ORIGINAL ARTICLE

# The occurrence and accumulation of D-pinitol in fenugreek (*Trigonella foenum graecum* L.)

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## Abstract

This article presents changes in concentrations of D-pinitol (and other cyclitols as well as low molecular weight carbohydrates) in vegetative and reproductive organs of fenugreek (*Trigonella foenumgraecum* L.) during an entire plant growing period. D-Pinitol was the major cyclitol in all tested organs, representing 43–94% of total cyclitols and 2–77% of total soluble carbohydrates. The highest concentration of D-pinitol was found in pods (14–23 mg g$^{-1}$ of dry weight, DW), lower in leaves and stems (5–20 and 9–10 mg g$^{-1}$ DW, respectively), and the lowest in maturing seeds (2–5 mg g$^{-1}$ DW). Although maturing seeds accumulate α-D-galactosides of D-pinitol (galactosyl pinitols, up to 6.6 mg g$^{-1}$ DW), the major storage sugars were raffinose family oligosaccharides (RFOs, 65.37 mg g$^{-1}$ DW). Both RFOs and galactosyl pinitols are hydrolyzed during seed germination, releasing sucrose and D-pinitol, respectively. Accumulation of free galactose was not detected. Owing to the high concentration of D-pinitol (up to 23.70 mg g$^{-1}$ DW) and low concentration of soluble sugars, developing pods seem to be the best source of D-pinitol.

## Introduction

Fenugreek (*Trigonella foenum-graecum* L., Family: *Fabaceae*) is a herb grown widely in India, Egypt and the Middle East. It is one of the oldest medicinal plants, whose seeds, leaves or whole plants are used to prepare powders and extracts for medicinal use, as well as to make vegetable and food additives (Acharya et al. 2006; Zandi et al. 2015). Today, fenugreek is considered as one of the leading functional foods in markets in Asia, Africa and in the Mediterranean countries. Fenugreek seeds contain substantial amounts of several bioactive compounds, indicating therapeutic and

medicinal properties (Aher et al. 2016). It contains beneficial metabolites, including galactomannans (mucilaginous fibers), pyridine alkaloids (mostly trigonelline), steroidal sapogenins (spirostanols), amino acid 4-hydroxyisoleucine, antioxidants and flavonoids (Zandi et al. 2015). Thus, fenugreek seeds/leaves (or extracts) possess antioxidant, anti-inflammatory, hypocholesterolemic, anti-diabetic and hypoglycemic properties. Unripe, mature and germinating seeds of fenugreek have the potential to be used in treatment of diabetes (Gaddam et al. 2015), obesity (Basch et al. 2003) and cancer (Lin et al. 2013). Fenugreek seed extracts are tested (Deshpande et al. 2016) and used for treatment of some metabolic disorders (Rao et al. 2016). Possible therapeutic mechanisms and biological effects of some seed compounds were last summarized in reviews concerning the health-promoting properties of fenugreek seeds (Zandi et al. 2015; Aher et al. 2016).

Unexpectedly, data on the presence of *myo*-inositol and D-pinitol (both cyclitols indicating healthful properties and therapeutic activity similar to properties of fenugreek seeds/extracts) in fenugreek seeds and plants are rare. The potential role and therapeutic effects of *myo*-inositol (cyclitol ubiquitous in plants) in metabolic diseases were last reviewed by Croze and Soulage (2013) and Muscogiuri

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et al. (2016). *Myo*-inositol derivative, D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) also reveals anti-diabetic (Do et al. 2008), anti-hyperglycemic (Hernández-Mijares et al. 2013), anti-oxidative (Nascimento et al. 2006), anti-inflammatory (Sivakumar et al. 2010), lipid lowering (Choi et al. 2009), anti-cancer (Sethi et al. 2008; Lin et al. 2013) and immunosuppressive (Chauhan et al. 2011) properties. Thus, it can be supposed that therapeutic effects of fenugreek seeds and plants (Aher et al. 2016) partially arise from the biological properties of D-pinitol. Thus far, D-pinitol in fenugreek has been detected only in seeds (Shang et al. 2002).

Changes in the content of sucrose, reducing sugars and raffinose family of oligosaccharides (RFOs: raffinose, stachyose and verbascose) in developing (Singh et al. 1994), mature and germinating fenugreek seeds were reported earlier (Reid 1971). However, no data are available on the occurrence and accumulation of D-pinitol and its α-D-galactosides (galactosyl pinitols) in fenugreek plants. In our preliminary study, D-pinitol and galactosyl pinitols were identified as an important share of soluble carbohydrates (accounting for 10% of total soluble carbohydrates) in mature fenugreek seeds (unpublished data). The current study is the first one to have demonstrated changes in the concentration of D-pinitol (and other cyclitols as well as low molecular weight carbohydrates) in vegetative and reproductive organs of fenugreek plants during a whole plant growing period. Moreover, our results can support a choice of developing pods, seeds, germinating seeds or seedlings to be used as ingredients in functional foods (Thomas et al. 2011) and/or as a new source for isolation of D-pinitol (Poongothai and Sripathi 2013).

## Materials and methods

### Plant material

Seeds of fenugreek (*Trigonella foenum-graecum* L.) were obtained from the W. Legutko Seed Breeding and Production Company (Poland).

### Germinating seeds and seedlings

Mature fenugreek seeds (200 seeds in each of 20 replicates) were placed on wet sheet germination paper (Eurochem BGD, Poland), rolled and transferred into 250 ml glass cylinders. After addition of 50 ml of redistilled water, cylinders were incubated in a germination chamber (ILW 115-T STD, Pol-Eko-Aparatura, Poland) at 22 °C in the dark for 5 days. Seedlings were collected at 24 h intervals, weighed, frozen in liquid nitrogen, stored at − 76 °C (for a week) and freeze dried for 48 h (freeze dryer Alpha 1-2LD, Christ, Germany). The water content in tissues was calculated by subtracting the dry weight (DW, after freeze drying) from the fresh weight (FW) of the tissues.

### Vegetative organs

For analyses of low molecular weight carbohydrates in early developing plants, fenugreek seeds were sown (in March) in 1 dm$^3$ pots (five per pot), containing a mixture of soil and sand (3:1), and cultivated in a greenhouse (up to 30th day after sowing) without artificial illumination. After emergence, cotyledons grew into leaves and during the subsequent 6 weeks, plants developed six true leaves, reaching 10–15 cm in height. Cotyledons, leaves (blades with the petiole), the apical bud and stem were collected separately from ten plants (in each of the three replicates), weighed and freeze-dried.

Fenugreek was planted in April 2016, in an experimental field near Olsztyn (53.66°N, 20.48°E) in north-eastern Poland. Vegetative organs (leaves with leaf petioles and stems, separately) were separated from the stems of whole shoots, weighed and frozen in liquid nitrogen. Shoots were collected five times: at the onset of the reproductive development (at 56 day after sowing, DAS), then at 76, 86, 116 DAS and finally at 126 DAS (maturation of seeds). At each harvest time, leaves (fully opened trifoliolates with petioles) and stems were separated from shoots (ten plants in each of three replicates), weighed, frozen in liquid nitrogen and freeze-dried.

### Pods and maturing seeds

Pods with seeds were collected from July–September, at six stages of seed development, starting from day 30 after flowering (DAF) up to full maturity of seeds (80 DAF). Pods were collected from the first and second node. Pods (20 at each harvest time) and isolated seeds (Online resources, Plate 1) were weighed separately and freeze-dried.

### Analyses of soluble carbohydrates

Dry samples of plant tissues were pulverized in a mixer mill (MM200, Retsch, Verder Group, Netherlands). Soluble carbohydrates were extracted from 40 to 45 mg of flour with 800 μL of ethanol: water (1:1, v/v, at 90 °C for 30 min with continuous shaking at 300 rpm), containing 100 μg of xylitol (internal standard). Homogenates were centrifuged (20,000*g* at 4 °C for 20 min) and aliquots (400 μL) of clear supernatants were deionized (with a 300 μL of a mixture of Dowex ion-exchanger resins) and brought to dryness in a speed vacuum rotary evaporator. The dry residues were derivatized with a 200 μL of mixture of TMSI/pyridine (1:1, v/v) at 80 °C for 45 min. The TMS derivatives of soluble carbohydrates were analyzed with the high-resolution

gas chromatography method on a capillary column (Rtx-1, 15 m length, 0.25 mm diameter, 0.1 µm thickness of 100% dimethyl polysiloxane layer, Restek, USA) in a gas chromatograph (GC2010, Shimadzu, Japan), under conditions described previously (Lahuta and Górecki 2011). The carbohydrate content was calculated using the internal standard method. Standards of carbohydrates (fructose, galactose, glucose, sucrose, maltose, raffinose, stachyose and verbascose), xylitol and *myo*-inositol were purchased from Sigma (USA). Standards of other cyclitols (D-pinitol, D-*chiro*-inositol and D-ononitol) were obtained from Fine Chemicals (New Zealand), and galactinol was supplied by Wako Pure Chemicals Industries Ltd. (Japan). Standards of α-D-galactosides of D-pinitol (mono-, di- and tri-galactosyl pinitol A), commercially unavailable, were extracted from seeds of *Vicia villosa* Roth. (winter vetch) and purified as described previously (Szczeciński et al. 2000). Standards of α-D-galactosides of D-*chiro*-inositol (fagopyritols) were a gift from Prof. Marcin Horbowicz (Siedlce University of Natural Sciences and Humanities, Poland).

## Statistical analysis

The results were presented (in mg g$^{-1}$ of dry weight, DW) as means from three replications ± SD. The significance of differences in the concentrations of various carbohydrates was determined by one-way ANOVA with the Tukey's test.

## Results

The GC analyses of soluble carbohydrates revealed the presence of sugars (glucose, fructose, galactose and sucrose), cyclitols (*myo*-inositol, D-pinitol, D-ononitol and D-*chiro*-inositol), raffinose family oligosaccharides (RFOs: raffinose, stachyose and verbascose) and galactosyl cyclitols in all parts of fenugreek plants (Fig. 1). However, RFOs and galactosyl cyclitols were accumulated mainly in maturing seeds (Fig. 1a), being detected in trace amounts in vegetative tissues (leaves and stems) (Fig. 1b, c). D-Pinitol was the major cyclitol, regardless of the type of tissues and plant developmental stage. The qualitative and quantitative differences in soluble carbohydrates were found between plant organs and developmental stages.

## Changes in carbohydrates during seed germination

The concentration of total soluble carbohydrates (TSCs) in fenugreek seeds (used for germination) was 88.56 mg g$^{-1}$ DW (Table 1). RFOs, sucrose and D-pinitol were the major soluble carbohydrate (61.40, 6.41 and 4.43 mg g$^{-1}$ DW, respectively), constituting 81% of TSCs. The prevalent RFO was stachyose (85% of total RFOs). Seeds also
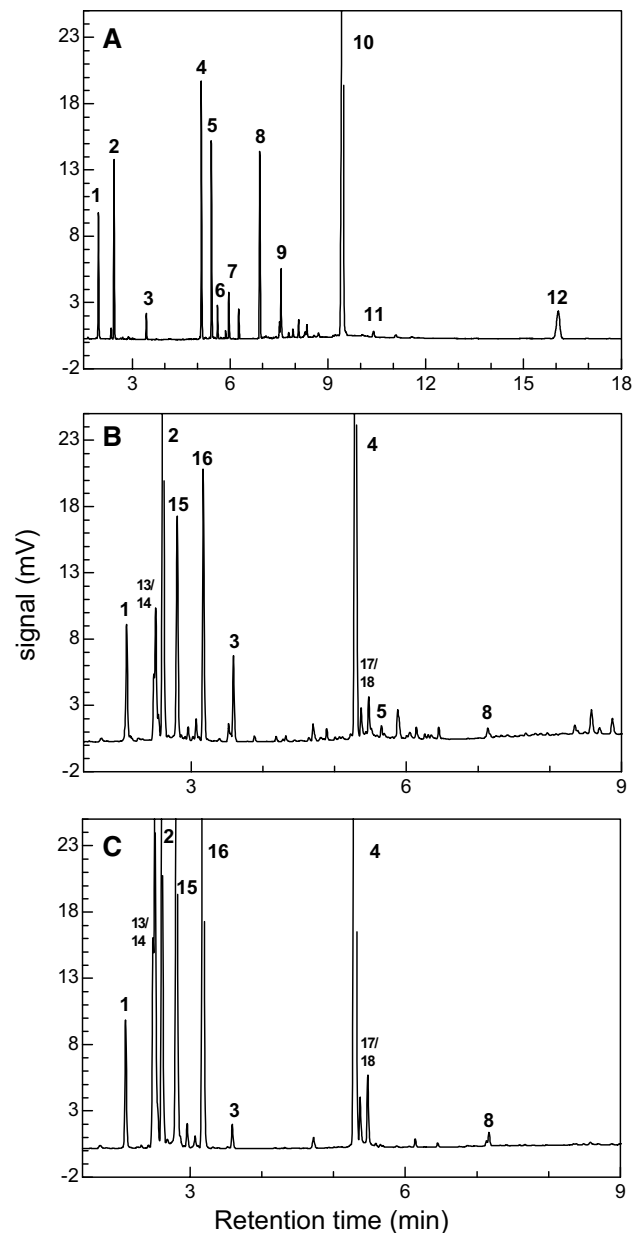


**Fig. 1** The GC chromatograms of TMS derivatives of soluble carbohydrates extracted from dry mature seeds (**a**), leaves and stems at 86 DAS (**b, c**, respectively) of fenugreek (*Trigonella foenum graecum* L.) plants. Denotations: 1—xylitol (internal standard), 2—D-pinitol, 3—*myo*-inositol, 4—sucrose, 5—GPA, 6—GPB, 7—galactinol, 8—raffinose, 9—ciceritol, 10—stachyose, 11—TGPA, 12—verbascose, 13 and 14—fructose, 15 and 16—glucose, 17 and 18—maltose

contained considerable amounts (11.25 mg g$^{-1}$ DW) of α-D-galactosides of D-pinitol (galactosyl pinitols, GPs), with galactosyl pinitol A (GPA) being prevalent (5.87 mg g$^{-1}$ DW, Online Resources, Table 1). Concentrations of the other identified galactosyl cyclitols: α-D-galactosides of *myo*-inositol (galactinol and di-galactosyl *myo*-inositol) and α-D-galactosides of D-*chiro*-inositol (fagopyritols) were

**Table 1** Changes in the concentration of: total soluble carbohydrates (TSCs), oligosaccharides (RFOs and GPs), major sugars (sucrose, glucose, fructose) and major cyclitols (D-pinitol, *myo*-inositol,) in fenugreek seeds and seedlings during 5 days of germination (DAI, days after imbibition)

| DAI (day) | TSCs* | Oligosaccharides (mg g$^{-1}$ DW ) | | Sugars (mg g$^{-1}$ DW ) | | | Cyclitols | |
|---|---|---|---|---|---|---|---|---|
| | | RFOs | GPs | Sucrose | Glucose | Fructose | D-Pinitol | *Myo*-Inositol |
| 0 | 88.56 ± 2.21$^{bc}$ | 61.40 ± 1.61$^a$ | 11.29 ± 0.34$^a$ | 6.41 ± 0.26$^d$ | 0.12 ± 0.02$^e$ | 0.81 ± 0.02$^d$ | 4.43 ± 0.15$^c$ | 0.67 ± 0.02$^d$ |
| 1 | 70.51 ± 2.21$^d$ | 42.48 ± 1.71$^b$ | 9.68 ± 0.18$^b$ | 9.56 ± 0.69$^d$ | 0.55 ± 0.21$^d$ | 0.79 ± 0.23$^d$ | 4.10 ± 0.09$^d$ | 0.57 ± 0.02$^d$ |
| 2 | 77.34 ± 4.77$^{cd}$ | 6.30 ± 1.08$^c$ | 3.38 ± 0.21$^c$ | 36.65 ± 1.49$^a$ | 8.67 ± 0.97$^c$ | 9.69 ± 1.06$^c$ | 6.32 ± 0.07$^b$ | 1.40 ± 0.03$^c$ |
| 3 | 105.56 ± 1.23$^a$ | 0.79 ± 0.09$^d$ | 0.64 ± 0.01$^d$ | 28.29 ± 0.67$^b$ | 36.26 ± 0.53$^b$ | 25.09 ± 1.41$^a$ | 7.77 ± 0.10$^a$ | 2.04 ± 0.06$^b$ |
| 4 | 101.43 ± 6.78$^{ab}$ | 0.33 ± 0.01$^d$ | 0.50 ± 0.01$^d$ | 16.39 ± 0.37$^c$ | 44.32 ± 3.28$^a$ | 23.65 ± 2.75$^a$ | 8.35 ± 0.09$^a$ | 2.14 ± 0.04$^{ab}$ |
| 5 | 78.83 ± 8.20$^{cd}$ | 0.00 ± 0.00$^d$ | 0.00 ± 0.00$^e$ | 7.61 ± 0.22$^d$ | 38.34 ± 4.61$^{ab}$ | 18.40 ± 3.04$^b$ | 8.07 ± 0.15$^a$ | 2.22 ± 0.07$^a$ |

Values (in mg g$^{-1}$ DW) are means ($n = 3$). Values with different superscripts (a–e) are significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for each compound separately (valid within columns only)

*TSCs—total soluble carbohydrates include: RFOs (raffinose, stachyose and verbascose), GPs, sugars, cyclitols, galactosides of *myo*-inositol and galactosides of D-*chiro*-inositol (presented in online resources, Table 1) and unknown compounds (data not shown)

much lower (2.28 and 0.40 mg g$^{-1}$ DW, respectively) than those of GPs (Online Resources, Table 1). Monosaccharides (glucose, fructose) and *myo*-inositol (Table 1) occurred at much lower concentrations (1.18 and 0.67 mg g$^{-1}$ DW). Moreover, seeds contained traces of D-*chiro*-inositol.

Dry seeds imbibed very fast—the fresh weight increased three-fold (from 13 up to 40 mg seed$^{-1}$) during the first day of seed imbibition (Online Resources, Plate 1A). Rapid degradation of RFOs and galactosyl pinitols started at the onset of seed germination (Table 1). Most of oligosaccharides were degraded during the first 2 days of seed germination, and RFOs and GPs were undetected in growing seedlings after the following 3 days (Table 1). The concentrations of sucrose, glucose, fructose and cyclitols (*myo*-inositol and D-pinitol) significantly increased ($P < 0.05$) between the first and second day of germination, due to the degradation of oligosaccharides and galactosyl cyclitols (Table 1). The concentration of sucrose was the highest (36.65 mg g$^{-1}$ DW) after 2 days of seed germination and decreased subsequently (to 7.61 mg g$^{-1}$ DW, after fifth day of germination, Table 1). Between the second and third day of seed germination, the concentrations of glucose and fructose dramatically increased (from 8.67 to 36.26 and from 9.69 to 25.09 mg g$^{-1}$ DW, for glucose and fructose, respectively). It is worth noting that the increase in the concentrations of glucose and fructose (Table 1) was much higher than the simultaneous decrease in the concentration of sucrose (from 36.65 to 28.29 mg g$^{-1}$ DW).

After the first day of seed germination, the concentration of free cyclitols decreased slightly (from 5.13 to 4.75 mg g$^{-1}$ DW, Table 1), despite the obvious onset of the degradation of all galactosyl cyclitols (by about 10%). The hydrolysis of individual types of galactosyl cyclitols: fagopyritols, galactosides of *myo*-inositol and galactosyl pinitols, finished after 2, 4 and 5 days of germination (Online Resources, Table 1).

Accordingly, the concentrations of free cyclitols increased: D-*chiro*-inositol—up to 0.10 mg g$^{-1}$ DW (after second day of germination, not presented data), *myo*-inositol—up to 2.14 mg g$^{-1}$ DW (fourth day), and D-pinitol—up to 8.07 mg g$^{-1}$ DW (fifth day, Table 1).

## Carbohydrates in stems and leaves during plant growth

Sucrose, glucose and D-pinitol were the main soluble carbohydrates in vegetative organs of fenugreek plants at the stage of shoot growth (six true leaves) (Online Resources, Table 2). The highest concentration of TSCs (76.61 mg g$^{-1}$ DW) was found in cotyledons (evolved into the first photosynthetic active leaves), whereas the lowest were in the stem (35.16 mg g$^{-1}$ DW). Sucrose made up 22–24% of TSCs in the stem, cotyledons and leaves, and 11.29% in the apical bud. Pinitol was the main carbohydrate in the stem (61% of TSCs), leaves (43%) and apical bud (58%, Online Resources, Table 2). The concentration of D-pinitol was higher in stems, leaves and the apical, but (20–26 mg g$^{-1}$ DW) than in cotyledons (12.29 mg g$^{-1}$ DW). Moreover, the high concentration of glucose in cotyledons (35.50 mg g$^{-1}$ DW, 43% of TSCs) is presumably a result of the degradation of transitory starch.

In plants collected at the initial stage of flowering (56 day after sowing, DAS) and subsequently up to seed maturation (126 DAS), the concentrations of carbohydrates differed between organs and plant developmental stages. The concentration of TSCs significantly ($P < 0.05$) increased in both the stem and leaves up to 76–86 DAS, and decreased thereafter (Table 2). The major sugars were sucrose, glucose and fructose. The concentrations of these sugars were higher in the stem than in leaves throughout the entire plant growing period. D-Pinitol and *myo*-inositol dominated among cyclitols, whereas D-ononitol and D-*chiro*-inositol were

**Table 2** Changes in the water content (WC), total soluble carbohydrates (TSCs), predominant sugars (sucrose, glucose, fructose) and cyclitols (D-pinitol, *myo*-inositol) in stems and leaves of fenugreek plants during generative period of plant vegetation (between 56th and 126th day after sowing, DAS, corresponding with 1–70th day after flowering, DAF)

| Plant organ | DAS | DAF | WC (% of FW) | TSCs | Sugars | | | Cyclitols | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Sucrose | Glucose | Fructose | D-Pinitol | *Myo*-inositol |
| | | | | mg g$^{-1}$ DW | | | | | |
| Stem | 56 | 1* | 88.56 | $32.87 \pm 2.76^d$ | $7.11 \pm 0.11^d$ | $11.03 \pm 2.30^b$ | $0.89 \pm 0.24^b$ | $9.50 \pm 0.09^b$ | $0.76 \pm 0.01^b$ |
| | 76 | 20 | 79.52 | $88.74 \pm 9.07^b$ | $32.54 \pm 0.56^b$ | $35.87 \pm 7.21^a$ | $4.70 \pm 1.39^b$ | $10.19 \pm 0.33^a$ | $0.82 \pm 0.03^a$ |
| | 86 | 30 | 76.01 | $112.91 \pm 8.56^a$ | $48.53 \pm 1.12^a$ | $36.57 \pm 4.78^a$ | $11.26 \pm 2.34^a$ | $10.57 \pm 0.25^a$ | $0.44 \pm 0.01^c$ |
| | 116 | 60 | 75.83 | $51.84 \pm 6.28^c$ | $22.10 \pm 5.39^c$ | $12.92 \pm 7.31^b$ | $4.26 \pm 3.16^b$ | $9.16 \pm 0.28^b$ | $0.43 \pm 0.03^c$ |
| | 126 | 70 | 72.99 | $39.41 \pm 4.56^d$ | $15.54 \pm 1.25^c$ | $5.37 \pm 2.23^b$ | $1.31 \pm 0.78^b$ | $9.01 \pm 0.07^b$ | $0.39 \pm 0.00^d$ |
| Leaves | 56 | 1* | 86.39 | $26.12 \pm 0.62^d$ | $8.07 \pm 0.14^b$ | $1.96 \pm 0.27^b$ | $0.88 \pm 0.18^b$ | $5.07 \pm 0.09^d$ | $3.99 \pm 0.10^a$ |
| | 76 | 20 | 85.36 | $63.03 \pm 3.29^{ab}$ | $21.26 \pm 0.77^a$ | $14.21 \pm 1.69^a$ | $2.03 \pm 0.07^b$ | $16.15 \pm 0.36^b$ | $2.85 \pm 0.06^b$ |
| | 86 | 30 | 85.33 | $70.68 \pm 5.61^a$ | $26.07 \pm 2.44^a$ | $15.22 \pm 0.98^a$ | $4.97 \pm 0.30^a$ | $14.23 \pm 1.37^c$ | $1.63 \pm 0.14^{cd}$ |
| | 116 | 60 | 82.17 | $60.88 \pm 2.47^b$ | $13.31 \pm 5.52^b$ | $12.02 \pm 4.26^a$ | $6.81 \pm 2.17^a$ | $17.94 \pm 0.50^b$ | $1.42 \pm 0.04^d$ |
| | 126 | 70 | 81.28 | $45.09 \pm 1.23^c$ | $11.10 \pm 0.05^b$ | $4.71 \pm 0.71^b$ | $1.96 \pm 0.44^b$ | $19.84 \pm 0.16^a$ | $1.76 \pm 0.01^c$ |

Values are means ($n = 3$) $\pm$ SD. Values with different superscripts (a–d) are significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for each compound separately (comparisons for stem and leaves separately, valid within columns only)

*One day after full opening of the first flower

present in trace amounts. The concentration of each cyclitol was higher in leaves than in the stem. Moreover, D-pinitol accumulated in leaves (to 19.84 mg g$^{-1}$ DW, at 126 DAS), remaining on a relatively stable level in the stem (9–10 mg g$^{-1}$ DW). Opposite to changes in D-pinitol, the concentration of *myo*-inositol decreased during plant growth in both leaves and stems (Table 2). Changes in the profiles of soluble carbohydrates in fenugreek shoots indicate that at 86 DAS plants were fully developing, whereas 30 days later, the ability of plants to synthesize soluble carbohydrates decreased (Table 2). At 126 DAS, senescence of leaves from the bottom of plants started, and those leaves were not collected for analyses.

## Changes in soluble carbohydrate during pod and seed maturation

The first sample of pods with developing seeds was collected at day 30 after flowering (DAF), corresponding to 86 DAS (Online Resources, Plate 1B). At this stage of the development of pod and seeds, the concentration of TSCs was the highest (111.08 and 181.76 mg g$^{-1}$ DW, in pods and seeds, respectively, Table 3). Sugars and cyclitols dominated among TSCs (Table 3). However, glucose and fructose dominated in pods (42.35 and 10.26 mg g$^{-1}$ DW, respectively), whereas sucrose was prevalent in seeds (149.75 mg g$^{-1}$ DW). During pod maturation, the concentration of TSCs gradually decreased (to 20.95 mg g$^{-1}$ DW, at 70 DAF) due to a decrease in the concentrations of sugars: sucrose and glucose (Table 3 and Online

Resources Table 3). Concentrations of cyclitols, RFOs and galactosyl cyclitols fluctuated. The concentrations of D-pinitol (constituting above 94% of total cyclitols), RFOs and galactosyl pinitols increased up to 50 DAF and diminished thereafter (Table 3). However, at 70 DAF, GPs were absent, RFOs were present in trace amounts only, whereas the level of D-pinitol was eight-fold higher (16.23 mg g$^{-1}$ DW) than that of total sugars (Table 3), including sucrose and glucose (Online Resources, Table 3).

In developing fenugreek seeds, the concentration of D-pinitol was the highest at 30 DAF (5.30 mg g$^{-1}$ DW) and decreased later on, down to 2.22–2.27 mg g$^{-1}$ DW (at 60–70 DAF, Table 3), most likely as a result of successive accumulation of galactosyl pinitols. Galactosyl pinitol A (GPA) was detected in fenugreek seeds at 40 DAF, di-galactosyl pinitol A (ciceritol)—at 60 DAF, and tri-galactosyl pinitol A TGPA—at 80 DAF (Online Resources, Table 3). However, the biosynthesis of galactosyl pinitols started later than that of RFOs and was less intensive.

Galactinol, raffinose and stachyose were detected in seeds at 30 DAF (Online Resources, Table 3). Raffinose and stachyose were accumulated up to 70 DAF, whereas the accumulation of verbascose, starting between 40 and 50 DAF, was continued to full seed maturity. In mature seeds, stachyose was the predominant RFO. It should be underlined that the accumulation of the highest homologues of GPA and raffinose (TGPA and verbascose, respectively) occurred most intensively during seed natural desiccation (between 70 and 80 DAF).

**Table 3** Changes in the fresh weight (FW), water content (WC), concentration of: total soluble carbohydrates (TSCs), D-pinitol, total sugars, total RFOs and total galactosyl pinitols (GPs) in maturing pods and seeds of fenugreek

| Plant organ | DAF | FW (mg part$^{-1}$) | WC (% FW) | TSCs (mg g$^{-1}$ DW ) | D-Pinitol (mg g$^{-1}$ DW ) | Total sugars (mg g$^{-1}$ DW ) | Total RFOs (mg g$^{-1}$ DW ) | Total GPs (mg g$^{-1}$ DW ) |
|---|---|---|---|---|---|---|---|---|
| Pod | 30 | 533.14 | 81.21 | 111.08 ± 11.83[a] | 14.49 ± 0.68[c] | 89.41 ± 10.70[a] | 3.61 ± 0.10[c] | 0.15 ± 0.01[c] |
| | 40 | 529.36 | 82.48 | 43.91 ± 2.43[b] | 21.94 ± 0.35[b] | 14.57 ± 2.05[b] | 5.12 ± 0.04[b] | 0.21 ± 0.01[b] |
| | 50 | 490.85 | 83.82 | 48.14 ± 0.34[b] | 23.70 ± 0.44[a] | 11.05 ± 1.36[b] | 9.94 ± 0.48[a] | 0.39 ± 0.03[a] |
| | 60 | 343.48 | 80.87 | 26.37 ± 0.59[c] | 19.66 ± 1.01[a] | 3.15 ± 0.16[b] | 1.41 ± 0.08[d] | 0.17 ± 0.01[bc] |
| | 70 | 75.60 | 58.33 | 20.95 ± 0.51[c] | 16.23 ± 0.66[c] | 2.25 ± 0.13[b] | 0.08 ± 0.01[e] | 0.00 ± 0.00[d] |
| Seed | 30 | 31.54 | 77.52 | 181.16 ± 7.53[a] | 5.30 ± 0.54[a] | 157.15 ± 7.77[a] | 1.44 ± 0.08[e] | 0.00 ± 0.00[d] |
| | 40 | 33,88 | 71.99 | 90.25 ± 8.16[d] | 3.84 ± 0.34[b] | 66.51 ± 5.84[b] | 9.84 ± 0.59[d] | 0.10 ± 0.02[d] |
| | 50 | 35.23 | 65.37 | 77.75 ± 2.43[d] | 2.61 ± 0.07[c] | 44.30 ± 1.22[c] | 21.25 ± 0.82[c] | 0.19 ± 0.01[d] |
| | 60 | 36.58 | 59.85 | 95.99 ± 0.76 [cd] | 2.22 ± 0.04[c] | 35.65 ± 0.10 [cd] | 50.05 ± 1.40[b] | 1.41 ± 0.07[c] |
| | 70 | 26.17 | 58.52 | 112.03 ± 0.83[b] | 2.27 ± 0.04[c] | 28.68 ± 0.37[d] | 68.54 ± 1.16[a] | 3.41 ± 0.06[b] |
| | 80[a] | 13.65 | 18.28 | 106.15 ± 1.66[bc] | 4.44 ± 0.11[b] | 15.41 ± 0.42[e] | 65.37 ± 2.44[a] | 6.56 ± 0.25[a] |

Values are means ($n = 3$) ± SD

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 for pods and seeds separately valid within columns only)

[a]Mature seeds

## Discussion

The composition of soluble carbohydrates in mature seeds of fenugreek was similar to that in soybean (Yasui and Ohashi 1990). The prevalent RFO in fenugreek seeds was stachyose (85% of total RFOs), which was consistent with data obtained by Reid (1971). Our data also indicate presence of considerable amounts of galactosyl pinitols (accounting for 13% of TSCs, Table 1), with prevalent GPA (Online Resources, Table 1). Changes in the concentrations of RFOs, sucrose and monosaccharides in germinating fenugreek seeds, found in our study (Table 1), correspond well with the results obtained earlier by Dirk et al. (1999) and are generally similar to the ones occurring in germinating legume seeds (Górecki et al. 2000; Kadlec et al. 2008; Martin-Cabrejas et al. 2008). Moreover, the fast degradation of galactosyl cyclitols confirms their role as reserve materials important for early germination of fenugreek seeds, same as in seeds of winter vetch (Lahuta and Goszczyńska 2009). The hydrolysis of RFOs and galactosyl cyclitols in fenugreek seeds coincided with an increase in the concentrations of sucrose (initially) and then (after the second day of germination) monosaccharides (glucose and fructose), *myo*-inositol and cyclitols. However, it can be expected that the level of galactose released from α-D-galactosides (both RFOs and galactosyl cyclitols) should be much higher than estimated in our study (up to 2.8 mg g$^{-1}$ DW, at second day of germination, data not presented). Initially, galactose is released from RFOs and galactosyl cyclitols (Lahuta and Goszczyńska 2009), but later on it originates from galactomannans (deposited in the endosperm tissues), which are hydrolyzed after 3–4 days of a fenugreek seedling's development (Reid 1971; Buckeridge and Dietrich 1996; Dirk et al. 1999). In starchless seeds of fenugreek, galactomannans are the main carbohydrate reserves, representing up to 50% of dry seed weight (Zandi et al. 2015). However, in our study, mannose and galactose were not accumulated during fenugreek seed germination and seedling development. Similarly, no accumulation of galactose and/or mannose in germinating fenugreek seeds was detected by Dirk et al. (1999). A much lower concentration of galactose (0.16–2.82 mg g$^{-1}$ DW) than that of glucose and fructose (Table 1) found in our study can be explained by the fast metabolic conversion of both galactose and mannose into substrates (UDP-glucose and fructose-6-phosphate, respectively) used for the synthesis of sucrose and cell wall compounds of growing seedling (Buckeridge et al. 2000).

The high concentration of sucrose after 2 days of seed germination (Table 1) can be a signal for both elongation of the hypocotyl (Penfield et al. 2004) and the synthesis of starch in cotyledons, as it was suggested by Dirk et al. (1999). Synthesis of starch in cotyledons is initiated early, i.e., during the first several hours of seed germination (Srivastava and Kayastha 2014), it increases up to 50–60 h and later on decreases (Dirk et al. 1999). Starch degradation in cotyledons occurs by the action of β-amylase (Srivastava and Kayastha 2014), releasing maltose. Maltose was detected at 1.25 mg g$^{-1}$ DW after the second day of fenugreek seed germination, after which it gradually increased, up to 2.27 mg g$^{-1}$ DW.

The significantly innovative aspect of our research lies in the determination of qualitative and quantitative changes in

cyclitols and their galactosides during fenugreek seed germination and plant vegetation. After the first day of seed germination, the concentration of free cyclitols decreased slightly (Table 1), despite the obvious onset of the degradation of all galactosyl cyclitols. However, the utilization of cyclitols at initial stages of seed germination remains to be explained. Leakage of free D-pinitol from imbibed seeds is possible, as it has been documented in soybean (Nordin 1984). The hydrolysis of individual types of galactosyl cyclitols: fagopyritols, galactosides of *myo*-inositol and galactosyl pinitols, finished after 2, 4 and 5 days of germination (Online Resources, Table 1). Accordingly, the concentrations of free cyclitols increased (Table 1). In germinating yellow lupin seeds, the concentration of D-pinitol decreased during the first 2 days of germination, and an increase in D-*chiro*-inositol (a product of D-pinitol demethylation) was noted (Zalewski et al. 2007). In germinating seeds of soybean (Gu et al. 2017), yellow lupin (Górecki et al. 1997) and winter vetch (Lahuta and Górecki 2011), the concentration of D-pinitol increased due to the degradation of galactosyl pinitols. However, high differences in concentrations of D-pinitol occur between species. A higher concentration of D-pinitol (up to 40–80 mg g$^{-1}$ DW) was found in the epicotyl and root of 7-day-old seedlings of winter vetch (Lahuta and Górecki 2011).

The average content of D-pinitol, as free and bound in total galactosyl pinitols (calculated on the basis of the molecular formula of individual galactosyl pinitols), in dry fenugreek seeds was 0.05 and 0.06 mg seed$^{-1}$, respectively. During 4 days of germination, the increase in the content of free D-pinitol in seedlings by 1.35 mg (to 1.40 mg seedling$^{-1}$) was much higher than the content of D-pinitol released from galactosyl pinitols, which indicates that the de novo synthesis of D-pinitol is initiated at early stages of fenugreek seedling development. Data documenting the beginning of D-pinitol accumulation during seedling development are rare (Górecki et al. 1997; Lahuta and Górecki 2011). The biosynthesis of D-pinitol occurs through four-step conversion of glucose phosphate precursor (Ishitani et al. 1996). At first, *myo*-inositol is synthesized from glucose-6-phosphate in two reactions catalyzed by *myo*-inositol 1-phosphate synthase (MIPS) and *myo*-inositol monophosphatase (IMP). The biosynthesis of D-pinitol occurs through transfer of the methyl group from S-adenosyl-L-methionine (SAM) to *myo*-inositol (in reaction catalyzed by high substrate specify inositol methyl transferase, IMT), producing D-ononitol (1D-4-*O*-methyl-*chiro*-inositol), and then ononitol is converted to D-pinitol through an epimerase reaction (Dittrich and Brandl 1987). However, that epimerase(-s) has yet to be isolated and characterized. The methyl transfer rate from SAM to *myo*-inositol can be regulated by availability of *myo*-inositol, presence of functional IMT and the intracellular ratio of SAM to SAH (S-adenosyl homocysteine),

a potent competitive inhibitor of SAM-dependent methyl transferase reactions (Sengupta et al. 2008).

Many reports documented accumulation of free *myo*-inositol, its isomers and methylated ethers (mainly D-pinitol and D-ononitol) in different plant species under abiotic stresses, like drought (Ahn et al. 2011; Sanchez et al. 2012; Yates et al. 2014), high salinity (Kumari et al. 2015; Slama et al. 2015), elevated (Deslauriers et al. 2014) or lowered temperature (Turner and Pollock 1998; Dauwe et al. 2012). The expression of IMT genes seems to be stress up-regulated (Vernon and Bohnert 1992; Sengupta et al. 2008). Several studies, conducted on transgenic plants expressing *MIPS* or *IMT* genes (and accumulating elevated amounts of *myo*-inositol or D-ononitol/D-pinitol, respectively), confirm the protective role of cyclitols under stress conditions (Sheveleva et al. 1997; Sengupta et al. 2008; Patra et al. 2010; Joshi et al. 2013; Kaur et al. 2013). Cyclitols can function as "compatible solutes" (Valluru and Van den Ende 2011), osmolytes (Matos et al. 2010) and antioxidants (scavenging of hydroxyl radicals, Nishizawa et al. 2008). Moreover, cyclitols can regulate the antioxidant systems activity (Tan et al. 2013; Zhang et al. 2017) and facilitate ion transport under salinity stress (Sengupta et al. 2008). However, under stress conditions affecting osmotic potential in cells, the short-term turnover of cyclitols is very low (Smith and Philips 1982; Merchant and Richter 2011). In leaves and stems of different plant species, both herbaceous (Bieleski 1994; Streeter et al. 2001) and woody plants (Adams et al. 2010; Warren et al. 2011; Dauwe et al. 2012; Lintunen et al. 2016), the concentration of D-pinitol (or other species-specific cyclitols) is as high or higher than that of sucrose, the main photoassimilate transported from sources to sink tissues. Pinitol can be translocated through both phloem (Kordan et al. 2011) and xylem (Krishnan et al. 2011), and diurnal changes in its concentration were detected (Krishnan et al. 2011). Thus, it is most likely that an important role of D-pinitol is to act as a carbon sink, enabling the allocation of carbon from carbohydrates and supporting primary metabolism during changes in resource availability at changing environmental conditions (Merchant and Richter 2011). Indeed, the differences in the concentrations of D-pinitol between organs of juvenile fenugreek plants (higher in stems, leaves and the apical bud – 20–26 mg g$^{-1}$ DW, than in cotyledons – 12.29 mg g$^{-1}$ DW, Online Resources, Table 2) seem to provide confirmation of the upward transport of D-pinitol from sources (leaves, cotyledons) to growing tissues (apical bud), as it was suggested by Kawai et al. (1985). During fenugreek plant's vegetation, D-pinitol and *myo*-inositol dominated among cyclitols, whereas D-ononitol and D-*chiro*-inositol were present in trace amounts. The concentration of each cyclitol was higher in leaves than in the stem. The low concentration of D-ononitol, as intermediate in biosynthesis of D-pinitol (Dittrich and Brandl 1987), indicates efficient conversion of

D-ononitol into D-pinitol. However, the lowest concentration of D-*chiro*-inositol (in all parts of fenugreek shoot), a product of D-pinitol demethylation, needs explanation in the future. Moreover, there are no data documenting metabolic route of D-pinitol in sink tissues.

The accumulation of D-pinitol in leaves throughout fenugreek plant growth (up to 19.84 mg g$^{-1}$ DW, at 126 DAS) and its relatively stable level in the stem (9–10 mg g$^{-1}$ DW) can be explained by the lowering sink demand of ageing organs, like roots and senescent leaves. Contrary to changes in D-pinitol, the concentration of *myo*-inositol decreased during plant growth in both leaves and stems (Table 2). The concentration of D-pinitol in soybean (Streeter et al. 2001) and *Cicer arietinum* (Matos et al. 2010) was similar to the one determined in fenugreek in our study, and ranged from 15 to 30 mg g$^{-1}$ DW of leaf blades, depending on genotypes and rainfall levels. Moreover, there was a D-pinitol gradient in soybean leaf blades from the bottom to the top of the plant, suggesting translocation of D-pinitol from lower to upper nodes during plant development (Streeter et al. 2001). In leaves of red clover, the concentration of D-pinitol ranged from 14 to 20 mg g$^{-1}$ DW under optimal water conditions, and up to 110–120 mg g$^{-1}$ DW under drought stress (Yates et al. 2014). An increase in the D-pinitol concentration under drought stress was also found in soybean (Streeter et al. 2001), *Cicer arietinum* (Matos et al. 2010), alfalfa (Kang et al. 2011) and *Medicago truncatula* (Zhang et al. 2017). In our study, fenugreek plants grew under suitable watering conditions. Changes in the profiles of soluble carbohydrates in fenugreek shoots indicate that at 86 DAS plants were fully developing, whereas 30 days later, the ability of plants to synthesize soluble carbohydrates decreased (Table 2), presumably due to lowering photosynthetic activity.

The pods, covering developing seeds, play an important dual function: (i) they participate in the regulation of transport of water, minerals and nutrients from the shoot to seeds, and (ii) as transitory photosynthetic active organs they synthesize additional pool of reduced carbon supplying nutrition of seeds (Patrick and Offler 2001). In maturing pods of fenugreek, the concentration of TSCs gradually decreased from 89.41 (at 30 DAF) to 2.25 mg g$^{-1}$ DW (at 70 DAF), as result of a decrease in levels of sucrose and glucose (Online Resources Table 3). It can mean that sugars were transferred into developing seeds (Weber et al. 1997). The concentrations of D-pinitol (constituting above 94% of total cyclitols), RFOs and galactosyl pinitols increased up to 50 DAF and diminished thereafter (Table 3). The physiological significance of the transitory biosynthesis of RFOs and galactosyl pinitols in tissues of developing pods remains to be explained. Neither type of α-D-galactosides is transported through the phloem in legume plants. Moreover, RFOs and galactosyl pinitol were degraded during pods desiccation in contrast to their fate in developing seeds. Additionally,

a relatively high concentration of D-pinitol in naturally aged (and dehydrated) pods remains intriguing. It can be expected that during seed maturation, most of soluble carbohydrates in pods, not only sugars (Lemoine et al. 2013) but also cyclitols, should be remobilized from pod tissues and transported into developing/maturing seeds. In fact, the concentration of D-pinitol in seeds doubled between 70 and 80 DAF, that is at the later stages of seed maturation.

The increasing concentration of D-pinitol in maturing seeds can lead to an increase in the biosynthesis of galactosyl pinitols in embryos, as it was earlier documented in developing seeds of soybean (Gomes et al. 2004), vetch (Lahuta et al. 2005a, b, 2010), pea (Lahuta and Dzik 2011), buckwheat (Ma et al. 2005) and grains of cereals (Lahuta and Goszczyńska 2010). In developing fenugreek seeds, the concentration of D-pinitol was the highest at 30 DAF (5.30 mg g$^{-1}$ DW) and decreased later on, down to 2.22–2.27 mg g$^{-1}$ DW (at 60–70 DAF, Table 3), most likely as a result of successive accumulation of galactosyl pinitols. Galactosyl pinitol A (GPA) was detected in fenugreek seeds at 40 DAF, di-galactosyl pinitol A (ciceritol)—at 60 DAF, and tri-galactosyl pinitol A TGPA—at 80 DAF (Online Resources, Table 3). The biosynthesis of galactosyl pinitols started later than that of RFOs and was less intensive, like in seeds of Vicia spp. (Lahuta et al. 2005), yellow lupine (Górecki et al. 1997), soybean (Obendorf et al. 1998) and chickpea (Gangola et al. 2014). Typically, seeds accumulate more RFOs than galactosyl pinitols (Obendorf and Górecki 2012), except seeds of some Vicia spp. accumulating equal amounts of both RFOs and galactosyl pinitols (Lahuta 2006) or more galactosyl pinitols than RFOs (Lahuta et al. 2010).

Galactinol (a galactosyl donor in the biosynthetic pathway for both RFOs and GPs; Peterbauer and Richter 2001), raffinose and stachyose were detected in seeds at 30 DAF (Online Resources, Table 3). Raffinose and stachyose were accumulated up to 70 DAF, whereas the accumulation of verbascose, starting between 40 and 50 DAF, was continued to full seed maturity. In mature seeds, stachyose was the predominant RFO, as it was noted earlier (Reid 1971). It should be underlined that the accumulation of the highest homologues of GPA and raffinose (TGPA and verbascose, respectively) occurred most intensively during seed natural desiccation (between 70 and 80 DAF). Although the concentration of D-pinitol also increased (almost two-fold) during seed desiccation (Table 3), the possible utilization of D-pinitol for the synthesis of elevated amounts of its galactosides was presumably restricted by the water loss and lowering rate of cellular metabolism.

It can be speculated that the preferential accumulation of RFOs was a result of a much higher concentration of sucrose than D-pinitol in seeds throughout seed development and maturation (Table 3 and Online Resources Table 3). Both sucrose and D-pinitol are the first galactose acceptors in the

biosynthetic pathway of RFOs and GPs. Thus, the higher concentration of sucrose enabled preferential biosynthesis of raffinose and, later on, stachyose and verbascose (Peterbauer and Richter 2001; Obendorf and Górecki 2012).

Some observations in our study: (1) relatively high concentration of D-pinitol in all parts of fenugreek shoot, (2) much lower changes in its concentration in leaves, stem and pods, as compared to the concentrations of sucrose and monosaccharides and (3) occurrence of D-*chiro*-inositol, a product of D-pinitol demethylation at extremely low concentration, lead us to the conclusion that the metabolic route of D-pinitol utilization in sink tissues needs explanation.

## Conclusions

Concentrations of D-pinitol change in different plant organs during fenugreek growth and development. The increase in D-pinitol at early stages of seed germination is a result of the hydrolysis of galactosyl pinitols, but later on it occurs as a result of the de novo synthesis of D-pinitol in photosynthetic active organs, i.e., cotyledons/leaves. D-Pinitol seems to be transported (like sucrose) from the source (leaves) to sink organs (pods/seeds). D-Pinitol reaches the highest concentration in pods at 96–106 DAS (40–50 DAF), but 3 weeks later its content peaks in leaves—even at 116–126 DAS (60–70 DAF). Because of the high concentration of D-pinitol and the simultaneously low level of low molecular carbohydrates, pods seem to be the best source of D-pinitol.

**Author contribution statement** LBL: determining the purpose of research, developing research methodology, participating in chromatographic analyzes, interpretation and discussion of results. JS: collection and analyses (using gas chromatography method) of plant material. MC: collection and analyses of plant material. RJG: discussion of results.

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