USE OF RAPD TECHNIQUE FOR MOLECULAR CHARACTERIZATION OF SOME *BRASSICA SP.* CULTIVARS AND *BRASSICA RAPA CAMPESTRIS* IN ORDER TO ANALYSE GENE FLOW POLLINATION DUE TO *APIS MELLIFERA*

UTILIZAREA TEHNICII RAPD PENTRU CARACTERIZAREA MOLECULARĂ A UNOR SOIURI DE RAPIȚĂ AMELIORATĂ (*BRASSICA RAPA*) ŞI A RAPIȚEI SĂLBATICE (*BRASSICA RAPA CAMPESTRIS*) ÎN VEDEREA ANALIZEI TRANSFERULUI ORIZONTAL DE GENE DATORAT SPECIEI *APIS MELLIFERA*

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Abstract. *RAPD technique was used to put in evidence molecular polymorphism to 6 improved breeds of Brassica sp. cultivars: Astrid, Eldo, Orkan, Gabriella, Dexter, Alaska and its wild relative Brassica rapa campestris. The protocol used for genomic DNA extraction was described by Lodhi et al. (1994), improved by Rodica Pop et al. (2003). The quality of extracted DNA corresponded quantitative and qualitative for optimal RAPD analysis. After agarose gel image analyzing using TL 120 v2006e and TL 100 DM soft were found that from the total of 41 primers, 9 did not generate amplification products, 20 were mono-morphic and respectively 12 were polymorphic. UPGMA dendrograma of genetical differences betrayed that there are some differences between improved breeds and its wild relative. Regarding the intra-specific differences between the 6 breeds, Eldo breed shows some differences comparing with the other 5. The following step of the study is gene flow pollination due to Apis mellifera.*

Rezumat. *Tehnica RAPD a fost utilizată pentru a evidenția polimorfismul molecular la şase soiuri (Astrid, Eldo, Orkan, Gabriella, Dexter, Alaska) de rapiță ameliorată (Brassica sp.) şi la specia sălbatică (Brassica rapa campestris). Protocolul de extracție utilizat pentru izolarea ADN-ului genomic, descris de Lodhi şi colab. (1994) şi modificat de Rodica Pop şi colab. (2003) a permis obținerea unui ADN care a corespuns calitativ şi cantitativ analizelor RAPD efectuate la materialul biologic studiat. În urma analizei imaginilor gelurilor de agaroză cu pachetul de programe TL120 v2006e şi TL100 DM s-a constatat că din totalul de 41 primeri folosiți, 9 primeri nu au generat produşi de amplificare, 20 au fost monomorfi, respectiv 12 au dat polimorfism. Dendrograma UPGMA alcătuită pe baza distanțelor genetice indică faptul că există unele diferențe între specia sălbatică şi soiurile cultivate de rapiță. În ceea ce priveşte soiurile analizate se observă că soiul Eldo a fost diferit genetic de celelalte cinci soiuri luate în studiu, fapt confirmat şi de imaginea obținută în unele geluri de agaroză. Datele experimentale obținute cu ajutorul tehnicii RAPD sunt parte integrantă a unui studiu privind transferul de gene realizat prin intermediul speciei Apis mellifera.*

Key words: RAPD, *Brassica sp.*, *Brassica rapa campestris*, gene flow

INTRODUCTION

Three *Brassica* species are important oilseed crops globally - *Brassica napus* (rapeseed, oilseed rape), *Brassica rapa* (turnip rape, sarson), and *Brassica juncea* (indian mustard, oriental mustard). The first two are referred to collectively as rapeseed, while *Brassica juncea* is considered a mustard. The term, "canola," describes grain from any of the above species that has less than 2% erucic acid (C22:1) in seed oil, and less than 30 micromoles per gram of aliphatic glucosinolates in the oil-free meal (Canola Council of Canada website www.canola-council.org). Grain from any of these species that does not meet canola quality standards is referred to as "rapeseed," or in the case of *Brassica juncea*, as "mustard grain."

There is some experimental evidence to suggest that the stigmas of oilseed rape flowers are partly sheltered from windborne pollen trajectories. For example, the field measurements of out-crossing from caged and naturally exposed oilseed rape plants (Ramsay *et al*., 1999) show that pollination can be dramatically reduced when insects are excluded by cages. Landscape-scale studies of out-crossing (Thompson *et al.*, 1999; Squire *et al.*, 1999) suggest that the greatest risk of GM pollen transport is from insect pollination closest to the source, and honeybees have been identified as the principal pollinator for oilseed rape at large distances. Observations of honeybee flights of 1 – 2 km to collect nectar and pollen have been known for some time (Eckert, 1933) and recent studies have recorded bees foraging on oilseed rape at 5 km from a hive (Ramsay *et al.*, 1999).

During the last years molecular techniques became increasingly used for the identification of gene flow *via* pollination. All these studies required a high purity of the expected DNA. The presence of contaminants in DNA preparation often makes the samples viscous and renders DNA unrestrictable in endonuclease digestion and unamplificable in PCR (polymerase chain reaction). These contaminants are usually removed by inclusion of polyvynilpyrrolidone (PVP-40), diethyldithiocarbamic acid (DIECA), sodium bisulfite, ascorbic acid or beta-mercaptoethanol in the extraction buffer (Weising *et al.*, 1995).

MATERIAL AND METHODS

Biological material used for DNA isolation was represented by young plants grown in pots and originated from six cultivars of oilseed rape. DNA was extracted from young leaves of *Brassica sp.* of the following cultivars: Astrid, Eldo, Orkan, Gabriella, Dexter and Alaska. Prior to isolation, approximately 400 mg of leaves were grind in liquid nitrogen into a fine powder. Three isolation protocols were tested: **A.**, a modified protocol described by Doyle and Doyle 1990, **B.** a protocol described by Roger *et al.*,1988 and **C.** a modified version (Rodica Pop *et al.*, 2003) of the protocol published by Lodhi *et al.* (1994). Protocol A has the following composition of the extraction buffer: 100 mM Tris-HCl, 20 mM sodium EDTA, pH = 8, 1,4 M $NaCl_2$, 2% (w/v) CTAB and 0,2% of beta mercaptoethanol added just before use. Extraction buffer in protocol B has the similar composition except the 1% PVP-40 who was also added just before use. Extraction buffer in protocol C has the similar

composition as in protocol B except for the PVP concentration that was enhanced to 2%. This buffer was also supplemented with 5 mM ascorbic acid and 4 mM DIECA.

DNA concentration was measured through the spectrofotometric method with the Nanodrop BioPhotometer. The BioPhotometer is used for rapid, simple and convenient measurement of the most common methods in research labs in the fields of molecular biology and biochemistry. The experiments concerning DNA purity was made in three replications and interpreted as a bifactorial test with A factor (cultivar) with six graduations and fator B (method) with three graduations.

The obtained data were computed according to analysis of variance in bifactorial experiments (Ardelean *et al.*, 2002).

RESULTS AND DISCUSSIONS

Table 1 shows the average of DNA concentration of analyzed cultivars:

Table 1

Mean of DNA concentration of analysed cultivars isolated with three protocols

Cultivar	Mean of DNA concentration (ng/µl) isolated by		
	Protocol A	Protocol B	Protocol C
Astrid	2045	1970	2836
Eldo	2846	3047	2948
Orkan	1978	2334	2683
Gabriella	1445	1978	2164
Dexter	2389	2003	2314
Alaska	1890	1893	3339

As it can be seen in table 1 the average yields of isolated DNA ranged from 1445 to 3339 ng/µl of fresh tissue. These results demonstrated that the protocols we used are suitable for extraction of high quantity DNA from young leaves of *Brassica sp*. The results of analysis of variance for data obtained in bifactorial experiments concerning the influence of cultivar and protocol of DNA isolation upon the purity of extracted DNA is showed in *table 2*.

Data obtained by Duncan test shows that DNA isolation protocols affected significantly the purity of isolated DNA and the extraction protocol C are obviously superior compare to the other two methods (table 2). The factor cultivars had no significant influence upon experimental data. This suggests that the used protocols are valid for any of the tested cultivars.

Table 2

The effect of cultivar and DNA isolation protocol upon the purity of extracted DNA at 6 cultivars of oilseed rape (*Brassica rapa*)

Cultivars	DNA isolation protocols			Mean for cultivars
	A	B	C	
Astrid	1,75 b*	1,56 b	2,03 a	1,78 **A**
Eldo	1,40 bc	1,35 bc	1,82 a	1,52 **A**
Orkan	1,32 bc	1,49 bc	1,92 a	1,76 **A**
Gabriella	1,20 bc	1,30 bc	1,95 a	1,48 **A**
Dexter	1,64 b	1,42 bc	1,87 a	1,64 **A**
Alaska	1,33 bc	2,15 a	1,80 a	1,76 **A**
Mean for DNA isolation protocols	1,44 **M**	1,54 **M**	1,88 **N**	-

*The differences between two variants with a common letter are not significant

SD 5% for cultivars: 0,33-0,42;
SD 5% for DNA isolation protocols: 0,38-0,4;
SD 5% for cultivars x DNA isolation protocol interaction: 0,5-1,6;

The interaction of the two experimental factors (cultivars and DNA isolation protocols) shows that the lowest value of DNA purity (1, 20) was obtained at Gabriella cultivar when DNA was isolated by A protocol. As it can be seen in table 2, the best value of DNA purity was registered at Astrid cultivar isolated by C protocol.

Regarding the polymorphism of the random amplified fragments (RAPD), there are 12 primers from the total of 41 which are polymorphic and the image analysis using TL120 v 2006e and TL100 DM program shows that there are primers with a number of fragments between 3 and 5 (*table 3*).

Table 3

The number of polymorphic fragments generated by the RAPD primers to *Brassica rapa* and *Brassica rapa campestris*

Nr. crt.	Polymorphic primers	Number of polymorphic fragments/primer
1.	OPA 03	3
2.	OPA 09	4
3.	OPAB 18	3
4.	OPB 08	5
5.	OPB 17	3
6.	OPB 18	5
7.	OPC 02	4
8.	OPC 04	3
9.	OPD 16	5
10.	OPD 19	3
11.	OPF 20	3
12.	OPP 18	4

The appearance of polymorphic fragments suggests that there are some genetic differences between the 6 improved cultivars of *Brassica sp.* and its wild relative *Brassica rapa campestris* (*fig. 1, 2* and *3*). That requires the application of further molecular techniques as ISSR and SSR (Inter Simple Sequence Repeat-ISSR or Simple Sequence Repeat-SSR) and sequencing in order to detect very precise these genetic differences.

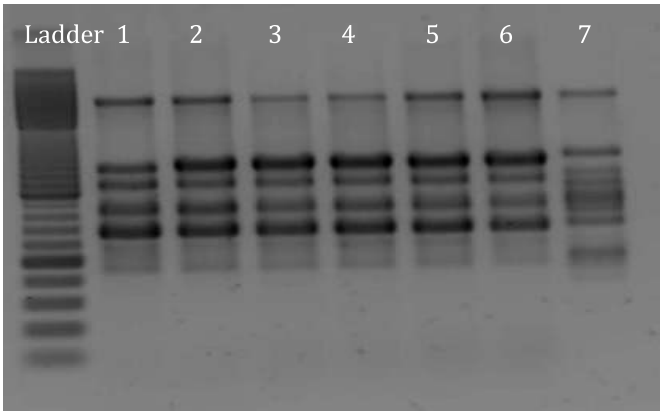

Fig. 1. Electrophoretic profile of amplified fragments using OPC 16 primer
1. Astrid; 2. Eldo; 3. Orkan; 4. Gabriella; 6. Dexter; 7. *Brassica rapa campestris*

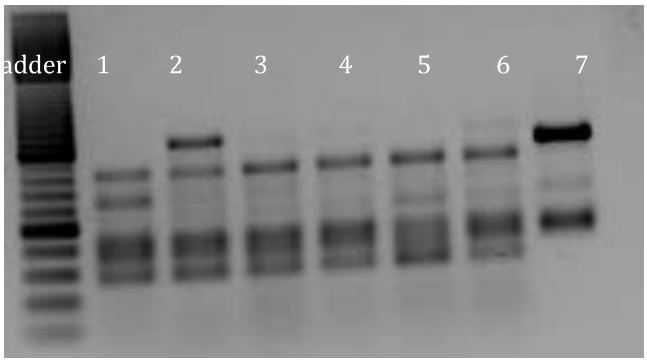

Fig. 2. Electrophoretic profile of amplified fragments using OPC 04 primer
1. Astrid; 2. Eldo; 3. Orkan; 4. Gabriella; 6. Dexter; 7. *Brassica rapa campestris*

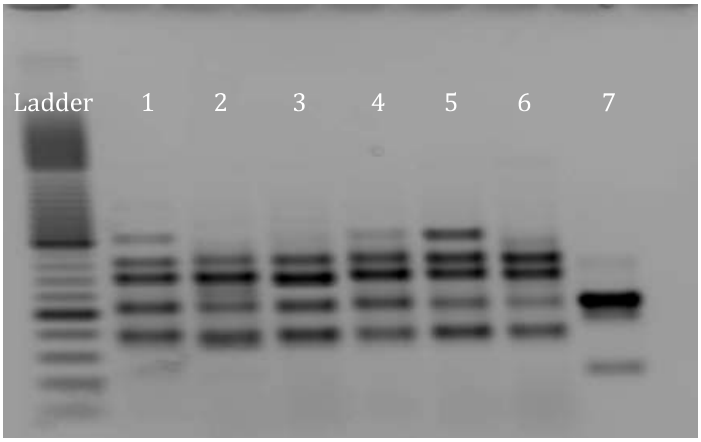

Fig. 3. Electrophoretic profile of amplified fragments using OPD 19 primer
1. Astrid; 2. Eldo; 3. Orkan; 4. Gabriella; 6. Dexter; 7. *Brassica rapa campestris*

The dendrogram UPGMA (FREETREE soft) indicate that Eldo cultivar is genetically different from the other 5 cultivars, and all of improved cultivars are significantly different by *Brassica rapa campestris* (*fig. 4*).

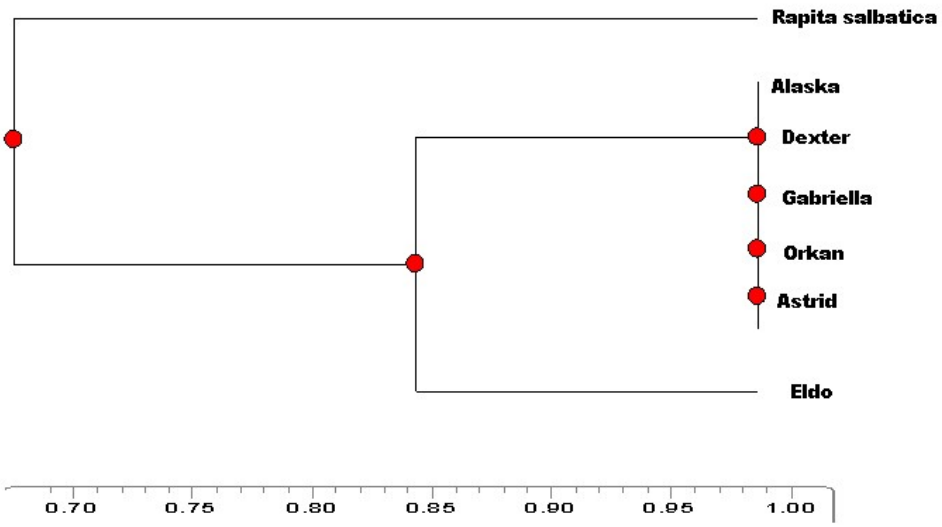

Fig. 4. UPGMA dendrogram to *Brassica sp.* and *Brassica rapa campestris*

CONCLUSIONS

✓ DNA isolation protocol **C.** represented a modified version (Rodica Pop *et al.*, 2003) of the protocol published by Lodhi *et al.* (1994) yields DNA purity value significant higher than protocol **A.** and **B.**;

✓ The use of PVP-40, DIECA and ascorbic acid in the extraction buffer in protocol **C.** minimized the damage caused de contaminants like polyphenols and polysaccharides to the nucleic acids;

✓ DNA extracted by protocol **C.** can be successfully used in our future studies concerning RAPD and AFLP analysis for revealing gene flow *via* pollination of oilseed rape;

✓ Eldo cultivar and *Brassica rapa campestris* can be used like markers in order to analyze gene flow pollination due to *Apis mellifera*;

✓ There are required further molecular techniques to get thoroughly into genetic structure of analyzed cultivars, such ISSR, SSR and sequencing.

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REFERENCES

1. Lodhi M.A., Y. Guan-Ning N.F. Weeden, B.I. Reisch. 1994 - *Plant Molecular Biology Reporter* 12(1): 6-13;

2.Mărghitaş L. Al. 2005 - *Albinele şi produsele lor*. Ed. Ceres. Bucureşti;

3.Mesquida, J., Renard, M. 1982 - *Study of the pollen dispersal by wind and of the importance of wind pollination in rapeseed (Brassica napus var. oleifera Metzger) (English summary)*. *Apidologie* 4: 353-366;

4. Metz, P.L.J., Jacobsen, E., Stiekema, W.J. 1997 - *Aspects of the biosafety of transgenic oilseed rape (Brassica napus L.)*. *Acta Bot. Neerl.* 46, 51-67;

5. Pop Rodica, M. Ardelean, D. Pamfil, Ioana Marina Gaboreanu. 2003 - *The efficiency of different DNA isolation and purification protocols in ten cultivars of Vitis vinifera, Buletinul USAMV-CN*, 59/2003 (259-261);

6. Ramsay G., C.E. Thompson, S. Neilson, G.R. Mackay 1999 - *Honeybees as vectors of GM oilseed rape pollen. In: Gene Flow and Agricultural Relevance for Transgenic Crops.* (Ed. Lutman PJW). pp. 209-214. *BCPC Symposium Proceedings*, no. 72;

7. Simpson E.C., Norris C.E., Law J.R., Thomas J.E., Sweet J.B. 1999 - *Gene flow in genetically modified herbicide tolerant oilseed rape (Brassica napus) in the UK. In: Gene Flow and Agricultural Relevance for Transgenic Crops.* (Ed. Lutman, PJW) pp. 75-82. *BCPC Symposium Proceedings*, no. 72;

8. Squire G.R., J.W. Crawford, G. Ramsay, *et al.* 1999 - *Gene flow at the landscape level. In: Gene Flow and Agricultural Relevance for Transgenic Crops* (Ed. Lutman PJW) pp. 57-64. *BCPC Symposium Proceedings*, no. 72;

9. Thompson C.E., Squire G.R., Mackay G.R., *et al*. 1999 - *Regional patterns of gene flow and its consequences for GM oilseed rape. In: Gene Flow and Agricultural Relevance for Transgenic Crops.* (ed. Lutman PJW) pp. 95-100. *BCPC Symposium Proceedings*, no. 72;
website-www.canola-council.org;

10. Weising K., H. Nybom, K. Wolff, W. Meyer. 1995 - *DNA Fingerprinting in Plants and Fungi*, *CRC Press Inc.*, 51-55;