RAPD MARKERS ASSOCIATED WITH LINOLENIC ACID SYNTHESIS IN SEVERAL *Boraginaceae* PLANT SPECIES

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Abstract: The polyunsaturated fatty acids are highly recommended in the human diet, but unfortunately they are not synthetized *de novo* in mammals and are almost exclusively plant derived. Some plant species contain high levels of polyunsaturated fatty acids, therefore this study aimed the investigation of several *Boraginaceae* species from the Romanian cultivated and spontaneous flora by RAPD markers associated with linolenic acid synthesis. Eight RAPD primers previously employed for *Brassica rapa* ssp. *oleifera* were applicable for *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis* but were not suitable for *Nonea lutea*. The analyses showed that most primers generated polymorphic patterns in all species, while the number of bands was generally higher than in *Brassica rapa* ssp. *oleifera* and the fragments size was different as well. The primers OPB-18, OPG-16, OPH-11, OPH-12, OPI-14,OPJ-20, OPK-20, OPL-03 and OPP-19 generated at least two fragments in most of the *Boraginaceae* species, but in *Brassica rapa* ssp. *oleifera* only the primers OPB-18, OPH-11 and OPH-12 generated two fragments. These RAPD markers are associated with the *fad3*gene, the predominant gene responsible for the synthesis of linolenic acid in seeds. Thus, such markers, could be valuable tools for a rapid screening of plant species producing linolenic acid or other polyunsaturated fatty acids as biochemical analysis of these compounds is difficult to achieve.

Keywords: PUFA; linolenic acid; RAPD markers; *Cynoglossum officinale*; *Echium vulgare*; *Anchusa officinalis*; *Nonea lutea*.

INTRODUCTION

Dietary fats and oils represent a significant percentage of the daily caloric intake comprising 33% of total calories. The polyunsaturated fatty acids (PUFA) - α linolenic acid (ALA; ω-3; 18:3; 9,12,15-octadecatrienoic acid) and linoleic acid (LA; ω-6; 18:2; 9,12-octadecadienoic acid) are known to be essential for humans [5].

Numerous studies documented the connection of dietary fats and the development of some diseases, including cardiovascular diseases and stroke [2, 16, 18], atherosclerosis [20], cancer [17], arthritis [15, 21] and different dermatological diseases [28]. In fact, some studies showed that a diet rich in omega-3 fatty acids not only lowers bad cholesterol, known as LDL, but also lowers triglycerides that circulate in the blood [16]. Not surprisingly, different health organizations have recently made dietary recommendations that focused not only on the quantity but also on the types of fats included in the diet recommending substitution of saturated fatty acids (SFA) with monounsaturated and polyunsaturated fats (PUFA) [26], knowing that PUFA are not synthesized de novo in mammals, they must be derived from diet [27]. Dietary PUFA are mostly plant derived, where they are produced from saturated fatty acids (SFA). SFA are progressively desaturated to form monosaturated fatty acid-oleic acid (OA) [18:1(n-9)] and polyunsaturated acids-linoleic acid (LA) [18:2(n-6)] and linolenic acid (ALA) [18:3(n-3)]. According to the position of the first double bond in the fatty acid molecule, polyunsaturated acids are classified in omega-3 (ω-3), omega-6 (ω-6) and omega-9 (ω-9) fatty acids.

The essential fatty acids are all omega-3 and omega-6 methylene-interrupted fatty acids. The PUFA biosynthetic pathway occurs in all plant cells, hence, omega-6 and omega-3 fatty acids are present in varying proportions in leaves or seeds [1, 26].

Gamma-linolenic acid (GLA) known as an essential fatty acid for human diet (with delta-6-desaturase deficiency) and is a precursor of prostaglandins, prostacyclins and thromboxanes with a demonstrated anti-inflammatory and antitumoral effect. Only few seed oils contain GLA despite the high contents of the precursor linoleic acid (from which it is obtained by dehydrogenation). For example, borage (*Borago officinalis*) [13], evening primrose (*Oenothera biennis*) [3] and black currants (*Ribes nigrum*) [4, 12, 24] are among the few plants that produce appreciable amounts of linolenic acid, but only *Oenothera* and *Borago* are cultivated as commercial source for GLA. There are several plants species, among them *Ribes nigrum* L., which contains glycerolipids in leaves, but their composition is considered unusual in that alpha-linolenic acid (α-18:3) occurs together with cis-7,10,13-hexadecatrienoic acid (16:3) and lower amounts of stearidonic acid (18:4) and gamma-linolenic acid (γ-18:3) [27]. Their leaves also contain other compounds, such as flavonoids that prevent peroxidation of polyunsaturated fatty acids. Thus, those species containing both unsaturated fatty acids and antioxidants are important in the pharmaceutical biotechnology.

Due to the complex methodology for PUFA content determination in different tissue types it is difficult to use this kind of analysis for a rapid screening of germplasm. However, this could be easily achieved by

the use of molecular markers linked to linolenic acid content, such as RFLPs (restriction fragment length polymorphisms), RAPDs (random-amplified polymorphic DNAs) or SCARs (sequence-characterized amplified regions). RAPD markers have been employed in many genetic studies because of their speed and simplicity and mostly of the universal primers which make them useful in different genomes analysis [25].

The main objective of this study was to test several RAPD markers associated with the linolenic acid content in leaves of some *Boraginaceae* species in order to find a panel of markers serving as valuable tools for rapid screening of germplasm collections comprising a wide range of plant species.

MATERIALS AND METHODS

Plant material

The plant material, represented by seeds of *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis* and *Nonea lutea* was provided by the Alexandru Borza Botanical Garden, from Cluj-Napoca, Romania. *Nonea lutea* is a spontaneous species in Russia and Caucasus, while in the rest of Europe is cultivated and occasionally become subspontaneous [19]. The other three species are spontaneous, xerophilous, considered weeds and are characteristic for two orders or alliances of *Artemisietea vulgaris* class [6], consists of ruderal xerophilous communities dominated by biennial or perennial herbs. *Echium vulgare* has a broad coenological distribution, being common to all herbaceous anthropogenic communities, from the plains to hills, rarely in the submontane belt and is considered as diagnostic species for the *Dauco-Melilotion* alliance. *Cynoglossum officinale* has an Eurasiatic distribution and it is characteristic for *Onopordion acanthii* alliance, while *Anchusa officinalis* is an European species characteristic for the *Onopordetalia* order [6]. Seeds were germinated in soil and leaves harvested from 20 different plants were used for molecular analysis.

RAPD analysis

Genomic DNA was isolated from leaves using the CTAB method described by Doyle and Doyle [7]. For RAPD analysis a total of eight primers previously used in *Brassica rapa* ssp. *oleifera* [26] were used (Table 1). PCR amplifications were performed in 0.2 ml tubes containing 2 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP, 1.5 U of Taq polymerase (Fermentas) and 25 ng of genomic DNA in a final volume of 25 µL. DNA amplification was performed according to the following program: 1. initial denaturation, T = 94°C, 5 min; 2. T = 94°C, 30 s; 3. primer annealing at 34°C, 30 s; 4. elongation T = 72°C, 45 s; the steps 2-4 were repeated 35 times. Amplicons were separated on 1.5% agarose gel, stained with 0.5 µg ml^{-1} ethidium bromide. DNA markers (Thermo Scientific) with 200, 500, and 1000 bp were used as control. At least 2 independent PCR amplifications were performed for each primer.

Table 1. Characteristics of RAPD primers used for DNA amplification [26]

Primer	Sequence 5'→3'
OPB-12	CCTTGACGCA
OPB-18	CCACAGCAGT
OPB-20	GGACCCTTAC
OPG-16	AGCGTCCTCC
OPH-11	CTTCCGCAGT
OPH-12	ACGCGCATGT
OPI-14	TGACGGCGGT
OPJ-20	AAGCGGCCTC
OPK-20	GTGTCGCGAG
OPL-03	CCAGCAGCTT
OPP-17	TGACCCGCCT
OPP-19	GGGAAGGACA

RESULTS

All primers amplified well in all tested species, except *Nonea lutea*, for which no primers ensured the DNA amplification. Most of the primers generated polymorphic patterns in all species and the number of bands was generally higher than in *Brassica rapa* ssp. *oleifera* (Table 2). *Cynoglossum officinale* was the only species for which all the RAPD primers amplified well and reproducible bands were obtained. The highest number of bands were obtained with the primer OPB-18 in *Cynoglossum officinale* and with the primer OPI-14 in *Anchusa officinalis* (six bands of 300-900 pb). The primers OPB-18, OPI-14 and OPH-12 generated four bands in *Cynoglossum officinale* (490-1200 pb), *Echium vulgare* (300-1000 pb) and *Anchusa officinalis* (350-1000 pb). The primers OPJ-20 generated three bands in *Cynoglossum officinale* (400-950) and *Anchusa officinalis* (350-1000 pb). The following primers generated two bands in different species: OPG-16, OPH-11, OPK-20, OPP-19 in *Cynoglossum officinale*, OPG-16 in *Echium vulgare*, OPP-18, OPG-16 and OPL-03 in *Anchusa officinalis*. In *Brassica rapa* ssp. *oleifera* most of the primers generated only one band except the primers OPB-18, OPH-11 and OPH-12 that generated two bands. The size of the bands was higher in *Brassica rapa* ssp. *oleifera* that in the tested *Boraginaceae* species, but these RAPD primers were informative in these species as well except *Nonea lutea*. The patterns of amplification with different RAPD primers in *Boraginaceae* species are shown in Fig. 1.

DISCUSSION

The objective of this work was to test the use of RAPD markers associated with the linolenic acid content in different *Boraginaceae* plant species from the Romanian flora, such as *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis* and *Nonea lutea*. It was reported that a high level of polyunsaturated fatty acids was found in different species of *Boraginaceae* [13] and *Brassicaceae* [11, 23].

Table 2. Size and number of the bands amplified by RAPD primers in different *Boraginaceae* species in comparison with *Brassica rapa* ssp. *oleifera*

Primer	Bands (pb)/species				
	Cynoglossum officinale	***Echium vulgare***	***Anchusa officinalis***	***Nonea lutea***	***B. rapa* ssp. *oleifera* [26]**
OPB-12	225	-	770	-	970
OPB-18	490/740/850/1200	730/750	730750	-	1010/1020
OPB-20	800	-	-	-	2760
OPG-16	300/500	320/500	600/700	-	450
OPH-11	350/800	-	-	-	1810/1910
OPH-12	450	-	350/400/450/1000	-	1250/1310
OPI-14	200	300/450/850/1000	300/450/500/650/850/900	-	1620
OPJ-20	400/650/950	-	300/400/750	-	930
OPK-20	500/850	-	-	-	800
OPL-03	600	400	400/700	-	750
OPP-17	450	1000	-	-	1390
OPP-19	400/600	500	450	-	870

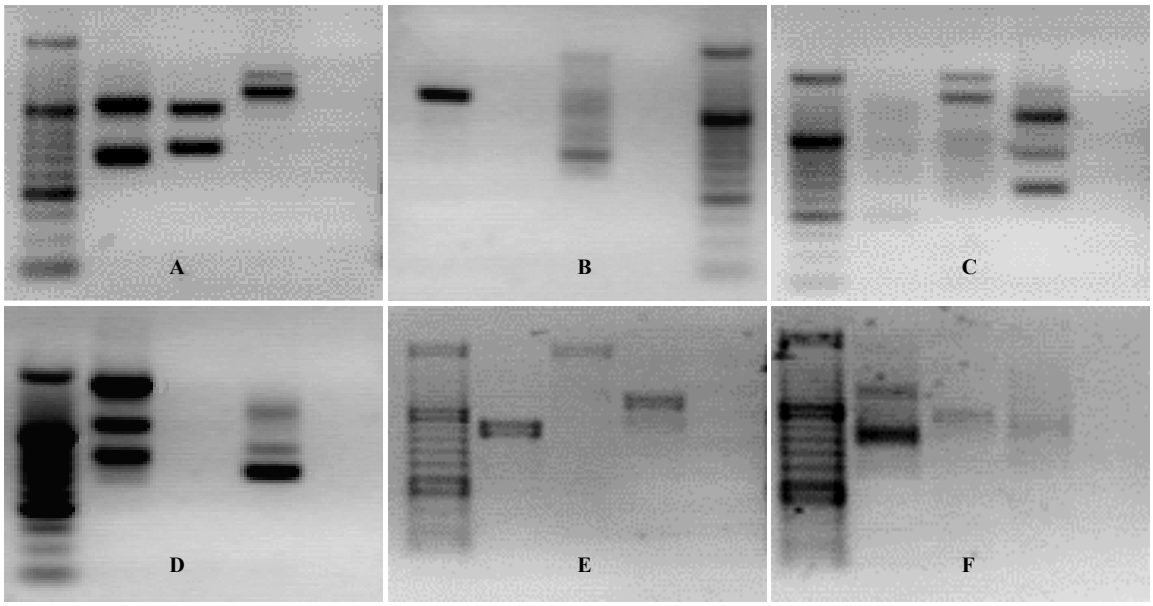

Fig. 1. Amplification pattern of *Boraginaceae* species using different RAPD primers (**A**-OPG16; **B**-OPH-12; **C**-OPI-14; **D**-OPI-20; **E**-OPP-17; **F**-OPP-19. Molecular marker control bands of 1000, 500, 200 pb. Order of samples: *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis*, *Nonea lutea*). Separation on 1.5% agarose gel stained with 0.5 μg/mL ethidium bromide.

Boraginaceous plants are characterized by high levels of polyunsaturated fatty acids and show a high ratio ω-3/ω-6 fatty acids.

There are only few studies reporting the screening of local *Boraginaceae* species for their compounds in fatty acids [9, 10, 18, 30]. The fatty acids concentration varies between genera of *Boraginaceae* family. *Echium* contains the highest amount of total ω-3 PUFA (47.1%), predominantly ALA (36.6%) and SDA (10.5%) combined with GLA (10.2%). Other *Boraginaceae* species rich in both SDA and GLA are *Omphalodes linifolia* (8.4%, 17.2%, respectively), *Cerinthe minor* (7.5%, 9.9%, respectively) and *Buglossoides purpureocaerulea* (6.1%, 16.6%, respectively). *Alkanna* genus has comparable amounts of ALA (37.3%) and GLA (11.4%) with *Echium*, but lower SDA content (3.7%) [14]. Guil-Guerrero et al. [8] found high GLA amounts in the seeds of more *Boraginaceae* species with a maximum of 20.25% total fatty acids in *Myosotis nemorosa*. Variable amounts of stearidonic acid (18:4-ω3, SDA) ranging from 0.08% of the seeds fatty acids were found in *Anchusa azurea* to 21.06% in *Echium asperrimum*. SDA was also very abundant in all organs of *Asperugo procumbens*. The study of the lipids content in three *Boraginaceae* species (*Cynoglossum officinale*, *Echium vulgare*, and *Lappula squarrosa*), discovered four polyunsaturated acids: linoleic (LA), γ-linolenic (GLA), α-linolenic (ALA), and stearidonic (SA) [29].

The quality of seeds oils is determined by their fatty acids composition. Linolenic acid (C18:3) is one of the main fatty acids in the seeds oils. Linolenic acid is synthesised from the desaturation of linoleic acid (C18:2) and also perhaps from the elongation of C16:3. Several genes seem to control the linolenic acid level but the predominant gene responsible for the synthesis of linolenic acid in seed triacylglycerols is *fad3* (fatty acid desaturation), the structural gene for a microsomal 18:2 desaturase. It is unlikely that the other genes encode for chloroplast desaturases, but they could represent members of a *fad3* family [12]. RAPD markers tested in our study were previously used in *Brassica rapa* ssp. *oleifera* [26] and it was shown that they are associated with linolenic acid synthesis. We also obtained several bands in the studied *Boraginaceae* species, but it is necessary to sequence

these fragments to prove the identity of these markers, in order to obtain valuable RAPD markers useful for rapid screening in germplasm collections. The RAPD markers used in this study are associated with *fad3* gene in linkage groups 9 and 10 (LG9, LG10) in *Brassica rapa* ssp. *oleifera*. The gene for palmitic acid content is also located in LG9, thus it is possible that the palmitic acid locus influences linolenic acid content as well [22]. In our study, the number of bands generated by each primer was generally higher in the tested species than in *Brassica rapa* ssp. *oleifera* and the size of the fragments was different as well, thus the sequencing becoms indispensable. The primers OPB-18, OPG-16, OPH-11, OPH-12, OPI-14, OPJ-20, OPK-20, OPL-03 and OPP-19 generated at least two fragments in *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis*, while in *Brassica rapa* ssp. *oleifera* only the primers OPB-18, OPH-11 and OPH-12 generated two fragments.

In conclusion, the RAPD markers associated with linolenic acid synthesis previously used in *Brassica rapa* ssp. *oleifera* could also be applied for the molecular characterization of *Cynoglossum officinale*, *Echium vulgare*, *Anchusa officinalis* but were not suitable for *Nonea lutea*. Other markers should be developed for *Nonea lutea*.

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