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Reproduction of *Sedum drymarioides*, an Endangered Rare Species, by Micropropagation

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Abstract

Sedum drymarioides, an endangered plant, was micro-propagated, and the regenerated plants showed the CO_2 exchange pattern of a C_3-plant. Stem and leaf explants were cultured on solid MS medium containing various concentrations of 1-naphtaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), resulting in callus formation at 0.1-1 mg l^{-1} NAA. From the stem callus, the best differentiation of root and bud was observed at 1 mg l^{-1} of NAA plus 0.1 mg l^{-1} of BAP and 0.1 mg l^{-1} of NAA plus 1 mg l^{-1} of BAP, respectively. From the leaf callus, the differentiation was most efficient with the combination of 1 mg l^{-1} of NAA plus 1 mg l^{-1} of BAP and 1 mg l^{-1} of NAA plus 10 mg l^{-1} of BAP, respectively. The buds further multiplied on medium containing 0.1 mg l^{-1} of NAA and 10 mg l^{-1} of BAP. They developed into rosette plantlets and grew to normal size on phytohormone-free MS medium in glass bottles, where they bolted and flowered. The regenerated plantlets were successfully transferred to pots where fertile seeds were obtained.

Key words: *Sedum drymarioides*, Crassulaceae, distinct-fear species, micropropagation, C_3-photosynthesis.

Introduction

The genus *Sedum* (Crassulaceae) comprises ca. 400 species, distributes all over the world except Australia and the Pacific islands, and lives usually on rocks where other plants can not survive. Many species are now facing extinction because of drastic changes in environmental conditions in the past 50 years.

S. drymarioides var. *Toyamae* Hara (Japanese name, Nanatugama-mannengusa) found in 1960 (Hara) is one of the most endangered species, having survived only the restricted spot on the wall of lime rocks in the stalactite grotto in Nagasaki prefecture, Japan. They grow in the shade and usually very wet conditions. *S. drymarioides* Hance (Japanese name, Hakobe-mannengusa) has been found in Taiwan and China (Chinese Sciences Plant Institution, 1972) on limestone as well. Ooba (1981) reported that no morphological difference was found between *S. drymarioides* var. *Toyamae* Hara in Japan and *S. drymarioides* Hance in China.

Some species of *Sedum* are used as folk medicine in Maderia and Porto Santo Islands (Rivera and Obon, 1995). Anti-inflammatory and immunologically active polysaccharides were found in *S. telephium* plants (Sendl *et al.*, 1993). Piperidine alkaloids together with nicotine were isolated from *S. acre* (Niemann *et al.*, 1976; Francis and Francis, 1977), and various flavonoids were obtained from 7 species of *Sedum* (Kim *et al.*, 1996). However, chemical and bioactive substances have not been examined from the very rare species *S. drymarioides* due to material restrictions. Many species of the genus *Sedum* are known as CAM (Crassulacean Acid Metabolism) plants and some acquire a C_3-CAM transposable mechanism (Osmond and Ziegler, 1975; Kluge, 1977; Kluge and Ting, 1978). With respect to *S. drymarioides*, no information on the type of photosynthesis has been available.

Biologically and pharmaceutically interesting species are likely to disappear without trace in the near future. The establishment of micropropagation methods is one of the most effective ways to overcome this crisis; it makes it possible to conserve the species and examine its chemical and biological features. The cultivation and conservation of endangered species using seeds and/or cuttings are alternative methods, but in the case of *S. dryma-*

rioides, the lack of materials prevents their use. The germination of *S. drymarioides* seeds has never been examined to the best of our knowledge. In contrast, *in vitro* propagation makes it possible to provide as many plants as required all the time from very small explants and to conserve wild species *in vitro* without worrying about disease and harmful insects.

Here we report on the clonal propagation of the endangered species *S. drymarioides* and an analysis of its photosynthetic type using the regenerated plants. In the Crassulaceae family, the genus *Kalanchoe* has been most actively studied in terms of regeneration *in vitro* as ornamental plants (Frello *et al.*, 2002). To date, the regeneration of *Sedum roseum* (synonym: *Rhodiola rosea*) (Furmanowa *et al.*, 1995), *S. dasyphyllum*, and *S. rubrotinctum* (Frello *et al.*, 2002) has been achieved in the *Sedum genus.*

Materials and Methods

Plant materials

A specimen of *S. drymarioides* var. *Toyamae* was collected from its natural habitat with permission from Mr. K. Sasamoto, the manager of Nanatugama, on July 27, 1997. It was transferred into a pot containing peat moss and soil (1:4), and cultured under fluorescent light (ca.20 μmol m^{-2} s^{-1}) at 25 ℃ according to Yamada (1984). When it grew to 12 cm in height after 5 months of cultivation, the shoot was cut and then separated into stem and leaves. The material was surface-sterilized with 70% ethanol for 5 s and then 0.5% sodium hypochlorite for 5 min followed by washing three times with sterile water.

Culture methods

The stem was cut into small pieces about 1-2 mm in length and put on a solid (0.2% gellan gum) basal Murashige-Skoog (1962) (MS) medium containing various combinations of 1-naphtaleneacetic acid (NAA) (0, 0.1, 1, and 10 mg l^{-1}) and 6-benzylaminopurine (BAP) (0, 0.1, 1, and 10 mg l^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving at 121 ℃ for 15 min. The explants (5 explants / treatment) were cultured in glass tubes containing a nutrient medium at 25 ℃ in the dark for 6 months without medium exchange except bud producing cultures. The cultures with buds were transferred under continuous fluorescent light (15-20 μmol m^{-2} s^{-1}, hereafter the same lighting conditions were employed unless mentioned otherwise) after 4 months and then continuously cultured for an additional two months. Then callus formation and organ differentiation were determined. In some cases, the number of explants decreased from 5 to 4 or 3 due to contamination during culture. The number of explants showing a response / number of explants treated was determined. In addition, callus growth and the number of differentiated organs (buds and roots) were checked by eye and expressed as intensity (**Table 1** and **2**).

The buds formed above were transferred to fresh medium, and maintained under continuous fluorescent light for 6 months without medium change. Multiple buds were obtained from explants cultured with 0.1 mg l^{-1} of NAA and 10 mg l^{-1} of BAP, which were divided and transferred to the fresh medium with the same hormone combination for further propagation. Single buds were developed with 0.1 mg l^{-1} of NAA and 1 mg l^{-1} of BAP. Rooted plantlets were obtained when buds (> 5 mm in diameter) were transferred to a phytohormone-free MS medium and cultured under the same lighting conditions for more than one month.

Leaf segments from plantlets regenerated *in vitro* were also provided for micropropagation experiments, where they were cultured with NAA (0, 0.1, and 1 mg l^{-1}) and BAP (0.1, 1, and 10 mg l^{-1}) (5 explants / treatment) under continuous light.

Gas exchange measurements

Regenerated rosette plants transferred to pots were acclimatized in rooms at 25 ℃ for 2-3 months under light. Then they were cultured under different light-dark cycles to determine their type of photosynthesis: 1) continuous fluorescent light (25 ℃, 15-20 μmol m^{-2} s^{-1}); 2) 16 h light (25 ℃) / 8 h dark (25 ℃); 3) 12 h light (25 ℃) / 12 h dark (5 ℃); 8 h light (25 ℃) / 16 h dark (5 ℃). The plants were also cultivated under waterless conditions: no water or watered with 0.3 M NaCl for 2 weeks. For gas exchange measurements, Ciras 1 (Koito Co.) was employed with a photosynthetic photon flux density (400-700 nm) of about 80 μmol m^{-2} s^{-1} during light periods.

Alkaloid analysis

Alkaloid extraction and TLC analysis were performed by the method of Kitamura *et al.* (1985).

Results and Discussion

Unlike other species belonging to Crassulaceae, *S. drymarioides* has very soft stems and leaves. Since the sterilization of leaf explants was not successful, stem explants were used for the first experiments and then the leaf explants of plants established from stem segments *in vitro* were provided as materials

for subsequent experiments.

Regeneration from stem segments of in vivo plants

Stem segments (ca. 5-9 mg) were treated with various combinations of NAA (0-10 mg l^{-1}) and BAP (0-10 mg l^{-1}) under dark conditions. Callus formation started two months later and after 3- to 4-months root and bud differentiation from the calli was found. The frequency and intensity of callus, root and bud formations were determined after 6 months of culture (**Table 1**).

The formation of callus mainly depended on the concentration of NAA(0.1-1 mg l^{-1}), although stem segments died on the media containing 10 mg l^{-1} NAA and remained unchanged on the media without NAA. The BAP concentration also affected the formation of callus. Organs only differentiated from explants showing calli. The differentiation of roots from calli was promoted at 0.1-1 mg l^{-1} of NAA together with 0.01-0.1 mg l^{-1} of BAP and at 1 mg

Table 1. Effect of NAA and BAP on callus formation and organ differentiation from stem segments of *S. drymarioides*

Plant hormone			Response					
			callus formation		root formation		bud formation	
NAA (mg l^{-1})	BAP (mg l^{-1})	No. of explants treated	No. of explants that responded	Intensity	No. of explants that responded	Intensity	No. of explants that responded	Intensity
0	0	5	0	-	0	-	0	-
	0.01	4	0	-	0	-	0	-
	0.1	5	0	-	0	-	0	-
	1	5	0	-	0	-	0	-
	10	5	0	-	0	-	0	-
0.01	0	5	0	-	0	-	0	-
	0.01	5	0	-	0	-	0	-
	0.1	5	0	-	0	-	0	-
	1	5	3	+	0	-	0	-
	10	5	0	-	0	-	0	-
0.1	0	5	3	+	0	-	0	-
	0.01	5	2	++	1	+	0	-
	0.1	5	5	+++	3	+	0	-
	1	5	4	+++	0	-	2	++
	10	3	1	+	0	-	1	+
1	0	5	2	+	1	+	0	-
	0.01	3	1	++	1	+	0	-
	0.1	5	3	+++	3	++	0	-
	1	5	1	++	0	-	0	-
	10	5	0	-	0	-	0	-
10	0	5	0	-	0	-	0	-
	0.01	5	0	-	0	-	0	-
	0.1	5	0	-	0	-	0	-
	1	5	0	-	0	-	0	-
	10	5	0	-	0	-	0	-

Most explants were cultured on solid MS medium for 6 months under darkness except bud forming cultures. After 4 months, explants producing buds were transfered to light and cultured an additional two months. The intensity of callus formation was expressed as callus growth. -, no growth; +, slight growth, callus volume was < 5 times the volume of initial explants ; ++, good growih, callus volume was 5 - 10 times that of initial explants; +++, vigorous growth, callus volume was > 10 times that of initial explants. The intensity of bud and root formation was expressed as the number of differentiated roots and buds. -, no differentiation; +, a few, organ number/explant was < 5 ; ++, many, organ number/explant was 5 - I O ; +++, a lot, organ number/explant was > 10 .

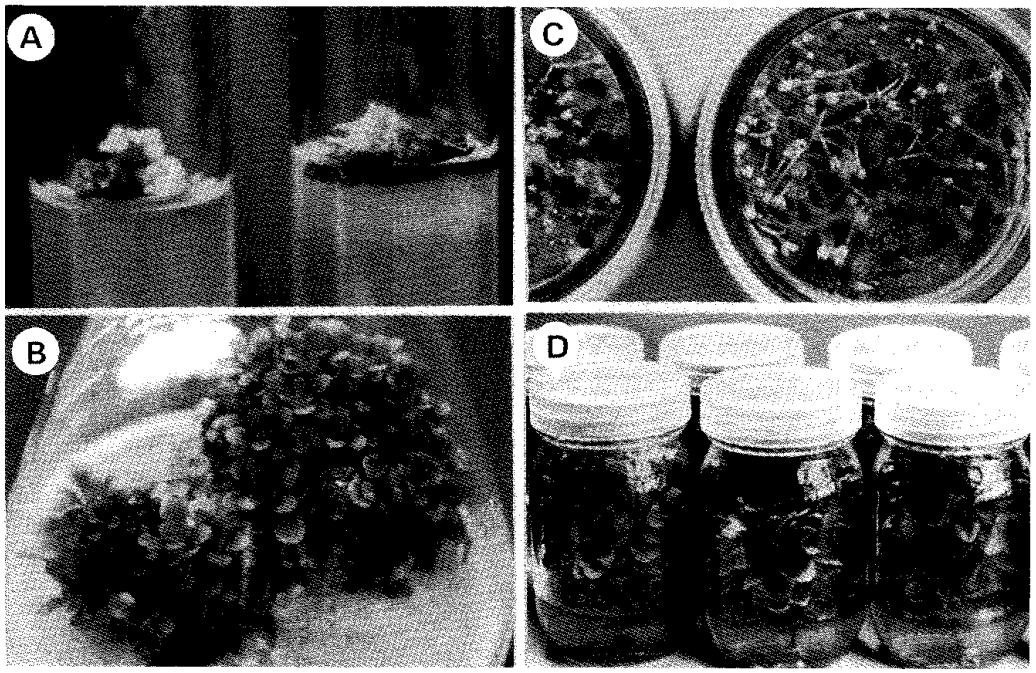

Fig. 1 *In vitro* cultures of *S. drymarioides*. **A** Differentiated buds (left) and roots (right) from stem callus cultures. **B** Multiple buds on medium containing NAA (0.1 mg l^{-1}) and BAP (10 mg l^{-1}). **C** Regenerated flowering plants in glass bottles (seen from the top). **D** Regenerated flowering plants in glass bottles (seen from the side).

l^{-1} of NAA alone, whereas buds formed at 1-10 mg l^{-1} of BAP combined with 0.1 mg l^{-1} of NAA (**Fig. 1A**). For root induction, a combination of 1 mg l^{-1} of NAA and 0.1 mg l^{-1} of BAP was best, whereas for bud induction, 0.1 mg l^{-1} of NAA and 1-10 mg l^{-1} of BAP was suitable.

Buds that formed at 0.1 mg l^{-1} of NAA together with 1 and 10 mg l^{-1} of BAP were subcultured in fresh medium, and maintained continuously under light. After 6 months, multiple buds were formed when cultured with 0.1 mg l^{-1} of NAA and 10 mg l^{-1} of BAP (**Fig. 1B**). With 0.1 mg l^{-1} of NAA and 1 mg l^{-1} of BAP, single buds developed. Buds developing leaves > 5 mm in diameter sometimes produced roots from the base.

Buds (> 5 mm in diameter, leaf number: 6-12) with or without roots (20 buds each) were transferred to phytohormone-free MS medium, where all of them developed into rosette plants during 3 months of culture. Leaf numbers increased to 12-18 and the rosette sizes to 15-30 mm in diameter. Further cultivation in glass bottles resulted in the development of rosette plants, bolting, and then flowering within 6 months (**Fig. 1C** and **D**).

Regeneration from leaf segments of in vitro plants

Leaf segments from plantlets obtained from stem callus *in vitro* were supplied for regeneration experiment. Based on the results obtained with stems mentioned above, phytohormone combined with NAA (0, 0.1, and 1 mg l^{-1}) and BAP (0.1, 1, and 10 mg l^{-1}) was supplied and cultured under continuous light (**Table 2**). Leaf explants cultured under light responded to the phytohormone and produced callus and organs similar to stem segments cultured in the dark, though they seemed to require higher concentrations of NAA and BAP than stem explants. The regeneration of buds was found at 0.1-1 mg l^{-1} of NAA together with 1-10 mg l^{-1} of BAP, and the best combination was 1 mg l^{-1} of NAA and 10 mg l^{-1} of BAP. Root formation occurred 0.1-1 mg l^{-1} of BAP with or without NAA. Interestingly, roots formed directly from leaf segments supplied with just BAP (0.1 and 1 mg l^{-1}), which has never been found in stem explants cultured in darkness. Usually the roots that formed from calli supplied with NAA were hairy, but those directly formed from leaf explants without NAA were thick. Buds formed at 0.1-1 mg l^{-1} of NAA and 10 mg l^{-1} of BAP were able to propagate on the same fresh medium repeatedly every 6 months. When single buds (> 5 mm in diameter) were transferred to hormone-free MS medium, they produced roots and plantlets developed into rosette plants during 3 months of culture. They also bolted and flowered in glass bottles under further continuous culture within 6 months.

It has been reported that regenerated plants can be established from shoot tips but rarely from leaf segments of *Sedum rosea* (Furmanowa *et al.*, 1995).

Table 2. Effect of NAA and BAP on callus formation and organ differentiation from leaf segments of *S. drymarioides*

Plant hormone		No. of explants treated	Response					
			callus formation		root formation		bud formation	
NAA (mgl^{-1})	BAP (mgl^{-1})		No. of explants that responded	Intensity	No. of explants that responded	Intensity	No. of explants that responded	Intensity
0	0.1	5	0	–	2[1)]	+	0	–
	1	5	0	–	2[1)]	+	0	–
	10	5	0	–	0	–	0	–
0.1	0.1	5	4	+	5[2)]	+	0	–
	1	5	5	++	4[2)]	+	4	+
	10	5	5	++	0	–	5	++
1	0.1	5	5	+	5[2)]	++	0	–
	1	5	5	++	5[2)]	+++	1	+
	10	5	5	+++	0	–	5	+++

Cultured on solid MS medium for 3 months under light.
The intensity of callus formation was expressed as callus growth of responding explants. –, no growth; +, slight growth, callus volume was < 5 times that of initial explants ; ++, good growth, callus volume was 5 – 10 times that of initial explants; +++, vigorous growth, callus volume was > 10 times that of initial explants. Intensity of bud and root formation was expressed as the number of differentiated roots and buds. –, no differentiation; +, a few, organ number/explant was < 5 ; ++, many, organ number/explant was 5 – 10 ; +++, a lot, organ number/explant was > 10 . [1)]thick roots. [2)]hairy roots.

From the leaf segments of *S. dasyphyllum* and *S. rubrotinctum*, shoots were produced on medium K22 which constitutes a basal MS medium containing 0.4 $mg\,l^{-1}$ of IAA and 0.5 $mg\,l^{-1}$ of TDZ (Frello *et al.*, 2002). Callus formation of *S. dasyphyllum* and *S. rubrotinctum* was not mentioned, but in *S. rosea* roots, differentiation and plant formation from callus were observed with NAA and BAP and with NAA and IBA, respectively. In the case of *S. drymarioides*, shoots, roots and calli formed from both stem and leaf segments with NAA and BAP; these responses depended on the balance between NAA and BAP concentrations. Higher concentrations of BAP and lower concentrations of NAA produced shoots, whereas the opposite produced roots. The shoots of *S. drymarioides* regenerated roots easily, but only a few shoots rooted in *S. dasyphyllum* and *S. rubrotinctum*. The phytohormone used for shoot formation might affect later responses of rooting.

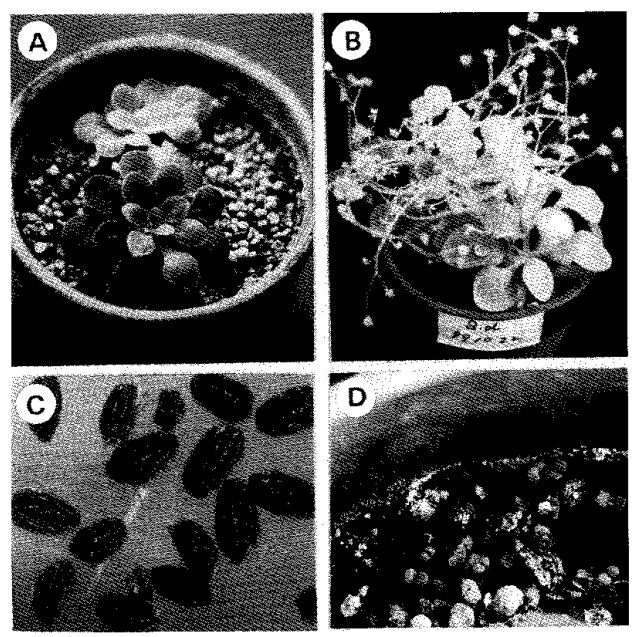

Fig. 2 *S. drymarioides* established under non-sterile conditions. **A** Rosette plants in a pot. **B** Regenerated plants with flowers in a pot. **C** Seeds from regenerated plants. **D** Germinated seedlings.

Cultivation under non-sterile conditions

Regenerated plantlets were successfully transferred to pots containing sand and soil (1:4) covered with glass beakers for an initial 7–12 days. Twelve plants *in vitro* were transferred to pots, all of which survived in the shade. They grew well to be 20–26 in leaf number and 30–60 mm in diameter (**Fig. 2A**) in the shade/under dim light, but not in direct sunshine. Within 4 months 11 of 12 plants started bolting, and then flowered to show fructification (**Fig. 2B**). The rest flowered after one year. One fruit contained 10 immature seeds, but the produc-

tion of matured seeds was restricted to ~7 and often 5 per fruit.

The seeds are an extremely small torpedo-shape (0.27-0.41 x 0.15-0.19 mm), are brownish black in color, and have cavernous vertical lines on their surface (**Fig. 2C**). The seeds were sown in soil under two different conditions: 1) in a pot in a green house with shade and 2) in a natural stand area. In both, seeds germinated. The seeds in pots germinated after 1-2 months of sowing (**Fig. 2D**) and the germination rate was 14%, though we have not determined the rate in the natural habitat. The seedlings developed into normal plants under cultivated conditions as well as in the natural habit.

Characterization

Using acclimatized regenerated plants, we determined whether their photosynthetic type was C_3, CAM, or C_3-CAM transposable. Plants in pots were cultured under long-day (24 h light, 25 ℃)/ (16 h light, 25 ℃-8 h dark, 25 ℃), short-day (8 h light, 25 ℃-16 h dark, 5 ℃), and water stress (no water or watered with 0.3 M NaCl for 2 weeks, 12 h light, 25 ℃-12 h dark, 5 ℃) conditions. Typical C_3 photosynthesis was observed in the leaves of plants under long-day treatment (**Fig. 3**). Even under water stress conditions as well as short-day conditions, the CO_2 exchange pattern of *S. drymarioides* did not change, though Jco_2 was below 0 throughout the dark-light cycle. They did not show any CO_2 exchange under very serious water-deficient conditions (data not shown). In the case of *S. acre*, C_3 photosynthesis was observed under normal conditions, but under water-deficient conditions, they behaved as CAM plants (Kluge, 1977). Since both *S. acre* and *S. drymarioides* grow in the Temperate Zone of the Northern Hemisphere, we expected that *S. drymarioides* might change from the C_3 to CAM mode under serious water-deficient conditions. However, we could not detect the CAM type of photosynthesis under the conditions employed. Instead, very weak C_3-photosynthesis was observed under water-deficient condition (**Fig. 3**).

It is known that the stoma density in leaves of CAM plants is much lower than that of C_3 plants and the average numbers of stoma in CAM plants and C_3 plants are 2,680 cm^{-2} and 10,000 cm^{-2}, respectively (Kluge and Ting, 1978). We pealed the epidermis of a *S. drymarioides* leaf, took photographs under a microscope, and measured stoma number and size. The stoma of *S. drymarioides* averaged 8,200 cm^{-2} in number and 18 × 27 μm in length. The number was far from that known in CAM plants (1,000-6,500 cm^{-2}) (Kluge and Ting, 1978) and close to values for herbaceous plants of shady habitats (5,000-10,000 cm^{-2}) (Larcher, 1995).

S. acre is a perennial herb, but *S. drymarioides* is an annual/ biennial herb. In the case of *S. drymarioides*, fertile seeds are easily produced so that the plants can survive under waterless conditions as seeds. They probably need not develop a C_3-CAM transposable system to survive.

Since piperidine alkaloids were detected in many other species of *Sedum*, we analyzed alkaloids in *S. drymarioides*. According to a previous method (Kitamura *et al.*, 1985), alkaloids were extracted and detected by TLC, resulting in a few Dragendorff's reagent-positive spots in the extract from the regenerated plantlets/plants at the initial stage. However, the positive spots disappeared in the extract from the regenerated plants repeatedly established for further analysis. In the case of *S. morrisonensis*, some plants accumulated piperidine alkaloids, but others did not (Kim *et al.*, 1996). As for these differences, stressful environmental factors such as viral infection and heavy metals may contribute to the induction of alkaloid biosynthesis in *Sedum* spp. A mutation in alkaloid production during regeneration might be possible in *S. drymarioides*.

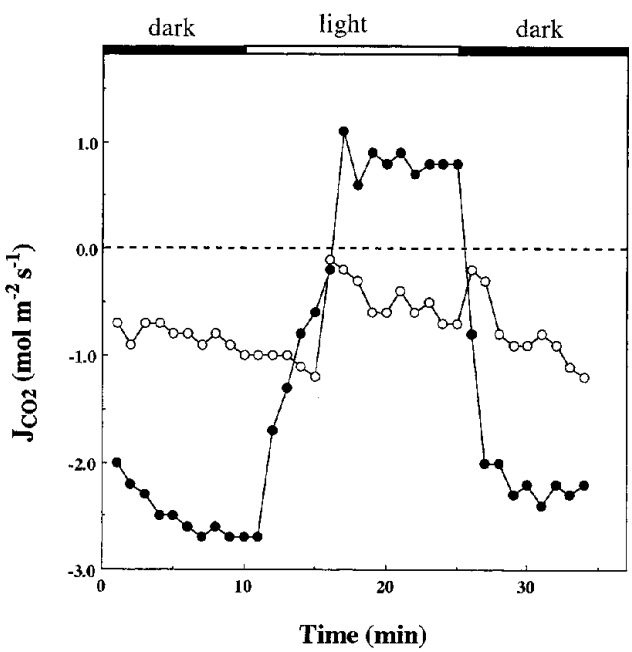

Fig. 3 Net CO_2 exchange (Jco_2) during dark/ light cycles in leaves of *S. drymarioides* measured under the following conditions: leaf area 2.5 cm^2; photosynthetic photon flux density 80 μmol m^{-2} s^{-1}. Before measuring, plants were cultivated under different diurnal cycles: ● light (25 ℃, 16 h) / dark (25 ℃, 8 h); ○ light (25 ℃, 12 h) / dark (5 ℃, 12 h) under water-deficient conditions.

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