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Crambe aspera plants *in vitro* propagation and its effect on fatty acids and phenolic compounds content and genome stability

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In vitro culture can be used for endangered species preservation but its effect on biochemical properties and genetic stability of plants requires further research. **Aim.** The study on fatty acids content, phenolic compounds and phylogenetic relationships of *Crambe aspera* plants upon aseptic cultivation *in vitro*. **Methods.** Morphogenic potential of *Crambe aspera* leaf, root and petiole explants was studied in the Murashige and Skoog (MS) medium with different concentrations of growth factors. Fatty acids (FA) content was determined by gas chromatography-mass spectrophotometry of FA ethers, phenolic compounds were determined by spectrophotometry. Polymerase chain reactions were used to study phylogenetic relationships. **Results.** The efficient protocols of seed surface sterilization as well as methods of fast microclonal multiplication and obtention of C. aspera callus tissue for were developed. **Conclusions.** Leaves of both *in vitro* and *in vivo* cultured plants had a high content of α-linolenic acid whereas erucic acid was absent. At the same time, the difference in biochemical composition between the plants grown in aseptic and non-aseptic conditions was shown. *In vivo* populations of *C. aspera* showed a high level of polymorphism but its genome did not undergo changes after the *in vitro* establishment.

K e y w o r d s: *Crambe aspera*, regeneration, fatty acids, phenolic compounds, SSR and ISSR markers.

Introduction

Crambe aspera M. Bieb. can be used as a fodder crop, food and oil culture [1]. At the same time, it is listed in the Red data book of Ukraine (vulnerable) and requires conservation measures. Application of *in vitro* techniques results in a relatively high propagation coefficient even for the species with problematical *in situ* and *ex situ* reproduction. These methods

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provide long-term conservation of plant species outside their natural habitats (in seed banks, under introduction conditions, in cryobanks and *in vitro* collections) for thorough study and it can be a significant addition to the global system of plant biodiversity conservation [2]. *In vitro* cultivation may cause somaclonal variation in plants [3, 4] therefore the *C. aspera* genome study for possible considerable changes after *in vitro* establishment is advisable. The *Brassicaceae* plants biochemistry study is fairly important due to a high economic value of this family [5]. The long-chain FA analysis of green mass is important for the researches as these acids are the components or precursors of numerous specialized metabolites synthesized in specific cell types and widely used in medicine [6, 7]. Phenolic compounds are the plant metabolites that take part in growth, pathogenic resistance, predetermine different plant parts pigmentation, have antioxidant activity and protect membrane lipids from oxidation [8]. They also have a protective effect against the bacterial pathogens [9, 10]. Therefore, the determination of FA composition and phenolic compounds content in threatened native species grown *in vitro* can enlighten possible applications of the *C. aspera* plants.

Materials and Methods

The *C. aspera* seeds were used for initializing *in vitro* culture. The seed surface sterilization protocol established in our previous works was conducted to obtain the *C. aspera* aseptic culture [11].

The plants explants (leaf, petiole and root) *in vitro* morphogenic potential was studied on Murashige and Skoog (MS) medium [12] with the addition of 6-Benzylaminopurine (BA) (1–5 mg/L), Naphthaleneacetic acid (NAA) (0.1–1.5 mg/L), and kinetin (1–5 mg/L) in different combinations (Table 1). The callusogenesis was estimated (from no visible callus to more than 10 mm of callus all over the explant). Each experiment was repeated twice with three replicates

Regeneration frequency (RF) was estimated after 30–45 days and determined by formula:

$$RF = (Nr/N) \times 100\ \%,$$

where Nr – is a number of regenerated explants; N – number of explants used in the study of each medium [13].

Determination of phenolic compounds was conducted on ULAB 108UV spectrophotometer according to [14] for quantitative estima-

Table 1. **Media used for morphogenic potential study**

Growth regulators	Medium number and its growth regulators content, mg/L											
	№1	№2	№3	№4	№5	№6	№7	№8	№9	№10	№11	№12
BA	1	1	1	1	2.5	2.5	2.5	2.5	5	5	5	5
NAA	0.1	0.5	1	1.5	0.1	0.5	1	1.5	0.1	0.5	1	1.5
Growth regulators	Medium number and its growth regulators content, mg/L											
	№13	№14	№15	№16	№17	№18	№19	№20	№21	№22	№23	№24
Kinetin	1	1	1	1	2.5	2.5	2.5	2.5	5	5	5	5
NAA	0.1	0.5	1	1.5	0.1	0.5	1	1.5	0.1	0.5	1	1.5

tion of polyphenolic compounds content (with pirogalol used as a reference), according to [15] for hydroxycinnamic acids content (with chlorogenic acid used as a reference) and according to [16] for estimation of total amount of flavonoids (with luteolin used as a reference). Air dried leaves from plants grown *in vitro* (on hormone-free solid MS medium at 24°C with a 16-h photoperiod and recurrent transplantation on the fresh medium every 30 days) and *in vivo* (plant material was gathered in May with average monthly temperature of 21°C) were used.

For the gas chromatography-mass spectrophotometry (GC/MS) analyses fresh apical leaves from plants grown *in vitro* and *in vivo* were used. *In vivo* plants were grown in M.M. Gryshko National Botanic Garden and provided by Prof. Dzhamal B. Rakhmetov.

The FA extraction and methylation were carried out stepwise accordingly to R.°Garces [17]. FA composition was determined using GC/MS system Agilent 6890N/5973inert (Agilent Technologies, USA). Identification was performed by means of mass spectrum library NIST 02 and standard bacteria methyl fatty acid ethers solution (Supelco). Heptadecanoic acid (C17:0) (chemically pure, ABCR, Germany) was used as an inner standard.

All data were expressed as a mean (M) ± SD (m).

Molecular genetic analysis. Total DNA was isolated with the modified CTAB method [18]. Polymerase chain reaction (PCR) of 20 μl included 0.5 μM of forward and reverse primers (Metabion, Germany), 1 × Reaction Buffer B (Solis BioDyne, Estonia), 2 mM $MgCl_2$, 0.2 μM of each deoxyribonucleoside-3-phosphate (Thermo Fisher Scientific, USA), 1 unit of FIREPol® DNA Polymerase (Solis BioDyne, Estonia), 30 ng of purified total plant DNA.

The primer sequences for SSR loci *gHGL218091*, *gHGL110933*, *gH032602* [19], Na10-F08 [20] and ISSR primers UBC827, UBC864, UBC890 [21], A17898, B17899, IS-05 [22] were used in the study.

The PCR products were separated by electrophoresis in 1.5 % and 2 % agarose gels in lithium borate buffer, 0.1 μg/ml ethidium bromide [23, 24]. The gels were visualized in UV-light with a photosystem Canon EOS 600D. Phylogenetic relationship was calculated by the unweighed pair-group method using arithmetic averages cluster analysis (UPGMA) with the DARwin 6.0.010 software [25].

Results and Discussion

The applied method of seed surface sterilization provided 90 % of aseptic seed material, 46 % of those germinated in 2–4 days after the sterilization procedure.

There are no available data on the *C. aspera in vitro* microclonal propagation. Recent reports on *Crambe* species microclonal propagation were based on the hypocotyls and roots (rarely leaves) regeneration potential study [26–28].

Leaf explants grown on the medium containing 1 mg/L BA with 0.1–1.5 mg/L NAA (№1–4) had no plantlets formed during cultivation period. Regeneration occurred on the medium containing 2.5 mg/L BA with 0.1 mg/L NAA (№5) or 1.5 mg/L NAA (№8) (Fig. 1). Cultivation on the medium with kinetin in combination with 0.1–0.5 mg/L NAA (№13, 14, 18, 19, 21, 22) showed the regeneration

activity of leaves up to 40 %. Further increase in the NAA content suppressed the plantlets formation. At the same time, the leaf explants cultivated on the medium with kinetin and NAA (№13-24) had very high rhizogenic potential (11 %-100 %) in contrast with the ones grown in the BA presence (fig. 1). Callus proliferation was noted on all leaf explants cultivated on the medium with BA and NAA but only on cut ends (2.5 mm). The kinetin and NAA presence in the medium resulted in small callus proliferation at cut ends only.

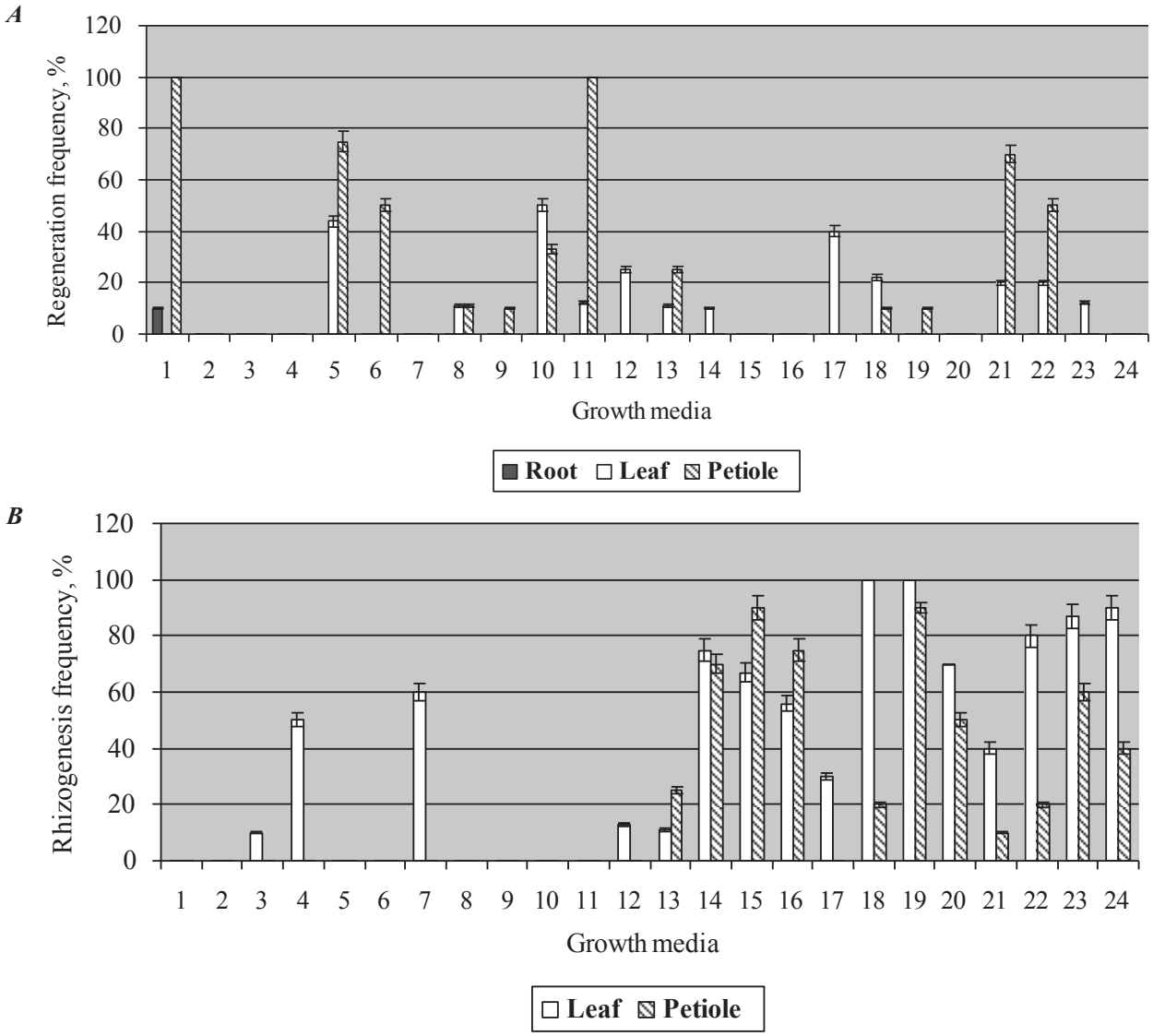

Fig. 1. *C. aspera* different explants morphogenesis on solid MS media supplemented with growth regulators: *A* – regeneration frequency; *B* – rhizogenesis frequency. Data shown as M ± m.

For another *Crambe* species *C. tataria* the leaf explants were reported to have high callusogenic activity and no ability to form plantlets on the MS medium with BA and NAA as well as with kinetin and NAA [28].

Petiole explants grown on the MS medium supplemented with BA and NAA showed high regeneration frequency (Fig. 1). Petiole explants cultivation on the medium with the lowest BA and NAA content (№1) resulted in the highest morphogenic activity – practically all used explants formed plantlets. A further increase of NAA content suppressed the plantlets formation. On the media №5, 6 (with BA 2.5mg/L and NAA 0.1-0.5 mg/L) petiole explants also had relatively high regeneration activity. 100 % of plantlets formation rates from petioles were observed on the medium №11 (5 mg/L BA and 1 mg/L NAA). Kinetin and NAA addition to MS medium resulted in a high rhizogenic activity of *C. aspera* petiole explants. The rhizogenic activity rate was directly proportional to the NAA content in media up to 1 mg/L (Fig. 3). The highest rhizogenic activity was observed on the medium №15 (1 mg/L kinetin and 1 mg/L NAA). Thus, the petiole regeneration study revealed its highest potential for fast microclonal propagation. Callus tissue was formed on most of the used petiole explants but for those grown on the media with kinetin addition the callus proliferation occurred only at the cut ends (rarely on a whole explant).

Root explants showed the lowest regeneration ability. However, the reports on other *Crambe* species *in vitro* propagation (*C. tataria* and *C. maritima*) show rather high organogenic activity of root explants on media containing auxins with cytokinins [27, 28]. Callus formation on the contrary was rather high for *C. aspera* roots grown on the media supplemented with BA and NAA (№1-12). On the media with kinetin and NAA (№13-24) callusogenesis occurred at cut ends only.

After the estimation of three types of explants regeneration ability, we assumed petioles to be the most promising material for fast microclonal propagation of *C. aspera* aseptic plants. The dissemblance in different plant genus and even habitats in response to the same growth regulators (with the use of same explants) has been previously reported [13, 29]. It can be explained by big influence of the plants genotype on regeneration – each genotype in the same conditions has various morphogenic response [13, 29]. Those findings suggest the necessity of further study on the content of *Crambe* species endogenous growth regulators in different plant parts.

Phenolic compounds content. Evaluation of different phenolic compounds in the plants grown *in vitro* and *in vivo* revealed great difference due to the cultivation in aseptic conditions (Fig. 2). The total amount of flavonoids for the *in vitro* cultured plants was approximately three times higher than for those grown *in vivo*. The quantitative estimation of polyphenolic compounds content revealed even greater difference – six times more polyphenols accumulated in the aseptic platns. Therefore, the study on hydroxycinnamic acids content showed the greatest increase in plants due to *in vitro* cultivation (Fig. 2). A number of phenolic compounds are the auxin synergists, they stimulate plant growth due to the Indolyl-3-acetic acid-oxidase inhibition [30]. The aseptic green mass for estimating phenolic compounds content was taken from *in vitro*

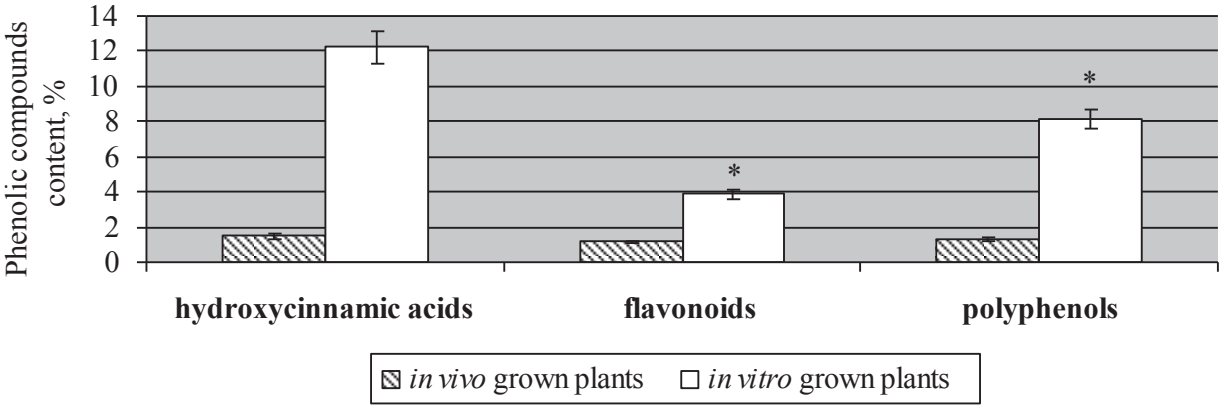

Fig. 2. Phenolic compounds content in *Crambe aspera* plants grown *in vitro* and *in vivo* (Data shown as M ± m; * – $p \leq 0.05$).

cultured plants previously cultivated on hormone-free MS medium for 30 days. Thus, we assume that only endogenous growth regulators were present in the plants. Therefore, as phenolic compounds stimulates plants growth it is clear that *in vitro* plants which are in the state of active growth and vegetation have higher content of all studied phenolic compounds than *in vivo* grown plants.

Fatty acid content. FA can be divided by the unsaturation degree into two groups: saturated (SFA) and unsaturated (USFA) (monounsaturated and polyunsaturated). FA ethers gas-spectra of the samples from leaves of the aseptic and not aseptic plants showed the presence of such SFA as lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0) and lignoceric acid (C24:0). Monounsaturated FA (oleic acid (C18:1 Δ9, ω 9) and polyunsaturated FA (linoleic (18:2 Δ9, 12, ω6), α-Linolenic (18:3 Δ9, 12, 15, ω 3) were detected. Leaf samples of aseptic plants had higher FA content (4.78 ± 1.93 mg/g) than not aseptic plants (3.56 ± 0.06 mg/g). Extracts from *in vitro* plants had almost twice as much USFA as SFA but extracts from *in vivo* plants had approximately three times more USFA than SFA. Therefore, we can assume that aseptic plants accumulate FA in higher amount than not aseptic ones and SFA to USFA ratio is different too (Table 2).

Table 2. **Total amount of FA, SFA, USFA in leaves of** *C. aspera* **grown** *in vitro* **and** *in vivo***.**

Fatty acids content	*In vivo* plants	*In vitro* plants
FA, mg/g	3.56±0.06*	4.78±1.93
SFA, mg/g	0.95 ± 0.03*	1.61 ± 0.37
USFA, mg/g	2.68 ± 0.10*	3.16 ± 1.56

Note: data shown as M ±m, * – $p \leq 0.05$.

Further FA ethers gas-spectra study showed [the] lauric acid presence in leaf samples (Fig. 3). While [the] *in vivo* plants had insignificant amount of C12:0 (0.34 ± 0.01 mol%) a part of lauric acid in [the] *in vitro* plants was higher (4.22 ± 2.02 mol%). Palmitic acid represented the majority of saturated FA in aseptic leaf samples (29.24 ± 3.72 mol%).

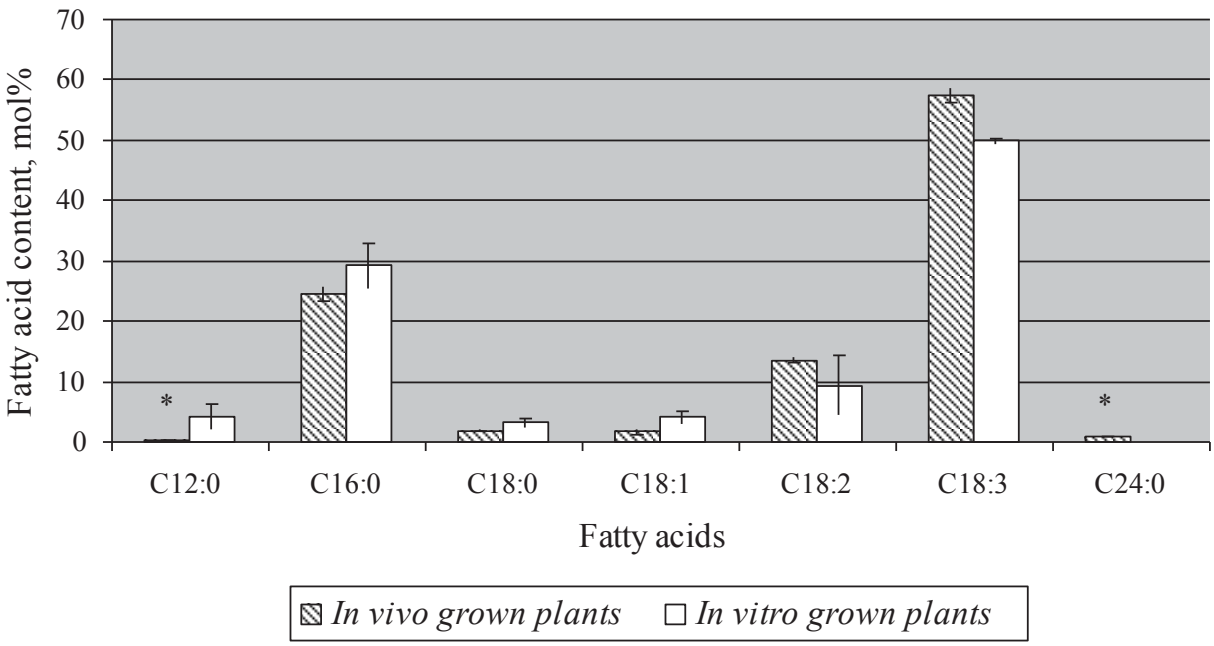

Fig. 3. Fatty acid content of *in vitro* and *in vivo* cultured *Crambe aspera* plants (Data shown as M ± m; * - $p \leq 0.05$).

The amount of C16:0 was also high for not aseptic plants (24.48 ± 1.22 mol%). Also, leaves had higher content of C18:0 (3.19 ± 0.81 mol% for *in vitro* and 1.92 ± 0.17 mol% for *in vivo* plants). Only leaf samples from *in vivo* cultivated plants had lignoceric acid (C24:0) (0.90 ± 0.12 mol%) while *in vitro* cultivated samples had no traces of that FA. The data from other research also show the presence of C24:0 in plants cultivated *in vivo* [31].

Some polyunsaturated FA were detected in leaf samples. Linoleic and α-Linolenic acids are essential fatty acids. Therefore, food with high content of these acids should be included in human diet [32, 33]. The amount of C18:2 in *C. aspera in vivo* grown plants was relatively high (13.54 ± 0.47 mol%) while aseptic plants contained 4.06 ± 1.12 mol% of C18:2 only. α-Linolenic acid content was the highest among all FA in the leaf samples for *in vivo* (57.37 ± 1.17 mol%) and *in vitro* (49.86 ± 0.39 mol%) cultured plants. Oleic acid content had the lowest amount of C18 FA group for both *in vitro* and *in vivo* plants (Fig. 3).

Molecular genetic analysis. In vitro cultivation could cause polyploidy, mutations or somaclonal variations due to different factors – long-term cultivation, type of explants, indirect organogenesis, medium composition, stress, *etc.* [3, 4]. There are practically no literature reports on the application of molecular markers to study the *in vitro* cultivation effect on the genome stability of *Crambe* plants. Therefore, the present evaluation of *C. aspera* plants from *in vitro* and *in vivo* with preselected 4 SSR and 7 ISSR markers [34] was conducted for the first time.

Table 3. **Characteristics of ISSR and SSR marker systems implemented for** *C. aspera* **genotyping.**

ISSR marker	Amplified bands	Polymorphic bands	Polymorphism, %*
A17898	10	6	60.00
B17899	8	8	100.00
HB10	-	-	-
IS-O5	13	10	79.92
UBC827	11	1	9.09
UBC864	11	3	27.27
UBC890	20	13	65.00
SSR marker			
gHGL218091	2	2	100
gHGL110933	3	3	100
gH032602	2	2	100
Na10-F08	3	3	100

Note: Polymorphism was calculated as the correlation between the number of polymorphic and amplified bands in percentages.

Most of the marker systems utilized in this study, remarkably well, formed clear and reproducible bands. The ISSR and SSR primers provided amplification of DNA fragments within 270-2300 base pairs (bp) and 80-190 bp, respectively. In this case, the ISSR primers generated 73 amplified fragments, among which 41 were polymorphic. The SSR primers produced 7 polymorphic amplified fragments (Table 3). The sequences A17898, B17899 and UBC890 were the most informative among the ISSR markers for genotyping *C. aspera.* Primer HB10 did not amplify the DNA clearly.

The results of ISSR and SSR analyses were used in the study on genetic relationship for 3 *in vivo* and 7 *in vitro C. aspera* genotypes. The denrogram (Fig. 4) was assembled by means of the UPGMA method using DARwin software.

According to the dendrogram the genotypes were distributed into two main clusters. The first cluster group included 7 genotypes from *in vitro* and a genotype from *in vivo*; the second – 2 genotypes from *in vivo*. Seven genotypes obtained from *in vitro* were identical and formed a separate subcluster group.

High level of polymorphism in *C. aspera* plants is common for wild populations which was established in other researches [35, 36]. However, the results obtained by us denote that *C. aspera* genome does not undergo de-

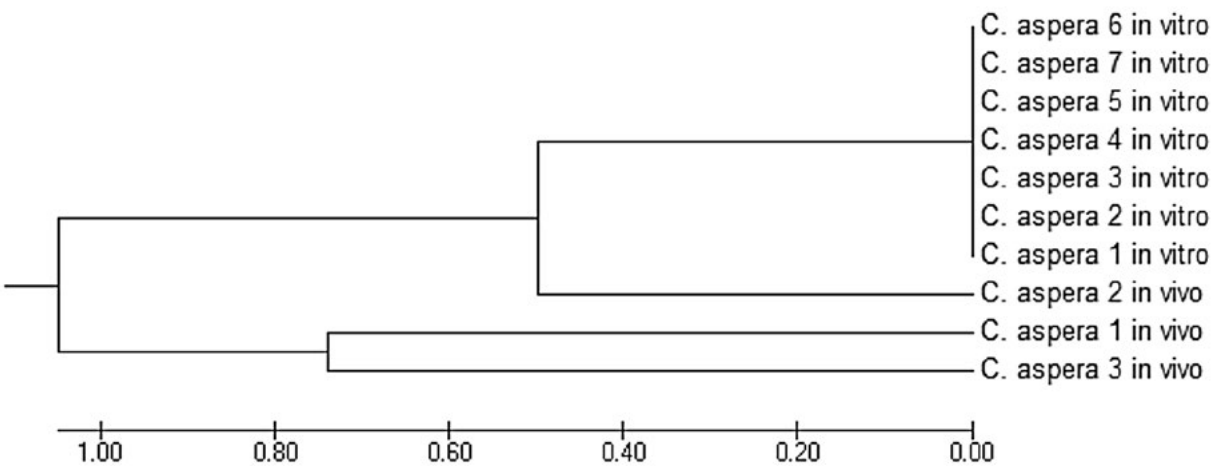

Fig. 4. Dendrogram based on the ISSR and SSR analyses for 10 *C. aspera* genotypes.

tectable changes after *in vitro* establishment under optimal conditions.

Conclusions

The study on morphogenic potential of leaf, petiole and root explant showed that the use of petiole for the fast microclonal propagation was highly advisable due to its greatest potential to form plantlets (MS with BA 1 mg/L and NAA 0.1 mg/L or MS with BA 5 mg/L and NAA 1 mg/L).

C. aspera plants grown *in vitro* and *in vivo* leaf samples FA ethers gas-spectrum analysis suggested that the aseptic conditions benefited to SFA accumulation but reduced the USFA amount in plant material. *In vitro* cultivation causes an increase of polyphenolic compounds and hydroxycinnamic acids content as well as an increase of total amount of flavonoids in *C. aspera* plants.

For the first time, 6 ISSR and 4 SSR markers were validated for *C. aspera* genotyping. The obtained data confirmed that the *in vitro* methods could be surely applied for biodiversity conservation as they did not cause any detectable genome changes in *Crambe aspera* under optimal conditions.

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Вплив розмноження *in vitro* рослин виду *Crambe aspera* на вміст жирних кислот, фенольних сполук та генетичну стабільність

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Застосування культури *in vitro* для збереження рідкісних видів має ряд переваг. **Мета.** Дослідження вмісту жирних кислот, фенольних сполук та філогенетичних зв'язків рослин *Crambe aspera* може допомогти оцінити вплив культивування *in vitro.* **Методи.** Було досліджено морфогенний потенціал листкових, кореневих та черешкових експлантів *Crambe aspera* на середовищі MS з різним вмістом регуляторів росту. Вміст жирних кислот визначали за допомогою газової хроматомас спектрометрії ефірів жирних кислот. Вміст фенольних сполук визначали за допомогою спектрофотометра. Полімеразна ланцюгова реакція була застосована для дослідження філогенетичних зв'язків. **Результати.** Було встановлено ефективні протоколи поверхневої стерилізації насіння разом з методами швидкого мікроклонального розмноження та отримання калюсної культури для виду *C. aspera.* **Висновки.** Отримані результати свідчать про високий вміст α-ліноленової та відсутність ерукової кислоти у листках рослин що культивували як *in vitro,* так і *in vivo*. В той же час, було встановлено різницю біохімічного складу між рослинами з асептичних та грунтових умов. Популяції *C. aspera* що зростали в умовах *in vivo* показали високий рівень поліморфізму але їх геном після введення культуру *in vitro* був стабільним.

Ключові слова: *Crambe aspera,* регенерація *in vitro*, жирні кислоти, фенольні сполуки, SSR- та ISSR-маркери.

Вплив розмноження *in vitro* рослин виду *Crambe aspera* на вміст жирних кислот, фенольних сполук та генетичну стабільність

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Использование культуры *in vitro* с целью сохранения редких видов имеет ряд преимуществ. **Цель.** Исследование состава жирных кислот, фенольных соединений и филогенетических связей растений *Crambe aspera* может помочь в оценке влияния культивирования *in vitro*. **Методы.** Было установлено морфогенный потенциал листовых, корешковых и черешковых эксплантов *Crambe aspera* на среде MS с различным составом регуляторов роста. Содержание жирных кислот определяли с помощью газовой хромато-масс спектрометрии эфиров жирных кислот. Содержание фенольных соединений определяли на спектрофотомере. Полимеразная цепная реакция была использована для исследования филогенетических связей. **Результаты.** Было установлено эффективные протоколы поверхностной стерилизации семян вместе с методами быстрого микроклонального размножения и получения калюсной культуры для вида *C. aspera.* **Выводы.** Полученные результаты свидетельствуют о высоком содержании α-линоленовой и отсутствии эруковой кислоты в листьях растений культивируемых как *in vitro,* так и *in vivo*. В то же время была установлена разница биохимического состава между растениями из асептических и грунтовых условий. Популяции *C. aspera* произрастающие в условиях *in vivo* показали высокий уровень полиморфизма, но их геном после введения в культуру *in vitro* был стабильным.

Ключевые слова: *Crambe aspera,* регенерация *in vitro*, жирные кислоты, фенольные соединения, SSR- и ISSR-маркеры.

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