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Seed Structure Characteristics of *Orobanche cumana* Populations

Abstract: Sunflower broomrape *Orobanche cumana* Wallr. is a rapidly growing threat to the oil crop production in many countries. Fast adaptation to new environments and increasing host resistance suggests that phenotypically distinctive populations of the weed may have evolved. The classification of the species and the differentiation of such populations on the base of seed micromorphological characters were attempted. Morphometric measurements allowed the distinction of *O. cumana* from several other *Orobanche* and *Phelipanche* species. An irregularly thickened cell wall of the anticlinal testa cells differentiated *O. cumana* and *O. cernua* from *O. caryophyllacea, O. crenata*, *O. minor*, *P. aegyptiaca*, *P. arenaria* and *P. ramosa*. However, populations of sunflower broomrape from five European countries and China could not be separated from each other on the base of micromorphological seed characters. In contrast, length to width measurements indicated that the Asian samples had a slightly different seed shape which was less elongated than the European samples. However, this seemingly geographic effect may as well be a consequence of sampling which comprised a higher rate of the so-called modern races E-H in the European samples.

Keywords: Broomrape, *Helianthus annuus*, *Orobanche cumana*, *Phelipanche*, seed micromorphology, sunflower

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Introduction

Seed morphology has been recognized as a suitable feature for the differentiation of parasitic weeds of *Orobanche* and *Phelipanche* species (Joel, 1987, 1988b; Abu Sbaih

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and Jury, 1994; Deif *et al*., 2000; Plaza *et al*., 2004; Domina and Colombo, 2005). While size and shape of entire seeds revealed to be highly variable, the micromorphology of the testa was shown to provide characteristics that are distinctive for the separation of the two genera, which were formerly placed in two sections (sect. *Trionychon* and sect. *Orobanche*) of the genus *Orobanche sensu lato*. For sunflower broomrape, *O. cumana*, transmission and scanning electron microscopic investigations had unravelled a complex composition of layers forming the typical reticulate ornamentation of the testa (Thomas *et al*., 1999) and similar structures were also found in the outer cell layers of *P. aegyptiaca* seeds (Joel *et al*., 2012). The usually oblongoid to ellipsoid *Orobanche* seeds have a surface composed of an outer layer of large, thick-walled cells with pitted periclinal inner walls which become visible when the thin outer wall layer collapses due to the loss of water. Attempts to use seed size and micromorphology for classification of broomrape species have been published for various agro-economically important taxa (Abu Sbaih and Jury, 1994; Deif *et al*., 2000; Plaza *et al*., 2004; Domina and Colombo, 2005), but infra-specific studies for the differentiation of races (or pathotypes) lack. The fast spreading of sunflower broomrape into new areas and the occurrence of new highly aggressive races of *O. cumana* within the past 20–30 years (Vrânceanu *et al*., 1986; Škorić *et al*., 2010; Fernández-Martínez *et al*., 2012) has become a major threat for sunflower production in Europe and Asia. Hence a classification system which allows the fast identification of such races would be very helpful, in particular since the so far used infection bioassays based on differential sunflower lines with defined resistance genes fail to differentiate races which overcome the OR5 gene (Alonso *et al*., 1996; Molinero-Ruiz and Melero-Vara, 2004; Škorić *et al*., 2010). The current study, therefore, aims to identify classifying characters in the seed morphology of sunflower broomrape and to explore their value for the infraspecific differentiation of populations from distant origin and different races.

Materials and methods

Sample preparation for scanning electron microscopy (SEM)

Dry seeds of five *Orobanche* species and three *Phelipanche* species from field accessions of different origin (see Table 1) were transferred on aluminum stubs covered with conductive carbon adhesive tabs. The samples were sputter coated with gold/palladium (SCD 040, Balzers Union, Switzerland) and the morphology and micromorphological characters were examined with a scanning electron microscope (DSM 940, Zeiss, Germany) at 5 kV.

Measurements and statistics

Size measurements (length and width) of 50 seeds per sample were recorded on scanning electron micrographs (Adobe Photoshop CS2) and statistically treated (mean value, standard deviation, minimum and maximum value). Analysis of variance (ANOVA) was conducted on seed length and width and the ratio of seed length/width with InfoStat (Version 2013, InfoStat Group, University of Córdoba, Argentina). Effects were considered significant if $p < 0.05$ in the Tukey test.

Further emphasis was put on seed shape, number of testa cells and micromorphological traits of the testa, i.e. periclinal and anticlinal cell wall features.

Plant material

The used seed samples are indicated in Table 1.

Table 1: Seed samples used for scanning electron microscopy. Sampling sites, host species and race determination are listed according to information obtained from sample providers (see footnote of Table).

#	Taxon	Collecting date	Origin	Host/Race
	P. aegyptiaca	2009	Israel (Maro Hawa)[1]	On *Solanum lycopersicum*
	P. arenaria	2011	Germany (Stuttgart)[2]	On *Artemisia campestris*
	P. ramosa	2008	Germany (Neupotz)	On *Nicotiana tabacum*
	O. caryophyllacea	2014	Germany (Pfullingen)	Host not determined
	O. cernua	2006	Spain (Níjar, Lucainena)[3]	On *Artemisia barrelieri*
	O. crenata	2007	Israel (Merahusia)[1]	On *Daucus carota*
	O. minor	2012	Germany (Bretten)	Host not determined
ES1	*O. cumana*	2003	Spain (Córdoba)[4]	EC403
ES2	*O. cumana*	2005	Spain (Córdoba)[4]	Not determined
ES3	*O. cumana*	2008	Spain (Córdoba)[4]	TOM 2008
ES4	*O. cumana*	1994	Spain (Carmona)[3]	Race E
RS1	*O. cumana*	2012	Serbia (Novi Sad)[5]	On sunflower genotype NS Slatki
RS2	*O. cumana*	2012	Serbia (Lipar)[5]	Race E or higher
RS3	*O. cumana*	2012	Serbia (Supliak)[5]	On non-resistant host
RS4	*O. cumana*	2012	Serbia (Sombor)[6]	Race E or higher
RO1	*O. cumana*	2012	Romania (Alexandria)[7]	Not determined
RO2	*O. cumana*	2012	Romania (Cuza-Voda)[7]	Not determined
RO3	*O. cumana*	2012	Romania (Iazu)[7]	Not determined

(*continued*)

Table 1: (*Continued*)

#	Taxon	Collecting date	Origin	Host/Race
MD1	*O. cumana*	2011	Moldova (Chisinau)[8]	Race G
MD2	*O. cumana*	2011	Moldova (Chisinau)[8]	Race G
MD3	*O. cumana*	2011	Moldova (Balti)[8]	Race F
RU1	*O. cumana*	2012	Russia (Krasnodar, Gulkevitchy)[9]	Race F or higher
RU2	*O. cumana*	2012	Russia (Rostov, Morozovskiy)[9]	Race H or higher
RU3	*O. cumana*	2012	Russia (Stavropol, Krasnogvardeysky)[9]	Race H or higher
CN1	*O. cumana*	2013	China (MinQin)[10]	Race A
CN2	*O. cumana*	2013	China (Zhaochun)[10]	Race D
CN3	*O. cumana*	2013	China (Gaomiao)[10]	Race E
CN4	*O. cumana*	2013	China (Dongdiyidui)[10]	Race F

Notes: Samples were generously provided by: [1]J. Hershenhorn, Hebrew University Rehovot, Israel; [2]B. Schäfer, Wilhelma, Stuttgart, Germany; [3]L. Velasco, [4]D. Rubiales and M. Molinero-Ruiz, Inst. for Sustainable Agriculture, Córdoba, Spain; [5]D. Miladinović and B. Dedić, Inst. of Field and Vegetable Crops, Novi Sad, Serbia; [6]S. Maširević, Dep. Environmental and Plant Protection, Novi Sad, Serbia; [7]M. Păcureanu-Joiţa, Nat. Agric. Res. Inst., Fundulea, Romania; [8]A. Glijin, Academy of Science of Moldova, Chisinau, Moldova; [9]T. Antonova, All-Russia Research Institute of Oil Crops by the name of V.S. Pustovoit, Krasnodar, Russia; [10]D. Ma, Beijing Sunrise Agritec Corp, Beijing, China.

Results

Comparison of seed shape and testa micromorphology

The seed shape of all species varied considerably, but a clear preference for roundish to pear shaped forms was observed in the *Phelipanche* samples (Figure 1(a)–(c)), whereas in *Orobanche* ovate (Figure 1(d)–(g)) to elongate (Figure 1(h)–(i)) seeds dominated. *O. cumana* seeds were narrow elongated to longish pear shaped. They significantly differed from *O. caryophyllacea*, *O. crenata*, *O. cernua* and *O. minor* in their length to width proportion (Figure 3(b)) and in having more elongated and fewer testa cells. Seeds of *O. cumana* showed a testa consisting of ca. 20–30 cells (mean value 23.8, SD 4.16) which often reached a length to width ratio of 5–6. In contrast, the testa of the closely related species *O. cernua* consisted of 30–45 cells (mean value 35.2, SD 6.55).

On dry seeds of all samples the anticlinal cell walls of the testa were visible, because the thin outer layer attached to the inner, pitted and thickened

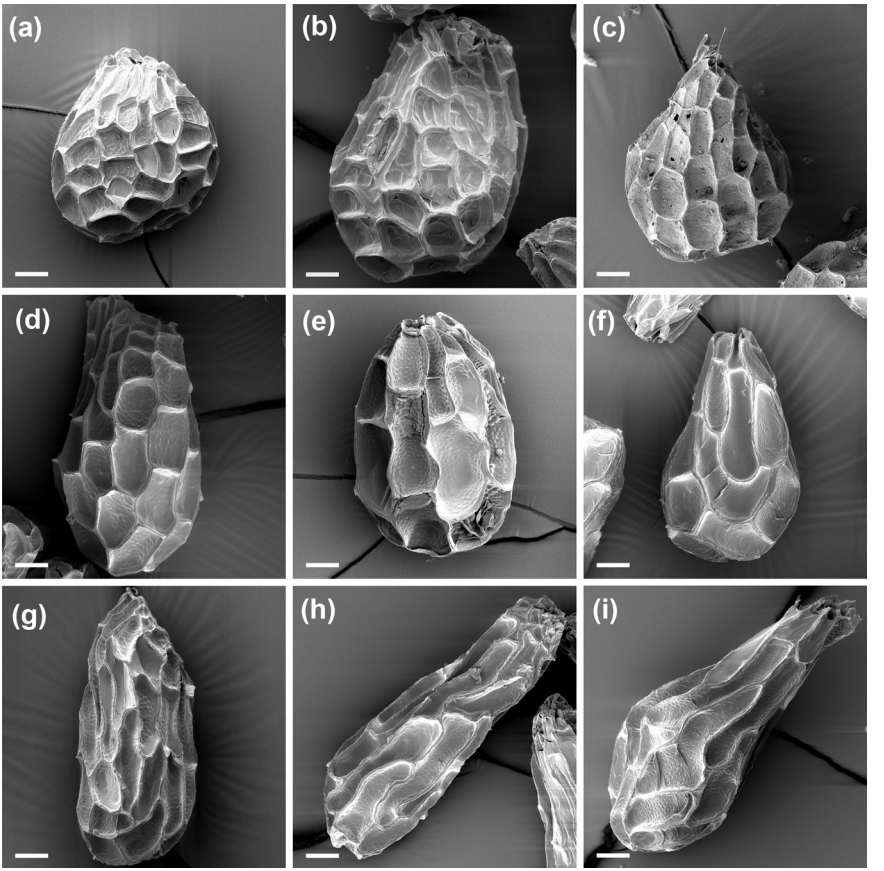

Figure 1: Seed shapes of *Phelipanche* and *Orobanche* species. SEM micrographs show typical seed shapes of *Phelipanche* species (a-c, roundish to pear shaped) and *Orobanche* species (d-g, ovate, and h-i elongated or longish pear shaped). a: *P. aegyptiaca*, b: *P. arenaria*, c: *P. ramosa*, d: *O. caryophyllacea*, e: *O. crenata*, f: *O. minor*, g: *O. cernua*, h: *O. cumana*, i: *O. cumana*. Scale bar: 50 µm.

periclinal cell wall thus giving the testa a reticulate appearance (Figure 2). The *Phelipanche* species were characterized by a fibrous texture of the outer periclinal cell wall which sometimes overlocked the pitted structure of the inner wall (e.g. *P. ramosa*, Figure 2(c)). In contrast, the *Orobanche* species had a smooth periclinal surface which occasionally appeared granulate, but never fibrous (Figure 2(d)–(i)).

A significant difference was observed in the structure of the anticlinal cell wall. The outer edge was crenated in all samples except for the two closely related species *O. cumana* and *O. cernua*, where this part was irregularly

thickened (Figure 2(g)–(i), arrows). The pitted structure of the testa cells in *O. cernua* apparently consisted of larger pits separated by smaller bars than in *O. cumana* (Figure 2(g)–(h), white arrow heads); however, we observed considerable variability of this feature. Seed samples of the 21 populations of *O. cumana* did not show differences in the their overall micromorphology which would have allowed infraspecific differentiation (data not shown).

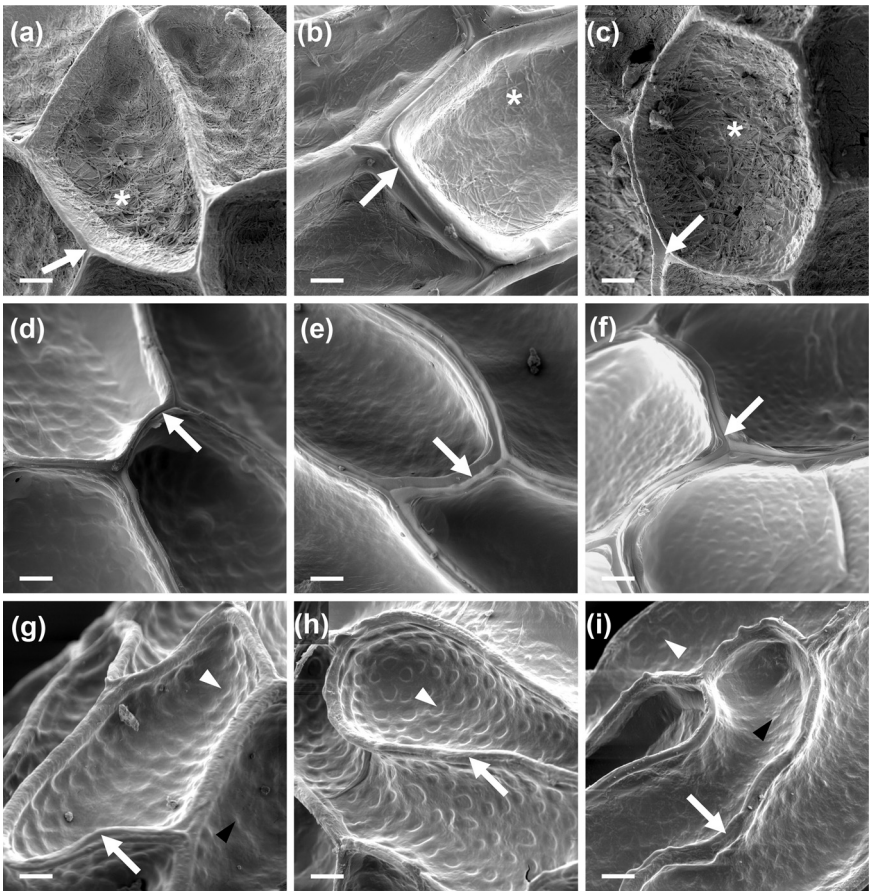

Figure 2: Testa micromorphology of *Phelipanche* and *Orobanche* species. SEM micrographs show typical features of testa cells. Arrows indicate crenated anticlinal walls for *Phelipanche* (a–c), *O. caryophyllacea, O. crenata* and *O. minor* (d–f), whereas *O. cernua* (g) and *O. cumana* (h, i) show an irregularly thickened anticlinal wall. Periclinal cell walls of *Phelipanche* seeds show a fibrous texture (asterisks), which does not occur in *Orobanche* seeds. White arrowheads indicate pits in inner periclinal cell walls which in some cases were overlocked by the granulate surface of the outer cell wall (black arrowheads). a: *P. aegyptiaca*, b: *P. arenaria*, c: *P. ramosa*, d: *O. caryophyllacea*, e: *O. crenata*, f: *O. minor*, g: *O. cernua*, h: *O. cumana*, i: *O. cumana*. Scale bar: 10 µm.

Morphometric data

Length and width measurements of the seeds revealed significant differences between the species tested (Table 2, Figure 3). So the seed length of the three *Phelipanche* species was generally about 10–20% lower than of *Orobanche* species, with the exception of *O. minor*. On the other hand, *Phelipanche* seeds reached similar or even higher widths when compared to *Orobanche* samples. This resulted

Table 2: Seed size of *Orobanche* and *Phelipanche* samples as analyzed by scanning electron microscopy.

#	Taxon	Seed length [µm]	Seed width [µm]	Length/width
	P. aegyptiaca	(220-) 295 ± 41 (–379)	(158-) 207 ± 26 (–259)	1.43 ± 0.23
	P. arenaria	(183-) 296 ± 59 (–487)	(124-) 190 ± 39 (–288)	1.58 ± 0.29
	P. ramose	(211-) 285 ± 32 (–356)	(165-) 205 ± 19 (–240)	1.40 ± 0.20
	O. caryophyllacea	(266-) 354 ± 36 (–423)	(122-) 181 ± 22 (–242)	1.99 ± 0.36
	O. cernua	(198-) 321 ± 45 (–416)	(119-) 174 ± 23 (–231)	1.87 ± 0.30
	O. crenata	(257-) 346 ± 44 (–447)	(153-) 216 ± 33 (–283)	1.63 ± 0.24
	O. minor	(173-) 297 ± 36 (–382)	(124-) 168 ± 18 (–209)	1.78 ± 0.27
ES1	*O. cumana*	(281-) 365 ± 42 (–472)	(113-) 160 ± 19 (–195)	2.31 ± 0.37
ES2	*O. cumana*	(250-) 339 ± 42 (–420)	(97-) 150 ± 20 (–202)	2.30 ± 0.37
ES3	*O. cumana*	(264-) 360 ± 51 (–476)	(89-) 150 ± 26 (–202)	2.45 ± 0.42
ES4	*O. cumana*	(241-) 342 ± 43 (–418)	(108-) 163 ± 24 (–204)	2.13 ± 0.27
RS1	*O. cumana*	(286-) 414 ± 59 (–517)	(136-) 178 ± 21 (–229)	2.37 ± 0.44
RS2	*O. cumana*	(279-) 371 ± 71 (–623)	(116-) 167 ± 44 (–341)	2.27 ± 0.34
RS3	*O. cumana*	(256-) 351 ± 36 (–428)	(130-) 167 ± 36 (–213)	2.12 ± 0.29
RS4	*O. cumana*	(271-) 382 ± 44 (–425)	(124-) 166 ± 19 (–209)	2.33 ± 0.40
RO1	*O. cumana*	(280-) 354 ± 40 (–435)	(113-) 150 ± 21 (–211)	2.40 ± 0.38
RO2	*O. cumana*	(248-) 332 ± 45 (–417)	(110-) 151 ± 19 (–201)	2.23 ± 0.37
RO3	*O. cumana*	(312-) 381 ± 40 (–452)	(128-) 175 ± 26 (–250)	2.22 ± 0.35
MD1	*O. cumana*	(257-) 359 ± 47 (–448)	(116-) 166 ± 25 (–216)	2.21 ± 0.41
MD2	*O. cumana*	(212-) 323 ± 52 (–489)	(100-) 140 ± 18 (–175)	2.34 ± 0.48
MD3	*O. cumana*	(274-) 352 ± 39 (–431)	(133-) 170 ± 21 (–206)	2.09 ± 0.31
RU1	*O. cumana*	(297-) 381 ± 39 (–457)	(121-) 165 ± 21 (–213)	2.36 ± 0.42
RU2	*O. cumana*	(333-) 418 ± 43 (–537)	(145-) 200 ± 24 (–267)	2.11 ± 0.26
RU3	*O. cumana*	(317-) 401 ± 38 (–478)	(134-) 173 ± 23 (–240)	2.36 ± 0.36
CN1	*O. cumana*	(266-) 363 ± 32 (–417)	(151-) 187 ± 18 (–233)	1.96 ± 0.24
CN2	*O. cumana*	(269-) 326 ± 28 (–401)	(147-) 173 ± 15 (–213)	1.90 ± 0.22
CN3	*O. cumana*	(237-) 312 ± 39 (–418)	(128-) 169 ± 21 (–219)	1.87 ± 0.27
CN4	*O. cumana*	(300-) 379 ± 45 (–469)	(137-) 174 ± 17 (–228)	2.20 ± 0.35

Notes: Values represent the mean ± standard deviation of $n = 50$ seeds in µm. Numbers in brackets indicate minimum and maximum values of each sample. The geographic origin of *O. cumana* samples is listed (ES: Spain, RS: Serbia, RO: Romania, MD: Moldova, RU: Russia, CN: China).

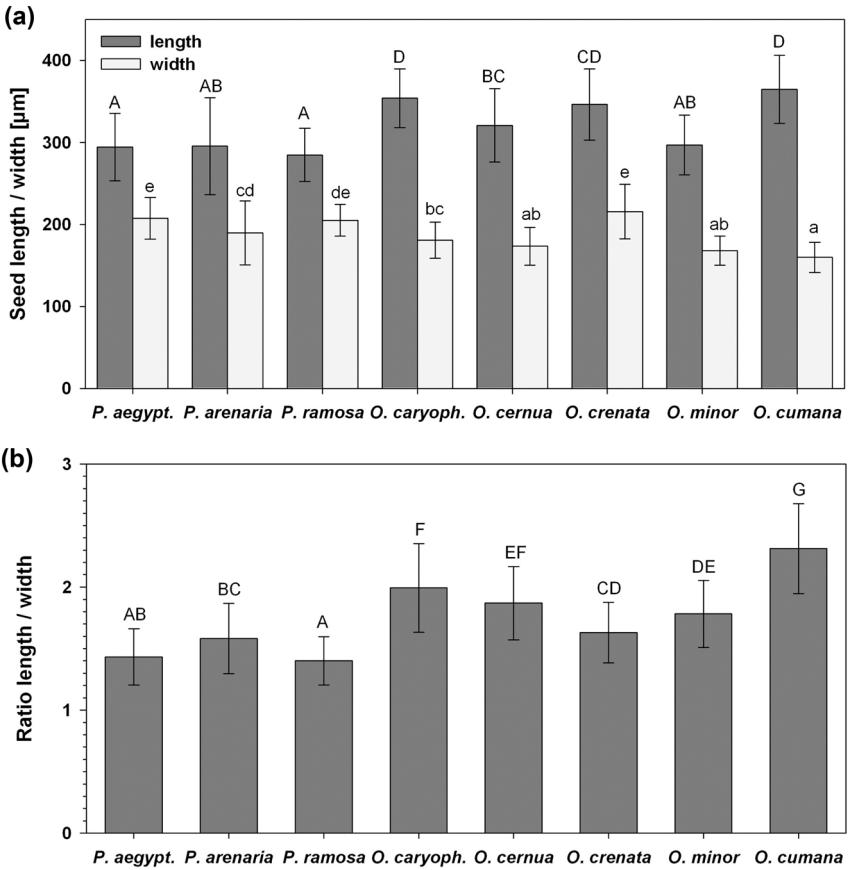

Figure 3: Seed size of *Phelipanche* and *Orobanche* species. a: seed length and width in µm, b: ratio length/width. Columns represent mean values and SD of $n = 50$ seeds per population. *O. cumana* ES2 is given as representative sample of sunflower broomrape. Columns marked with different letters indicate that differences between seed length, seed width and length/width ratios were statistically significant (Tukey test, $p < 0.05$).

in characteristic length to width ratios which ranged between 1.4–1.6 for *Phelipanche* and 1.6–2.2 for *Orobanche* (Figure 3(b)). *O. cumana* reached a mean length of 362 (SD ± 50) µm, width of 166 (SD ± 25) µm and a ratio of 2.22 (SD ± 0.39) in the 21 populations investigated. This ratio, reflecting the elongated, narrow shape of *O. cumana* seeds, significantly differed from all other studied species.

The size variation in between the populations of *O. cumana* was tested for field isolates from Spain, Serbia, Romania, Moldova, Russia and China (Figure 4). Seed length and width were quite variable, but there was no

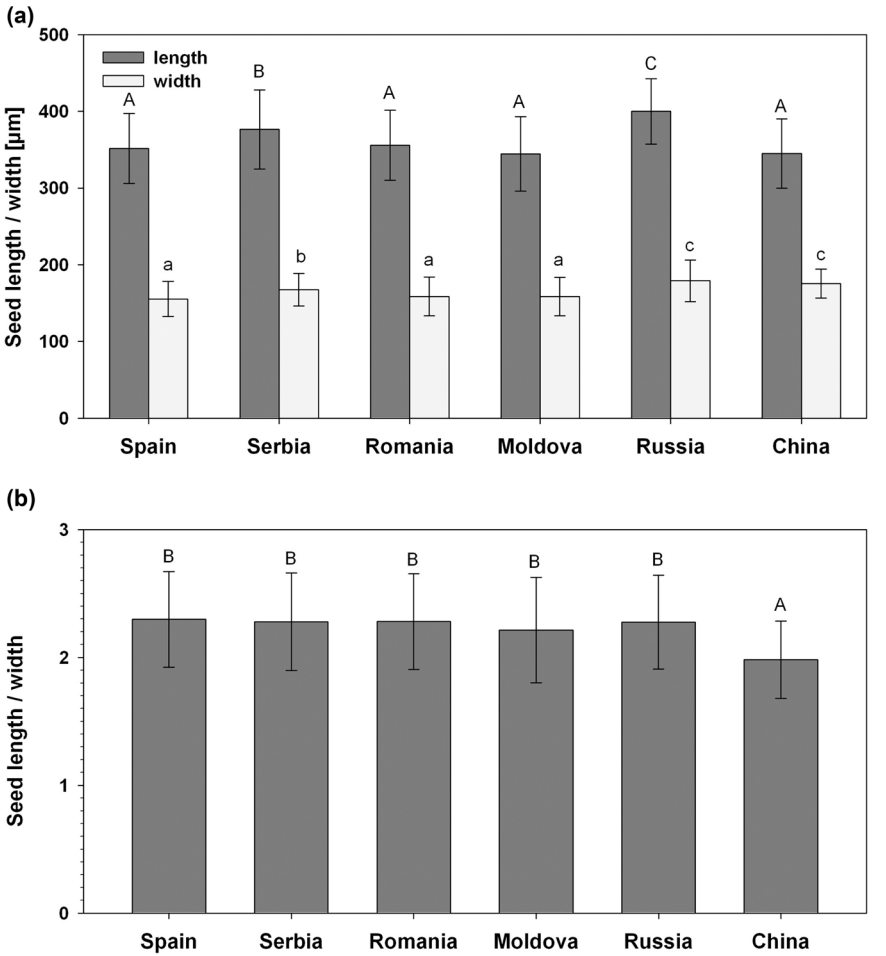

Figure 4: Seed size of *O. cumana* populations from different regions. a: seed length and width, b: ratio length/width. Columns represent mean values and SD of $n = 150–200$ seeds, i.e. 3–4 populations per region. Columns marked with different letters indicate that differences between seed length, seed width and length/width ratios were statistically significant (Tukey test, $p < 0.05$).

statistically significant (Tukey test $p < 0.05$) difference between the length to width ratio of the European samples. The four samples from China showed a statistically significant reduced length to width ratio. This difference was enhanced, when one of the Chinese samples, classified as race F, was separated (Figure 5). The length to width ratio of the European samples of races E-H was very similar and clearly above 2, whereas it was below 2 in the race A-E samples obtained from China.

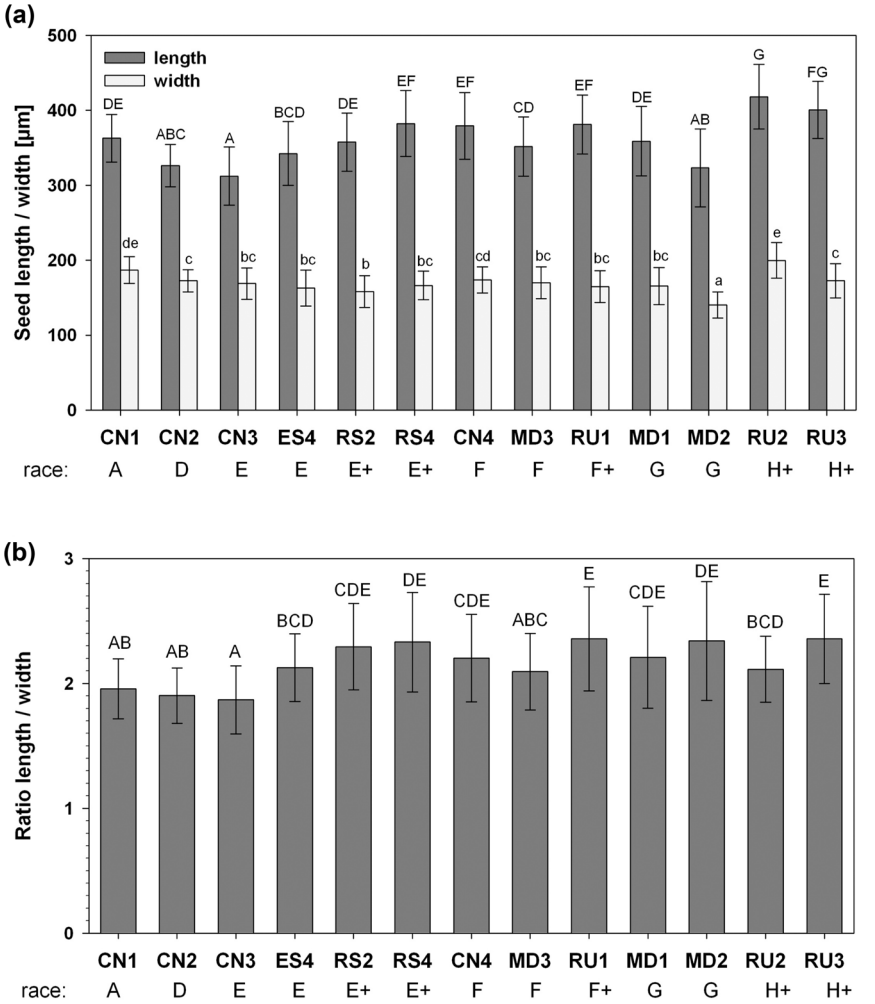

Figure 5: Seed size of *O. cumana* populations of different races (pathotypes). a: seed length and width, b: ratio length/width. Columns represent mean values and SD of $n = 50$ seeds per population. Columns marked with different letters indicate that differences between seed length, seed width and length/width ratios were statistically significant (Tukey test, $p < 0.05$). ES: Spain, RS: Serbia, RO: Romania, MD: Moldova, RU: Russia, CN: China.

Discussion

The progression of broomrape incidences in European and Asian sunflower cultivation (Parker, 2009) and the fast occurrence of new and not uniformly defined races

of *O. cumana* within the past two decades (Škorić *et al.*, 2010; Fernández-Martínez *et al.*, 2012) necessitated and enforced the search for classifying characters on the species and infraspecific level. Previous investigations had indicated that the seed morphology of broomrape species can provide reliable and fast accessible characters for species classification (Joel, 1988a; Abu Sbaih and Jury, 1994; Thomas *et al.*, 1999; Plaza *et al.*, 2004; Domina and Colombo, 2005; Joel *et al.*, 2012), but morphological differentiation between populations of the same species has not been attempted yet. The current study on seeds of more than 20 isolates of *O. cumana* is the first investigation which tested the variability of such morphological features between accessions from different geographic regions and of different race phenotypes. It could be shown that the structure of the seed testa and morphometric measurements are suitable to differentiate sunflower broomrape from related species of *Orobanche* and its sister genus *Phelipanche*. The investigation confirmed the characteristically smooth, pitted inner cell wall of *Orobanche* in comparison to the fibrous texture of *Phelipanche* as previously reported by several groups (Abu Sbaih and Jury, 1994; Thomas *et al.*, 1999; Plaza *et al.*, 2004; Domina and Colombo, 2005; Joel *et al.*, 2012). An irregularly thickened anticlinal wall of the testa cells in contrast to a typically crenated wall was firstly described as a marker to distinguish *O. cumana* and *O. cernua* from other *Orobanche* species by Joel (1988b). This was confirmed in our study and accounted for all 21 accessions of sunflower broomrape, whereas *O. caryophyllacea*, *O. crenata*, *O. minor* and all three *Phelipanche* species showed the typical split between the testa cells. The crenated type of wall has been described for several other *Orobanche* species as well (e.g. Plaza *et al.*, 2004; Domina and Colombo, 2005). An unequivocal differentiation between *O. cumana* and its closest relative *O. cernua* based on micromorphological characters was not achieved. The pit diameter and the distance between the pits in the inner periclinal wall which was suggested by Joel (1988b) for differentiation of the two species appeared inverse in some of our *O. cumana* samples and the variability of this parameter in *O. cernua* could not be analyzed due to the lack of additional samples. However, our investigation suggested that the number of testa cells which form the seed surface is clearly lower in *O. cumana* than in *O. cernua* and that their shape is more elongated. It will be necessary to test the variability of this character for *O. cernua* by screening more samples in future studies. Among the 21 samples of *O. cumana*, no cellular characters were found suitable for the differentiation of geographically defined populations or races.

Morphometric data obtained from seed measurements provide a second source of data for species classification. The size values found for three Phelipanche and five Orobanche species investigated in this study were well in the range reported for other samples of these taxa (Abu Sbaih and Jury, 1994; Plaza *et al.*, 2004; Domina and Colombo, 2005). This particularly accounts for

O. cumana, for which Plaza *et al.* (2004) reported size measurements of 360 × 160 to 500 × 250 µm from populations in Spain. The Spanish samples in our study averaged 360 × 155 µm but also contained single larger seeds up to 476 µm in length and 202 µm in width. Such absolute size differences might be attributed to external factors such as the nutritional situation, climate or unintentional selection of harvested material. In addition, the maturity of seeds at the date of harvest could have an influence. We therefore preferred the comparison of the size ratio between length and width, as we found that this parameter is less affected by the mentioned factors. Taken this into account, *O. cumana* clearly differed from the other species studied by showing a length/width ratio of well above 2, whereas the other taxa reached values around 1.5 for *Phelipanche* and ca. 1.6–2.0 for the *Orobanche* species. *O. cernua* showed a ratio of 1.87 and therefore could clearly be distinguished from the average of *O. cumana*. This corroborates the measurements of previous studies for *O. cernua* which reported a ratio of 1.4–1.6 (Abu Sbaih and Jury, 1994; Domina and Colombo, 2005). The multiple collections of *O. cumana* from different European countries were very similar to each other in the length to width ratio, but differed from three of the four Chinese samples which had less elongated seeds. Interestingly, the broader type of seeds was correlated with the race phenotype of "older" races, whereas the F race sample from China closely resembled the European samples of race F and higher. It is certainly too early to draw further conclusions from this coincidence before additional samples of races A-E have been studied, but this may be an additional feature indicating that the evolutionary background of sunflower broomrape points to *O. cernua* (Román *et al.*, 2003; Manen *et al.*, 2004). Nevertheless, morphometric measurements did not provide characteristics significant enough for the differentiation of races E-H which dominate the European areas of sunflower production. This is in line with several recent reports on the molecular diversity of sunflower broomrape, in which SSR markers (Pineda-Martos *et al.*, 2013) and RAPD amplicons (Molinero-Ruiz *et al.*, 2014) were screened but also failed to detect race correlation.

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