

Variations of naphthoquinone levels in micropropagated *Drosera* species in vitro, under greenhouse and outdoor growth conditions

**Christoph Wawrosch*, Eva Vackar, Brigitte Grauwald
and Liselotte Krenn**

Department of Pharmacognosy, University of Vienna, Althanstr. 14, A-1090
Vienna, Austria

Abstract

The naphthoquinone levels in clones of the sundew species *Drosera communis*, *D. madagascariensis*, *D. peltata* and *D. rotundifolia* were determined under in vitro, greenhouse, and outdoor growth conditions. *D. rotundifolia* revealed a lower naphthoquinone content in vitro which rose upon transfer ex vitro. *D. communis* and *D. madagascariensis* exhibited higher levels in vitro which decreased in the greenhouse and further under outdoors conditions. Decreased naphthoquinone levels were found in *D. peltata* when in vitro cultures were moved to the greenhouse, which increased again when the plants were cultivated outdoors. The results underline that in the cultivation of the medicinally useful carnivorous genus *Drosera* species-specific differences in the biosynthesis of secondary metabolites under different environmental conditions have to be taken into consideration.

Key words

sundew, medicinal plant, 7-methyljuglone, plumbagin, HPLC

Introduction

The genus *Drosera* (sundew; Droseraceae) includes nearly 150 species which are present throughout the world, particularly in Australia, Africa and South America

[1]. In Middle Europe the species *D. rotundifolia* L., *D. intermedia* HAYNE ex DREWES and *D. anglica* HUDSON occur, of which especially the former has been used medicinally since ages [2]. The application and excellent performance of *Droserae Herba* in the therapy of infections of the respiratory system like bronchitis or whooping-cough have generally been attributed to 1,4-naphthoquinones (NQ) as major secondary compounds [3], although recently flavonoids were shown to be responsible for antispasmodic and anti-inflammatory effects of extracts of *D. rotundifolia*, too [4-6]. Either or both of the naphthoquinones 7-methyljuglone (7-MJ) and plumbagin (P) occur in several, although not in all *Drosera* species [7].

In the past decades the European sundews have become increasingly rare and are protected by law in several countries. This is due to the fact that the natural habitats of these carnivorous plants are nutrient poor areas like bogs and these highly sensitive ecological systems have been massively disturbed by human activities. Hence, for a long time mainly other non-European *Drosera* species have been used for pharmaceutical purposes [8], namely *D. madagascariensis* DC. and *D. peltata* Sm. [9,10]. While for *D. rotundifolia* NQ levels between 1.0 and 2.3 % have been reported [11-14], much lower amounts of max. 0.063 % were detected in various samples of *D. madagascariensis* by Krenn et al. [9,10]. The latter authors also showed that nearly all commercial samples of *Droserae Herba* contain *D. madagascariensis* and a only few consist of *D. peltata* with up to 0.614 % P. Apart from the low NQ contents in the commercial drug, large amounts of up to 20 t of *D. madagascariensis* are exported from Madagascar annually [15] which is likely to cause ecological problems in the near future.

While due to the specific bog conditions cultivation of the European taxa is still in an experimental stage [12], two approaches are considered to improve the availability of good quality drug: in vitro-cultivation of *Drosera* species as well as identification of other medicinally usable sundews. Respective findings suggest that on a qualitative level most, but not all of the non-European species contain NQ [7,16]. However, quantitative data are only available for a few taxa with samples

from both in vivo and in vitro origin having been investigated. An overview on NQ contents of different *Drosera* species is given in Table 1.

Species	origin ^a	NQ (% dry matter)	reference
<i>D. rotundifolia</i>	in vivo	1.78	11
- " -	in vitro	6.01	11
- " -	in vivo	1.0 - 3.1	14
- " -	in vitro	2.6 - 5.2	14
- " -	in vitro	0.6	24
<i>D. intermedia</i>	in vivo	2.12	11
- " -	in vitro	10.55	11
<i>D. anglica</i>	in vivo	0.89 ^b	12
<i>D. binata</i>	in vivo (GH)	4.3 ^c	17
<i>D. binata</i>	in vitro	1.4 - 2.6 ^d	24
<i>D. capensis</i>	in vivo (GH)	1.25 ^c	17
- " -	in vivo	0.7	16
- " -	in vivo (GH)	0.048 ^c	25
- " -	in vitro	0.005 ^c	25
- " -	in vitro	0.5	26
- " -	in vitro	0.5	24
<i>D. communis</i>	in vitro	0.17	27
<i>D. madagascariensis</i>	in vivo (CS)	0.007 - 0.063	10
<i>D. natalensis</i>	in vivo	0.025 ^c	25
- " -	in vitro	0.01 ^c	25
<i>D. peltata</i>	in vivo (CS)	0.614	10

^a CS: commercial sample; GH: greenhouse cultivation

^b average of original data which are given separately for leaf, flower and stem

^c estimated values assuming a fresh matter : dry matter ratio of 10:1 (original data in % fresh matter)

^d depending on method of cultivation

Table 1. Naphthoquinone (NQ) contents in *Drosera* species of in vivo and in vitro origin.

It is apparent that the values within one species can vary (*D. rotundifolia*, *D. capensis*). This can be explained with seasonal [17] and/or genotype dependent variation [14]. However, different contents have also been reported for samples of in vivo origin (plants grown outdoors or in a greenhouse) and material derived from

in vitro cultures. Interestingly, NQ values found in in vitro samples were described to be either higher (*D. rotundifolia*, *D. intermedia*) or lower (*D. capensis*, *D. natalensis*) than in corresponding in vivo originated material.

To find possible correlations and trends between growth conditions and NQ contents the respective levels were investigated in clones maintained in vitro, after acclimatization and transfer to the greenhouse, and after further transfer to outdoor conditions. The results obtained with clones of *D. rotundifolia*, *D. madagascariensis*, *D. peltata* and *D. communis* are reported in the present paper.

Results and discussion

In an attempt to elucidate the putative influence of growth conditions on NQ formation in sundews a total of 7 clones of the species *Drosera rotundifolia*, *D. madagascariensis*, *D. peltata* and *D. communis* were investigated. Newly sub-cultivated in vitro cultures were transferred ex vitro after 20 weeks. The second growth period of 15 weeks included 2 weeks of hardening in a mist chamber plus 13 weeks in the greenhouse. Subsequently plantlets of each clone were grown for further 15 weeks in an outdoor peat bed. At the end of each of the three periods the contents of NQ were determined.

Although *Drosera rotundifolia* is practically not available at the herbal drug market, we included it into our study because the round-leaved sundew was the most important species for centuries [2] and the production of crude drug through cultivation seems to be feasible [12]. In the examined 3 clones of this species we determined a NQ content of up to 0.52% in vitro (Table 2). After transfer and subsequent 15 weeks cultivation in the greenhouse the NQ levels rose to nearly 1%. But, after further cultivation of 15 weeks in the outdoor peat beds slightly less NQ were determined - however, in 2 of the 3 clones this decrease was not significant. *Drosera madagascariensis* and the 2 clones of *D. communis* gave comparable results: Initially high NQ levels in vitro decreased significantly after transfer to the greenhouse, with a further drop upon cultivation outdoors. The tuberous species *D. peltata* has been described to yield about 0.6 % NQ [10]. In the

clone used in our study we detected more than 0.8 % NQ in vitro and in greenhouse originating plants. The level rose to well over 1 % in the outdoor samples.

	in vitro	greenhouse	outdoors
<i>D. rotundifolia</i> A	0.521 ^a	0.940 ^b	0.706 ^c
<i>D. rotundifolia</i> B	0.357 ^a	0.968 ^b	0.840 ^b
<i>D. rotundifolia</i> C	0.481 ^a	0.991 ^b	0.927 ^b
<i>D. madagascariensis</i>	0.901 ^a	0.733 ^b	0.642 ^c
<i>D. peltata</i>	0.872 ^{ab}	0.709 ^a	1.167 ^b
<i>D. communis</i> B	0.624 ^a	0.503 ^b	0.380 ^c
<i>D. communis</i> C	0.547 ^a	0.496 ^b	0.369 ^c

Table 2. Naphthoquinone content (% dry matter) in shoots of clones of *Drosera* species in vitro, after acclimatization and 15 weeks in the greenhouse, and after further 15 weeks outdoors in peat beds (within rows, values followed by the same letter are not significantly different at $P \leq 0.05$, Duncan's Multiple Range Test).

The results found with *D. rotundifolia* were different to those reported by Kämäräinen et al. [14] who detected higher NQ levels in vitro than ex vitro. However, the authors did not provide informations about the duration of the in vitro culture. The specific in vitro conditions have been described as simulating natural stress conditions [18]. This seems evident because even half strength MS medium as used by Kämäräinen et al. [14] or in our own investigations is of distinctly higher ionic strength than the peat substrate in a typical *Drosera* habitat [19]. Hence, at the beginning of an in vitro culture period possibly the formation of NQ is stimulated while after long term cultivation (which was the case for our clones) the NQ level drops. In turn, the transfer to ex vitro conditions may again be a stress factor which in our clones led to increased NQ formation. The changing irradiation conditions

were another factor which might have influenced NQ formation. Recently the possible role of many plant phenolics as protection against photodamage in addition to the classic function as defence against herbivores has been discussed [20]. Thus, the transfer to the greenhouse with its altered light intensity and composition may trigger a temporarily changed rate of NQ biosynthesis.

The changes in NQ formation which we found in *D. madagascariensis* and *D. communis* were different to those of the *D. rotundifolia* clones. For these species the in vitro culture obviously was a permanent instead of a temporary stress situation which led to increased NQ levels – the origin of these species from a completely different geographical and climatical habitat might be an explanation for this observation. It was of interest that irrespectively of the culture conditions our clone of *D. madagascariensis* had a much higher NQ content than typical market drug as described by Krenn et al. [9,10]. Besides of probable genotype-based differences post-harvest treatment and/or long term storage may have led to the very low NQ levels described by these authors. It has been demonstrated that the drying procedure as well as a long-term storage can influence the NQ contents in some sundew species [10].

As a tuberous sundew, *Drosera peltata* is particularly adapted to arid habitats with short wet seasons. Together with the factors discussed above this might be an additional parameter influencing secondary product formation under different growth regimes. From a statistical point of view the NQ levels in both greenhouse and outdoor grown plants did not significantly differ from the NQ amount in vitro. But, considering the significantly higher NQ content outdoors compared to greenhouse conditions our results indicate that NQ formation in this species shows more similarities with *Drosera rotundifolia* than with the other sundews.

We assume that differing environmental factors in the various culture systems lead to species-specific adaptations, one of which is a change in the biosynthesis rate of NQ.

It has been repeatedly shown for various species that the production of typical secondary metabolites in vitro is lower than in the intact in vivo growing plant [21].

Previous findings suggest that this is not the case for the genus *Drosera* (Table 1): while *D. capensis* or *D. natalensis* indeed produce less NQ in vitro, high levels have been detected in vitro with other taxa. Our results indicate that this subject may be species-specific, with both the possibility of lower or higher NQ contents in vitro.

Concerning the production of commercial medicinal drug, some implications can be derived from this study and earlier knowledge of NQ production in sundews:

The statement of [14] was confirmed that testing of NQ production (e.g. for the selection of clones with high contents for further cultivation) has to be performed with field grown material. Micropropagation might be used for the quick mass production of genetically uniform plantlets which can subsequently be cultivated outdoors. Field cultivation in Europe as suggested by Galambosi et al. [12] seems to be useful for frost tolerant species such as *D. rotundifolia*. Material of species which exhibit a high NQ formation in vitro might possibly be produced biotechnologically. However, this would only be economically feasible when the costs of in vitro-culture can be lowered [22].

In any case the determination of NQ in *Drosera* species for which quantitative data on NQ have not been published yet, have to be carried out with outdoor material. Although this can also be a momentary snapshot only (seasonal variations have e.g. been described by Caniato et al. [17] for a wild growing population of *D. rotundifolia*), at least possible misinterpretations due to differing NQ formation under in vitro or greenhouse conditions can be avoided.

Experimental

In vitro and ex vitro culture

Shoot cultures of *Drosera rotundifolia* (clones A, B and C), *D. madagascariensis*, *D. peltata* and *D. communis* (clones B and C), which at the time of our investigations had been maintained in vitro for at least 5 years, were kept on half strength MS medium [23] supplemented with 10 g l⁻¹ sucrose and 0.8% MERCK agar. After adjusting the pH to 5.7 portions of 40 ml of medium were dispensed to baby food jars closed with MAGENTA B-Caps® and autoclaved at 121

°C for 20 min prior to use. For subculture single rosettes were transferred to fresh medium regularly at intervals of ca. 20 weeks. The cultures were kept at 25 ± 1 °C and a 16-h photoperiod (SYLVANIA Gro-Lux[®] fluorescent tubes at an intensity of $45 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Rooted shoots (rosette diameter ca. 4 cm) were transferred to pots filled with sphagnum peat which was soaked with an aqueous solution of the fungicide Previcur[®] (0.1 % v/v). For 2 weeks the pots were kept at 25 ± 1 °C in a mist chamber with an initial relative humidity of 90 % which was gradually reduced to 60 %. Subsequently the acclimatized plantlets were transferred to the greenhouse where they were grown for 15 weeks. Finally they were planted in a sphagnum peat bed outdoors where they were maintained for further 15 weeks.

Naphthoquinone quantification

For the NQ analyses samples of the different clones were taken a) at the end of a 20 weeks subculture, b) after a 15 weeks period in the greenhouse, and c) after further 15 weeks outdoors. Shoots from the different sources were harvested and dried at room temperature. After pulverisation 1.0 g of the drug was moistened with 5 ml water and after addition of 50 ml petroleum ether (b.p. 40 - 60°C) the mixture was sonicated for 15 min. The extract was filtered over sodium sulfate, which was washed with 25 ml petroleum ether. After evaporation at 240 mbar and 30°C the residue was dissolved in 2 ml of the solution of internal standard (6 mg juglone in 10 ml acetonitrile).

The naphthoquinone content was determined by HPLC as described previously [10]. Briefly, the stationary phase was a 5 μm Hypersil BDS (250 x4.6 mm, Shandon, Runcorn, Great Britain) column, the mobile phase contained acetonitrile with 5 % (v/v) tetrahydrofuran (A) and 0.2 M acetic acid (pH adjusted to 3.0 with triethylamine) (B). Isocratic elution with 38% A and 62 % B was performed at a flow rate of 1.0 ml min^{-1} and with detection at 425 nm.

For each of the studied clones 2 samples were extracted and each extract was subjected to analysis twice. Hence the resulting values are averages of 4 analyses.

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