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Raffinose family oligosaccharides in seeds of common vetch (*Vicia sativa* L. ssp. *sativa*)

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ABSTRACT

The composition and content of low-molecular weight soluble carbohydrates in seeds of 66 accessions of common vetch (*Vicia sativa* L. ssp. *sativa*) stored in the Polish and German gene banks, originating from Europe, Asia, Middle East and North Africa, were analyzed using a gas chromatography method. Genotypes showed differences in the composition of carbohydrates depending on the origin of seeds. Common features of accessions were: *i*) the predominance of raffinose family oligosaccharides (RFOs) in soluble carbohydrates, and *ii*) verbascose as the main oligosaccharide in RFOs. Several accessions with lowered RFOs content were identified. They may be suitable for breeding focused on diminishing RFOs, as antinutritional compounds in seeds.

Key words: Common vetch, Seed, Raffinose oligosaccharides, *Vicia*.

INTRODUCTION

The species common vetch (*Vicia sativa* L. ssp. *sativa*) is widely cultivated in many areas of the world, playing an important role both as feed for livestock and food for humans, especially in regions with unfavorable conditions as a substitute for the cultivation of other perennial or annual legumes (Ford *et al*. 2008). Grains of common vetch are a rich source of high quality protein (28-32%), carbohydrate, fibre and phytochemicals and are considered to be a quality feed for ruminant animals, such as sheep and cattle. However, some seed compounds (i.e. phenolics, enzyme inhibitors, lectins, saponins) restrict their broader use for humans and monogastric animals (Campos-Vega *et al*. 2010). The most dangerous are glucosidic aminopyrimidine derivatives (vicine and convicine) and β-cyanoalanie (and its *g*-glutamyl peptide), common also in seeds of some other Vicia species: *V. angustifolia*, *V. faba*. Two aglycones (divicine and isouramil), released from vicine/convicine upon digestion in the large intestine and cecum (*via* microbial hydrolysis), are the main factors of favism, whereas β-cyanoalanine indicates neurotoxic properties. The concentration of vicine/convicine in seeds is below 1% of dry matter (d.m.), whereas β-cyanoalanine constitues around 1% of d.m. (Ford *et al*. 2008). Thus, breeders focus mainly on eliminating or decreasing the content of the above toxins in seeds (Duc *et al*. 1999; Ford *et al*. 2008).

Much less attention is paid to non-toxic raffinose family oligosaccharides (RFOs), present in considerable amounts in seeds of legumes (up to 12% of dry weight-DW). In seeds of some *Vicia* species, the RFOs content reaches up to 10% DW (Lahuta *et al*. 2018). RFOs are α-D-galactosides of sucrose, containing 1-4 units of galactose linked by α-1,6 linkages (they are: raffinose, stachyose, verbascose and ajugose, respectively). RFOs are not digested in the upper part of the gastrointestinal tract of humans and monogastric animals, due to the absence of endogenous α-D-galactosidase and are therefore available for bacterial fermentation in the colon, causing flatulence and digestive discomfort (Martínez-Villaluenga *et al*. 2008). In non-ruminant animals, consumption of high concentrations of oligosaccharide-rich feed can reduce potential weight gain and can be a major cause of methane gas production in confined spaces (Polowick *et al*. 2009). Moreover, RFOs have the biggest deterrent effect on people, preventing them from increasing the legume component of their diet, especially in Western countries (Polowick *et al*. 2009).

Decrease in the RFOs content seems to be an additional way towards higher consumption of common vetch, especially in regions of the world with shortage of feed and

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dietary protein. The screening of the composition and content of RFOs in the seeds of important legume species from gene banks enables the detection of genotypes or mutants with a reduced content of RFOs, useful for further breeding. Such studies have already brought results in soybean (Mozzoni *et al*. 2013), chickpea (Gangola *et al*. 2013), lentil (Tahir *et al*. 2012) and pea (Gawłowska *et al*. 2017) production. In soybean, low raffinose and low stachyose genotypes were found (Mozzoni *et al*. 2013), but the content of RFOs can be modified not only by genetic but also by environmental factors. The RFOs concentration in lentil was generally higher in domesticated than in wild genotypes (Tahir *et al*. 2012). In chickpea, the activity of enzymes of RFOs pathway is positively correlated with an accumulation of higher amounts of RFOs in seeds. In pea, increased accumulation of RFOs is stimulated by disturbances in starch biosynthesis.

The reduction of RFOs in seeds seems to be achievable by genetic engineering *via* downregulation of key enzymes of RFOs biosynthetic pathway (Bock *et al*. 2009) or over-expression of α-D-galactosidase in seeds (Polowick *et al*. 2009), but the use of genetically modified crops is restricted or undesirable by consumers in some countries. Another way of lowering the content of RFOs may be to increase the accumulation of galactosyl cyclitols (α-D-galactosides of cyclitols), which are synthesized in the same pathway as RFOs. It may be possible in species naturally synthesizing *myo*-inositol derivatives (like D-pinitol or D-*chiro*-inositol) in vegetative tissues. In lentil seeds, a negative correlation between accumulation of verbascose and ciceritol (di-galactosyl pinitol A) has been found (Frias *et al*. 1999). Beside *myo*-inositol, seeds of some *Vicia* species accumulate D-pinitol and their α-D-galactosides - galactosyl pinitols (Lahuta *et al*. 2018). The accumulation of higher amounts of galactosyl pinitols correlates with the reduction of RFOs, especially verbascose and stachyose (Lahuta *et al*. 2010a, b). However, common vetch seeds accumulate *myo*-inositol as the only cyclitol and, beside RFOs, they accumulate traces of α-D-galactosides of *myo*-inositol-galactinol and di-galactosyl *myo*-inositol (Lahuta *et al*. 2018). Thus, the reduction of RFOs in common vetch seeds seems to be possible *via* selection of genotypes with a lowered RFOs content or lowered content of verbascose, the predominant oligosaccharide in common vetch (Lahuta *et al*. 2018). In the present study, the RFOs composition and content in seeds of *Vicia sativa* ssp. *sativa* L. (domesticated and wild accessions) from different geographical regions were screened, using a gas chromatography method (Sharma and Gaur, 2019). Results of our study may be useful for breeders to choose genotypes with a reduced RFOs content.

MATERIALS AND METHODS

Seeds of *Vicia sativa* L. ssp. *sativa* were derived from a collection of the Institute of Plant Genetics Polish Academy of Sciences in Poznañ (Poland) and Gene Bank in Gatersleben (Germany). The details of codes and number of accessions are given in Table 1. Seeds were grouped according to their "geographical" and state origin: Asia, the Balkan Region and Turkey (BRT), Central Europe (CE), Eastern Europe and the Baltic States (EEBS), Eurasia, Middle East and North Africa (MENA), Mediterranean Pool (MP) and Western and Northern Europe (WNE). The geographical origin has been defined more conventionally, based on the evolution and likely widespread distribution of Fabeae from the Eastern Mediterranean region across Eurasia. The investigated accessions were grown at the Experimental Field, Institute of Plant Genetics Polish Academy of Sciences located in Cerekwica (51°55′N, 17°21′E) in 2015, in one replication. The seeds were sown in rows (3 m long) and spacing of 50 cm between rows. Dry

Table 1: Symbols used for grouping of *Vicia* accessions due to their geographical and state origin.

Group symbol	Geographical region	State, accession code
Asia	Asia	India (VIC5045), Mongolia (VIC664, VIC703).
BRT	Balkans Region and Turkey	Albania (VIC120, VIC121), Romania (VIC1122), Yugoslavia (VIC542), Turkey (VIC101, VIC108).
CE	Central Europe	Czech Republic (VIC325, VIC356, VIC386, Kraj O³omun), Hungary (VIC335, VIC365, VIC390, VIC538), Poland (Alba, Fama, Ina, Jaga, Kamiko, Kwarta, Niwa; breeding lines: 91/A/10/25, LGR51A, LGR284/4, LGR72/8), Slovakia (Ebena, Fatima, Hera, Lyra, Toplesa).
EEBS	Eastern Europe and the Baltic States	Latvia (VIC541), Lithuania (VIC388), Russia (VIC287, VIC363, VIC375, VIC381), Ukraine (VIC369, VIC389, VIC549, VIC749).
Eurasia	Eurasia	Armenia (VIC820), Georgia (VIC937, VIC1103, VIC1149), Turkmenia (VIC671, VIC691).
MENA	Middle East and North Africa	Iran (VIC346, VIC400), Syria (VIC383), Tunisia (VIC1148).
MP	Mediterranean Pool	France (VIC555, VIC584), Greece (VIC284), Italy (VIC117, VIC337), Spain (VIC878, VIC970).
WNE	Western and Northern Europe	Germany (VIC259, VIC260, VIC266, VIC267), Sweden (VIC273, VIC342).

The accessions codes (or cultivars) are shown in brackets.

pods with seeds, collected from several plants, were combined and used for analyses of soluble carbohydrates.

The composition and content of soluble carbohydrates were analyzed by the high resolution gas chromatography method as described by Lahuta *et al*. (2018). Each carbohydrate was quantified by using authentic standards (sugars, cyclitols, oligosaccharides and galactinol) purchased from Sigma-Aldrich (USA). Standard of digalactosyl *myo*-inositol (DGMI) was isolated from seeds of *Vicia cracca* by the method described previously (Szczeciński *et al*. 2000) and was tentatively identified after their hydrolysis to galactose and *myo*-inositol (2 N trifluoroacetic acid, 80°C, 2 hours). The content of the analyzed carbohydrates was calculated by the internal standard method.

Multivariate analysis of variance (MANOVA) and one-way analyses of variances (ANOVA) were performed in order to verify the zero hypothesis on a lack of differences between the regions. For individual traits, mean values and standard deviations were calculated. The Fisher's least significant differences (LSDs) were also estimated at the significance level α=0.01. The relationships between the observed traits were assessed on the basis of the correlation coefficients. A parallel coordinate plot has been proposed as an efficient tool for visualization of means of the regions (Bocianowski *et al*. 2015; Lekshmanan and Vahab, 2018). The analysis of canonical variables was applied in order to present a multi-trait assessment of similarity of the tested regions in a lower number of dimensions with the least possible loss of information. This makes it possible to illustrate variation in the regions in terms of traits in a graphic form. The Mahalanobis' distance was suggested as a measure of "poly-traits" species similarity (Seidler-Łożykowska and Bocianowski, 2012) and its significance was verified by means of critical value D_{α} called "the least significant distance" (Mahalanobis, 1936). All the analyses were conducted using the GenStat v. 18 statistical software package.

RESULTS AND DISCUSSION

The mean content of soluble carbohydrates in seeds of 66 accessions of common vetch used in the study has been reported earlier (Lahuta *et al*. 2018), but without analyzing the relationship between a genotype's origin and its composition of carbohydrates. In the present study, the results of MANOVA indicate that the regions were significant (Wilk's λ=0.1870; $F_{7,190}$=5.68; P<0.0001) with respect to differences in the content of each carbohydrate, as well as the total soluble carbohydrates (Total SCs) and total RFOs. Results of ANOVA confirm variation among the tested regions at the significance level α=0.01 for all traits. The concentration of total SCs ranged from 46.60 (VIC555) up to 68.05 mg g^{-1} DW, (Kwarta). Raffinose family oligosaccharides were the main fraction in SCs, constituting 62-77% of SCs (Table 2). Large variation was found for the RFOs content: from 35.76 (mean for BRT region) up to 46.99 mg g^{-1} DW (mean for MENA region). In the seeds of broad bean, a species closely related to common vetch, even greater variation in the content of RFOs (14-2 mg g^{-1} DW) was documented by Duc *et al*. (1999). In seeds of five Polish

Table 2: The concentration of total soluble carbohydrates (Total SCs) and among them total raffinose family oligosaccharides (Total RFOs), in common vetch seeds.

Group	Total SCs	Total RFOs	RFOs in SCs
	mg g^{-1} DW		%
Asia	62.26±3.37^{a}(61.07-63.84)	38.84±4.06^{cd}(35.28-43.34)	62.39
BRT	52.14±3.59^{c}(49.44-57.51)	35.76±3.39^{d}(31.30-38.92)	68.59
CE	60.06±5.43^{ab}(48.94-68.05)	43.28±6.97^{ab}(26.66-52.37)	72.07
EEBS	57.70±3.37^{b}(53.72-62.55)	41.01±5.65^{bc}(33.00-49.25)	71.07
Eurasia	59.97±5.68^{ab}(53.95-67.07)	46.05±4.07^{a}(40.38-50.75)	76.80
MENA	60.94±3.75^{ab}(57.12-65.19)	46.99±3.42^{a}(43.43-51.69)	77.11
MP	53.66±4.91^{c}(46.60-59.01)	41.42±3.63^{bc}(37.08-46.58)	77.20
WNE	57.66±4.49^{b}(54.40-64.21)	40.58±4.45^{bc}(33.20-46.01)	70.38
$LSD_{0.01}$	**3.39**	**3.91**	

Values are means (n=3)±standard deviation. The same letters indicate no significant differences in mean.

The ranges of concentrations are in brackets, whereas the percentage of RFOs in Total SCs is shown in the last column.

Table 3: The mean concentration (mg g^{-1} DW) of soluble carbohydrates in common vetch seeds from eight regions.

Region	Asia	BRT	CE	EEBS	Eurasia	MENA	MP	WNE	$LSD_{0.01}$
Sucrose	21.50^{a}	14.48^{b}	14.90^{b}	14.84^{b}	11.60^{c}	11.95^{c}	10.13^{c}	15.53^{b}	2.91
Raffinose	3.47^{a}	2.31^{bc}	2.51^{b}	2.24^{bc}	2.17^{bc}	1.76^{cd}	1.45^{d}	2.32^{bc}	0.58
Stachyose	10.27^{a}	8.32^{bc}	9.13^{b}	8.35^{bc}	8.69^{bc}	8.04^{c}	6.97^{d}	8.43^{bc}	1.01
Verbascose	25.10^{d}	25.13^{d}	31.65^{bc}	30.43^{c}	35.20^{ab}	37.20^{a}	33.01^{abc}	29.83^{c}	4.23
Myo-inositol	0.53^{bc}	0.46^{cd}	0.51^{bc}	0.56^{ab}	0.71^{a}	0.47^{bc}	0.6^{ab}	0.38^{c}	0.17
Galactinol	0.44^{c}	0.53^{bc}	0.52^{bc}	0.49^{bc}	0.83^{a}	0.64^{b}	0.67^{ab}	0.41^{c}	0.18
DGMI	0.49^{abc}	0.60^{a}	0.49^{bc}	0.49^{bc}	0.49^{bc}	0.55^{ab}	0.48^{bc}	0.42^{c}	0.11

cultivars of field bean (*V. faba* var. *minor* Harz.), the RFOs content was 23-30 mg g^{-1} DW (Hejdysz *et al*. 2016). Despite the differences in the content of RFOs, the dominance of verbascose over stachyose and raffinose among RFOs is a feature shared by both the common vetch genotypes tested in our study (Table 3) and most of broad bean accessions and cultivars of field bean. The mass ratio of verbascose to stachyose in common vetch ranged from 2 (VIC273, Ina, Kamiko, Niwa, Toplesa, VIC388, VIC541, VIC664, VIC703) up to 6 (VIC980, VIC337, Alba, LGR284/40, VIC1149). In contrast to faba bean, where the content of raffinose was higher than that of stachyose or verbascose in two accessions (among 12 tested), the content of verbascose was higher than stachyose and the content of stachyose was higher than raffinose in seeds of all the studied common vetch accessions (Table 3). Moreover, high positive correlation (r=0.96) was found between sucrose and stachyose and between raffinose and stachyose. The content of verbascose highly correlated with total RFOs (r=0.94). Sucrose was negatively correlated with verbascose (r=-0.75) and galactinol (r=-0.73). This means that the accumulation of verbascose has a dominant influence on the final level of RFOs in common vetch seeds. Beside RFOs, seeds of common vetch contained a small amount of *myo*-inositol and its α-D-galactosides - galactinol and di-galactosyl *myo*-inositol (Table 3). Similar composition of soluble carbohydrate as in mature common vetch seeds was found earlier in seeds of other *Vicia* species (Lahuta *et al*. 2018) belonging to section *Vicia* (*V. angustifolia*, *V. cordata*, *V. macrocarpa*) and *Narbonensis* (*V. narbonensis*, *V. johannis*), as well as in seeds of *Vicia faba* (Duc *et al*. 1999) and *Vicia faba* var. *minor* (Lahuta *et al*. 2000).

The greatest diversity in terms of all the nine traits jointly (measured by the Mahalanobis distances) was found for BRT and Eurasia (3.891). The greatest similarity was found for CE and EEBS (0.964). The effect of the origin of common vetch accessions on the differences in the composition of soluble carbohydrates was clearly distinguished by the canonical variable analysis (Fig 1). The higher differences were found between BRT, Eurasia and Asia. Moreover, parallel coordinate plots enabled separation of origin due to differences in the content of carbohydrates (Fig 2). For example, genotypes from Asia contained the highest, whereas those from MP had the lowest amounts of raffinose, stachyose and sucrose.

The most favorable genotypes for breeding, owing to the lowest content of RFOs (mean 35.76 mg g^{-1} DW) and the narrowest range of the RFOs content (31.30-38.92 mg g^{-1} DW), distinguished accessions from the Balkans Region and Turkey region (Table 2). Among them, the lowest content of RFOs (31-33 mg g^{-1} DW) was found in VIC120 and VIC542. However, similarly low RFOs (33 mg g^{-1} DW) were detected in some genotypes from other regions: VIC273, VIC 541 and Kraj Ołomun. The lowest RFOs was found in seeds of cultivars Ina and Kamiko (26.7 and 30.2 mg g^{-1} DW, respectively).

In recent years, there have been many reports indicating the positive role of galactinol and RFOs in plant response to abiotic stresses (Van den Ende, 2013; Kalyan and Ameta, 2017). In developing seeds, accumulation of RFOs is a way to store galactose, important for early germination events (Lahuta and Goszczyñska, 2009) and RFOs increase seed desiccation tolerance, longevity and vigor. Thus, the reduction of RFOs may lead to a decrease in seed physiological quality. However, raffinose and stachyose are not required for soybean seed germination (Dierking and Bilyeu, 2009). Furthermore, the lowest RFOs content is not an obstacle to breeding common vetch cultivars (Ina, Kamiko, Kraj Ołomun). Therefore, we recommend to continue the cultivation of common vetch as a good research object for deeper exploration of the effects of a lower content of RFOs on seed quality. However, taking into account the

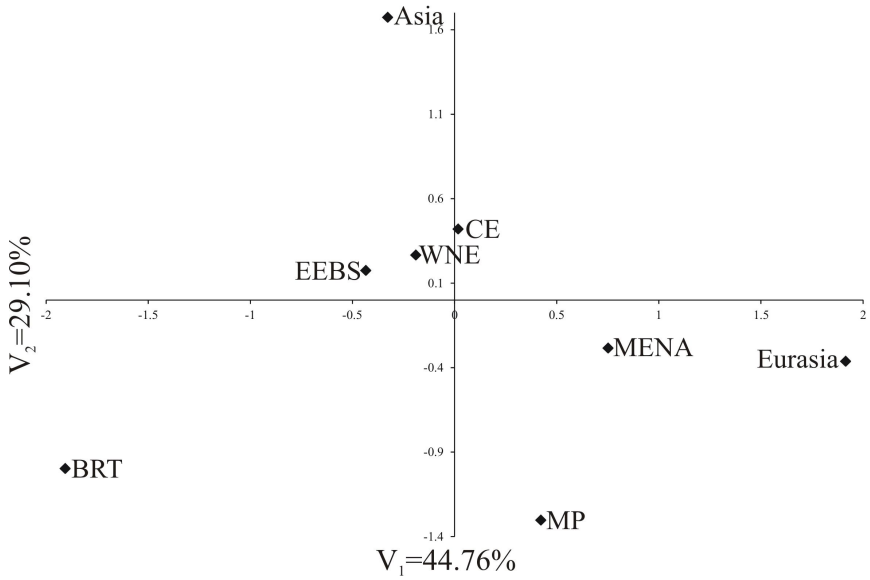

Fig 1: Distribution of eight regions studied in the space of two first canonical variables.

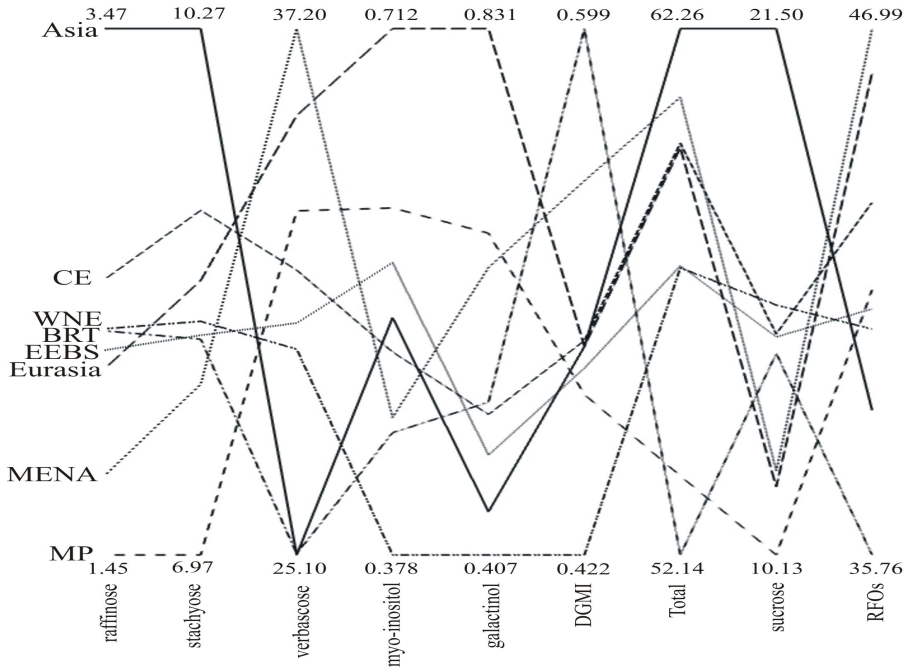

Fig 2: Parallel coordinate plots (PCPs) for eight studied regions and nine traits.

significant influence of environmental factors on the content of soluble sugars in seeds, as it was demonstrated in soybean (Hou *et al*. 2009), lentil (Tahir *et al*. 2011) and chickpea (Gangola *et al*. 2013), it seems necessary to further study on the impact of different locations at different climatic conditions on the content of sugars in seeds of selected vetch genotypes.

CONCLUSION

The composition and content of RFOs, a major soluble carbohydrate in vetch seeds, correlate with geographical seeds origin. Among 66 accessions, from eight origins, a few with decreased level of RFOs in seeds were identified. They are suitable for vetch breeding programs, focusing on decrease of RFOs, which can improve usefulness of common vetch seeds as feed or food.

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