Short Communication

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Effects of Macro-components and Sucrose in the Medium on *in vitro* Red-color Pigmentation in *Dionaea muscipula* Ellis and *Drosera spathulata* Labill.

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Abstract

Effects of the five macro-components and sucrose in half strength MS (1/2 MS) agar medium on red-color pigmentation were studied in the plant bodies of *Dionaea muscipula* and *Drosera spathulata* generated from multiple shoots *in vitro*. In 1/2 MS agar medium modified with 10.31 mM NH_4NO_3 and 9.40 mM KNO_3 and supplemented with 0.75 or 0% sucrose the subcultured plants continuously proliferated by multiple shoots and generated large, green-colored plants, while with dilution of those nitrogen components and increase of sucrose to 1.5% the red-color anthocyanin pigmentation spread from the glands or glandular hairs to the entire leaves and the plant sizes and dry weight decreased in inverse proportion to the depth of red color. The anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-*O*-glucoside which was new to the species and cyanidin 3-*O*-glucoside (chrysanthemin), and those of *Drosera spathulata*, studied here for the first time, consisted of cyanidin 3,5-di-*O*-glucoside (cyanin), cyanidin 3-*O*-galactoside (idaein), cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-galactoside, and pelargonidin 3-*O*-glucoside (callistephin).

Dionaea muscipula Ellis and *Drosera spathulata* Labill. in the Droseraceae are ornamental, carnivorous plants (Kondo and Kondo, 1983). The plant bodies of the two species vary in coloration from green to red. However, plants with stable red color are horticulturally more desirable than green ones. The commercialized cultivars of 'Red Dragon', 'Red Giant', 'Red Purple', and 'Royal Red' of *Dionaea muscipula* are artificially selected to bring out a stable, deep red-color pigment in whole plant body when cultivated *in vivo*.

Although anthocyanin pigmentation in leaves of a few species of *Dionaea* and *Drosera* is a common phenomenon, it has been poorly characterized with limited references; cyanidin-3-glucoside in *Dionaea muscipula* (Di Gregorio and Di Palma, 1961; Jay and Lebreton, 1972), cyanidin-glycoside, malvidin-glycosides, pelargonidin-glycoside, quercetin-3-galactoside, and qercetin-3-digalactoside, in a few species of *Drosera* other than *D. spathulata* (Gascoigne *et al.*, 1948; Paris and Denis, 1957; Paris and Delaveau, 1959; Bienenfeld and Katzmeister, 1966; Bendz and Lindberg, 1968; Bendz and Lindberg, 1970; Ayuga *et al.*, 1985).

Dionaea muscipula and *Drosera spathulata*, which are autotrophic plants, commonly occupy relatively closed ecosystems where the soil is poor in nutrient substances, wet and acid. They take in and absorb nutrients directly from small animal resources by way of carnivorous leaves (Lloyd, 1942). Darwin (1875) and some other workers (Kellermann and Raumer, 1878; Thum, 1988, 1989; Gibson, 1991) stated that the plants of some *Drosera* species fed small animals artificially through their leaves increased the number of flowers, total weight of seeds and vegetative organs. In contrast, the plants of *Dionaea muscipula* and some *Drosera* species fed mineral nutrients through their underground roots *in vivo* grew poorly (Robert and Oosting, 1958; Juniper *et al.*, 1989). A tissue culture

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of another carnivorous plant, *Utricularia praelonga* St. Hil., (Idei and Kondo, 1998) showed different organogenesis, micropropagation, growth forms, and so on by adjusting KNO_3 concentrations between 24.73 and 3 mM as well as BAP (N6-benzylaminopurine) concentrations in B5 (Gamborg *et al.*, 1968) liquid medium. However, studies on the correlation between nutrients and anthocyanin pigmentation in *Dionaea miscipula* and *Drosera spathulata* as well as other carnivorous plants are very much lacking.

Seeds of *Dionaea muscipula* and *Drosera spathulata* Kanto type collected in cultivation were surface-sterilized with 0.1% (w/v) benzalkonium chloride solution for 5 min, 1% (w/v) sodium hypochlorite solution for 5 min, 70% (v/v) ethanol for 30 s and were rinsed three times with sterile, distilled water before they were sown on half strength of MS (1/2 MS; Murashige and Skoog, 1962) medium supplemented with 0.8% sucrose. They germinated 30 to 60 days after they were sown.

Individual leaves of *Dionaea muscipula* used as explants were planted on 1/2 MS supplemented with 1.5% sucrose. After 5 months they propagated an average of 6 plants per explant by adventitious buds and multiple shoots. Each plant averaged 2 cm in diameter, 0.12 ± 0.04 g fresh weight, and 0.02 ± 0.00 g dry weight was used for the present experiment.

Individual plants of *Drosera spathulata* averaging 2.5 cm in diameter each were used as explants. They were planted and subcultured at intervals of 20 days on 1/2 MS supplemented with 1.5% sucrose. After 5 months they propagated numerous plants per explant by multiple shoots. Plants averaging 2.5 cm diameter, 0.14 ± 0.03 g fresh weight, and 0.02 ± 0.00 g dry weight were used for the present experiment.

All cultures were planted on 50 m*l* medium supplemented with no growth regulator at pH 5.5 in cylindrical-shaped, culture vials 80 mm diameter X 129.5 mm high, 450 m*l* capacity, air-tight with a transparent, clear lid at 25 ℃ under 3500 lux continuous illumination.

0.2 g fresh weight of plant bodies, especially leaves, per sample was utilized to extract anthocyanin pigments with 1 m*l* 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 μm (Tosoh) pre-cartridge. The composition of plant extracts was determined by the methods of Iwashina (1996).

Modified 1/2 MS media with less or no macro-components and with more sucrose induced red-color pigmentation in the inner surface of the trap lobe in *Dionaea muscipula* and in the glandular hair in *Drosera spathulata* and in the whole leaves of the both species after four months culture. However, they made plant growth worse. In contrast, 1/2 MS media with more to complete macro-components promoted deeper green color in the whole plant bodies and larger growth and more proliferation in the both species.

Moreover, the modified 1/2 MS media with no NH_4NO_3 resulted in some red colored glands, glandular tissues and sensitive hairs but green color in the other parts of the leaves in *Dionaea muscipula* after four months culture and the modified 1/2 MS medium with no NH_4NO_3 and no KNO_3 resulted red coloration in the inner surface of the trap lobe and relatively red color in the whole leaves and reduction of dry weight (**Fig. 1**). Thus, NH_4NO_3 among the macro-components of the 1/2 MS media could be the major nitrogen source for *Dionaea muscipula* to giving thin red-color pigmentation and more green color and to increasing plant growth. The natural habitat of *Dionaea muscipula* in North Carolina, U.S.A. has 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 NO_3^-, Ca and Mn (Robert and Oosting, 1958).

Similarly, the lack of NH_4NO_3 and KNO_3 among the macro-components of the 1/2 MS medium deepened red-color pigmentation in glandular hairs and the other parts of the leaves and reduced plant growth and dry weight in *Drosera spathulata*. 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 the balanced combination of N, P, K, and Ca. In contrast, when the 1/2 MS medium lacked $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 the leaf and shoot tips died perhaps due to an unbalanced combination of less Ca against more N and K. Thus, NH_4NO_3 and KNO_3 among the macro-components of the 1/2 MS media could be the major nitrogen sources for *Drosera spathulata* creating thin red-color pigmentation and more green color and increasing plant growth and even propagation.

The anthocyanin pigments of *Dionaea muscipula* consisted of delphinidin 3-0-glucoside and cyanidin 3-0-glucoside (chrysanthemin), and those of *Drosera spathulata* consisted of cyanidin 3,5-di-*0*-glucoside (cyanin), cyanidin 3-*0*-glucoside, pelargonidin 3-*0*-glucoside (callistephin). Delphinidin 3-0-glucoside was reported here in *Dionaea muscipula* for the first time, while the other one was already known in the species (Di Gregorio and Di

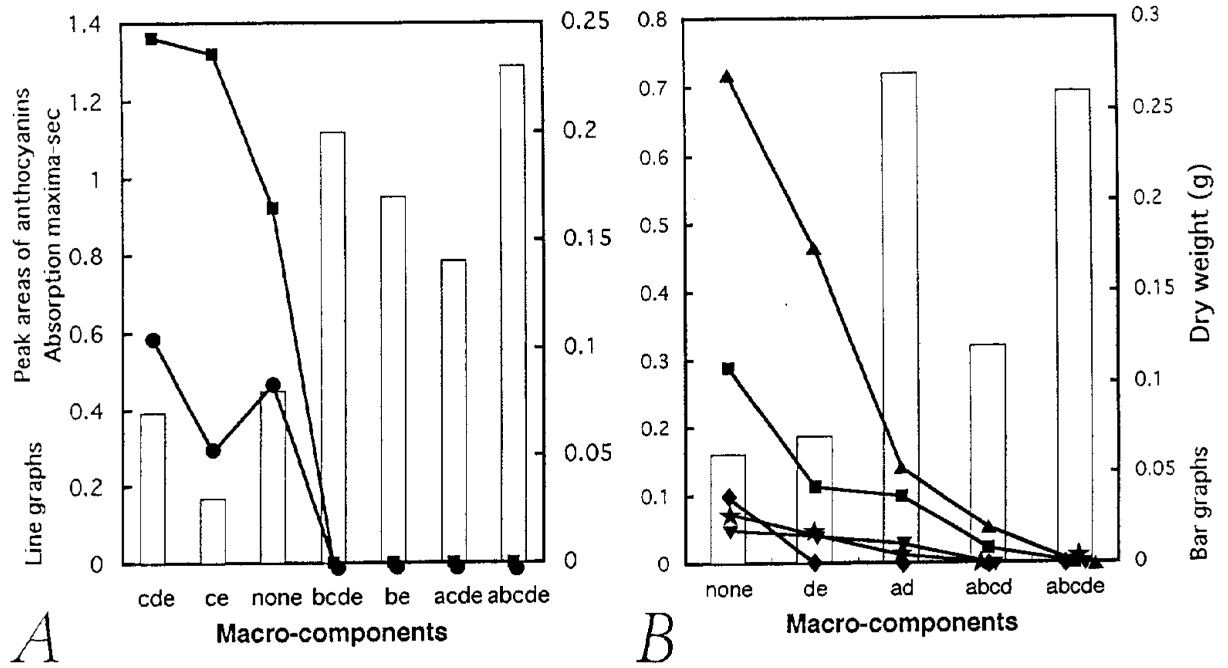

Fig. 2 Effects of macro-components of 1/2 MS medium in combination on red color pigmentations (line graphs) and growth in dry weight (bar graphs) in *Dionaea muscipula* (A) and *Drosera spathulata* (B). ●-● =delphinidin 3-*O*-glucoside. ■-■ =cyanidin 3-*O*-galactoside. ▼-▼ =cyanidin 3,5-di-*O*-glucoside. ★-★ =cyanidin 3-*O*-glucoside. ▲-▲ =pelargonidin 3-*O*-galactoside. ◆-◆ =pelargonidin 3-*O*-glucoside.
Relative peak areas of the anthocyanins (absorption maxima · sec) were measured at the wavelength of 510 nm by HPLC. Macro-component a: 10.31 mM NH_4NO_3; b: 9.40 mM KNO_3; c: 1.50 mM $CaCl_2$; d: 0.75 mM $MgSO_4 \cdot 7H_2O$; and e: 0.62 mM KH_2PO_4.

Palma, 1961; Jay and Lebreton, 1972). All of the anthocyanins found here in *Drosera spathulata* have been reported in other species of *Drosera* (Gascoigne *et al.*, 1948; Paris and Denis, 1957; Paris and Delaveau, 1959; Bienenfeld and Katzmeister, 1966; Bendz and Lindberg, 1968, 1970; Ayuga *et al.*, 1985).

Thus, dilution of NH_4NO_3 and KNO_3 and increase of sucrose up to 1.5% mainly promoted and could be in relatively inverse proportion to depths of red-color anthocyanin pigmentation spread from glands or glandular hairs to entire leaves in the both species (**Fig. 2-A, B**). 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; Dixon *et al.*, 1980; Watson *et al.*, 1982) that are somewhat smilar to the medium requirements studied here. Prey would be more attracted to and captured by red-colored plants of *Drosera* species than by green-colored ones. Generally, carnivorous plants might have adaptation strategies to barren, wet and low pH soil conditions by interaction between leaf carnivory and low root consumption of nutrients (Adamec, 1997). The present study suggests that the two species would turn red color when they became deficient in nitrogen compounds to make themselves attractive to prey and would catch more prey if they had too low a root consumption of nutrients to survive, grow and propagate. The anthocyanin pigmentation in the two species may make it possible to be biosensitive to nitrogen consumption uptake.

Since this phenomenon is observed throughout our tissue culture experience in *Drosera adelae* F. Muell., *D. anglica* Huds., *D. binata* Labill., *D. burkeana* Planch., *D. capensis* L., *D. peltata* Sm. *ex* Willd., *D. petiolaris* R. Br. *ex* DC., and *D. rotundifolia* L. in the Droseraceae (whole plant bodies), *Nepenthes mirabilis* Druce in the Nepenthaceae (pitchers especially peristomes), *Cephalotus follicularis* Labill. in the Cephalotaceae (whole pitchers), and *Sarracenia flava* L. and *S. purpurea* L. in the Sarraceaniaceae, this methodology may be generalized to the majority of the red-color pigmented carnivorous plants.

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