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Effective Techniques for Resynthesized Rapeseed Production of Contrasting Components via Ovule Culture and Flow Cytometry

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Introduction

Brassica crops display enormous diversity. They are used as a source of fodder, feed, mustard condiments, green manure vegetables, root and oil crops and the genus is highly polymorphic (Warwick et al. 2009, Gupta et al 2013). Among all, *Brassica* crops belong to the most important oilseeds in the World and their usage continues to increase. So called ‘‘Double Low (00) rapeseed’’ or ‘‘Canola’’ - with zero erucic acid in oil and low glucosinolate content - was bred from genetic variations within the population of *B. napus* or *B. rapa*. Initially, many other important agronomic traits of the first “00” or Canola cultivars were poorer than the traditional types of rapeseeds. While intensive breeding efforts in the past few decades increased seed yield as well as other agronomic traits (Rahman 2013), however, it also resulted in narrowed genetic diversity of breeding materials (Girke et al. 2012, Rahman 2013).

The breeding of oilseed rape (*B. napus*) focuses not only at the production of high-yielding varieties, but also at the specific composition of fatty acids profile in the oil and increased resistance to major diseases and other biotic and abiotic stressors. Due to permanent climate changes that accompany the increasing occurrence of droughts, torrential rains and atypical winter periods, it becomes also important to achieve the adaptability of varieties to specific soil and climatic conditions. Successful breeding of competitive varieties of both spring and winter oilseed rape requires constant innovation of breeding programmes. An important prerequisite appears to be sufficient genetic variability of initial materials.

To broaden the diversity within *Brassica napus* gene pool, studies on resynthesis of rapeseed through crossing of selected subspecies of *Brassica rapa* L. and *Brassica oleracea* L. and obtaining plants through *in vitro* culture of isolated embryos in the early stage of their development have been published (Rahman 2005, Wen et al. 2008, Sosnowska et al. 2010). It has been proved, that resynthesized plants are distinct from cultivars of winter oilseed rape which are bred and cultivated nowadays (Sosnowska et al. 2010), so they would serve as sources of new genetic variability in rapeseed breeding programmes, including cytoplasmic and nuclear male sterility, resistance to diseases, insect or nematode pests and tolerance to cold, salt and drought conditions (Warwick et al. 2009).

The aim of this study was to evaluate the crossability of selected one-sided crosses between different varieties and cultivars *of B. rapa* and *B. oleracea* and effectively produce hybrids for the selection of genetic material in winter oilseed breeding programmes.

Material and Methods

Accessions of winter turnip rape (*B. rapa*. ssp. *oleifera* f. *biennis)* Arktus, Brachina, Bulharska, Grubers Winterrübsen, Izumrudnaja K 193, Svalöfs Duro, Ludowy, Slezsky Krajový and Rapido, and accessions of spring turnip rape *(B. rapa*. ssp. *oleifera* f. *praecox)* Jumbuck, Ante-12, Ante-27, Evissa, and one breeding material of spring turnip rape (*B. rapa* ssp. *rapa*) V17, were used as female components, while accessions of cabbage *(B. oleracea* convar. *capitata*) Vysocké AIK, Zakamenné and winter curly kale (*B. oleracea* convar. *acephala* var. *sabellica*) Frosty, Kaderávek Zelený, Kapral Scarlet, Pentland Brig, Nero di Toscana and Vates were used for experiments as male components. Genotypes were selected by contrasting morphological (cabbage and curly kale) or agronomical traits (turnip rape).

Donor plants were grown in the greenhouse; plants of winter type accessions were vernalised (4 °C, and 12 h photoperiod, irradiance 20.4 μmol m^{-2} s^{-1}) for 8 weeks. Spring and vernalized winter type parental components were grown during flowering under a controlled environment in a growth chamber (light intensity 84 μmol m^{-2} s^{-1}, 17/15 °C day/night, and photoperiod 16/8 h). Fifty different crosses were made between selected *B. rapa* (female component) and *B. oleracea* (male component) accessions with at least 30 buds per one combination.

Pollination efficiency was computed as the ratio between the number of siliquae with at least one ovule developed and the number of flower buds pollinated; hybrid siliquae ratio (HSR) as the mean number of embryos per one cultured siliquae and hybrid production ratio (HPR) as the mean number of embryos obtained from one cross-pollinated bud. Percent data subjected to analysis of variance were modified by means of angular transformation to follow normal distribution of errors (Gomez and Gomez 1984). Homogeneity of variance was accessed via Levene's test, followed by tests of variance (parametric or non-parametric, according to Levene's test), multiple comparisons of means, Spearman correlation and simple linear regression, computed in the statistical software [StatSoft STATISTICA 10.0].

The relative DNA content of all parental components as well as hybrids was assessed by flow cytometric analysis. Ten randomly chosen plants from all parental cultivars have been analysed three times on different days. Up to five regenerants, originating from different embryos of identical crosses, were processed together in two replications. The two-step method according to Dolezel et al. (2007) was used. Approximately 1 cm^2 from each plant and an appropriate amount of internal standard (*Zea mays*, cv. CE-777, 2C = 5.43 pg; Lysak and Dolezel 1998) was chopped with a razor blade in 0.5 ml of Otto I buffer (0.1 M citric acid, 0.5 % Tween 20). The suspension was filtered through a 42 μm nylon mesh. After ten minute incubation at room temperature, 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), 4 μg ml^{-1} 4',6-diamidino-2-phenylindole and 2 μl ml^{-1} β-mercaptoethanol was added. Relative fluorescence intensity of at least 3,000 nuclei was measured using a CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Data were analyzed using FloMax software, version 2.4d (Partec, GmbH, Münster, Germany).

Morphology was assessed at the stage of at least five true leaves by the comparison of selected characteristics (preferably leaf shape and/or colour) of regenerants and female parental components. Obtained plants were also surveyed for possible morphological markers, inherited from the respective male component.

Excised root tips were pretreated in saturated solution of p-dichlorobenzene for 2 h. Pretreated samples were fixed in ethanol-acetic acid (glacial) 3:1 mixture at 5 °C overnight. After maceration in 1:1 hydrochloric acid:ethanol for 20 s and subsequent rinsing in deionized water for 15 minutes, the root tip cuttings were squashed in lacto-propionic orceine. The slides were evaluated by using Carl-Zeiss Jena NU microscope equipped with an Olympus Camedia C-2000 Z camera.

Results and Discussion

The first embryos appeared after approx. two weeks of culture, depending on the cross combination and/or replication; forty-two out of fifty combinations successfully produced embryos when counted on the 35th day of culture. Therefore, successful regeneration of embryos was achieved in 84% of crosses. The average production rate was 0.41 embryos/siliquae and 0.32 embryos/bud, respectively.

♀	♂	P. E.	HSR	HPR
Arktus	Frosty	90,00	1,45	1,40
Brachina	Frosty	90,63	1,00	0,25
Bulharska	Frosty	90,91	1,33	0,67
Grubers Winterrübsen	Frosty	85,00	0,33	0,17
Izumrudnaja k 193	Frosty	84,62	0,50	0,30
Svalöfs Duro	Frosty	91,43	0,18	0,04
Arktus	Kapral	88,57	0,03	0,10
Brachina	Kapral	86,11	0,06	0,06
Bulharska	Kapral	46,88	0,30	0,16
Grubers Winterrübsen	Kapral	64,29	0,00	0,00
Izumrudnaja k 193	Kapral	58,33	0,58	0,32
Svalöfs Duro	Kapral	95,24	1,81	1,65
Arktus	Kaderávek zelený	81,25	0,62	0,48
Brachina	Kaderávek zelený	86,49	0,18	0,22
Bulharska	Kaderávek zelený	90,63	0,62	0,54
Grubers Winterrübsen	Kaderávek zelený	64,71	0,14	0,09
Izumrudnaja k 193	Kaderávek zelený	100,00	0,19	0,19
Svalöfs Duro	Kaderávek zelený	88,89	0,85	0,75
Arktus	Scarlet	93,94	0,38	0,36
Brachina	Scarlet	67,65	0,13	0,09
Bulharska	Scarlet	70,37	0,56	0,29
Grubers Winterrübsen	Scarlet	87,10	0,67	0,58
Izumrudnaja k 193	Scarlet	70,97	1,27	0,85
Svalöfs Duro	Scarlet	57,69	0,14	0,15
Arktus	Nero di Toscana	55,56	0,00	0,00
Brachina	Nero di Toscana	84,11	0,41	0,15
Bulharska	Nero di Toscana	48,14	0,00	0,00
Grubers Winterrübsen	Nero di Toscana	65,87	0,39	0,35
Izumrudnaja k 193	Nero di Toscana	86,48	0,00	0,00
Svalöfs Duro	Nero di Toscana	64,25	0,00	0,00
Arktus	Pentland Brig	67,67	0,15	0,15
Brachina	Pentland Brig	75,56	0,32	0,19
Bulharska	Pentland Brig	72,23	0,18	0,14
Grubers Winterrübsen	Pentland Brig	56,00	0,00	0,00
Izumrudnaja k 193	Pentland Brig	55,56	0,00	0,00
Svalöfs Duro	Pentland Brig	46,85	0,00	0,00
Slezsky krajový	Nero Di Toscana	67,51	0,12	0,03
Brachina	Vates	86,11	0,06	0,06
Rapido	Vates	67,65	0,13	0,09
V17	Vysocké AIK	84,62	0,14	0,15
Jumbuck	Vysocké AIK	84,57	0,52	0,48
Ante-12	Vysocké AIK	91,25	1,15	1,28
Evissa	Zakamenné	89,87	0,37	0,52
Ante-12	Zakamenné	89,66	0,74	0,62
Ante-27	Zakamenné	73,17	0,66	0,38
Jumbuck	Zakamenné	88,89	0,14	0,32
Izumrudnaja k 193	Zakamenné	69,23	0,71	0,12
Svalöfs Duro	Zakamenné	85,71	0,42	0,33
Ludowy	Zakamenné	79,41	0,42	0,15
Brachina	Zakamenné	90,91	0,34	0,53

Table 1: Crossability of individual combinations, assessed by means of pollination efficiency (P. E.), hybrid siliquae ratio (HSR) and hybrid production ratio (HPR)

Our experiments avoided ovule counting and one-by-one ovule manipulation to shorten time requirements and manual labour. Moreover, our procedure (understood as superficial damage at blade cut and pulling out of siliquae content) may be beneficial as ovule perforations can increase water and nutrient uptake (Reed 2005). A relatively large spread between the worst and best mean pollination efficiency (46.85–100%, see Table 1) was found but no significant effect of a cross combination and no differences between combinations were detected, according to non-parametric Kruskal–Wallis one way analysis of variance and multiple mean comparisons at $P \leq 0.05$. The relatively large variances between individual crosses were likely due to differences in the physiological state of donor plants at the time of crossing and sampling pods. Nevertheless, we were able to identify several combinations, which were among those with the highest yields. In general, the technique used with these culture media was appropriate to obtain embryos in most of the evaluated combinations and therefore it is considered suitable for using a large number of different genotypes to produce resynthesized hybrids of oilseed rape. The performance of combinations with no production could have been caused by some specific post-fertilization barriers or genetic incompatibility in these combinations, not solvable by means of embryo rescue method and/or sexual hybridization (Wang et al. 2013), or they may be solvable by optimization and larger amount of crosses. Further studies will be necessary to confirm any of these hypotheses.

Due to highly significant differences in relative DNA content between all hybrid combinations and their respective parental components it was possible to reliably assess the

hybrid nature of all regenerants via flow cytometry. As the occurrence of the self-pollinated and/or somatic-tissue regenerated female parent was not detected, the hybridity of all regenerants was reliably verified. Morphological assessment of regenerated plants showed typical characteristics originating from both parental components, and further corroborated the results of flow cytometric analysis.

Conclusions and Outlook

It can be concluded, that the above procedure of oilseed rape resynthesis via ovule culture is sufficient enough to be applicable in breeding programmes, aimed at diversity expansion of winter oilseed rape gene pool, as the resynthesized embryos were derived in most combinations. Current research is aimed at evaluation of various antimitotic agents in terms of their efficiency to double chromosome number of obtained regenerants. Fertile regenerants are being self-pollinated and crossed with elite commercial lines. Crosses of perspective materials to obtain new sources of yellow-seeded *Brassica napus* are also planned.

Dedication

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