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Cytological analysis of *Fallopia japonica* and *Fallopia ×bohemica* shoots during growth season

Citološka analiza poganjkov japonskega in češkega dresnika med rastno sezono

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Abstract: *Fallopia japonica* and *Fallopia ×bohemica* are two very invasive plant species in Europe and North America. Their main mode of spread is vegetative reproduction. In spring new shoots emerge from the overwintering rhizome, grow rapidly and develop broad leaves which shade undergrowth plants. We studied cell size and starch accumulation in three stem regions at five sampling times during one growth season to determine possible differences in growth dynamics of both *Fallopia* species. On average *F. ×bohemica* had somewhat larger cells than *F. japonica* but the differences were not significant, except in the internodes of the middle stem region with differentiating cells. Also, cell growth dynamics of both species was similar and the only difference was detected at the 2nd sampling when cells of *F. ×bohemica* were more elongated. *F. ×bohemica* also accumulated starch earlier in the growth season and in younger tissues than *F. japonica*.

Keywords: *Fallopia japonica*, *Fallopia ×bohemica*, cell size, starch, growth

Izvleček: Japonski (*Fallopia japonica*) in češki dresnik (*F. ×bohemica*) sta v Evropi in Severni Ameriki zelo invazivni tujerodni rastlinski vrsti. Nespolno razmnoževanje je glavni način njunega razširjanja in vsako pomlad iz korenike požene brst, ki hitro zraste in razvije široke liste, ki zasenčijo podrast. V raziskavi smo preučevali velikost celic in tvorbo škrobnih zrn v treh območjih stebla pri petih vzorčenjih, ki smo jih opravili v eni rastni sezoni in tako ugotavljali morebitne razlike v dinamiki rasti pri obeh vrstah dresnika. V povprečju so bile celice češkega dresnika nekoliko večje kot celice japonskega dresnika, vendar razlike niso bile statistično značilne razen v členkih srednjega dela stebla z diferencirajočimi se celicami. Tudi dinamika celične rasti je bila podobna pri obeh vrstah dresnika razen pri 2. vzorčenju, ko so bile celice češkega dresnika bolj podaljšane. Češki dresnik je v primerjavi z japonskim kopičil škrob prej in v mlajših tkivih.

Ključne besede: japonski dresnik, češki dresnik, velikost celic, škrob, rast

Introduction

Fallopia japonica (Houtt.) Ronse Decr. (*Polygonaceae*) and its close relatives in the section *Reynoutria* are well known and problematic invasive plant species worldwide. In Slovenia are present octoploid Japanese knotweed (*F. japonica* var. *japonica* (Houtt.) Ronse Decr.), tetraploid giant

knotweed (*F. sachalinensis* (F. Schmidt) Ronse Decr.) and their hybrid hexaploid Bohemian knotweed (*F. ×bohemica* (Chrtek and Chrtková) J. P. Bailey) (Strgulc Krajšek and Jogan 2011). Japanese knotweed is cytologically and genetically uniform in Europe and has successfully spread since its early introduction in Europe in the 19th century. Now it is considered as one of the most invasive plant species. On the other hand, giant knotweed has limited genetic variation and is less invasive. Their hybrid Bohemian knotweed exhibits the highest genetic variation (Bailey et al. 2009) and recent study shows that it also has higher invasive potential than Japanese knotweed (Parepa et al. 2014). All three knotweed species are approx. 2-4 m tall herbaceous perennials with high growth rate and broad leaves which shade undergrowth plants (Herpigny et al. 2012). The main competitive traits of knotweeds against the native flora were shown to be their vegetative reproduction with rhizomes and high regeneration success, but allelopathy and genome plasticity also account. All these characteristics enable knotweeds successful colonisation of new habitats, especially ruderal habitats and river banks (Parepa et al. 2014).

Japanese knotweed is up to 2 m high and has up to 15 cm long leaves, giant knotweed is higher, from 2 to 4 m, and has up to 30 cm long leaves while Bohemian knotweed is intermediate in size (Vreš 2007, Bailey et al. 2009). The size and shape of organs are related to cell division, cell elongation and cell differentiation which take place in the meristematic and submeristematic tissues. Environmental (water and nutrient supply) as well as genetic factors control organ growth (Mizukami 2001, John and Qi 2008, Krizek 2009). Cell size can be correlated also to the ploidy level (Kondorosi et al. 2000, Sugimoto-Shirasu and Roberts 2003) and has been used in the taxonomic studies to estimate nuclear DNA content and ploidy level by the size of the stomatal guard cells (Šturm and Bačič 2013) even in the fossil plants (Masterson 1994). It was also shown that polyploidization can represent a mean to accelerate the growth of the plant species in niches that require and support fast development (Barow and Meister 2003) and can facilitate plant invasions (te Beest et al. 2012).

The aim of our study was to compare the growth of Japanese and Bohemian knotweed during one season. Different cytological traits (cell size, presence of starch grains) were determined and the following questions were asked: (i) Are the cells in the octoploid Japanese knotweed larger cells than in the hexaploid Bohemian knotweed? (ii) Is the cell size different in the meristematic, young and mature regions of the stem, which would lead to different growth dynamics in both *Fallopia* species? (iii) Is the starch accumulation species specific and correlated to cell size dynamics?

Material and methods

Knotweed sampling

Shoot samples of five plants of Japanese knotweed (*F. japonica* var. *japonica* (Houtt.) Ronse Decr.) and Bohemian knotweed (*F. ×bohemica* (Chrtek and Chrtková) J. P. Bailey) were collected in Ljubljana, Slovenia (46° 2′ 33.98″ N, 14° 27′ 0.91″ E and 46° 3′ 0.3″ N, 14° 28′ 44″ E, respectively) from April to October 2013. According to the developmental stage of the shoot, apical meristem (upper region, 1st internode), young (medium region, 5th internode) and mature internodes (lower region, 10th and 15th internode) were collected for further cytological analyses (Table 1).

Table 1: Sampling data.
Tabela 1: Potek vzorčenja.

Sampling	Date	Collected material	Developmental stage
1	15.4.2013	Shoot apex	Emerging shoot bud from the rhizome
2	24.4.2013	Shoot apex, internode 5	Young plants approx. 50 cm high
3	5./10.7.2013	Internodes 1, 5, 10	Fully grown plants approx. 2 m high
4	29.8.2013	Internodes 5, 10, 15	Flowering plants
5	25.10.2013	Internodes 5, 10, 15	Senescent plants at the end of the season

Fixation and preparation of microscopic slides

Approx. 1 cm long tissue samples were cut from the stem and fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% glacial acetic acid) for at least 24 hours at 4°C. For longer storage samples were transferred to 70% ethanol at -20°C.

Thin transversal and longitudinal sections of fixed material were hand-cut using a razorblade. Sections were put in a drop of distilled water on the objective glass, covered and analysed with a light microscope (Axioskop 2 MOT, Carl Zeiss, Germany) combined with a colour digital camera (AxioCam MRc, Carl Zeiss, Germany) and AxioVision 4.8 software (Carl Zeiss, Germany). For better resolution of cell walls, autofluorescence (at UV excitation 365/12 nm band pass and emission 397 nm long pass) was also recorded when necessary.

Starch identification

The presence of starch grains was determined by staining the sections with the indicator iodine solution (3% KI/I_2 (w/v)).

Image analysis and statistics

In the longitudinal sections, width and length of cells (Fig. 1) were measured for at least 20 cells using ImageJ software (Rasband, 1997-2014). The obtained data were used to calculate cell volume according to the formula $V=\pi r^2 l$ (r – half of the cell width, l - cell length). Mean values and standard errors were calculated and the samples were compared by t-test and ANOVA (GraphPad Prism). The level of significance was set at p-value < 0.05.

Results

Cell size of *F. japonica* and *F. ×bohemica* gradually increased during the growth season from the smallest meristematic cells at the shoot apex (average width 40.73 μm, average volume 30.95×10^3 μm^3) to the largest mature cells of the 10th and 15th internode (average width 64.38 μm, average volume 593.05×10^3 μm^3). Cell width in the middle shoot region increased to 123% and to 158% in the lower mature region when compared to the meristematic cells in the upper shoot region (Fig. 2A). Cell volume increased more intensely indicating higher level of cell elongation than expansion of cell width; the volume increased to 758% in the middle shoot region and to 1917% in the lower mature region when compared to the meristematic cells in the upper region (Fig. 2B).

The width and volume of the cells in both *Fallopia* species were not significantly different except for the volume of the cells in the middle shoot region (p=0.020). Dynamics of the cell growth during the season was estimated by a trend line and was similar in case of cell width and cell volume expansion for both *Fallopia* species (cell width trend line equation for *F. japonica* y=14.10x, R^2=-0.86; for *F. ×bohemica* y=15.18x, R^2=0.15; cell volume trend line equation for *F. japonica* y=99.76x, R^2=0.31; for *F. ×bohemica* y=110.13x, R^2=0.16).

Upper shoot region

In the upper shoot region cells of the shoot apical meristem and the youngest internodes (1st) were measured. These cells were collected only during first three samplings. Later in the growth season at the 4th and 5th sampling, the apical region developed in reproductive tissues without the shoot apical meristem (Tab. 1).

The cell width was very similar for both *Fallopia* species (p=0.974) but the difference was more pronounced in cell volume (p=0.351) where cells of *F. ×bohemica* were on average larger (39.66×10^3 μm^3) than of *F. japonica* (25.31×10^3 μm^3) (Fig. 3, Suppl. Tab. 2).

In this region, starch granules were present in *F.* x *bohemica* at all sampling times while in *F. japonica* starch occured only at the 3rd sampling when plants were fully developed (Fig. 4).

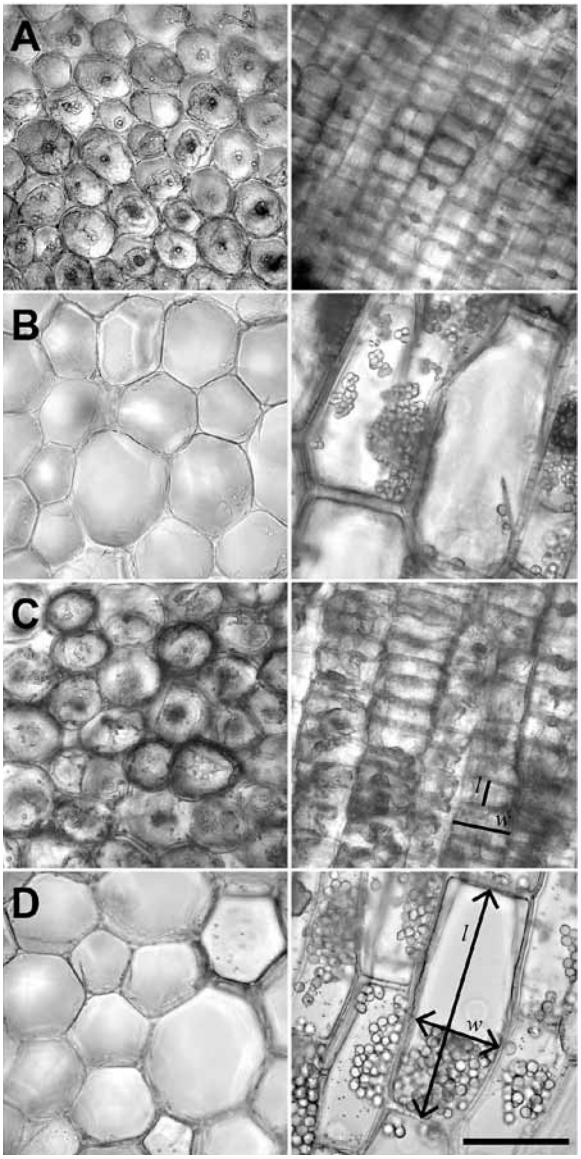

Figure 1: Transversal (left) and longitudinal (right) sections of *F. japonica* and *F. ×bohemica* shoots. **A** – Sections of *F. japonica* meristematic region at the 1st sampling; **B** - Sections of *F. japonica* mature shoot region at the 3rd sampling. **C** - Sections of *F. ×bohemica* meristematic region at the 1st sampling. **D** - Sections of *F. ×bohemica* mature shoot region at the 3rd sampling. Measurement of width (w) and length (l) of cells is indicated by lines in panels C and D. Bar represents 100 µm.

Slika 1: Prečni (levo) in vzdolžni (desno) prerez poganjka pri *F. japonica* in *F. ×bohemica*. **A** – Prereza zgornjega meristemskega območja pri *F. japonica* pri 1. vzorčenju. **B** - Prereza spodnjega zrelega območja pri *F. japonica* pri 3. vzorčenju. **C** - Prereza zgornjega meristemskega območja pri *F.* x *bohemica* pri 1. vzorčenju. **D** - Prereza spodnjega zrelega območja pri *F.* x *bohemica* pri 3. vzorčenju. Meritev širine (w) in dolžine (l) celic je prikazana z oznakami na slikah C in D. Merilo predstavlja 100 µm.

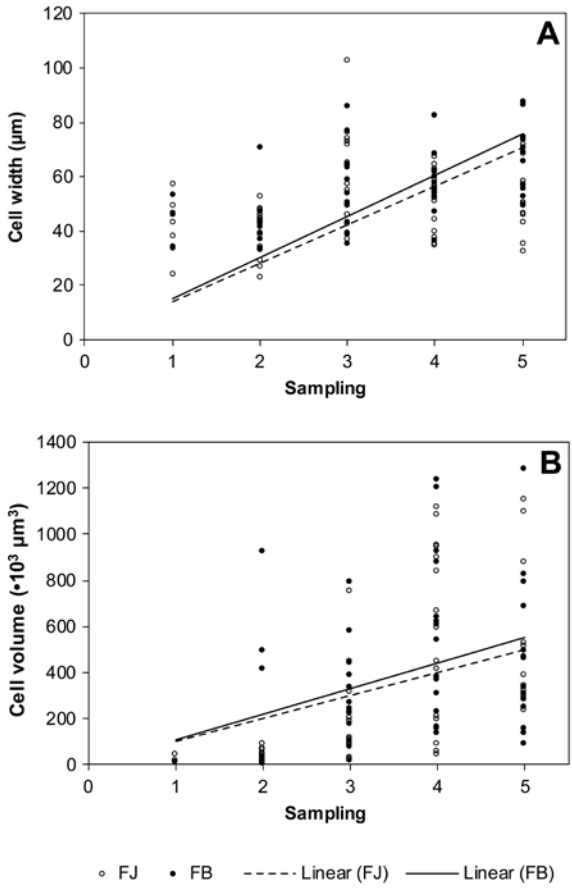

Figure 2: Dynamics of cell growth of *F. japonica* (FJ) and *F. ×bohemica* (FB) during growth season. Data present **A -** cell width and **B -** cell volume (N=5-25). Trendline is a linear regression line (intercept set at 0.0).

Slika 2: Dinamika rasti celic pri *F. japonica* (FJ) in *F. ×bohemica* (FB) med rastno sezono. Rezultati prikazujejo **A -** širino in **B** - prostornino izmerjenih celic (N=5-25). Trendna črta je linearna regresijska premica (izhodišče nastavljeno na 0,0).

Table 2: Cell size in the upper shoot region/meristem of *F. japonica* and *F. ×bohemica.* Mean value ± standard error are presented (N=5-10).

Tabela 2: Velikost celic v zgornjem/ meristemskem delu stebla pri *F. japonica* in *F. ×bohemica*. Rezultati prikazujejo povprečno vrednost ± standardno napako (N=5-10).

Sampling	*F. japonica*		*F. ×bohemica*	
	Volume (×10³ µm³)	Width (µm)	Volume (×10³ µm³)	Width (µm)
1	19.33±6.16	2.46±5.56	9.64±0.49	42.66±3.83
2	19.76±6.06	33.35±2.18	7.57±3.77	35.82±1.44
3	43.88±17.26	50.62±6.30	93.23±33.39	42.91±3.12

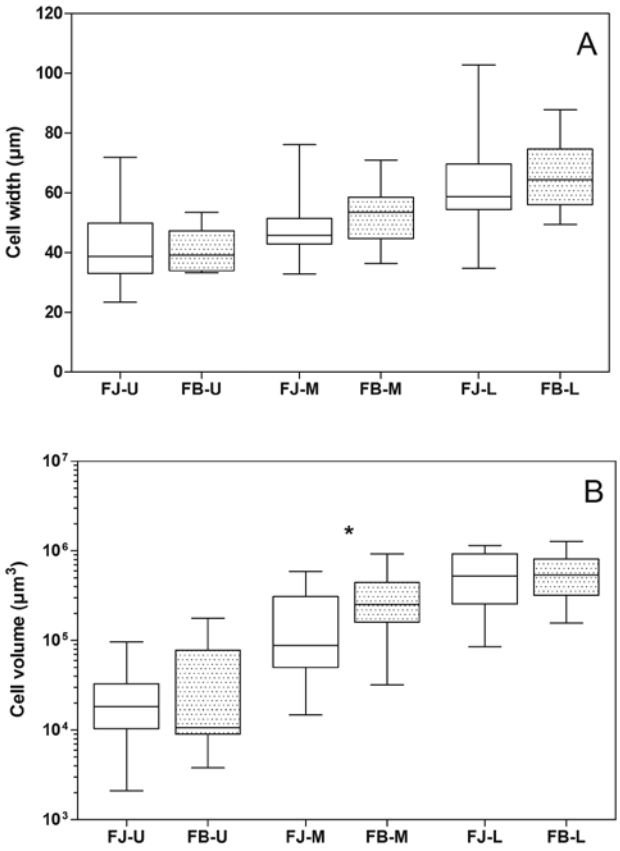

Figure 3: Cell size of *F. japonica* (FJ) and *F. ×bohemica* (FB) in the upper (U), medium (M) and lower (L) shoot region; **A** - cell width, **B** - cell volume.
* statistically significant ($p<0.05$) difference between *F. japonica* and *F. ×bohemica.*

Slika 3: Velikost celic pri *F. japonica* (FJ) in *F. ×bohemica* (FB) v zgornjem (U), srednjem (M) in spodnjem (L) delu poganjka; **A** - širina celic, **B** - prostornina celic.
* statistično značilna ($p<0.05$) razlika med vrstama *F. japonica* in *F. ×bohemica.*

Middle shoot region

In the middle shoot region, differentiating cells of the intermediate (5th) internode were measured. These cells were collected during four samplings and were missing only at the 1st sampling because plants were too small (Tab. 1).

The width of *F.* x *bohemica* cells was on average larger (52.95 µm) than of *F. japonica* cells (47.51 µm) and the difference was almost significant ($p=0.067$). On the other hand the cell volume was significantly ($p=0.020$) larger in *F. ×bohemica* (321.48×10^3 µm³) than in *F. japonica* (178.75×10^3 µm³) mostly because of the differences at the beginning of the growth season at the 2nd and the 3rd sampling. Later (4th and 5th sampling) the volume size was similar in both *Fallopia* species (Fig. 3, Suppl. Tab. 3).

In the middle region, there was no difference in the accumulation of the starch granules which were present in cells of both *Fallopia* species from the 3rd sampling on.

Table 3: Cell size in the middle shoot region of *F. japonica* and *F. ×bohemica*. Mean value ± standard error are presented (N=5-10).
***** statistically significant ($p<0.05$) difference between *F. japonica* and *F. ×bohemica*
Tabela 3: Velikost celic v srednjem delu stebla pri *F. japonica* in *F. ×bohemica*. Rezultati prikazujejo povprečno vrednost ± standardno napako (N=5-10).
***** statistično značilna ($p<0.05$) razlika med vrstama *F. japonica* in *F. ×bohemica*.

Sampling	*F. japonica*		*F. ×bohemica*	
	Volume ($\times 10^3$ µm^3)	Width (µm)	Volume ($\times 10^3$ µm^3)	Width (µm)
2	**51.96**±8.03*	45.97±0.94	**465.79**±182.65*	49.27±5.50
3	173.04±73.764	57.47±6.13	293.36±42.58	54.04±3.59
4	309.89±110.42	42.13±3.26	305.94±92.37	49.29±3.56
5	256.18±33.90	46.03±5.89	249.69±69.78	59.22±2.64

Lower shoot region

In the lower shoot region cells of fully differentiated and mature (10th and 15th) internodes were measured. These cells were collected in the fully developed plants from the 3rd sampling on (Tab. 1).

At the 4th and 5th sampling, the cells of *F. japonica* had significantly ($p=0.038$) larger volume than earlier in the growth season. The same pattern was observed also in *F. ×bohemica* but the difference was not significant ($p=0.292$). On the other hand, the cell width was similar during all mature period (Fig. 3, Suppl. Tab. 4).

In the lower region, starch granules were present in cells of both *Fallopia* species at all sampling times.

Discussion

Japanese and Bohemian knotweed are important invasive species in Slovenia and Europe. They form large and dense monospecific stands along rivers, railways, roads and other ruderal habitats, and severely decrease native biodiversity. In spring they restore the shoot from the underground rhizome and very quickly develop high stem and broad leaves. In our previous study (Strgulc Krajšek and Dolenc Koce 2015) it was shown that only octoploid Japanese knotweed (*F. japonica* var. *japonica*) and hexaploid Bohemian knotweed (*F. ×bohemica*) present highly invasive populations in Slovenia which form monospecific as well as mixed population with both species. Giant knotweed (*F. sachalinensis*) is less common and at the moment does not present such severe invasive threat. The same observations were reported also for Belgium (Herpigny et al. 2012).

Since level of ploidy can affect the cell and organ size as well as plant fitness (Barow and Meister 2003) we aimed to compare the dynamics of cell growth during the season in *F. japonica* and *F. ×bohemica* species to determine if cytological traits are correlated to morphological differences of adult plants (*F. ×bohemica* is taller and has bigger leaves than *F. japonica*).

Cell size gradually increased from the smallest apical cells to the fully developed cells in the lower shoot regions as plants developed during the growth season (Fig. 1). Cell width increased less than cell volume which indicates that cells primarily elongated. At the end of the season both *Fallopia* species had cells of similar size. The dynamics of cell growth shows that the only significant difference was measured at the 2nd sampling when middle size cells of *F. ×bohemica* had 896% higher volume than of *F. japonica* (Fig. 2). Nevertheless, when cell data for all shoot regions and all sampling times at are pulled together and compared for both *Fallopia* species it is revealed that *F. ×bohemica* has bigger cells than *F. japonica* and differences are close to significant ($p=0.065$ for

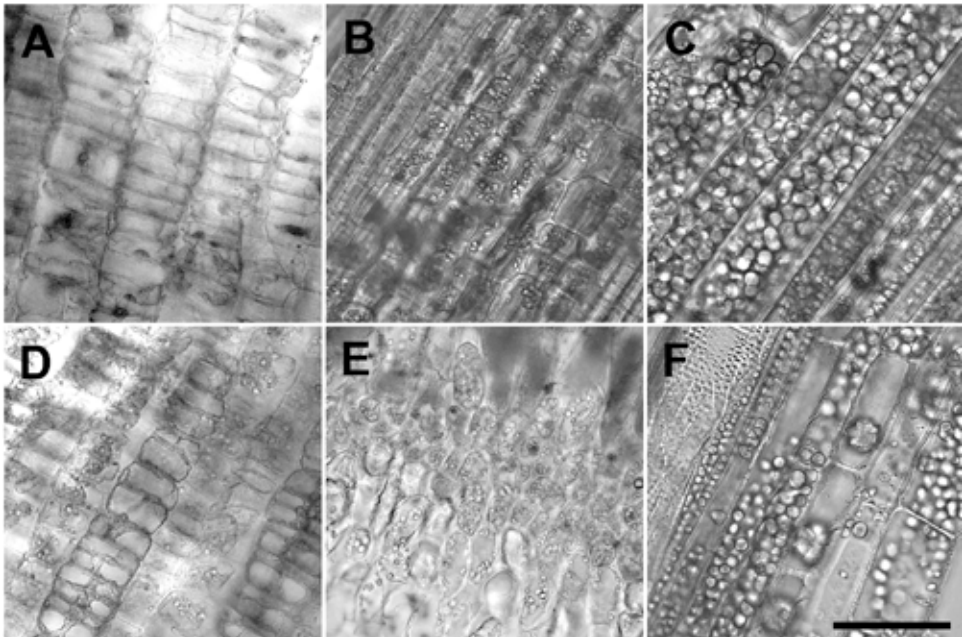

Figure 4: Stem cells with starch grains in *F. japonica* (**A-C**) and *F. ×bohemica* (**D-E**). **A, D -** meristematic cells at the 1st sampling; **B, E** - meristematic cells at the 3rd sampling; **C, F -** cells of the middle shoot region at the 4th sampling. Bar presents 100 µm.

Slika 4: Celice stebla s škrobnimi zrni pri *F. japonica* (**A-C**) in *F. ×bohemica* (**D-E**). **A, D -** meristemske celice pri 1. vzorčenju; **B, E** - meristemske celice pri 3. vzorčenju; **C, F -** celice v srednjem delu stebla pri 4. vzorčenju. Merilo predstavlja 100 µm.

cell width and p=0.135 for cell volume). Similar growth kinetics was described for tested *Fallopia* species in Belgium with *F. japonica* and *F. ×bohemica* having more comparable kinetics than *F. sachalinensis* with lower competitive ability for light and nitrogen (Herpigny et al. 2012). In that study it was also shown that *F. ×bohemica* had more variable growth and functional traits (shoot height, ramification, leaf size, foliar nitrogen concentration) which could be related to its hybrid origin. In our study, the variability of the cell width and cell volume was generally higher in *F. japonica* (CV range from 6.5 to 95.3%) than of *F. ×bohemica* (CV range from 8.0 to 78.4%) but only one cell trait was tested which is not enough to generalize the conclusion about cell growth.

We also tested correlation between cell size and ploidy level which was not significant therefore we conclude that polyploidy in investigated *F. japonica* and *F. ×bohemica* species had no effect on cell size and consequently on the size of the shoot.

Additionally, the presence of starch grains in all plant material was observed from the initial developmental phases until the fully developed plants. Cells of *F. ×bohemica* accumulated starch already at the beginning of the growth season which could contribute to its higher growth rate when compared to *F. japonica* plants. In the fully developed and mature stems there were no differences in starch accumulation between *Fallopia* species.

Conclusions

Fallopia japonica and *F. ×bohemica* have similarly large stem cells and their size is not correlated to the ploidy level.

Cell size of both *Fallopia* species gradually increased during the growth season from the smallest meristematic cells at the shoot apex to the

Table 4: Cell size in the lower/mature shoot region of *F. japonica* and *F. ×bohemica.* Mean value ± standard error are presented (N=5-10).
Tabela 4: Velikost celic v spodnjem/zrelem delu stebla pri *F. japonica* in *F. ×bohemica*. Rezultati prikazujejo povprečno vrednost ± standardno napako (N=5-10).

Sampling	*F. japonica*		*F. ×bohemica*	
	Volume ($\times 10^3$ μm^3)	Width (μm)	Volume ($\times 10^3$ μm^3)	Width (μm)
3	292.53±121.31	78.47±6.38	455.54±103.63	71.06±4.49
4	698.03±123.22	57.34±3.14	685.04±115.98	61.07±2.80
5	630.55±102.06	58.18±3.09	577.60±103.38	71.26±4.21

largest mature cells of the 10th and 15th internode

Cell volume increased more intensely indicating higher level of cell elongation than expansion of cell width

F. ×bohemica accumulated starch earlier in the growth season and in younger tissues than *F. japonica*.

Povzetek

Japonski (*Fallopia japonica*) in češki dresnik (*F. ×bohemica*) sta v Evropi in Severni Ameriki zelo invazivni tujerodni rastlinski vrsti. Spomladi iz prezimujoče korenike požene poganjek, ki hitro raste do končne višine (2-4 m) in ima široke liste, ki zasenčijo podrast. V raziskavi smo preučevali celično rast in tvorbo škrobnih zrn v treh območjih stebla, da bi ugotovili: (i) Ali ima oktoploidni japonski dresnik večje celice kot heksaploidni češki dresnik? (ii) Ali je velikost celic v meristemu, mladem in zrelem območju stebla različna in imata vrsti dresnika različno dinamiko celične rasti? (iii) Ali vrsti dresnika različno kopičita škrob?

V eni rastni sezoni smo petkrat (dvakrat na začetku rastne sezone, ob vegetativni zrelosti rastlin, v času cvetenja in ob zaključku rastne sezone) vzorčili celice stebla na vršičku, v srednjem delu stebla, kjer se celice še diferencirajo, in v nižje ležečih zrelih delih stebla. Rastlinski material smo fiksirali v fiksativu in ročno pripravili prečne in vzdolžne prereze za svetlobno mikroskopijo z vidno in UV-svetlobo. Na posnetih slikah smo z računalniškim programom za analizo slike (ImageJ) izmerili širino in dolžino celic, iz česar smo izračunali prostornino celic. Prisotnost škroba v tkivu smo dokazali z raztopino jodovice.

V povprečju so bile celice češkega dresnika nekoliko večje kot celice japonskega dresnika, vendar razlike niso bile statistično značilne razen v členkih srednjega dela stebla z diferencirajočimi se celicami. Tudi dinamika celične rasti je bila podobna pri obeh vrstah dresnika razen pri 2. vzorčenju, ko so bile celice češkega dresnika bolj podaljšane. Češki dresnik je v primerjavi z japonskim kopičil škrob prej in v mlajših tkivih.

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