MICROPROPAGATION OF SELECTED *OENOTHERA* SPECIES AND PRELIMINARY STUDIES ON THEIR SECONDARY METABOLITES

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ABSTRACT

A method was devised for the micropropagation of eight species of the genus *Oenothera* L. from shoot tips and shoot segments with nodes. Microshoot cultures were obtained from explants on Murashige and Skoog (MS) medium enriched with IAA and BA. Numerous shoots developed properly after they had been transferred onto MS medium without BA. When they had rooted under the influence of auxins (IAA or IBA), shoots were transferred to pots and then to the soil, where they matured.

In the seeds produced by these plants, the content of fatty acids was determined using the GC method. A preliminary analysis of flavonoid compounds and phenolic acids was made using the 2D TLC method (fingerprinting) in microshoots and leaves of soil plants regenerated in vitro.

KEY WORDS: *Oenothera* sp., micropropagation, shoot tips, nodal segments of shoots, callus, fatty acids, phenolic compounds.

INTRODUCTION

Species of the genus *Oenothera* are an object of interest to many scientific and industrial centres around the world thanks to the bio-oil present in their seeds, and especially to one of its components, the unsaturated-linolenic acid that has a broad range of biological effects (Horrobin 1990).

So far, in vitro cultures of *Oenothera* plants have been dealt with only sporadically. As follows from their patent reports, Japanese authors (Osamu and Tadashi 1987; Takeo et al. 1987) obtained callus of *O. biennis* L. probably as a source of γ-linolenic acid. Successful attempts have been made at the micropropagation of this species (Skrzypczak et al. 1994) and of *O. erythrosepala* Borbas (Suzuki et al. 1990), while *O. hookeri* de Vries was regenerated as a result of androgenesis (Martinez and Noher de Halac 1995). A book summarising the current knowledge about the genetics of *Oenothera* has also been published (Harte 1994).

The aim of our research was to establish the method of micropropagation of *Oenothera paradoxa* and other seven species. Thus, in order to determine whether the plants propagated in vitro contain the secondary metabolites, characteristic for the intact plants, we analysed in them the presence of fatty acids, flavonoids and phenolic acids.

Abbreviations
IAA – indole-3-acetic acid; BA – benzyladenin; GA_3 – gibberellic acid; IBA – indole-3-butyric acid; 2,4-D – 2,4-dichlorophenoxyacetic acid; kin – kinetin

MATRIALS AND METHODS

In vitro culture

In vitro cultures were initiated from seed collection of the Department of Pharmaceutical Biology and Botany of the Medical Academy in Wrocław. The seeds came from the following biennial species: *O. ammophila* Focke, *O. erythrosepala* Borbas, *O. fallax* Renner em Rostański, *O. paradoxa* Hudziok, *O. rubricaulis* Kleb., *O. salicifolia* Desf. ex G. Don., *O. silesiaca* Renner, and *O. acerviphila* Rostański (Rostański 1997).

Stratified seeds were sterilised using a 0.2% sublimate solution (7 min.) with an addition of 2 drops of Tween 80. From 350 to 500 seeds of each species were placed on an agar-solidified (0.8%) MS medium (Murashige and Skoog 1962). Explants used to initiate micropropagation and start the callus culture were obtained from 30-day-old seedlings. Shoot tips 5 mm long (for micropropagation) and also nodal segments of shoot about 5-8 mm long (for further multiplication) were used. Media were enriched with various growth regulators, from which a final choice was made of those listed in Table 1.

The cultures were placed in a growth chamber with a 16-hour photoperiod, in the light of a cool-white fluorescent tube

TABLE 1. Media used in different stages of *Oenothera* tissue culture.

A. MS + 1.0 mg/l IAA + 1.0 mg/l BA B. MS + 0.1 mg/l IAA + 1.0 mg/l BA	shoot multiplication
C. MS + 1.0 mg/l GA_3 D. MS	shoot elongation
E. MS + 1.0 mg/l IAA F. MS + 1.0 mg/l IBA G. MS	rooting
H. MS + 0.25 mg/l 2,4D + 1.0 mg/l BA I. MS + 0.5 mg/l 2,4D + 1.0 mg/l kin	callus induction and growth

having the intensity of 60 μmol/m²s at a temperature of 24°C ± 2°C and air humidity of 60-70%. In all the experiments the pH of the medium was adjusted to 5.6-5.8 and agar (0.8%) was added before autoclaving, which was carried out for 15 min. at 121°C. All the experiments were repeated three times, and there were ca. 20 replicates per combination in each set.

Shoot tips and nodal segments were placed on a shoot multiplication medium A or B. After 5 weeks the microshoot culture was transferred onto medium C to ensure further growth in length. Shoots 1.5-2 cm long were separated and rooted under in vitro conditions on medium E or F. Rooted plantlets were transferred to pots, to a sterile mixture of garden soil, peat and sand (1:1:1). In the initial growth stage the plantlets were covered with glass to increase humidity, and gradually acclimatised. Each year in May an average of 40 plantlets of the species under study were transferred to experimental plots of the Department of Pharmaceutical Biology and Botany of the Medical Academy in Wrocław. The plants developed normally, and when they reached maturity their seeds were collected for further studies.

The callus tissue was induced from fragments of seedlings on medium H or I which was passaged every 6 weeks onto the same medium. The callus from a stabilised culture intended for analyses was collected and dried at 60°C.

Chemical analysis

The content of fatty acids in dried seeds was determined using gas chromatography (GC). The oil was extracted by n-hexane, and the analysis of the fatty acid fraction was made after they had been converted into methyl esters with the help of boron fluoride (Skrzypczak et al. 1994).

Microshoots of some species and leaves of soil plants regenerated in vitro were used to test the presence of flavonoid compounds as well as ellagic and gallic acids. The method used was two-dimensional, thin-layer chromatography (2D-TLC) – fingerprinting. Shoots of individual species (2-4 g) were extracted with methanol three times for 1 hour. The combined extracts were reduced to dryness under lower pressure at 40°C. The solid residue was dissolved in hot water and shaken first with chloroform and then n-butanol. Butanol extracts were analysed (2D-TLC) on cellulose-covered aluminium plates (10 x 10 cm) (Merck) in the following dimensions: I – 15% acetic acid; II – butanol (2) – acetic acid – water (14:1:5) (Bartl 1975). The distribution of the spots of compounds in chromatograms was analysed in UV_{365} and UV_{254} light before and after spraying the plates with solutions of reagents characteristic of flavonoid compounds (1% ethanol solution of aluminium chloride; 0.1% β-aminoethanol ester of diphenylboric acid – Naturstoffreagenz A).

RESULTS AND DISCUSSION

Research on in vitro clonal propagation and tissue cultures of evening primroses was conducted in our laboratory in the years 1990-1996. Partial results of those studies were presented at conferences (Thiem et al. 1994; Thiem et al. 1995). The results of experiments on in vitro cultures and of chemical analyses are averages of three repetitions.

Sterile seeds germinated after 14-21 days at 90-100%. Shoot tips differentiated into microshoot cultures on MS medium supplemented with BA and IAA. After 5 weeks in the presence of 1.0 mg/l BA and 1.0 mg/l IAA, some 12 axillary shoots per explant developed (Fig. 1). The derived cultures consisted of compact green tissue with numerous shoot primordia, besides clusters of microshoots. The transfer of those cultures onto medium C or D without BA caused the elongation of microshoots, which grew into normal, healthy organs. The development of similar abundant, thickly growing dwarf shoots under the influence of a high BA level has been frequently noted (Wysokińska 1993). After 4 weeks, easy-to-separate shoots were cut off from the base tissue, which could then be transferred again onto medium C for the next set of buds to develop. The shoots thus obtained could be rooted directly, or used as a sterile source of further explants – shoot tips or nodal segments. Shoots of more than 1 cm in length were isolated and transferred onto medium E or F supplemented with 1 mg/l IAA or IBA, on which they rooted very well (100%) after 4 weeks (Fig. 2). The rooting of shoots on medium G without an auxin was sporadic and poor. Using this method, some 200 plantlets can be obtained from a single shoot tip in 100 days. Nodal segments of shoots on medium A also formed a microshoot culture, but a less abundant one (up to 8 per explant).

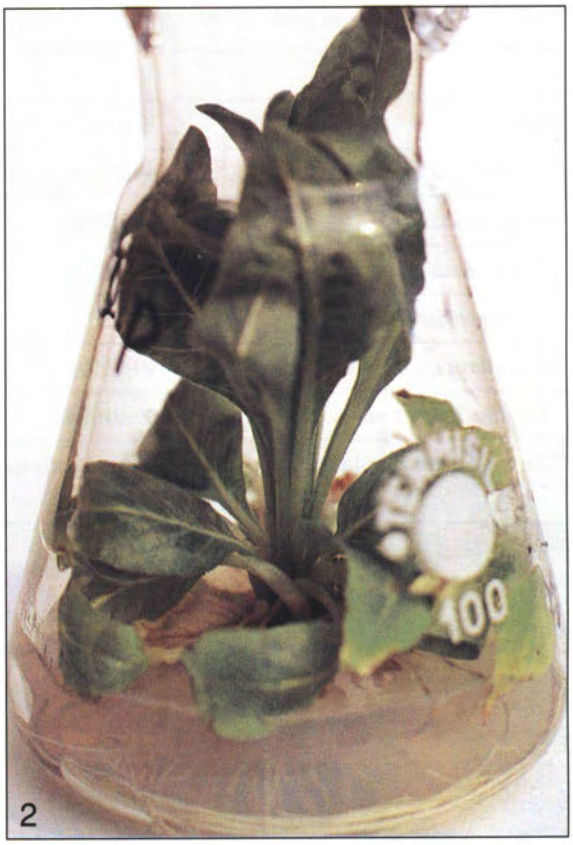
After potting and acclimatisation (Fig. 3) the plants were transferred to the soil where they developed correctly and produce seeds in the second season of vegetation (Fig. 4). They branched better than seed-derived plants. The described micropropagation method turned out to be useful in the propagation of all eight species.

On the media H and I callus of *O. paradoxa* was grown. In the light it become pale green, sometimes with a pink or red hue (Fig. 5).

In the seed of the selected species of the genus *Oenothera* the contents of fatty acids was determined using the GC method (Table 2). As it turned out, γ-linolenic acid, to the amount of 11%, occurs in the seeds of *O. ammophila* and *O. paradoxa* after the first season of vegetation. This content is comparable with those from intact plants (Lamer-Zarawska et al. 1989). Our interest was focused on the second species whose seeds are used in Poland in the production of medicinal oil, which is also a component of very many cosmetics.

Resently many articles concerning taxonomic of *Oenothera* (Rostański 1992, 1995, 1998), technological, analytical and medical issues have been published. As it was shown in a previous study of *O. biennis* L. (Skrzypczak et al. 1994), the seeds of plants propagated in in vitro cultures had a slightly higher content of the oil (24%) and γ-linolenic acid in it (8.2%) than those from intact plants growing in the same place (23% and 7.7%, respectively).

Encouraged by patent reports (Osamu and Tadashi 1987; Takeo et al. 1987) about the synthesis of γ-linolenic acid (6.7%) in the callus tissue of the species *O. biennis*, we determined the fatty acid in the callus tissue of *O. paradoxa* using a known method (Krzyżaniak and Segiet-Kujawa 1991). It turned out that in this material α-linolenic acid predominated,



Figs 1-4. Various developmental stages of *Oenothera paradoxa* Hudziok plantlets derived from shoot tip explants.
Fig. 1. Multiple shoot formation after 6 weeks on MS medium containing 1.0 mg/l IAA and 1.0 mg/l BA.
Fig. 2. 45-day-old plantlet with rooting induced by 1.0 mg/l IAA.
Fig. 3. Regenerated plantlets established in the soil after 3 months of transplantation.
Fig. 4. Flovering plant during the second year of vegetation in the field. Bars: 1 = 5 mm; 2 = 15 mm; 3 = 20 mm; 4 = 100 mm.

TABLE 2. Fatty acids in seeds of *Oenothera* plants regenerated in vitro.

		Content of fatty acids [%]			
Species	Season	palmitic	oleic	linoleic	γ-linolenic
O. paradoxa Hudziok	1993/1994	4.9	5.9	74.8	10.1
O. paradoxa	1994/1995	5.1	5.9	77.1	9.0
O. paradoxa	1995*	6.2	4.6	75.9	11.3
O. ammophila Focke	1995*	6.5	5.3	74.4	11.2
O. erythrosepala Borbas	1994/1995	5.1	12.5	71.9	7.7
O. fallax Renner em Rostański	1993/1994	5.4	10.4	70.1	9.6

*The seeds were produced already in the frist season of vegetation

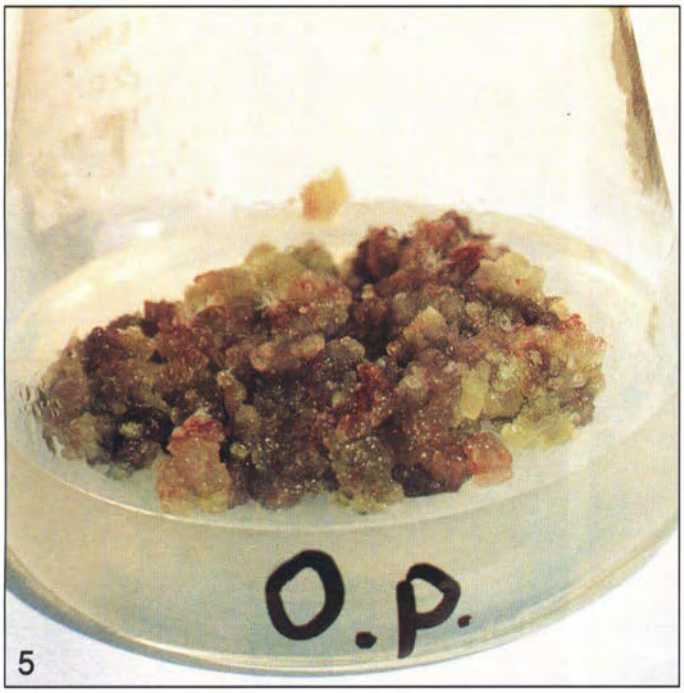


Fig. 5. Callus of *Oenothera paradoxa* Hudziok formed at the hypocotyl after 4 weeks on MS medium with 0.25 mg/l 2,4-D and 1.0 mg/l BA in the light. B = 16 mm.

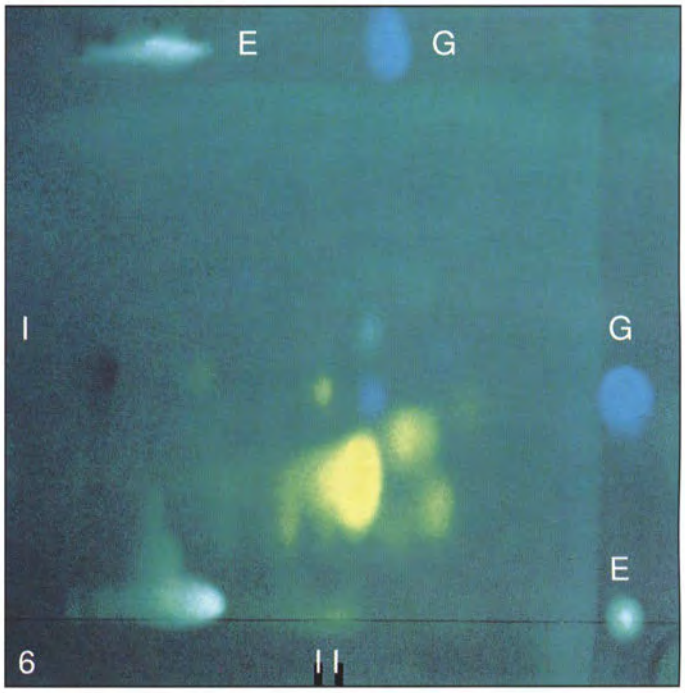


Fig. 6. Chromatogram of the butanolic fraction from leaves of *O. erythrosepala* Borbas regenerated in vitro and standard compounds. G – gallic acid; E – ellagic acid; cellulose plate, developing phases: **I** 15% OHAc, **II** BuOH (2) – OHAc-H_2O 14:1:5; UV_{254} after spraying with 0.1% ethanol solution of β-aminoethanol ester of diphenylboric acid.

while the content of the biologically active γ-linolenic acid was negligible.

In the leaves of species of the genus *Oenothera* from North America flavonoid compounds of varying structures were found to be present (Averett et al. 1987, 1988). *Oenothera* species also contain phenolic acids (Zinsmeister and Bartl 1971; Krzaczek and Bogucka-Kocka 1994; Krzaczek et al. 1995) and hydrolysable tannins which were termed Oenotheins. From the leaves of *O. erythrosepala* two ellagitannins were isolated – oenotheins A and B (Asanaka et al. 1988; Hatano et al. 1989, 1990) which also occur in *O. biennis* and *O. laciniata* besides other compounds of this type (Yoshida et al. 1991, 1995). The above tannins have anticarcinogenic properties (Miyamoto et al. 1987, 1993a, 1993b; Motoyama et al. 1988). The above reports encouraged us to test some species obtained in in vitro culture for the presence of flavonoid compounds as well as ellagic and gallic acids.

The results of chromatographic analyses indicate that there are flavonoid compounds in microshoots and leaves of soil plants regenerated in vitro. In turn, the results of characteristic reactions (Harborne 1991) justify the conclusion that besides ellagic and gallic acids, the material under examination may also contain their derivatives (Fig. 6).

On the basis of our research considering the genus *Oenothera* and results of other authors we conclude:

- Described method of micropropagation of plants from the existing meristems through axillary shoot formation, in the case of interesting selected varietes, especially of *Oenothera paradoxa*, may find practical application in mass production of plant material with genetic stability (Bajaj et al. 1998) of high pharmaceutical quality.

– The presence of the controlled secondary metabolites indicates that not only seeds, but also the leaves may be sources of the compounds endowed with a wide range of biological activity.

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MIKROROZMNAŻANIE WYBRANYCH GATUNKÓW *OENOTHERA* I WSTĘPNE BADANIA METABOLITÓW WTÓRNYCH

STRESZCZENIE

Opracowano metodę mikrorozmnażania ośmiu gatunków rodzaju *Oenothera* L. ze szczytów pędów i fragmentów pędów z węzłem. Kultury mikropędów otrzymano z eksplantatów na pożywce Murashige i Skooga (MS) wzbogaconej IAA i BA. Liczne pędy rozwijały się prawidłowo po przeniesieniu na pożywkę MS bez BA. Ukorzenione pod wpływem auksyn (IAA lub IBA) pędy wysadzano do doniczek i dalej do gruntu, gdzie rośliny osiągały dojrzałość. W zebranych nasionach oznaczono metodą GC zawartość kwasów tłuszczowych. Wstępną analizę związków flawonoidowych i kwasów fenolowych metodą 2D-TLC „odcisk palca" przeprowadzono w mikropędach i liściach roślin gruntowych zregenerowanych in vitro.

SŁOWA KLUCZOWE: *Oenothera* sp., mikrorozmnażanie, szczyty pędów, fragmenty pędów z węzłem, tkanka kalusowa, kwasy tłuszczowe, związki fenolowe.