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CRAMBE TATARIA SEBEÓK SEEDS AND PLANTS GROWN *IN VITRO* AND *IN VIVO* FATTY ACID COMPOSITION COMPARISON

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ABSTRACT

Methods of *in vitro* conservation offer a number of advantages for endangered species preservation. *Crambe tataria* Sebeók biochemistry study (fatty acid (FA) composition, antioxidant activity (AOA), polyfructan and total soluble protein content) is fairly importaint and could show the potential value of this species in agriculture, Food and Chemical Industry or pharmacology including its use as a source of valuble genetic material and could lead to new promising sources of biofuel discovery. Also, comparison of *in vitro* and *in vivo* cultured plants could point out to the effect of *in vitro* culture methods on plants biochemical composition Fatty acid (FA) content was determined using Gas chromatography-mass spectrophotometry (GC/MS) of fatty acid ethers. Antioxidant activity was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. Total soluble protein content was measured using Bradford method and polyfructan content determination was based upon ketosugars ability to color in the acidic environment with resorcinol. Plants that were grown under *in vitro* and *in vivo* conditions and seeds were used in this research. Obtained data showed that *C. tataria* plants had high AOA and total soluble protein content along with high total FA content along with high content of α-Linolenic acid and absence of erucic acid. Difference in biochemical composition between plants grown in aseptic and not aseptic conditions was shown.

Keywords: threatened species; *in vitro* culture; GC/MS mass spectrophotometry; fatty acids

INTRODUCTION

A number of *Brassicaceae* family plant species is known to play a significant role in the global oil and biofuel production **(Xiao-qin et al., 2011)**. Very-long-chain FA analysis is an important task for the researches as these acids are components or precursors of numerous specialized metabolites synthesized in specific cell types **(Haslam and Kunst, 2013)**. FA is also essential for plant natural processes and is widely used in medicine **(Taylor et al., 2009)**.

Biodiesel is manufactured mostly from vegetable oil (rapeseed, palm and coconut oils). It has some benefits over traditional types of fuel – it isn't toxic, it decomposes under natural conditions in relatively short term, has almost no sulfur and benzene and is obtained from renewable materials. Biodiesel has good environmental properties (lower emissions are produced by the combustion of biodiesel than for diesel) **(Angelovič et al., 2013)**. Taking into account all the benefits of the biofuel its active investigation and application in the USA, Japan, China, Canada and in the EU countries is understandable. International Energy Association predicts the rise of biofuel production up to 150 million tons of the oil energy equivalent till 2030. Ukraine is in beneficial conditions for biofuel production from agricultural materials. Total biodiesel volume that can be manufactured in Ukraine could reach 500 thousand tons that can ensure 60% of our country fuel needs and 10% of petrol needs **(Medvedkova and Trudayeva, 2013)**. Qualitative physico-chemical properties of vegetable oils used for biodiesel production study **(Angelovič at al., 2015)** often excludes potential sources of biofuel from mass production so a search of new effective sources of high quality biofuel is fairly important.

The *Crambe* L. genus belongs to *Brassicaceae* family and consists of about 29 species. These species are annual, biennial or perennial and have diverse application: as vegetable or forage plants, as oilseed, as the source of biofuels (seeds have up to 60% of erucic acid), in Food Industry for making pastry, in paint and varnish industry, in chemical industry **(Prakhova, 2013)**. All studies on *Crambe sp.* were focused on *C. abyssinica* cultures which is proven to be a valuable biofuel source because it doesn't hybridize with any known oilseeds which eliminates gene flow issues **(Gonçalves et al., 2013)**; its seeds have high content of slowly-drying oil with low Iodine number, rich with erucic acid; it has high crop capacity and low demands for soil quality; it is drought-resistant and has short vegetation period **(Askew, 1993)**.

A number of species of *Crambe* L. genus is threatened so they require conservation measures. *In vitro* techniques need a small number of plants and result in a relatively high propagation coefficient even for the species which have problematical *in situ* and *ex situ* reproduction and do not depend on the climatic conditions. These methods provide a long time conservation of plant species outside their natural habitats (in seed banks, under introduction conditions, in *in vitro* collections and cryobanks) with thorough study possibility and can be significant addition

to the global plant biodiversity conservation system **(Belokurova, 2010)**. *In vitro* and *in vivo* cultivated plants biochemistry comparison could lead to better understanding of the aseptic conditions influence on plant secondary metabolites synthesis and accumulation processes and could also estimate the efficiency of *in vitro* culture methods application for fast gaining of raw materials with high content of secondary metabolites.

The aim of this study is to determine *C. tataria* oil FA composition (from seeds and plant grown under *in vitro* and *in vivo* conditions) and study the effect of aseptic conditions on plant biochemical composition.

MATERIAL AND METHODOLOGY

Crambe tataria (*Brassicaceae* family) from the flora of Ukraine was used for this research. *C. tataria* is listed in the Red data book of Ukraine (Vulnerable) **(Didukh, 2009)**. For GC/MS analyses seeds and fresh apical leaves from plants grown under *in vitro* (on hormone-free solid MS medium **(Murashige and Skoog, 1962)** 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 (average monthly temperature 21 °C)) conditions were used. *In vivo* plants were grown in M.M. Gryshko National Botanical Garden of National Academy of Sciences of Ukraine and provided by prof. D. B. Rakhmetov. Seeds and *in vitro* plants were obtained from seed bank and *in vitro* collection of The Institute of Cell Biology and Genetic Engineering of National Academy of Science of Ukraine and also provided by research assistant (M. S. Kalista) of National Museum of Natural History of National Academy of Sciences of Ukraine.

Gas Chromatography-Mass Spectrophotometry of fatty acid ethers

FA extraction and methylation were conducted stepwise accordingly to **(Garces and Mancha, 1993)**. 50 mg of seeds and 200 mg of fresh leaves were used for extracts preparation. Seeds samples were ground in pounder and leaves were cut with defatted scissors. Then material was moved to glass tubes with spin caps and teflon gaskets. Reaction mixture which consisted of methanol: toluene: sulfuric acid (volume ratio 44:20:2) was added to the plant material first. Then, 1.7 mL of hexane was added (methanol, toluene, hexane – HPLC-grade, Sigma-Aldrich, Germany; sulfuric acid – chemically pure, Alfarus, Ukraine). Tubes were kept in water bath at 80 °C for 2 hours and then after cooling down to room temperature were gently shaken which led to separation of the liquid into two phases. Upper phase, which contained concentrated methyl fatty acid ethers, was gathered. The acidity of the solution was adjusted to neutral pH with saturated solution of 1M sodium phosphate. FA composition was determined using GC/MS system Agilent 6890N/5973inert (Agilent Technologies, USA) with capillary column DB-FFAP (length – 30 m; inner diameter – 0.25 mm; stationary phase thickness – 0.25 µm). Chromatographic fractionation occurred in gradient mode from 150 °C to 220 °C with a temperature gradient of 2 $°C.min^{-1}$. Helium was used as a carrier gas with flow rate of 1 $mL.min^{-1}$. Identification was done using 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 ±SD.

Acil-lipid ω9, ω6 and ω3 desaturases activity and saturation degree estimation

For saturation degree estimation in leaves and seeds the index of saturation (double bound index – DBI) was used **(Lyons, Wheaton and Pratt, 1964)**:

$$DBI = \sum Pjn/100,$$

where: Pj – the amount of FA (mol %), n – the number of double bounds in every unsaturated FA. The unsaturation index (K) – the ratio between total amount of unsaturated FA (UFA) and total amount of saturated FA (SFA) is also used. Acil-lipid ω9, ω6 and ω3 desaturases activity, which catalyzes formation of double bonds into the carbon chain of Oleic (C18:1), Linoleic (C18:2) and α-Linolenic (C18:3) acids respectively was determined by stearic- (SDR), oleic- (ODR) and linoleic- (LDR) desaturases ratio. These ratios were calculated by amount (mol% of total FA content) of C18 components:

$$SDR = (C18{:}1) / (C18{:}0 + C18{:}1)$$
$$ODR = (C18{:}2 + C18{:}3) / (C18{:}1 + C18{:}2 + C18{:}3)$$
$$LDR = (C18{:}3) / (C18{:}3 + C18{:}2),$$

where: C18:0, C18:1, C18:2 i C18:3 – mol% amount of Stearic (C18:0), Oleic (C18:1), Linoleic (C18:2) and α - Linolenic (C18:3) acids **(Jaworski and Stumpf, 1974)**.

Antioxidant activity, total soluble protein and polyfructan content

Fresh apical leaves from plants grown under *in vivo* and standard *in vitro* conditions were weighed (200 mg), homogenized with distilled water (0,7 mL) and centrifuged at 10000 g for 10 min. 100 µL of supernatant was taken and mixed with 100 µL of 0.1% alcohol solution of resorcinol (chemically pure, Alfarus, Ukraine) and with 100 µL of concentrated hydrochloric acid (chemically pure, Alfarus, Ukraine). Extraction of polyfructan was held in water bath at +80 °C for 20 min and measured on spectrophotometer (550 nm) (Eppendorf, USA). Calibration was made using fructose **(Maznik and Matvieieva, 2013)**. Antioxidant activity of extracts was measured by DPPH radical scavenging method **(Brand-Williams, Cuvelier and Berset, 1995; Adámková, Kouřimská and Kadlecová, 2015)**. Bradford method was used for total soluble protein determination **(Bradford, 1976)**.

RESULTS AND DISCUSSION

FA can be divided by the unsaturation degree into 2 groups: saturated (SFA) and unsaturated (USFA) (monounsaturated and polyunsaturated). GC/MS of the samples from seeds and leaves grown *in vitro* and *in vivo* showed presence of SFA (Lauric acid (C12:0), Palmitic acid (C16:0) and Stearic acid (C18:0); monounsaturated FA (Oleic acid (C18:1 Δ9, ω 9); polyunsaturated FA (Linoleic acid (18:2 Δ9, 12, ω 6) and α-Linolenic acid

Table 1 *Crambe tataria* seeds and plants grown *in vitro* and *in vivo* total amount of FA, saturation degree, unsaturation index and acil-lipid ω9, ω6 and ω3 desaturases activity estimation[1].

Indexes	*Crambe tataria* samples		
	***In vivo* leaf**	***In vitro* leaf**	**Seed**
FA, $mg.g^{-1}$ ±SD	2.65 ±0.09*	6.09 ±0.51	545.91 ±24.23
SFA, $mg.g^{-1}$ ±SD	0.67 ±0.04*	1.94 ±0.10	16.31 ±1.43
USFA, $mg.g^{-1}$ ±SD	1.95 ±0.11*	4.14 ±0.41	529.59 ±25.63
K ±SD	2.95 ±0.35	2.13 ±0.10	32.72 ±4.60
DBI ±SD	2.02 ±0.08*	1.81 ±0.009*	1.33 ±0.03*
SDR ±SD	0.33 ±0.03*	0.52 ±0.01*	0.98 ±0.008*
ODR ±SD	0.98 ±0.002*	0.94 ±0.009*	0.50 ±0.04*
LDR ±SD	0.80 ±0.017*	0.81 ±0.08*	0.24 ±0.04*

Note: total fatty acids (FA), saturated FA (SFA) and unsaturated FA (USFA) amount; saturation degree (DBI), unsaturation index (K) and acil-lipid ω9, ω6 and ω3 desaturases activity (SDR, ODR and LDR respectively).
* – significance level ($p < 0.05$).

(18:3 Δ9, 12, 15, ω3)). Only seed samples had Paullinic acid (C20:1 Δ13, ω7), Erucic acid (C22:1 Δ13, ω 9) presence. Nervonic acid (C24:1 Δ15, ω 9) was detected only in aseptic plants and Lignoceric acid (C24:0) only in not aseptic plants (Figure 1).

Total FA amount in seed and leaves was quite different (Table 1). *C. tataria* seeds had high content of total FA. Total FA amount in plants grown under in vitro conditions was more than two times higher than FA amount in plants grown *in vivo*. USFA total amount in seeds was 32 times higher than SFA (K – 32.72 ±4.60) but DBI was rather low (DBI – 1.33 ±0.03). Extracts from *C. tataria* plants grown *in vivo* had almost 3 times higher total amount of USFA then SFA (K – 2,95 ±0.35) and *in vitro* leaf samples had 2 times higher USFA amount then SFA (K – 2.13 ±0.10). It shows that aseptic plants had higher ratio of USFA than SFA compared to not aseptic plants. The DBI was higher for *in vivo* cultured plants then for plants grown *in vitro* as well (Table 1).Calculated SDR, ODR and LDR coefficient showed relevant desaturases (ω9, ω6, ω3) activity level. The activity of ω9 – desaturase that provides first double bound input was the lowest among others desaturases activity in aseptic plant samples, and its highest activity was determined in seed samples. ODR and LDR coefficients were very high in leaves of both aseptic and not aseptic plants that indicate high ω6- and ω3-desaturase activity but low in seeds. According to SDR, ODR and LDR coefficient that was calculated ω9-, ω6- and ω3-desaturases activity in plants grown in different conditions and seeds was different. Samples from seeds showed high ω9-desaturase and low ω6- and ω3-desaturases activity while samples from leaves, on the contrary, showed high ω6- and ω3-desaturases and low ω9-desaturase activity (Table 1).

Further FA ethers gas-spectrums of the samples from seeds and leaves study showed that Lauric acid was present in all seed and leaves samples. While seeds and *in vivo* plants had insignificant amount of C12:0 (seeds – 0.37 ±0.04 mol%; *in vivo* grown plants – 0.42 ±0.03 mol%) a part of Lauric acid in leaf samples from aseptic plants was higher (2.84 ±0.09 mol%). Palmitic acid had the biggest share of SFA content. It's amount was low in seeds – 2.80 ±0.11 mol% but leaf samples, on the contrarily, had high and almost equal content of C16:0

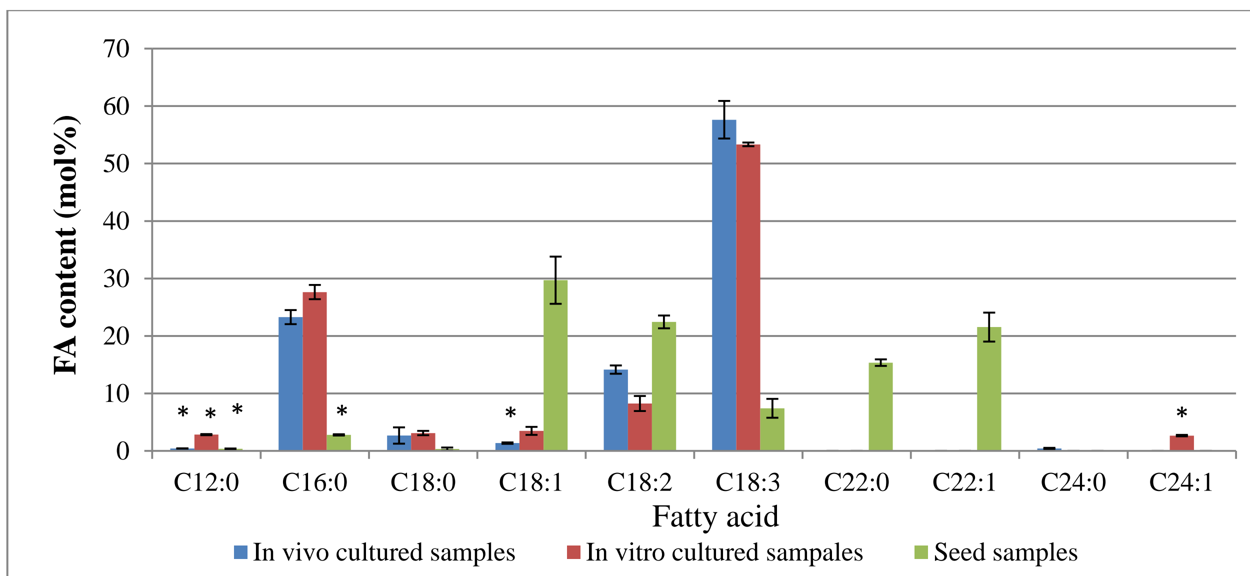

Figure 1 *Crambe tataria* seeds and plants grown *in vitro* and *in vivo* FA content comparison.
* significance level ($p < 0.05$).

(aseptic plants – 27.63 ±1.24 mol% and not aseptic plants – 23.29 ±1.22 mol%) (Figure 1).

Leaf samples had higher amount of C18:0 then seed samples (seeds – 0.33 ±0.26 mol%, aseptic plants – 3.10 ±0.37 mol%; not aseptic plants – 2.68 ±1.41 mol%) though its share in aseptic plants was little higher than in not aseptic ones.

Unsaturated FA have a preventive effect on the many diseases caused by modern lifestyles. It reduces the risk of cardiovascular diseases, modifies blood pressure, improves blood vessels elasticity and reduces the level of cholesterol and blood coagulation **(Frančáková et al., 2015)**. In seeds of studied species the biggest share of all FA had Oleic acid but in leaves its content was low (samples from seeds had 29.71 ±4.10 mol% of C18:1). *In vitro* cultured plants accumulated more than twice as much Oleic acid (3.49 ±0.69 mol%) as plants cultivated in the botanical garden (1.37 ±0.12 mol%) (Figure 1).

Seed samples had high content of monounsaturated omega-9 fatty acids – Gondoic acid (C20:1) and Erucic acid (C22:1) (Figure 1) while leaves from both aseptic and not aseptic plants had no traces of these acids. Those acids are harmful to human and animal health, so their absence in leaves samples indicates the possible use of *Crambe* species green mass as forage crops. At the same time, the use of their seeds as forage crops is not advisable due to high content of C22:1. The Erucic acid content in seed samples (21.54 ±2.52 mol%) was little lower then it was previously reported for *C. tataria* (27.0 – 29.87%) **(Rudloff and Wang, 2011)**. Gondoic acid content in seeds was high (15.35 ±0.56 mol%) compared to previously reported for the same species (7.70 – 21.0%) **(Rudloff and Wang, 2011)**. Some polyunsaturated FA were detected in *C. tataria* seed and leaf samples. Linoleic and α-Linolenic acids are essential fatty acids so foods with high content of these acids should be included in human diet **(Frančáková et al., 2015)**. The biggest amount of C18:2 was detected in *C. tataria* seed samples (22.45 ±1.11 mol%). Though *in vivo* grown plants had quite high amount of this FA (14.15 ±0.72 mol%) *in vitro* cultivated plants had the lowest C18:2 share of all studied samples (8.25 ±1.30 mol%). α-Linolenic acid content had the biggest share among SFA and UNFA in leaf samples from plants cultivated in both *in vitro* and *in vivo* conditions and its amount was approximately equal in both aseptic and not aseptic plants (*in vitro* plants – 53.32 ±0.32 mol% and *in vivo* plants – 57.62 ±3.27 mol%). In seed samples C18:3 content was rather low – 7.42 ±1.64 mol%.

Nervonic acid has a wide range application. It's used for ssymptomatic treatment De-myelinating diseases, Multiple Sclerosis, Parkinson, Schizophrenia, deficiencies associated with neurological disorders (Alzheimer's). Also it has many applications in nutritional markets (senility, memory aid etc.), for treatments of arthritis, liver diseases and obesity and is used as dietary supplements (in baby foods and formulas, in pre-term babies nutrition, in diet for pregnant or lactating women and in enriched energy supplements with neuro-protective effect for high-level training adults/athletes) **(Sandhir, Khan, Chahal and Singh, 1998; Taylor et al., 2010; Sargent, Coupland, and Wilson, 1994)**. Nervonic acid (C24:1) was detected only in aseptic plants and its content was rather low – 2.66 ±0.13 mol% but due to the high value of this FA this data suggest possible use of the studied *C. tataria* species as biotechnological object for C24:1 production.

Antioxidant activity, total soluble protein and polyfructan content

In vivo leaf samples had higher total soluble protein content (6.41 ±1.05 mg.g^{-1} of fresh weight) comparing to *in vitro* samples (4.50 ±0.10 mg.g^{-1}). It is interesting that for the most commonly used species of *Brassicaceae* family that were grown *in vivo*, total protein content was lower than for *Crambe* species also grown in not aseptic conditions (4.8 mg.g^{-1} of fresh weight for broccoli) **(Campbell et al, 2012)**.

AOA for *C. tataria* both *in vitro* and *in vivo* samples was very high comparing to the AOA of ascorbic acid solution (1 mg.mL^{-1} – 98.22%). Both aseptic and not aseptic leaf samples showed very high AOA (100%) which is higher than ascorbic solution that was used as a reference.

While AOA and total soluble protein content was roughly equal for both *in vitro* and *in vivo* plants their polyfructan content on the contrary was much different: aseptic plants – 8.17 ±3.98 mg.g^{-1} and not asepticplants – 1.47 ±0.48 mg.g^{-1}.

CONCLUSION

GC/MS of the samples from seeds and leaves study reviled an impact of aseptic conditions on the FA composition of studied *C. tataria* species. Quantitative FA analysis shows difference between plants grown in aseptic and not aseptic conditions and also suggests that aseptic conditions benefits to SFA accumulation and increases polyfructan content on the other hand it reduces USFA amount and so as plant material protein content. The study of ω9-, ω6- and ω3- desaturases activity showed that seed samples had high ω9- desaturase activity but very low ω6- and ω3-desaturases activity. Samples from leaves on the contrary showed high ω6- and ω3- desaturases activity and low ω9- desaturase activity.

The dominant FA in leaves samples was α-Linolenic acid and *in vitro* cultured plants had its highest content. The dominant FA of all present in seed samples was Oleic acid. *C. tataria* seeds had rather low content of erucic acid but it still could be as a source of biofuel. On the contrary Erucic acid hasn't been detected in both aseptic and not aseptic plant samples.

Determination of AOA showed very high potential of *C.tataria* as a source of natural antioxidants and could also point out to medical application of this species. Both *in vitro* and *in vivo* plants had almost equal AOA and total soluble protein content, but aseptic plants polyfructan amount was more than 5 times higher comparing to not aseptic plants. Therefore, we can assume that aseptic conditions can benefit to SFA synthesis and increase polyfructan content of *C. tataria* plants.

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