in certain microenvironments in tissue culture supports that the species would turn red color when they got deficient in nitrogen compounds to make the prey attractive and would catch more preys as leaf carnivory if they had too low root nutrient to survive, grow and propagate. The anthocyanin pigmentation in the two species may make it possible to be biosensor against nitrogen consumption uptake and furthermore degree of active movement of trap.

This methodology in culture medium has been partially patent pending by the patentee Bioparco Sanmei.

References

Adamec, L., 1997. Bot. Review, **63**: 273-299.

Darwin, C., 1878. Journ. Linn. Soc. Bot. (London), **17**: 17-23.

Darwin, C., 1899. "Insectivorous plants", D. Appleton and Co., N. Y.

Dixon, K. W., Pate, J. S., Bailey, W. J., 1980. Austral. Journ. Bot., **28**: 283-297.

Gamborg, O. L., Miller, R. A. and Ojima, K., 1968. Exp. Cell Res., **50**: 151-158.

Gibson, T. C., 1991. Am. Nat., **138**: 785-789.

Harborne, J. B. and Grayer, R. J., 1988. In "The Flavonoids: Advances in Research since 1980" (ed. by Harborne, J. B.), p 1-20, Chapman and Hall, London, UK.

Idei, S. and Kondo, K., 1998. Plant Cell Rep., **17**: 451-456.

Ichiishi, S., Nagamitsu, T., Kondo, Y., Iwashina, T., Kondo, K. and Tagashira, N.,

1999. Plant Biotech., **16**: 235-238.
Iwashina, T., 1996. Ann. Tsukuba Bot. Gard. **15**: 1-18.
Juniper, B. E., Robins, R. J. and Joel, D. M., 1989. "The Carnivorous Plants", Academic Press, London.
Kellermann, C. and Raumer, E., 1878. Bot. Zeitung, **36**: 209-218, 225-229
Kondo, K. and Kondo, T., 1983. "Carnivorous Plants of the World", Ienohikari Association, Tokyo.
Lloyd, F. E., 1942. "The Carnivorous Plants", The Ronald Press Co., N. Y.
Murashige, T. and Skoog, F., 1962. Physiol. Plant., **15**: 473-497.
Reichle, D. E., Shanks, M. H. and Crossley, D. A., 1969. Ann. Entomol. Soc. Amer., **62**: 57-62.
Robert, P. R. and Oosting , H. J., 1958. Ecol. Mongraphs, **28**:193-218.
Thum, M., 1988. Oecologia, **75**: 472-480.
Thum, M., 1989. Oecologia, **81**: 401-411.
Watson, A. P., Matthiessen, J. N. and Springett, B. P., 1982. Austral. Journ. Ecol., **7**: 13-22.