COMPARISON OF PROTEIN PATTERNS AFTER TWO-DIMENSIONAL GEL ELECTROPHORESIS FROM LEAVES OF IN VITRO CULTURES AND SEEDLINGS OF *RUBUS CHAMAEMORUS* L.

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ABSTRACT

Proteins from leaves of *Rubus chamaemorus* propagated in vitro were subjected to miniaturized 2-D electrophoresis. The 2-DE patterns of proteins showed qualitative differences between plants propagated in vitro and control seedlings. More proteins of a high molecular weight were observed in leaves of plants from in vitro culture.

A two-dimensional map of proteins from leaves provides detailed data concerning both polymorphism and protein patterns of this species. This makes it possible to start constructing a protein map of *R. chamaemorus*. The reasons for qualitative differences are discussed.

KEY WORDS: *Rubus chamaemorus*, in vitro culture, shoot culture, protein, two-dimensional electrophoresis.

INTRODUCTION

Cloudberry, *Rubus chamaemorus* L. (Rosaceae), is a common plant with boreal, circumpolar distribution. It inhabits mainly swamps and peat bogs of the northern hemisphere, but it also occurs in some mountain regions in Central Europe and Central Asia (Taylor 1971). In Poland, *R. chamaemorus* grows only in few localities as a glacial relict being under strict protection. Consequently it has been classified as a vulnerable species in the Polish Red Data Book of Plants (Kruszelnicki and Fabiszewski 1993). For this reason, a method of its in vitro propagation from shoot tips has been developed (Thiem 2001).

R. chamaemorus is a herbaceous, octoploid ($2n=8x=56$), dioecious plant producing edible fruit of economic value in Scandinavia (Naess et al. 1993). The plant reproduces itself both sexually and vegetatively, but mainly through clonal growth by branched rhizomes. As a consequence of vigorous vegetative propagation, clones of one individual can cover large areas. Although sexual reproduction is very slow

and infrequent, it is important as it forms new genotypes and the produced seeds can colonize new habitats (Taylor 1971; Mäkinen and Oikarinen 1974; Korpelainen 1994).

Three methods are currently used in research on plant cell cultures to analyse genetically determined variation and cell line identification. Electrophoretic analysis of isoenzymes and cytogenetic analysis have several drawbacks (Hoepfner et al. 1993), whereas DNA fingerprinting has been used successfully to distinguish different genotypes within populations of wild *Rubus* species (Nybom and Schaal 1990) and to monitor cell line identity in micropropagated raspberries (Hoepfner et al. 1993). Some genetic studies of *R. chamaemorus* populations have also been performed. Polyacrylamide gel electrophoresis of proteins has revealed very low levels of genetic variability in natural populations of this species (Korpelainen 1994). DNA analysis indicated that each of the investigated populations was composed of only two, three or four clones (Korpelainen et al. 1999). Although plants growing in different habitats display a marked phenotypic variation, the level of genetic variation appears to be fairly low (Lohi 1974).

Two-dimensional polyacrylamide gel electrophoresis (2-DE), as a sensitive method with a high discrimination value, is very useful for investigation of protein polymorphism (Humphrey-Smith et al. 1997). This method has been used for qualitative studies of genetic diversity, modifi-

Abbreviations:

2-DE – two-dimensional polyacrylamide gel electrophoresis, BA – 6-benzyladenine, GA₃ – gibberellic acid, IBA – indole-3-butyric acid, IEF – isoelectrofocusing, IP – isoelectric point, MS – Murashige and Skoog, MW – molecular weight

cation of the protein profile due to mutations, phylogenetic relationships, genetic mapping of plant species and other genetic analyses. These analyses concerned 2-DE resolution of proteins from diploid tissues, such as seeds, leaves, seedlings (e.g. Picard et al. 1997; Thiellement et al. 1999), and from pollen (Kalinowski et al. 2001). Recently, extensive use of DNA and protein polymorphism analyses to ascertain plant fidelity following micropropagation has been preferred (Ashmore 1997; Altman et al. 2000; Rival et al. 2000).

The aim of this study was to analyse electrophoretically peptides from leaves of *R. chamaemorus* propagated in a long-term in vitro culture. We were interested in studying protein polymorphism and in initiating the 2-DE protein map for this species.

MATERIALS AND METHODS

Plant material and culture conditions

The aggregate fruits of *Rubus chamaemorus* L. (so-called cloudberry) were collected from a natural locality of this species in Norway (Hade Land county). Seeds surrounded by hard endocarps (so-called pyrenes) were separated from the fleshy mesocarps and stored at 4°C for 3–6 months before the beginning of micropropagation. Cloudberry plants were micropropagated from shoot tip culture by initiation of axillary shoots, as described previously (Thiem 2001). Leaves collected from long-term shoot cultures of *R. chamaemorus* (19th passage) and leaves of seedlings obtained directly from seeds (control) were materials for this study (Fig. 1a–b). The shoot cultures were grown on MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg l⁻¹ 6-benzyladenine (BA) and 0.1 mg l⁻¹ indole-3-butyric acid (IBA); they were marked as samples **b**, **c**, **d**, **e** and **f** (Fig. 2b–f). The control (sample **a**) consisted of 45-day-old seedlings grown in vitro on hormone-free MS medium under the same conditions (Fig. 2a). The seedlings germinated from seeds of the same origin, stored at 4°C since the time of collection (i.e. for nearly 4 years). Five or six leaves

from each seedling or shoot culture were collected and stored at -70°C for few days until electrophoresis.

Extraction of proteins

Extraction of proteins was carried out according to Hurkman and Tanaka (1986). Leaves (25 mg) were ground with 400 µl of extraction buffer. Proteins were extracted for 15 min. at 4°C and the crude extract was shaken with 400 µl of water-saturated phenol. The proteins from the phenol phase were precipitated with cold ammonium acetate in methanol. Precipitated proteins were dried and then dissolved in 15 µl of a buffer containing urea, detergent Nonidet NP-40 and Servalyte pH 2–4 in water.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed as described by Hochstrasser et al. (1988). In the first dimension (isoelectrofocusing, IEF) a capillary 0.55 mm × 60 mm was used for polymerisation of a polyacrylamide column gel with Servalyte pH 3–10. Isoelectrofocusing was conducted at room temperature for 195 min (50–400 V). The proteins in the second dimension (SDS-PAGE) were separated in 13% polyacrylamide slabs (0.5 mm × 60 mm × 60 mm). Following electrophoresis, the peptides were stained with silver nitrate according to Heukeshoven and Dernick (1985).

The protein spots on the gels were scanned (SHARP JX-330) and subjected to processing by LabScan software and the data were further processed by Image Master 2-D Elite (Amersham Pharmacia Biotech) software. Pharmacia 2-D Electrophoresis Calibration Kit (17-0582-01) and Silver Stain SDS Molecular Weight Mixture (Sigma, M-5630) were used to standardise the gels. From both analysed control plants one synthetic electrophoregram was created, which included all detected peptides in the two samples (patterns). Characteristic groups of new proteins from the studied samples, different from the proteins present in control seedlings, were marked with solid circles on electrophoregrams.

Fig. 1a–b. *Rubus chamaemorus* – seedling (control) (a) and in vitro shoot culture (b). Bar: 1 cm.

Fig. 2a-f. Proteins of *Rubus chamaemorus* from leaves of control (a) and shoot cultures (b-f) after 2-D electrophoresis. New spots, compared to control, were marked with full circles. Common proteins revealed from all shoot cultures, but absent from the control, are marked with arrows.

RESULTS

The synthetic control 2-DE pattern is shown in Fig. 2a, while Fig. 2b-f shows protein patterns of leaves from in vitro shoot cultures. The peptides found in only one of the two control seedlings are marked in grey in the synthetic control electrophoregram (Fig. 2a). Fig. 2a₁-f₁ presents the original gels for samples **a-f**. The appearance of additional proteins in the 2-DE pattern of a sample reflects a qualitative difference from the control. The electrophoregrams also reveal that the samples of *R. chamaemorus* cultured in vitro

are polymorphic. In the control (Fig. 2a) the total number of detected peptides was 168, while shoot cultures had 130, 171, 171, 215 and 264 peptides, respectively (Fig. 2b-f). The 2-DE patterns of shoot cultures varied also in number of novel peptides, i.e. the peptides which were not detected in the control (Table 1). The molecular weight (MW) of peptides from control leaves did not exceed MW of 66 kDa, but in shoot cultures some proteins of a higher MW were detected. Sixteen proteins common to samples **b-f**, but absent from the control, are marked with arrows in Fig. 2b-f and with inboldface in Table 1.

TABLE 1. Electrophoretic parameters molecular weight (MW in kDa) and isoelectric points (IP at pH) of new peptides in shoot cultures (samples **b-f**), compared to the control pattern.

b	c	d	e	F
MW / pH	MW / pH	MW / pH	MW / pH	MW / pH
16, 33/4.7	13.5/6.7, 7.2	12, 24.5/7.4	12, 24.5/7.4	11, 5/4.1, 4.3
18.5/5.6	15/7.3	13, 5/6.7	12.5, 22/7.3	12/4.5, 7.4
20.5/5.3, 5.9	17, 57/8.1	15, 22/7.3	13/6.9	12.5, 20, 22/7.3
22/6.2, 7.3	18.5/5.3, 5.6	18.5 , 21/5.6	13, 5/6.7, 7.2, 7.5	13/6.9
23/6.9	19, 24/5.1	20.5/5.9	14, 67/7.7	13.5/6.7, 7.2, 7.5
23.5/6.1	20, 22/7.3	23/6.9	15/6.6, 7.7, 7.3	14, 63, 67/7.7
25/7.1	20.5/5.3, 5.9	24, 25/7.1	15.5/4.6, 7.6	15/6.6, 7.3
26/5.6, 7.0, 8.3	21.5, 29.5/5.7	25.5/7.2, 7.6	16, 28/4.7	15.5/4.6, 7.6
27/5.4, 6.0, 7.3	23/6.9	26/5.6, 8.3	17, 57/8.1	16/4.7
27.5/5.8	24, 25 , 58/7.1	26.5, 27.5, 52/5.8	18.5/5.6	17, 57/8.1
28.5/7.2	25, 26.5/5.2	30/6.3	20/7.3	18 , 5/4.9, 5.6
29/7.6	26/4.8, 5.1, 5.6, 8.3	32/5.5, 6.3	20.5/5.3, 5.9	19.5/4.9
29.5/8.2	27/5.4	33/4.7	21/5.6, 7.6	20.5/5.3, 5.9, 7.7
32/7.2, 7.9	30, 33/4.7	34/7.0	21.5/5.7	21/4.6, 7.6, 7.8
36, 42, 45/7.4	32/7.6, 7.9	35/7.2	23/4.7, 6.9	21, 5/5.7
38/7.5	35/7.2	38 , 70/7.5	23.5/6.1	22, 5/4.5, 8.0
39/5.3	37/6.0	39/5.3	24, 25 , 58/7.1	23/4.6, 4.7, 5.8, 6.9, 80
41/7.6	38/7.5	42 , 51/7.4	26/5.6, 8.3, 7.0	23, 23.5/6.1
54/5.9, 7.2, 8.0	39/5.3 , 6.0, 6.1	43/5.7, 6.7, 6.8	26, 5/5.2, 7.1	24/5.1, 7.1, 7.8
56/6.4	42, 45 , 51/7.4	45/7.4, 7.5	27/5.4, 6.0, 7.3	24, 5/4.4, 7.4, 7.9
57/8.1	43/6.7, 6.8	53/6.0	27.5/5.8, 7.6	25/5.2, 7.1, 7.6
60/7.6, 7.7, 7.8	52/5.8, 7.7, 8.4	54/5.8, 5.9, 7.2, 8.0	28.5, 70/7.5	25, 5/7.2, 7.6
70/7.5	54/5.8, 5.9, 7.2, 8.0	56/6.4	29/7.6, 7.9	26/5.6, 7.0, 8.3
	56/6.4	57/8.1	29.5/8.2	26.5/5.2, 7.1
	60/7.6, 7.7, 7.8	64/6.6	30, 33/4.7	27/5.4, 6.0, 7.3
	61/7.0	66/6.5	32/7.7, 7.9	27.5/5.8, 7.6
	63, 67/7.7		34/7.9	28/4.7, 6.4
	64/6.6		35/7.2, 8.2	28.5/7.2, 7.5
	66/6.5, 7.2		36, 42 , 51/7.4	29/7.6, 7.9
			38/7.5, 8.3	29.5/5.7, 8.2
			39/4.6, 5.3 , 6.0, 6.1, 7.6	30/4.7, 6.1, 6.3
			43/5.7, 6.8	32/5.5, 6.3, 7.2, 7.77.9
			45/7.4, 7.5	33/4.7, 5.3
			52/7.7, 8.0	34/7.9
			54/5.8, 5.9, 7.2, 8.0	35/7.2
			60/7.6, 7.7	38/5.2, 5.4, 7.5
			66/6.5	39/4.6, 5.3 , 7.5, 7.6, 7.9, 6.0, 6.1
				42, 45 , 51/7.4
				43/5.7, 8.4
				52/5.8, 7.7, 8.0
				53/6.0
				54/5.8, 5.9, 7.2, 8.0
				56/6.4
				58/7.1
				60/7.6, 7.7, 7.8
				61/7.0
				66/6.5, 7.2
				69/6.6
				72/6.3
				74/6.7

Table 1 presents electrophoretic parameters (MW in kDa and IP at pH) of all new peptides, which are absent in the control. Below we mention only the most numerous groups of distinguishing peptides. In sample **b** 130 peptides were detected (Fig. 2b), of which 71.5% (93 spots) were identical with those of the control. Among the 37 peptides absent from the control there were 7 peptides with high MW of 54-70 kDa and isoelectric points (IP) corresponding with pH 7.0-8.1 and five peptides with MW of 18.5-23.5 and IP at pH 5.3-6.2. In sample **c** 171 peptides were revealed (Fig. 2c), of which 66% (113 spots) corresponded with the peptides found in the control extract. Numerous peptides absent from the control and detected in this sample included 10 peptides of 52-66 kDa (IP at pH 6.9-8.1). Similarly, sample **d** (Fig. 2d) had 171 peptides, too, of which 75% (128 spots) were identical to those found in the control. Among 43 new peptides the most characteristic are six with high MW of 54-66 kDa and IP at pH 6.4-7.3. From the leaves of sample **e** 215 proteins were isolated, of which 64% (138 spots) corresponded with peptides from control seedlings (Fig. 2e). Among the remaining 77 peptides, the group of seven peptides with high MW of 52-66 kDa and IP at pH 6.5-8.0 were found and eleven peptides with low MW of 12-15 kDa and pH 6.5-8.0. In sample **f** 264 peptides were detected (Fig. 2f), of which 57% (150 spots) were the same as in the control. The numerous peptides absent from the control seedlings and detected in this sample included ten peptides with MW 12-15, IP at pH 6.5-8.0 and five peptides of 66-74 kDa (IP at pH 6.3-8.0).

DISCUSSION

Miniaturized 2-DE electrophoresis of proteins from leaves of *R. chamaemorus* revealed many qualitative differences between the studied samples: shoots micropropagated in vitro (in the presence of plant growth regulators) and control seedlings grown in vitro (in the absence of plant growth regulators). The different numbers of separated proteins in the leaves from shoot cultures might be caused by physiological and epigenetic variation resulting from numerous factors present in the in vitro culture environment (Rivali et al. 2000; Cassells and Curry 2001). In vitro cultures of *R. chamaemorus* were initiated using shoot tips of seedlings growing under sterile conditions from excised embryos. The leaves subjected to our study were collected from plantlets propagated in vitro from the existing meristems (Thiem 2001). For micropropagation of *R. chamaemorus* shoots we used relatively low concentrations of plant growth regulators: BA (0.5 mg l⁻¹) and IBA (0.1 mg l⁻¹) because higher concentrations might increase protein polymorphism. The chosen method of micropropagation from shoot tip culture by the initiation of axillary buds maintains genetic stability of the obtained plants (Bajaj et al. 1988; Rice et al. 1992; Cassells and Curry 2001). On the other hand, it has been suggested recently that the clonal fidelity of micropropagated plants of *Populus tremuloides* cannot always be assured even when organized meristems are used as the explant source (Rahman and Rajora 2001).

In the shoot cultures of *R. chamaemorus*, expression of silent genes may have occurred, too, or variation in the number of peptides has been attributed to the effect of regulators controlling expression of structural genes (Colas

des Francs and Thiellement 1985; Zivy et al. 1992). Another possible reason contributing to changes in the protein profile may be the action of homoeologous genes responsible for synthesis of the same product. An example is hexaploid wheat in which the absence of homoeologous genes intensifies protein synthesis by up to 30-80% (Aragoncillo et al. 1978). Cloudberry is an octoploid plant so the products of a multiplied gene dose in *R. chamaemorus* are likely to act as suppressors causing low protein synthesis. Detection of new peptides in samples of shoot cultures may be a result of post-translation modification of proteins, such as phosphorylation or glycosylation, which may change their isoelectric points (Zanis and Breslow 1981). Moreover, changes in protein profiles may be also due to formation of complexes with phenolic compounds (Bates 1979). Secondary metabolites, like phenolic compounds, appearing in large amounts in *R. chamaemorus* (Törrönen 1997, 2000; Thiem 1998) are likely to form complexes with proteins, changing their electrophoretic characteristics.

The long-term shoot cultures of *R. chamaemorus* used in our study, were in the 19th passage, while a recommended maximum number of subcultures is 10-15. Despite so many passages the multiplication rate of shoot cultures remained at a similar level and amounted to 8 new shoots per explant. During three years' maintenance of in vitro shoot cultures no morphological changes were observed. Our previous studies showed phenotypic fidelity of plantlets propagated in vitro by the axillary culture systems, as the plantlets did not differ in their morphological structure from intact plants (Thiem 2001). Homogeneity of the obtained plantlets was also suggested by the results of two-dimensional thin-layer chromatography (2D-TLC), which revealed a pattern of phenolic compounds corresponding to that obtained from naturally growing plants (Thiem 1998).

However, it is known that plants propagated in vitro may show somaclonal variation caused by structural rearrangements and variable number of chromosomes (Larkin and Scowcroft 1981; Bajaj 1990). Our preliminary flow cytometric studies of nuclear DNA content of in vitro shoot cultures of *R. chamaemorus* (20th passage) did not reveal any changes in ploidy, as compared to control seed-derived plants (Thiem and Śliwińska 2002). Similarly, polypeptide patterns and estimation of nuclear DNA content of oil palm propagated in vitro have been investigated (Rival et al. 1997).

Wild populations of cloudberry have been previously described, and the results of standard electrophoretic analysis of their proteins disclosed a very low level of genetic variation (Korpelainen 1994). Studies involving molecular methods (DNA fingerprinting) also confirmed the exceptionally low genetic variation of *R. chamaemorus* in natural populations (Korpelainen et al. 1999).

An interesting discussion on clonal stability and seasonal periodicity of long-term micropropagated plants can be found in a paper by Altman et al. (2000). A marked seasonal effect on specific protein expression has been detected in long-term cultures of aspen (*Populus tremula* L.). The persistence of growth periodicity in long-term cultures suggests that the actual rate of phenotypic polymorphism in plants is often far less frequent than DNA and protein band polymorphism.

The results of the present study, obtained by highly sensitive method, suggest that shoot cultures of *R. chamaemorus*

1715 after several subcultures of vegetative propagation in vitro from existing organized meristem tissues of shoot tips, have produced plantlets with varied protein profiles. However, in all samples of shoot cultures sixteen proteins with the same or very close electrophoretic parameters were detected. Their identity can be confirmed only by protein sequencing. The method of 2-D electrophoresis, is characterised by high resolution and reproducibility, and it allows simultaneous analysis of several thousand features (O'Farrell 1975).

Future research should explain whether the differences between the 2-DE patterns of the micropropagated shoots and control plants are caused by genetic origin, physiological stage of examined leaves, duration of in vitro culture or presence/absence of roots. Moreover, cloudberry plants are octoploid and dioecious, so the material used for propagation could be heterogeneous genetically.

Regardless of the observed protein polymorphism of the leaves acquired from in vitro cultures, the results of 2-DE analyses have supplied significant information on the examined species. An optimized method of protein extraction from cloudberry leaves was found. The isolation of soluble and membrane proteins according to Hurkman and Tanaka (1986), combined with miniaturized 2-D electrophoresis, enabled the visualisation of peptides on the gels, although the extract from leaves of *R. chamaemorus* contains low levels of proteins. The detailed 2-DE peptide patterns of the analysed leaves may help to start to create a protein map of this species, as suggested by Thiellement et al. (1999). It seems to be of interest, as *R. chamaemorus* is a rare and protected species both in Poland and some other European countries.

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PORÓWNANIE BIAŁEK PO DWUKIERUNKOWEJ ELEKTROFOREZIE NA ŻELU Z LIŚCI KULTUR IN VITRO I SIEWEK *RUBUS CHAMAEMORUS* L.

STRESZCZENIE

Metodą zminiaturyzowanej 2-D elektroforezy badano białka z liści *Rubus chamaemorus* mnożonych w kulturach in vitro. 2-DE profil białkowy wskazał na wyraźne różnice jakościowe pomiędzy roślinami mnożonymi in vitro, a kontrolą. Większą liczbę polipeptydów o względnie wysokich masach cząsteczkowych obserwowano w liściach roślin z kultur in vitro.

Dwukierunkowy rozdział białek z liści maliny moroszki dostarczył szczegółowych danych świadczących o polimorfizmie, jak również pozwolił opisać profil białkowy tego gatunku. Zebrane wyniki mogą w przyszłości być wykorzystane przy konstruowaniu mapy białkowej *Rubus chamaemorus*. W niniejszej pracy dyskutowano przyczyny powstałych różnic w obrazie białek liści roślin mnożonych w kulturach in vitro i kontrolnych młodych roślinkach otrzymanych z nasion.

SŁOWA KLUCZOWE: *Rubus chamaemorus*, kultury in vitro, kultury pędów, białka, dwukierunkowa elektroforeza. *