RESEARCH ARTICLE

Diversity of the composition and content of soluble carbohydrates in seeds of the genus *Vicia* (Leguminosae)

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Received: 15 March 2017 / Accepted: 14 August 2017 / Published online: 30 August 2017
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Abstract Low molecular weight carbohydrates of seeds of 10 species of *Vicia*, namely: *V. angustifolia*, *V. articulata*, *V. cordata*, *V. ervilia*, *V. johannis*, *V. macrocarpa*, *V. monantha*, *V. narbonensis*, *V. pannonica* and *V. sativa* were analyzed by the high resolution gas chromatography method. Seeds of the investigated species contain common (glucose, fructose, *myo*-inositol, sucrose, galactinol, di-galactosyl *myo*-inositol and raffinose family oligosaccharides—RFOs) and species-specific carbohydrates (*D*-pinitol

and its α -*D*-galactosides—in *V. articulata*, *V. monantha* and *V. pannonica* or *D*-ononitol and its galactoside—in *V. ervilia*). Among the species containing in seeds RFOs as the main α -*D*-galactosides (*V. angustifolia*, *V. cordata*, *V. johanensis*, *V. macrocarpa*, *V. narbonensis* and *V. sativa*), an additional subgroup can be separated, which contains a set of unknown compounds (found in *V. angustifolia*, *V. cordata* and *V. macrocarpa*). Moreover, several other unidentified carbohydrate-containing compounds were detected exclusively in seeds of *V. ervilia*. The concentrations of total soluble carbohydrates (TSCs), including sugars, RFOs, cyclitols and galactosyl cyclitols and unknown compounds, in seeds differ significantly ($P < 0.05$) among the species. RFOs and sucrose are the main fractions in TSCs, with the exception of *V. pannonica* seeds, containing more galactosyl pinitols, and *V. ervilia*, in which unknown compounds account for 50% of TSCs. In *V. ervilia* RFOs occur at the lowest concentration (9.32 mg g⁻¹ seeds), and the concentration of DGMI (di-galactosyl *myo*-inositol) is comparable with that of RFOs, which is unusual among all the investigated *Vicia* species.

Electronic supplementary material The online version of this article (doi:[10.1007/s10722-017-0552-y](https://doi.org/10.1007/s10722-017-0552-y)) contains supplementary material, which is available to authorized users.

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Keywords *Vicia* · Seeds · Chemotaxonomy · Carbohydrate · Raffinose oligosaccharides · Pinitol

Introduction

The genus *Vicia* L. comprises about 160 annual and perennial species, which are distributed in Europe,

Asia, Americas and tropical East Africa (Schaefer et al. 2012). A few important annual species, such as *V. narbonensis* L. (narbon vetch), *V. sativa* L. subsp. *sativa* (common vetch), *V. ervilia* (L.) Willd. (bitter vetch) and *V. villosa* Roth subsp. *dasycarpa* (hairy-pod vetch) are grown for livestock feed in non-tropical dry areas. The grains of these vetches can be used as less costly sources of proteins and energy for ruminants and non-ruminants. However, seeds of some *Vicia* species contain also toxins and antinutritional compounds, restricting their use as food and feed (Enneking and Wink 2000). Moreover, the presence of raffinose family oligosaccharides (RFOs: including raffinose, stachyose, verbascose, ajugose), ubiquitous for seeds of all legumes (Obendorf and Górecki 2012), is an additional factor decreasing the use of vetch seeds by monogastric animals and humans. RFOs are α -D-galactosides of sucrose, containing 1–4 units of galactose linked by α -1,6 linkages. They are not digested in the upper part of the gastrointestinal tract, 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 seeds of some legumes, including several *Vicia* species, beside RFOs, α -D-galactosides of cyclitols (galactosyl cyclitols, GalCs) are present (Yasui and Ohashi 1990), whose flatulent potential seems to be lower than RFOs. The physiological effects of GalCs are not known. However, cyclitols (*myo*-inositol, *D-chiro*-inositol, *D*-pinitol), released during GalCs hydrolysis in the digestive tract, indicate some health benefit properties (insulin-mimetic, antioxidative, anti-inflammatory and anticancer activity) and can be used as diet supplements with therapeutic properties (Croze and Soulage 2013). Thus, legume seeds can be used not only as foodstuff, but also as a source of health-promoting cyclitols.

The content and composition of α -D-galactosides in seeds was characterized in detail in major legume crops: soybean (*Glycine max* [L.] Merr.), pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* L.) and chickpea (*Cicer arietinum* L.). The concentration of RFOs is within the range of 2–12% of dry matter and stachyose or verbascose are the prevalent oligosaccharides in seeds (Guillon and Champ 2002). The concentration of GalCs occurring in seeds of lentil, chickpea, soybean and lupin (*Lupinus* L.) is lower (up to 3–4%, in chickpea) than

that of RFOs (up to 12%, in lupin, Gulewicz et al. 2014). Galactosyl pinitols (ciceritol and galactopinitol A) predominate among GalCs (Obendorf et al. 2012).

Infrageneric variation of RFOs and GalCs in seeds of *Vicia* genus was characterized by Yasui et al. (1987). In the chemotaxonomic studies, these authors analyzed 29 species, including 19 accessions of common vetch and 3 accessions of narbon vetch. In the seeds of the two latter species, RFOs are the only α -D-galactosides, and verbascose was the predominant oligosaccharide (1.2–2.0% of dry mass). Verbascose is also a major oligosaccharide in bitter vetch (Muzquiz et al. 2012). Seeds of few other *Vicia* species accumulate considerable amounts of *D*-pinitol and its galactosides (Yasui et al. 1987; Lahuta et al. 2005a). The higher concentration of galactosyl pinitols than RFOs in seeds is a unique feature and has been found only in the subgenus *Cracca*, in seeds of *V. villosa* Roth, *V. cracca* L. (Yasui et al. 1987; Lahuta et al. 2005a; Lahuta 2006) and *V. tenuifolia* Roth (Lahuta et al. 2010a). The characterization of low molecular weight carbohydrates in seeds of legumes from different taxa allowed Yasui and Ohashi (1990) to conclude that the accumulation of RFOs is a primary evolutionary attribute of legumes, whereas the synthesis of *myo*-inositol methyl derivatives: *D*-ononitol, *D*-pinitol and *D*-bornesitol and their α -D-galactosides, is a secondary trait. The confirmation of this hypothesis can be found in catalytic properties of enzymes engaged in RFOs biosynthetic pathway and the lack of identification of enzymes responsible exclusively for the synthesis of galactosyl cyclitols (Peterbauer et al. 2003). The biosynthetic pathway of RFOs is determined by the activity of galactinol synthase (GolS), catalyzing the synthesis of galactinol from UDP-galactose and *myo*-inositol. Galactinol is the main galactosyl donor for the synthesis of raffinose, stachyose and verbascose (Peterbauer and Richter 2001). Raffinose synthase (RS), transferring the galactosyl moiety from galactinol to sucrose (producing raffinose), and stachyose synthase (STS), transferring the galactosyl moiety from galactinol to raffinose and stachyose (producing stachyose and verbascose, respectively), indicate multifunctional properties. They are able to transfer galactose to *D*-pinitol/*D*-ononitol and their galactosides (Peterbauer et al. 2002a, b). Moreover, STS from pea indicates an ability to catalyze the synthesis of verbascose from two molecules of stachyose (Peterbauer et al. 2003).

The indirect confirmation of the synthesis of both RFOs and galactosyl pinitols by the same set of enzymes was found in developing *Vicia villosa* seeds (Lahuta 2006). The profiles of enzyme activity and the accumulation of RFOs and galactosyl pinitols were very consistent. Additionally, in seeds of *Vicia* species naturally accumulating only *myo*-inositol and RFOs, and not *D*-pinitol/galactosyl pinitols, the activity directed towards the synthesis of ciceritol and tri-galactosyl pinitol A was found (Lahuta et al. 2010b). The accumulation of higher amounts of galactosyl pinitols in seeds may depend on *D*-pinitol (1*D*-3-*O*-methyl-*chiro*-inositol) at sufficiently high concentrations (Lahuta et al. 2005a, b, c, 2010a). This cyclitol is synthesized from *myo*-inositol by *myo*-inositol methyl transferase (IMT), catalyzing the synthesis of *D*-ononitol (1*D*-4-*O*-methyl-*myo*-inositol), which is later epimerized (presumably in a two-step reaction) to *D*-pinitol (Peterbauer and Richter 2001). The synthesis of *D*-ononitol/*D*-pinitol was found in vegetative tissues, but not in seeds (Obendorf and Górecki 2012). Thus, the accumulation of galactosyl pinitols/ononitols in seeds seems to depend on the transport of cyclitols from vegetative tissues into developing seeds. In fact, exogenously applied *D*-pinitol (via stem-leaf-pod explants feeding with solution containing cyclitols) affects the biosynthesis of RFOs, leading to the accumulation of galactosyl pinitols and decline in the level of RFOs (Gomes et al. 2004; Lahuta et al. 2005b, c, 2010a). The negative correlation between the accumulation of RFOs and galactosyl pinitols in seeds of *Vicia* species (Lahuta et al. 2010b) and lentil seeds (Frias et al. 1999) justifies the suggestion that the diminishing of RFOs content by their replacement with galactosyl cyclitols is achievable by breeding. However, for this purpose it will be necessary to discover genotypes with the most favorable composition of galactosides in seeds. Moreover, in *Vicia* species containing only RFOs, the discovery of genotypes with reduced levels of RFOs should be also helpful in breeding programs.

In the present study, the composition and content of soluble carbohydrates was analyzed in seeds of 166 accessions of 10 *Vicia* species. Although we focused on the RFOs/GalCs levels in seeds of three *Vicia* species: *V. sativa*, *V. narbonensis* and *V. ervilia*, the composition of soluble carbohydrates in seven wild *Vicia* species has not been previously demonstrated and can provide additional data to assist in the

establishment of the taxonomy of species of the genus *Vicia*.

Materials and methods

Material

Seeds of 10 *Vicia* species were derived from Gene Bank in Gatersleben (Germany), whereas 17 cultivars and breeding lines of *Vicia sativa* L. were derived from a collection of the Institute of Plant Genetics in Poznań (Poland). The investigated *Vicia* species belong to subgenus *Vicia*, *Cracca* and *Ervum*. Seven species from the sections: *Vicia* (*V. angustifolia* L., *V. cordata* Wulfen ex Hoppe, *V. macrocarpa* (Moris) Bertol and *V. sativa* L.), *Narbonensis* (*V. narbonensis* L. and *V. johannis* Tamamsch.) and *Hypechusa* (*V. pannonica* Crantz) in the subgenus *Vicia*, two species (*V. articulata* Hornem. and *V. monantha* Retz.) from the section *Cracca* in the subgenus *Cracca*, and one (*V. ervilia* (L.) Willd.) from the section *Ervilia* in the subgenus *Ervum* were used. The Latin and English names of the investigated species, details of seeds' origin, codes and number of accessions are given in Online Resources (Table 1). The highest number of accessions was analyzed among species: *V. sativa* (66), *V. ervilia* (13) and *V. narbonensis* (11). Investigated accessions grown in Experimental Field Polish Academy of Sciences located in Cerekwica (51°55'N, 17°21'E) in 2015 year, in 1 replication. The seeds were sown in rows (3 m long) and spacing of 50 cm between rows. Dry pods with seeds, collected from several plants, were combined and used for analyses of soluble carbohydrates.

Analyses of soluble carbohydrates

The composition and content of soluble carbohydrates were analyzed by the high resolution gas chromatography method, as described earlier (Lahuta 2006). Seeds (1 g) were pulverized in a mixer mill MM200 (Retsch, Germany) for 2 min at the frequency of 22 Hz. Carbohydrates were extracted from pulverized material (40–45 mg each, in 3 replications) with 900 µL of 50% aqueous ethanol solution containing xylitol (100 µg) as an internal standard. After heating at 90 °C for 30 min (with continuous shaking at 300 rpm), samples were centrifuged (21,000×*g* for

Table 1 The content of total soluble carbohydrates (TSCs) and sub-fractions: total sugars (fructose, glucose, sucrose), total RFOs (raffinose, stachyose, verbascose), total cyclitols (*myo*-inositol, D-pinitol, D-ononitol) and their α -D-galactosides (GalCs), total unknown compounds in seeds of *Vicia*

Sub-genus	Section	Vicia species (number of accessions)	Sub-fractions in TSCs					mg g ⁻¹ seeds		
			TSCs	Total sugars	Total RFOs	Total cyclitols and GalCs	Total UNK			
<i>Vicia</i>	<i>Vicia</i>	<i>V. angustifolia</i> (7)	78.57 ^{de} (62.32–93.76)	5.72 ^e (3.13–8.81)	61.57 ^{bc} (50.49–72.84)	2.98 ^c (2.38–3.84)	8.29 ^b (5.92–10.64)			
		<i>V. cordata</i> (12)	71.13 ^e (57.41–94.45)	10.97 ^{de} (5.86–15.26)	49.02 ^{de} (34.71–65.76)	2.14 ^c (1.27–3.23)	9.00 ^b (4.76–12.02)			
		<i>V. macrocarpa</i> (11)	70.57 ^e (60.47–76.98)	10.53 ^{de} (8.19–14.96)	47.83 ^e (43.54–53.52)	2.16 ^c (1.50–2.75)	10.05 ^b (5.98–15.00)			
		<i>V. sativa</i> (66)	58.37 ^f (46.60–68.05)	14.60 ^{bcd} (6.77–27.24)	42.19 ^e (26.66–52.37)	1.58 ^c (0.78–3.18)	0 ^c			
		<i>Narbonensis</i>	102.93 ^b (77.73–123.80)	14.38 ^{bcd} (9.56–22.31)	86.50 ^a (65.47–102.69)	2.05 ^c (1.25–4.22)	0 ^c			
<i>Cracca</i>	<i>Cracca</i>	<i>V. johannis</i> (6)	75.96 ^{de} (62.05–91.08)	10.12 ^{de} (6.55–13.60)	63.07 ^{bcd} (51.61–74.64)	2.77 ^c (2.18–4.31)	0 ^c			
		<i>V. pannonica</i> (12)	95.34 ^{bc} (79.24–122.50)	20.97 ^b (6.89–32.40)	31.93 ^f (17.56–38.90)	41.51 ^a (25.92–70.51)	0.93 ^c (0.64–1.36)			
		<i>V. articulata</i> (8)	121.87 ^a (108.29–137.90)	33.45 ^a (26.45–39.94)	70.98 ^b (62.97–77.29)	16.74 ^b (12.52–21.24)	0.70 ^c (0.52–0.79)			
<i>Ervum</i>	<i>Ervilia</i>	<i>V. monantha</i> (10)	88.14 ^{cd} (71.02–104.43)	18.49 ^{bc} (11.37–30.94)	50.31 ^{cde} (36.87–60.19)	18.03 ^b (8.19–44.76)	0.31 ^c (0.16–0.57)			
		<i>V. ervilia</i> (13)	115.82 ^a (102.68–132.49)	37.53 ^a (27.37–50.24)	9.32 ^g (5.79–15.91)	11.43 ^b (9.04–14.39)	57.54 ^a (34.86–87.34)			

Values are means (in mg g⁻¹ of seeds) for accessions in each of species. The range of content is given in parentheses. Values with different superscripts (a–f) are significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for each compound separately (comparisons valid within columns only)

Sugars—sucrose represented >95% of total sugars; UNK—the content of each unknown compound was calculated according to regression coefficient of the known standard with the closely related retention time

Fig. 1 Gas chromatograms of soluble carbohydrates extracted from seeds of four groups of *Vicia* species: **A** *V. johannis* and *V. narbonensis*, **B** *V. angustifolia*, *V. cordata*, *V. macrocarpa* and *V. sativa*, **C** *V. articulata*, *V. monantha* and *V. pannonica*, **D** *V. ervilia*. This figure demonstrates the separation of soluble carbohydrates from seeds of species showing differences in sugars, RFO, cyclitols, galactosyl cyclitols, and unknown compounds by gas chromatography. Identification of

compounds: *IS* xylitol (internal standard), *F* fructose, *G* glucose, *MI* myo-inositol, *Su* sucrose, *Go* galactinol (galactosyl myo-inositol), *1* di-galactosyl glycerol (presumably), *Ra* raffinose, *DG* di-galactosyl myo-inositol, *St* stachyose, *V* verbascose, *PI* D-pinitol, *GA* galactosyl pinitol A, *GB* galactosyl pinitol B, *CI* ciceritol (di-galactosyl pinitol A), *TGA* tri-galactosyl pinitol A, *ON* D-ononitol, *GN* galactosyl ononitol, 2–23 unknown compounds

30 min at 4 °C) and 400 µL of homogenate was deionized with the mixture (300 µL) of Dowex ion exchange resins for 45 min (with shaking at 1250 rpm). After centrifugation, a portion of the clear extract (200 µL) was concentrated (in 2 ml gas chromatography vials containing glass inserts) in a rotary evaporator until dry. Dry residues were derivatized with a mixture of TMSI (trimethylsilyl-imidazole) and pyridine (1:1, v/v) at 80 °C for 45 min. TMS-derivatives of carbohydrates were separated in a ZB-1 capillary column (15 m length, 0.25 mm diameter, 0.1 µm thickness, Phenomenex, USA) in a GC 2010 gas chromatograph (Shimadzu, Japan). The temperature of the injector and detector (flame ionization detector) was 325 and 350 °C, respectively. The column was heated from 150 to 350 °C at

different rates of temperature increase. Helium was used as a carrier gas. Chromatograms were analyzed with an integrator in the CHROMA 3.2 application (Pol-Lab, Poland). Each carbohydrate was quantified by using authentic standards (sugars, cyclitols, oligosaccharides and galactinol) purchased from Sigma-Aldrich (USA). Standards of galactosyl pinitols: galactosyl pinitol A (GPA), galactosyl pinitol B (GPB), di- and tri-galactosyl pinitol A (ciceritol and TGPA, respectively), commercially unavailable, were extracted and purified from seeds of *Vicia villosa* Roth (Szczeciński et al. 2000).

Di-galactosyl myo-inositol (DGMI) and galactosyl ononitol, isolated from seeds of *Vicia cracca* and *Vigna angularis* by the method described previously (Szczeciński et al. 2000) were tentatively identified

after their hydrolysis to galactose and *myo*-inositol/*D*-ononitol (2 N trifluoroacetic acid, 80 °C, 2 h). The content of the analyzed carbohydrates was calculated by the internal standard method. The contents of unidentified compounds (peaks no 1–23, Fig. 1) were calculated according to the regression coefficients for the known carbohydrate standards with the closest retention time. Thus, these calculations are only approximate.

Statistical analysis

The results were presented (in mg g⁻¹ of seeds) as means from three replications. The significance of differences in the concentrations of various carbohydrates was determined by one-way ANOVA with the Tukey's test. Data were also analyzed using multivariate methods. The canonical variable analysis was applied in order to present multitrait assessment of similarity of the tested species in a lower number of dimensions with the least possible loss of information (Rencher 1992). This makes it possible to illustrate variation in species in terms of all observed traits in a graphic form. Mahalanobis' distance was suggested as a measure of "polytrait" species similarity (Seidler-Łożykowska and Bocianowski 2012), whose significance was verified by means of critical value D_α called "the least significant distance" (Mahalanobis 1936). Mahalanobis' distances were calculated for species. All the analyses were conducted using the GenStat v. 17 statistical software package.

Results and discussion

The carbohydrate composition in *Vicia* seeds

The composition of soluble carbohydrates in seeds of the analyzed *Vicia* species has not been reported earlier, except for a few genotypes of *V. angustifolia* (Lahuta et al. 2005a), *V. sativa* and *V. narbonensis* (Yasui et al. 1987; Lahuta et al. 2005a). In the present study, the gas chromatographic analyses revealed distinct composition of soluble carbohydrates in seeds among the investigated *Vicia* species (Fig. 1). Common carbohydrates (fructose, glucose, sucrose, raffinose, stachyose, verbascose, *myo*-inositol, galactinol and DGMI) and species-specific cyclitols: *D*-pinitol, *D*-ononitol and their α -*D*-galactosides, were determined

in the seeds (Fig. 1). Moreover, several compounds (containing sugar moieties) found in GC-chromatograms were not identified. Their identity with other galactosyl cyclitols (GalCs) occurring in some legumes (galactosides of *D*-*chiro*-inositol or *D*-ononitol) can be excluded because of: (1) differences between retention times of the unidentified compounds and retention times of galactosyl cyclitols standards and (2) absence of *myo*-inositol isomers/methyl derivatives (excluding *D*-pinitol/*D*-ononitol).

According to the differences in the composition of soluble carbohydrates in seeds, *Vicia* species can be classified into four groups (A–D, Fig. 1). In the first group (A), fructose, glucose, *myo*-inositol, sucrose, RFOs, galactinol and DGMI were present (Fig. 1A). Beside two compounds, di-galactosyl glycerol (peak no. 1 on Fig. 1A) and presumably pyrimidine glycoside, vicine (2,6-diamino-4-hydroxy-5-pyrimidinyl β -*D*-glucopyranoside, peak no. 2 on Fig. 1B), common for all *Vicia* species (Yasui and Ohashi 1990), no other carbohydrates were found. Such composition of carbohydrates characterized seeds of *V. johannis* and *V. narbonensis* (section *Narbonensis*). Among RFOs, verbascose was the dominant oligosaccharide.

In the second group: *V. angustifolia*, *V. cordata*, *V. macrocarpa* and *V. sativa* (section *Vicia*), the share of verbascose in RFOs was the highest (84–88%) among all the investigated *Vicia* species, and seeds contained also considerable amounts of a few unidentified compounds (peaks no. 2–6, Fig. 1B). The higher concentration of verbascose in seeds of *V. angustifolia* than in seeds of different *Vicia* species (in the subgenera *Vicia* and *Vicilla*) was described earlier by Lahuta et al. (2010b). Some accessions of *V. sativa* differed in the content of an unknown compound (peak no 6, Fig. 1B), presumably vicianine (α -cyanobenzyl 6-*O*- α -*L*-arabinopyranosyl- β -*D*-glucopyranoside), occurring in this *Vicia* species (Yasui and Ohashi 1990).

Seeds of the third group, including *V. articulata*, *V. monantha* (section *Cracca*) and *V. pannonica* (section *Hypechusa*), beside having RFOs contained *D*-pinitol and its galactosides: GPA, GPB, ciceritol and TGPA, (Fig. 1C). In some species from the section *Cracca* (*V. cracca*, *V. hirsuta*, *V. villosa*) and *Hypechusa* (*V. tetrasperma*) the relative proportions of mono- di- and tri-galactosyl pinitols are different and species-specific (Yasui et al. 1987; Lahuta et al. 2005a, b, c, 2010b; Lahuta 2006). Our results confirm the accumulation of *D*-pinitol and galactosyl pinitols in seeds as a common

Table 2 The content of sucrose, raffinose, stachyose and verbascose in seeds of *Vicia*

Sub-genus	Section	<i>Vicia</i> species	Sucrose	Raffinose	Stachyose	Verbascose
			mg g ⁻¹ seeds			
<i>Vicia</i>	<i>Vicia</i>	<i>V. angustifolia</i>	5.47 ^f (2.96–8.49)	0.71 ^d (0.50–0.86)	6.47 ^{ef} (4.72–8.33)	54.39 ^a (45.20–63.65)
		<i>V. cordata</i>	10.65 ^d (5.62–14.74)	0.76 ^d (0.40–1.46)	6.84 ^e (4.41–10.06)	41.42 ^c (29.74–55.41)
		<i>V. macrocarpa</i>	10.41 ^d (8.09–14.86)	1.04 ^d (0.71–1.29)	6.60 ^e (5.69–7.45)	40.19 ^c (35.04–46.09)
		<i>V. sativa</i>	14.26 ^c (6.48–26.72)	2.29 ^c (0.81–4.36)	8.61 ^e (5.40–12.07)	31.29 ^d (14.27–42.86)
	<i>Narbonensis</i>	<i>V. narbonensis</i>	13.96 ^{de} (9.04–21.92)	6.82 ^a (4.70–10.02)	31.32 ^b (21.37–48.01)	48.36 ^b (35.67–66.95)
		<i>V. johannis</i>	9.92 ^{cd} (6.36–13.40)	1.23 ^d (0.88–1.72)	9.51 ^e (6.68–11.53)	53.34 ^{ab} (43.94–61.39)
	<i>Hypechusa</i>	<i>V. pannonica</i>	20.71 ^b (6.77–31.98)	3.83 ^b (1.82–6.14)	19.62 ^d (9.26–26.07)	8.48 ^f (6.40–16.71)
		<i>V. articulata</i>	33.17 ^a (26.20–39.74)	7.38 ^a (5.52–10.52)	47.14 ^a (38.07–53.58)	16.46 ^e (10.41–20.42)
<i>Cracca</i>	<i>Cracca</i>	<i>V. monantha</i>	19.01 ^{bc} (10.75–30.28)	7.42 ^a (2.96–10.81)	26.10 ^c (19.03–39.52)	16.79 ^e (2.99–28.82)
		<i>V. ervilia</i>	37.24 ^a (27.1–49.89)	0.78 ^d (0.56–1.05)	4.15 ^f (2.89–6.24)	4.40 ^f (2.22–8.77)

Values are means for accessions in each of species. In parentheses the range of concentrations was showed. Values with different superscripts (a–f) are significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for each compound separately (comparisons valid within columns only)

feature of species belonging to both sections: *Cracca* and *Hypechusa*.

In seeds of the last group (one species *V. ervilia*), D-pinitol was absent, whereas D-ononitol—a precursor in the biosynthetic pathway of D-pinitol (Peterbauer and Richter 2001) and galactosyl ononitol were detected (Fig. 1D). Both compounds were identified earlier in seeds of *V. tetrasperma*, belonging to the section *Ervum* and *V. dumetorum* from the section *Vicilla* (Yasui et al. 1987). However, seeds of both *V. tetrasperma* and *V. dumetorum* contained also D-pinitol and its galactosides (Yasui et al. 1987; Lahuta et al. 2005b), which were absent in *V. ervilia* (our study). Although Muzquiz et al. (2015) reported the presence of galactopinitol and ciceritol in seeds of two cultivars of *V. ervilia* (Villanueva and Taranto), our results (13 accessions) contradict the occurrence of D-pinitol and galactosyl pinitols in *V. ervilia* (Fig. 1D). Moreover, the retention times of two peaks, present in GC chromatograms of extracts from *V. ervilia* seeds (peaks no. 10 and 11, between myo-inositol and sucrose, Fig. 1D), were identical with retention times of two compounds occurring in seeds of *V. hirsuta* and *V. sylvatica* only (Yasui et al. 1987; Lahuta et al. 2010b). Other unique features of the composition of carbohydrates in seeds of *V. ervilia* were: (1) the lowest amount of RFOs, as compared to other *Vicia* species; (2) the highest content of DGMI and (3) the considerable amounts of several unidentified compounds (Fig. 1D, peaks no. 8–23), with retention times

differing from those unknown compounds found in seeds of the second *Vicia* group (Fig. 1B).

The content of soluble carbohydrate in seeds

The content of soluble carbohydrates in seeds (means and a range for species) is presented in Tables 1, 2, 3. Species are listed in the order of their current taxonomic classification (Leht 2005; Jaaska 2008; Schaefer et al. 2012; Smýkal et al. 2015). In view of a very low content of fructose and glucose (<1 mg g⁻¹ seeds), these data were omitted.

The significantly ($P < 0.05$) highest content of total soluble carbohydrates (TSCs) was found in seeds of *V. articulata* (section *Cracca* in subgenus *Cracca*) and *V. ervilia* (section *Ervilia* in subgenus *Ervum*): 121.87 and 115.82 mg g⁻¹, respectively (Table 1). The reason was the high content of sucrose and RFOs, or sucrose and unknown compounds, in *V. articulata* and *V. ervilia*, respectively (Tables 1, 2). In seeds of all accessions of *V. articulata* and *V. ervilia*, the level of TSCs was above 100 mg g⁻¹. A two-fold lower content of TSCs in seeds of *V. sativa* (subgenus *Vicia*, section *Vicia*)—58.37 mg g⁻¹ was found (Table 1). The range of TSCs in this species was also low: 46.60–68.05 mg g⁻¹.

The main sub-fraction of soluble carbohydrates consisted of raffinose oligosaccharides (RFOs), making up from 57% (*V. monantha*) to 84% of TSCs (*V. narbonensis*). The significantly ($P < 0.05$) highest

Table 3 The content of cyclitols (*myo*-inositol and *D*-pinitol) and their galactosides (galactinol and DGMI; GPA, GPB, ciceritol and TGPA, respectively) in seeds of *Vicia*

Section	<i>Vicia</i> species	Cyclitols and their α -D-galactosides		D-Pinitol and its α -D-galactosides (galactosyl pinitols)					
		<i>Myo</i> -inositol and its α -D-galactosides		Galactinol	DGMI	D-Pinitol			
		<i>Myo</i> -inositol	mg g ⁻¹ seeds			GPA	GPB	Ciceritol	TGPA
<i>Vicia</i>	<i>V. angustifolia</i>	0.51 ^{cd} (0.4–0.7)	1.63 ^b (1.3–2.1)	0.84 ^b (0.6–1.1)	– [*]	–	–	–	–
	<i>V. cordata</i>	0.59 ^c (0.2–0.9)	0.92 ^d (0.6–1.6)	0.62 ^b (0.4–0.8)	–	–	–	–	–
	<i>V. macrocarpa</i>	0.61 ^c (0.3–0.9)	0.91 ^d (0.5–1.2)	0.64 ^b (0.4–0.8)	–	–	–	–	–
	<i>V. sativa</i>	0.53 ^c (0.2–1.6)	0.56 ^e (0.2–1.3)	0.50 ^b (0.2–0.9)	–	–	–	–	–
<i>Narbonensis</i>	<i>V. narbonensis</i>	0.59 ^c (0.2–1.3)	1.09 ^d (0.6–2.0)	0.37 ^b (0.2–1.0)	–	–	–	–	–
	<i>V. johannis</i>	0.73 ^{bc} (0.5–1.2)	1.20 ^{cd} (0.8–1.9)	0.84 ^b (0.5–1.2)	–	–	–	–	–
<i>Hypochaeris</i>	<i>V. pannonica</i>	0.31 ^d (0.1–0.5)	0.50 ^e (0.2–0.9)	0.60 ^b (0.2–1.0)	6.51 ^a (2.8–17.6)	2.70 ^b (1.5–4.6)	1.01 ^a (0.5–1.9)	25.19 ^a (16.9–39.4)	4.68 ^a (2.1–7.9)
<i>Cracca</i>	<i>V. articulata</i>	1.39 ^a (1.1–1.9)	2.30 ^a (1.4–2.9)	0.66 ^b (0.2–0.8)	5.75 ^a (2.9–10.8)	2.27 ^{ab} (1.4–3.3)	0.84 ^a (0.5–1.2)	3.17 ^c (1.7–5.1)	0.24 ^b (0.1–0.5)
	<i>V. monantha</i>	0.81 ^b (0.4–1.1)	0.88 ^d (0.4–2.7)	0.71 ^b (0.2–2.8)	4.25 ^a (2.3–13.5)	1.34 ^b (0.7–2.7)	0.79 ^a (0.5–1.3)	8.20 ^b (2.9–22.4)	0.99 ^b (0.1–6.4)
<i>Ervum</i>	<i>V. ervilia</i>	0.54 ^c (0.3–0.7)	1.46 ^{bc} (0.9–1.9)	8.57 ^a (6.6–10.8)	–	–	–	–	–

Values are means for objects in each of species. In parentheses the range of concentrations was shown. Values with different superscripts (a–e) are significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for each compound separately (comparisons valid within columns only)

DGMI di-galactosyl *myo*-inositol, GPA and GPB galactosyl pinitol A and galactosyl pinitol B, ciceritol di-galactosyl pinitol A, TGPA tri-galactosyl pinitol A

* Not detected

content of RFOs (mean 86.50 mg g^{-1}) was found in seeds of *V. narbonensis* (Table 1). In seeds of other economically important *Vicia* species—*V. sativa* (common vetch) and *V. ervilia* (bitter vetch), the content of RFOs was two-fold and ca 10-fold lower (respectively) than in *V. narbonensis* (Table 1). The high level of RFOs in *V. narbonensis*, found in our study (Tables 1, 2), is consistent with results obtained by Rolletschek et al. (2002) and Martín-Pedrosa et al. (2016). In a wild type of *V. narbonensis*, the level of RFOs was 70.2 mg g^{-1} dry mass, whereas in lines with a disrupted starch biosynthesis pathway, the level of RFOs increased up to 94 mg g^{-1} dry mass (Rolletschek et al. 2002). In our study, some accessions of *V. narbonensis*, suitable for a breeding program dedicated to the lowering of the level of RFOs in seeds, were identified. The lowest content of RFOs ($65\text{--}67 \text{ mg g}^{-1}$) was found in accessions: VNAR154 (from Italy) and VNAR139 (Spain). In all the accessions of narbon vetch, verbascose was the predominant oligosaccharide in RFOs.

In common vetch, a few genotypes (out of 66 analyzed) with a decreased content of RFOs ($26.6\text{--}35.9 \text{ mg g}^{-1}$) in seeds were found. They are two Polish cultivars, Ina and Kamiko, the Czech cultivar Kraj Otomun and accessions from Albania (VIC121), Latvia (VIC541), Sweden (VIC273), Yugoslavia (VIC542), Turkey (VIC101), Mongolia (VIC664) and Ukraine (VIC749). Verbascose was the predominant oligosaccharide (Table 2). In our previous study, a low content of RFOs (36.7 mg g^{-1} dry mass) was found in seeds of *V. sativa* cv. Kamiko and Kwart (Lahuta et al. 2010a, b). The mean content of RFOs in seeds of accessions of *V. sativa* is as high (42.19 mg g^{-1}) as in seeds of *Vicia faba* (Yasui et al. 1987; Guillon and Champ 2002) and *V. faba* var. *minor* (Zalewski and Lahuta 1998).

Seeds of bitter vetch contained the lowest amount of RFOs (mean for 13 accessions 9.32 mg g^{-1}) among 10 investigated *Vicia* species (Table 1). Similarly low levels of RFOs were estimated recently by Martín-Pedrosa et al. (2016) in seeds of two bitter vetch cultivars. Such a low level of RFOs is unique among legumes (Kadleec et al. 2001). Moreover, in 3 of the 13 bitter vetch accessions analyzed: ERV93 (France), ERV524 (Bulgary) and ERV94 (Afghanistan), the content of RFOs was below 7 mg g^{-1} , whereas in 4—it was above 10 mg g^{-1} (data not presented). Verbascose or stachyose were main oligosaccharides in

RFOs. The low amount of RFOs coincided with the highest content of sucrose (Table 2) and di-galactosyl myo-inositol (DGMI, Table 3). Such composition indicates that synthesis and accumulation of DGMI in *V. ervilia* seeds occurs concurrently with the synthesis of RFOs. The transfer of the galactosyl moiety from galactinol to sucrose (producing raffinose) and then to raffinose and stachyose seems to be impaired by the transfer to galactinol, producing DGMI. Thus far, no data have been made available that would identify the enzyme controlling this reaction. It can be supposed that multifunctional STS (Peterbauer and Richter 2001; Peterbauer et al. 2002b) and RS (Peterbauer et al. 2002a) can catalyze the transfer of the galactosyl moiety from one molecule of galactinol to another. D-Pinitol was absent in *V. ervilia*, but its seeds contained D-ononitol (0.42 mg g^{-1}), which can be used by STS as a substrate in the synthesis of galactosyl ononitol (Peterbauer and Richter 1998). In fact, galactosyl ononitol was detected in seeds of *V. ervilia* (0.43 mg g^{-1} , data not presented). The question remains whether some of secondary metabolites, occurring in *V. ervilla* at high concentrations (49% of TSCs, Fig. 1d; Table 1), can regulate the activity of enzymes engaged in the biosynthesis of RFOs. Moreover, the presence of D-ononitol (precursor in biosynthesis of D-pinitol, Peterbauer and Richter 2001) and galactosyl ononitol in seeds, which is a trait of only few legumes (Yasui and Ohashi 1990; Peterbauer and Richter 1998), and the absence of D-pinitol implicate that the epimerization of D-ononitol to D-pinitol does not operate in *V. ervilia*. Thus, the role of *V. ervilia* as a presumed ancestor of other *Vicia* species, accumulating D-pinitol and galactosyl pinitols beside RFOs, awaits clarification. The presence of galactopinitol and ciceritol in seeds of cultivated bitter vetch, reported by Martín-Pedrosa et al. (2016), is not possible without the presence of D-pinitol, and can be a result of incorrect identification of some peaks in GC-chromatograms (Fig. 1d), due to the lack of appropriate standards.

Among the investigated *Vicia* species, D-pinitol and galactosyl pinitols were accumulated in seeds of *V. pannonica*, *V. articulata* and *V. monantha* (Table 3). Galactosyl ononitol was absent, whereas D-ononitol was below the detection limit or only in trace amounts (not presented data). In seeds accumulating D-pinitol, the concentration of its intermediate, i.e. D-ononitol, is

generally very low (Yasui et al. 1987; Lahuta et al. 2005b; Obendorf et al. 2012). Seeds of *V. pannonica* contained a significantly higher ($P < 0.05$) amount of galactosyl cyclitols (41.51 mg g^{-1}) than seeds of *V. articulata* and *V. monantha* (6.49 and 11.32 mg g^{-1} , respectively, Table 1). Ciceritol was the main galactosyl pinitol (Table 3). Seeds of *V. pannonica* also contained the highest amount of TGPA (4.68 mg g^{-1}). In seeds containing D-pinitol, the content of its galactosides significantly increased the participation of total GalCs in TSCs (up to 36.2% in *V. pannonica*). In seeds of *V. pannonica*, the high level of galactosyl pinitols (33.58 mg g^{-1} , Table 3) coincided with the low level (31.93 mg g^{-1}) of RFOs, as compared to the other species of the subgenera *Vicia* and *Cracca* (Table 1). A negative relationship between the level of RFOs and GalCs was found earlier in seeds of *V. tenuifolia* and *V. cracca* (Lahuta et al. 2010a). The content of galactosyl pinitols in seeds of *V. pannonica* (Table 3) was lower (33.58 mg g^{-1}) than in *V. cracca* and *V. tenuifolia*, 44.7 and 70.1 mg g^{-1} , respectively (Lahuta et al. 2010a), and similar to *V. villosa* (Lahuta et al. 2010b). However, tetra-galactosyl pinitol A, present in seeds of *V. tetrasperma*, *V. villosa*, *V. cracca* and *V. tenuifolia* (Lahuta et al. 2010a, b), was not detected in *V. pannonica*.

Statistical analyses of our results confirmed the taxonomic classification of the investigated *Vicia* species. The *Vicia* species can be separated according to the results of a canonical variable analysis of the content of all soluble carbohydrates (including unidentified compounds) in seeds (Fig. 2). The first two canonical variables accounted for 88.69% of the total multivariate variability between *Vicia* species (Fig. 2). The greatest variability in terms of all the analyzed traits expressed jointly with the greatest Mahalanobis distance was recorded for the *V. ervilla* and *V. articulata* (Mahalanobis distance equal to 22.605) as well as between *V. ervilla* and *V. angustifolia* (20.979). In turn, the strongest phenotypic similarity was observed for *V. sativa* and *V. cordata* (2.391), *V. narbonensis* and *V. johannis* (2.526) as well as for *V. sativa* and *V. macrocarpa* (2.671) (Online resource, Table 1). The separation of *V. ervilla* from other *Vicia* species is a result of its lowest level of RFOs, highest content of DGMI, absence of D-pinitol (and its galactosides) and presence of unknown compounds at high concentrations

(Tables 1, 2, 3). Seeds of *V. cordata*, *V. monantha* and *V. pannonica* accumulate higher amounts of sucrose, stachyose (Table 2), D-pinitol and its galactosides (Table 3). The differences in the composition of carbohydrates in the remaining 6 *Vicia* species are smaller (Fig. 2).

The presence of D-pinitol/D-ononitol (and galactosyl cyclitols) or unknown compounds seems to be a trait that substantiates some preliminary confirmation of the taxonomic classification of *Vicia* species. However, the phylogenetic tree constructed according to myo-inositol, galactinol, DGMI, raffinose, stachyose and verbascose (cyclitol and α -D-galactosides common for 10 *Vicia* species) indicated an evolution of two descendants—the subgenus *Cracca* and an unknown one, from which presumably the subgenera *Ervum* and *Vicia* evolved. The latter one differentiated into the sections *Vicia* and *Narbonensis* (Fig. 3).

The infrageneric variation of low molecular weight carbohydrate composition in *Vicia* (as well in all Fabaceae) can be an effect of both evolutionary trends and domestication, as suggested Yasui and Ohashi (1990). Although narbon and common vetch belong to legumes domesticated early (Schaefer et al. 2012; Smýkal et al. 2015), they did not develop enzymatic pathways leading to the synthesis of myo-inositol isomers/methyl derivatives. Such an important evolutionary trait developed in bitter vetch, another *Vicia* species domesticated early (Mikić et al. 2015). The primitive ancestors accumulated RFOs in seeds, whereas the ability to synthesize galactosides of myo-inositol methyl derivatives (D-ononitol, D-pinitol, D-bornesitol) developed later, as a result of the synthesis of different cyclitols in vegetative tissues, presumably due to the pressure of unfavorable environmental conditions. The accumulation of methyl derivatives of myo-inositol can be an element of mechanisms ensuring tolerance to abiotic stresses such as: salinity, drought and cold (Merchant and Richter, 2011). Developing embryos are unable to convert myo-inositol into methyl derivatives, or even to epimerize of D-ononitol to D-pinitol (Obendorf and Górecki 2012). Thus, the accumulation of such cyclitols and the biosynthesis of appropriate galactosyl cyclitols in seeds arise from the synthesis of cyclitols in vegetative tissues and their temporary transport from the mother plant to seeds. Consequently, cyclitols can redirect the accumulation of galactose from RFOs into the GalCs pathway only at

Fig. 2 Distribution of ten *Vicia* species studied in the space of two first canonical variables. Original data set: all identified carbohydrate and unidentified compounds

Fig. 3 Phylogenetic tree for 10 *Vicia* species based on the content of *myo*-inositol, galactinol, DGMI, raffinose, stachyose and verbascose in seeds

sufficiently high concentrations. Both galactinol synthase (GolS) and two enzymes synthesizing RFOs, i.e. RS and STS, indicate the ability to use specific cyclitols as galactosyl acceptors (Obendorf and Górecki 2012). The accumulation of GalCs in legumes instead of RFOs has not been documented yet. RFOs are mostly replaced by α -D-galactosides of D-*chiro*-inositol, named fagopyritols only in seeds of buckwheat (*Fagopyrum esculentum* Moench, *Polygonaceae*) (Horbowicz et al. 1998). Beside galactinol (from UDP-galactose and *myo*-inositol), the two forms of GolS also demonstrate an ability to synthesize fagopyritol B1 (α -D-galactoside of D-*chiro*-inositol) or a mixture of fagopyritols (A1 and B1) from UDP-galactose and D-*chiro*-inositol (Ueda et al. 2005). Similar activity was found in *Vicia* seeds (Lahuta et al. 2005c). Some mutations in enzymes of the RFOs pathway found in soybean (Obendorf et al. 2008) lead to a decrease in RFOs and an increase in the amount of galactosyl cyclitols, which can enhance the imbibitional chilling tolerance of seeds (Obendorf et al. 2008). Karner et al. (2004) found a significant role of *myo*-inositol at elevated concentrations for the accumulation of higher amounts of RFOs in pea seeds. However, recessive mutations in main pea seed genes (*R*, *A* and *I*) seems to be more important for an increased content of RFOs (Gawłowska et al. 2017). Among 248 accessions of pea (derived from Polish *Pisum* Genebank) the highest content of RFOs were stated for accessions with wrinkled seeds (*r* and *rb* genes), whereas the lowest content for seeds of the wild species *P. fulvum* Sibth. et Sm. It was found that the content of total RFOs was the most highly, frequently, and positively correlated with a stachyose and verbascose. In seeds of forty faba bean (*Vicia faba* L.) populations the concentration of RFOs ranged from 25 to 75 mg g⁻¹ of dry mass, and verbascose was the predominant oligosaccharide (Landry et al. 2016). In chickpea and lentil occur D-pinitol and its galactosides (mainly ciceritol). In seeds of both species the concentration of ciceritol is as high as stachyose (the predominant RFO) (Xiaoli et al. 2008; Muzquiz et al. 2012). In lentil crosses, a large negative correlation was found between the level of verbascose and ciceritol in seed (Frias et al. 1999). However, they are not available data on diversity of D-pinitol and galactosyl pinitols among the more numerous accessions of genus *Cicer*

and *Lens*. Thus, plants of the genus *Vicia* can be used as an excellent object for research on the evolution of the biosynthetic pathway of *myo*-inositol methyl derivatives.

Conclusions

The GC separation of TMS-derivatives of soluble carbohydrates can be a useful tool for preliminary identification of species belonging to different *Vicia* subgenera, which can be important during breeding programs of *Vicia*. Beside obvious carbohydrates: sucrose, *myo*-inositol, galactinol and raffinose family oligosaccharides (RFOs), seeds of some *Vicia* species contain species-specific cyclitols and their α -D-galactosides. The presence of D-pinitol and its galactosides in seeds of *V. pannica*, *V. articulata* and *V. monantha* has been demonstrated for the first time. Galactosyl ononitol was detected at a low concentration only in seeds of *V. ervilia*. Seeds of *V. ervilia* contained the lowest amount of RFOs, but the highest quantity of digalactosyl *myo*-inositol among all the analyzed *Vicia* species. Moreover, considerable amounts of unknown compounds were found in *V. ervilia*. Among cultivated species of *Vicia*, seeds of narbon vetch accumulate the highest amount of RFOs (86.5 mg g⁻¹), whereas the level of RFOs in common vetch is two-fold lower. Some accessions/cultivars of common vetch and narbon vetch with lowered levels of RFOs were found. The differences in soluble carbohydrates in seeds support the current taxonomic classification of the analyzed *Vicia* species.

Acknowledgements This study was partially supported by the programme “Improvement of native plant protein feeds, their production, trade turnover and utilization in animal feed” of the Ministry of Agriculture and Rural Development of Poland.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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