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SEED COAT PATTERNS IN RAPID-CYCLING *BRASSICA* FORMS

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The micromorphological typology of seed surfaces was investigated in rapid-cycling *Brassica* (RCBr) forms, using scanning electron microscopy. Four types of basic ornamentation pattern were recognized: reticulate (*B. rapa*, *B. juncea*), reticulate-foveolate (*B. nigra*), randomly reticulate (*B. oleracea*, *B. napus*) and reticulate-rugose (*B. carinata*). The seed coats showed variation in the shape and size of the testa epidermal cells and the structure of the outer periclinal and anticlinal cell walls. The surface patterns in RCBr seeds were less exposed and were moderately reticulated compared to cultivated *Brassica* species. The micromorphological characteristics of the seed coats may provide valuable additional diagnostic criteria for delimitation of RCBr forms, and can be used in identification of seeds.

Key words: Rapid-cycling *Brassica* (RCBr), micromorphology, sculpture, seed surface pattern, testa, SEM.

INTRODUCTION

The seed coat (often referred to as testa) is the outer covering of every mature seed. It is therefore the main modulator of interactions between the internal structures of the seed and the external environment. Most seed coats exhibit complex and highly diverse morphology and anatomy, providing valuable taxonomic characters (Barthlott, 1981, 1990). These characters, especially the microstructural features of the seed surface, can be useful for assessing phenetic relationships and delimiting taxa at various levels (Hufford, 1995; Karcz et al., 2000).

Economically, cultivated *Brassica* species are the most important group within the genus *Brassica*. These species include three diploid species and three derived allotetraploid species. The evolution of the brassicas and the phylogenetic relationships among diploids and allotetraploids are well illustrated by the classic "U-triangle" (U, 1935). The triangle shows how the genomes of the diploid progenitors *B. rapa* (2n = 20, AA), *B. nigra* (2n = 16, BB) and *B. oleracea* (2n = 18, CC) are combined in the allotetraploids *B. juncea* (2n = 4x = 36, AABB), *B. napus* (2n = 4x = 38, AACC) and *B. carinata* (2n = 4x = 34, BBCC). In recent years, extensive research at the molecular level has examined the phenetic relationships among these species (Bhatia et al., 1996; Warwick and Black, 1997; Somers et al., 2001; Panda et al., 2003).

In *Brassica* species, relatively numerous reports concerning the seed coat structure have been published (Mulligan and Bailey, 1976; Buth and Roshan, 1983; Setia and Richa, 1989; Ren and Bewley, 1998). Several recent studies of different seed coat characters have employed scanning electron microscopy (SEM). For example, Koul et al. (2000) gave detailed descriptions of seed morphology in 44 species of the subtribes Brassicinae, Raphaninae and Moricandiinae, and elucidated the phylogenetic relationships between taxa. More recently, Zeng et al. (2004) examined testa topographic patterns during seed development in the cultivated forms of these species.

Although seed coat pattern studies on cultivated brassicas have been done, little is known about seed morphology in the rapid-cycling *Brassica* forms. These forms, selected from six cultivated brassicas, possess many desirable attributes for a model plant system: a short life cycle of 5–6 weeks, small size, rapid seed maturation, and the absence of seed dormancy (Williams and Hill, 1986). Because of their relevance to

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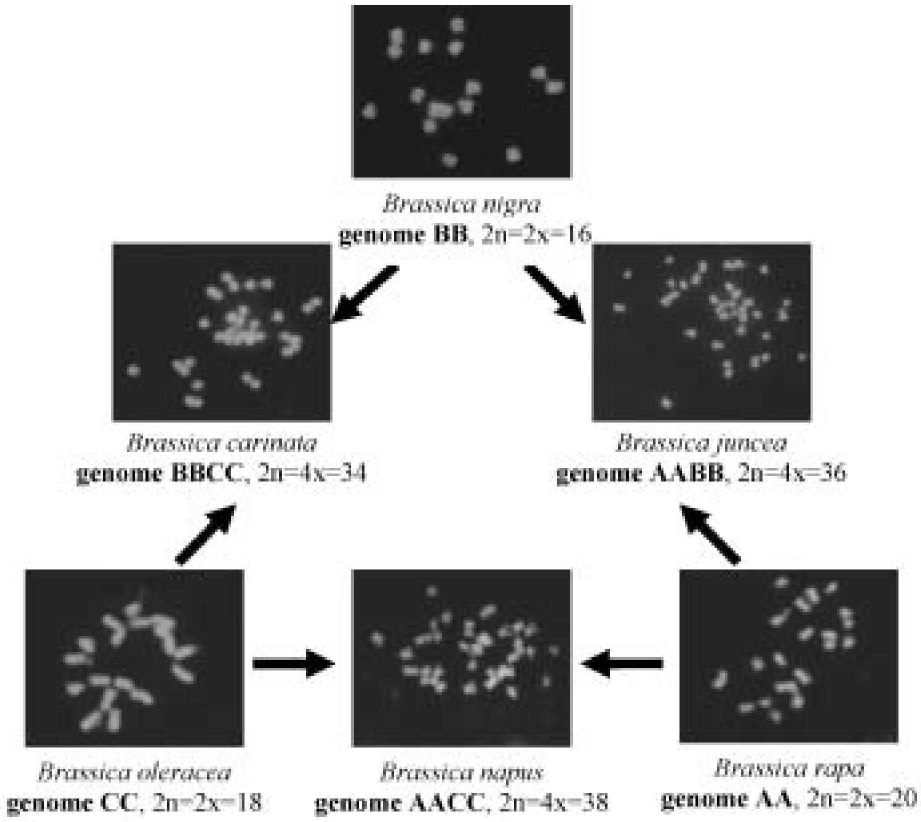


Fig. 1. Scheme showing "U-triangle" of rapid-cycling *Brassica* forms. Mitotic chromosomes stained with DAPI.

commercial *Brassica* species, rapid-cycling *Brassica* populations, especially RC. *B. rapa* and RC. *B. oleracea*, have seen wide application in plant biology research and education, in fields such as genetics, molecular biology, plant breeding, physiology, ecology and agricultural biotechnology (Koh and Loh, 2000). Adding to the popularity of these small short-lived plants for research applications is their extensive use in teaching at all levels of the educational system.

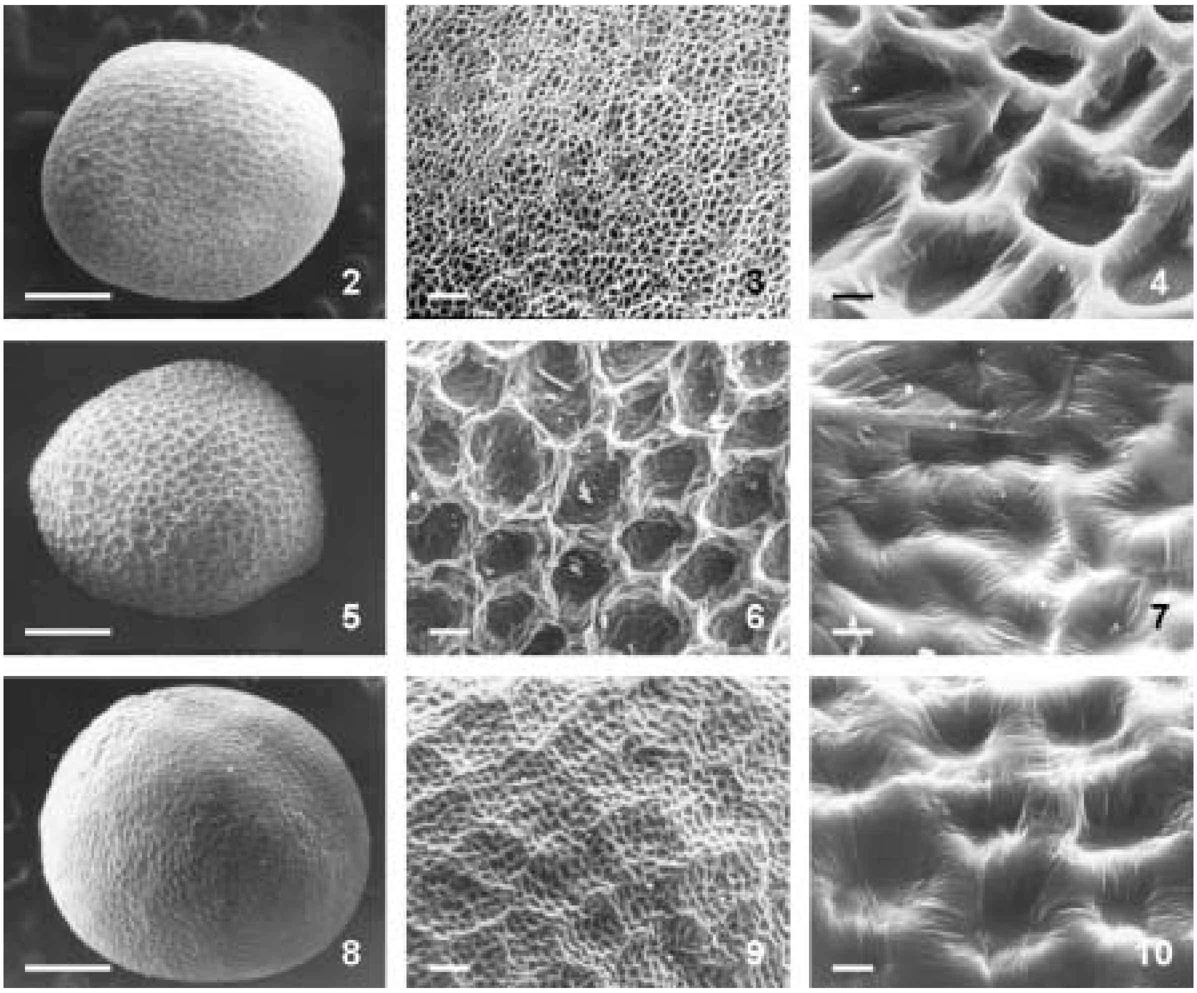
TABLE 1. List of rapid-cycling (RC) *Brassica* accessions studied for seed coat pattern; Voucher data – Crucifer Genetics Cooperative (CrGC), Madison, U.S.A.

Species*	Code No.	Genome
Diploid forms		
RC. *B. rapa*	Bpl 1–1	AA (2n = 20)
RC. *B. nigra*	Bpl 2–1	BB (2n = 16)
RC. *B. oleracea*	Bpl 3–1	CC (2n = 18)
Poliploid forms		
RC. *B. juncea*	Bpl 4–1	AABB (2n = 36)
RC. *B. napus*	Bpl 5–1	AACC (2n = 38)
RC. *B. carinata*	Bpl 6–1	BBCC (2n = 34)

*Collection used for chromosome preparation.

The data on the general plant morphology of RCBr forms have been summarized (Musgrave, 2000; Himelblau et al., 2004). In these papers the phenotypic characterization of some vegetative and generative organs has been given (e.g., plant stature, leaf shape and petiole length, flower color, male sterility and silique shape). Recently, epicuticular wax structure on leaves and stems of RCBr plants at developmental stages in vivo and in vitro was described (Karcz et al., 2004). On the other hand, the morphology of seed structure within RCBr forms is not documented, though some physiological and biochemical studies have been done on RCBr seeds (Shattuck, 1993; Bettey et al., 1998; Sinniah et al., 1998). This lacuna has prompted the present investigation.

To date, SEM has not been used to examine the morphology of seeds of RCBr forms. The present work studies the external morphology of mature seeds of rapid-cycling *Brassica*, with the aim of providing new diagnostic features for characterization of the RCBr group.



Figs. 2–10. SEM micrographs of diploid rapid-cycling (RC) *Brassica* seeds. **Figs. 2–4.** RC *B. rapa*. **Fig. 2.** Mature seed. **Fig. 3.** Reticulate seed coat pattern. Testa cells with weakly raised anticlinal cell boundaries. **Fig. 4.** Polygonal pits of outer periclinal cell walls with finely striated secondary ornamentation. **Figs. 5–7.** RC *B. nigra*. **Fig. 5.** Mature seed. **Fig. 6.** Reticulate-foveolate seed coat pattern showing hexagonal cells with concave outer periclinal walls and distinctly raised anticlinal boundaries. **Fig. 7.** Outer periclinal cell wall with visible shallow depressions and finely striated surface. **Figs. 8–10.** RC *B. oleracea*. **Fig. 8.** Mature seed. **Fig. 9.** Randomly reticulate surface pattern with weakly outlined polygonal cells. **Fig. 10.** Pitted outer periclinal cell wall with striated secondary sculpture. Bars = 500 μm in Figs. 2, 5, 8; 50 μm in Figs. 3, 6, 9; 5 μm in Figs. 4, 7, 10.

MATERIALS AND METHODS

The seed material of rapid-cycling *Brassica* forms is given in Table 1.

CHROMOSOME PREPARATION AND DAPI STAINING

Seeds were germinated on moist filter paper. Seedlings (2–3 days old) were treated with 2 mM 8-hydroxyquinoline for 3.5 h at room temperature, fixed in an ethanol-glacial acetic acid mixture (3:1) and stored at -20˚C until required. Fixed seedlings were washed in 0.01 M citric acid-sodium citrate buffer (pH 4.8) for 15 min and digested in 2% (w/v) cellulase (Calbiochem) and 20% (v/v) pectinase (Sigma) for 1.5 h at 37˚C. The root tips were squashed in a drop of 45% acetic acid. After freezing, the coverslips were removed; the slides were air-dried and then stained with 2 μg/ml 4'-6-diamidino–2-phenylindole (DAPI) solution (Serva) and mounted in an antifade buffer (AF1 Citifluor, Ted Pella Inc.). The fluorescent images were acquired using an Olympus Camedia C–4040Z digital camera attached to a Leica DMRB microscope, and then processed using Micrographx Picture Publisher software.

SCANNING ELECTRON MICROSCOPY (SEM)

Mature dry seeds (without fixation) were mounted on aluminum stubs with double-sided adhesive carbon type and sputter-coated with gold using a Pelco SC-6

TABLE 2. Seed morphological characters in rapid-cycling (RC) *Brassica* forms

Species	Seed color	Seed shape	Cellular pattern	Outer seed coat cells		
				Shape of cells	Periclinal walls	Anticlinal walls
Diploids						
RC. *B. rapa*	brown	ovate	reticulate	irregular polygons	concave with polygonal pits and smooth or finely striated interstices	weakly raised and pitted
RC. *B. nigra*	brown	ovate	reticulate-foveolate	regular to irregular pentagons or hexagons	depressed with shallow pits; smooth or finely striated interstices	distinctly raised, undulated and finely striated
RC. *B. oleracea*	brown to grey	ovate	randomly reticulate	weakly outlined polygons	slightly concave with polygonal or circular pits; smooth or finely striated interstices	slightly raised and pitted
Polyploids						
RC. *B. juncea*	light brown to brown	ovate	reticulate	regular polygons, mostly hexagons	concave with polygonal pits, finely striated interstices	raised and slightly pitted
RC. *B. napus*	dark grey	ovate	randomly reticulate	irregular polygons	slightly concave with polygonal pits and wide interstices	unevenly raised, undulated
RC. *B. carinata*	brown	ovate	reticulate-rugose	differently shaped polygons	concave with irregular depressions	raised and undulated

sputter coater. For each genotype, at least 20 seeds were randomly selected and studied. The coated materials were examined and photographed with a Tesla BS 340 scanning electron microscope at an accelerating voltage of 20 kV. In the micromorphological description of seed surface texture, the following concepts were used: arrangement of epidermal cells, primary sculpture, and secondary sculpture (Barthlott, 1981).

RESULTS AND DISCUSSION

CHROMOSOME NUMBER

The diploid and tetraploid karyotypes of the investigated rapid-cycling *Brassica* forms are presented in Figure 1. The number of chromosomes in allopolyploids is equal to the sum of chromosome numbers in their diploids. The allotetraploids arose by spontaneous interspecific hybridization from putative diploid ancestors, and during evolution their genomes were subjected to changes and chromosome rearrangements. Recently, *Brassica* have been the subject of extensive molecular and cytogenetic studies contributing to genome analyses of allopolyploids and their phylogenesis (Maluszynska and Hasterok, 2005).

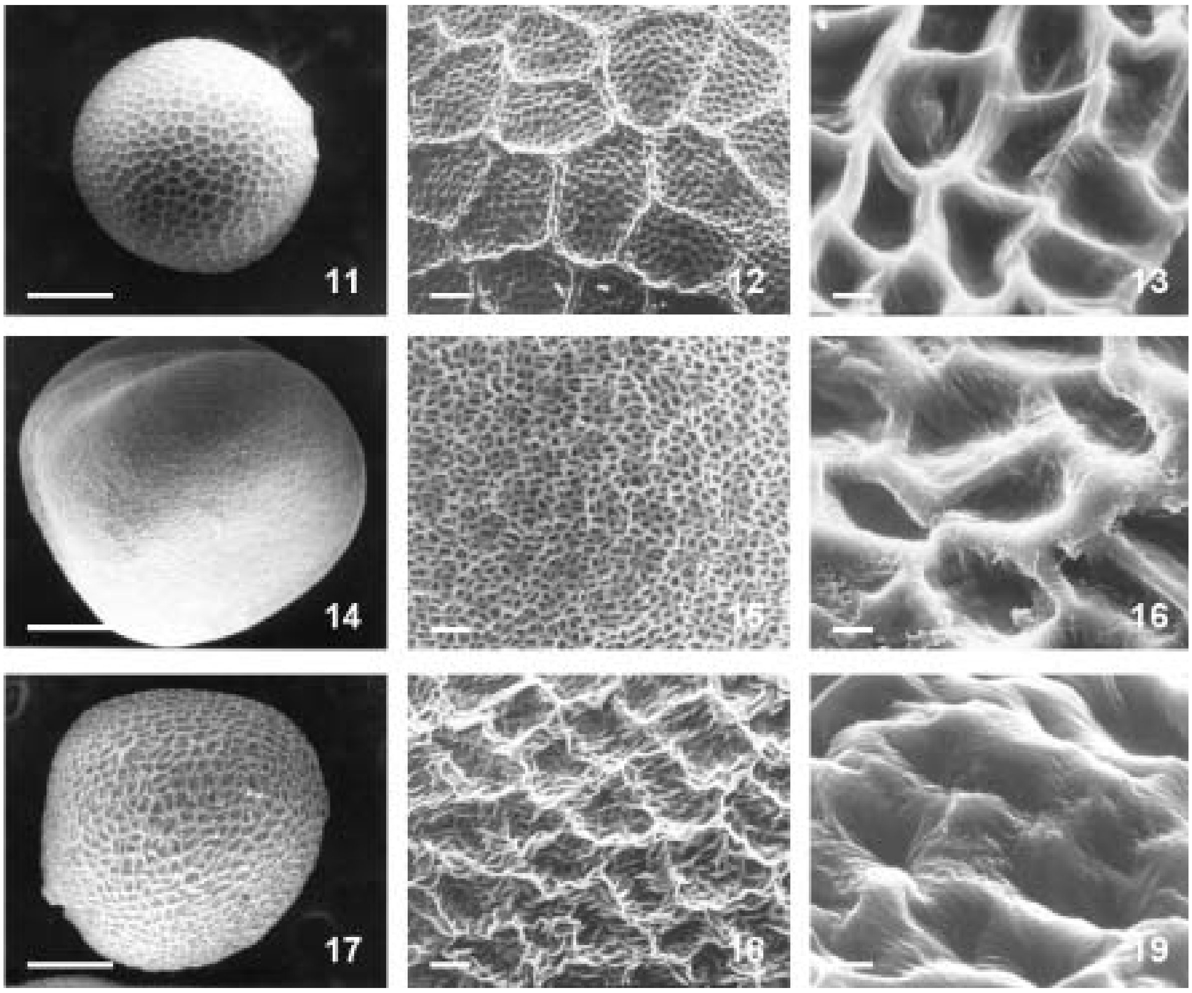
SEED MORPHOLOGY

The seed is one of the distinctive features in taxa diagnosis. The work of Koul et al. (2000) indicated that seed surface features can help in demarcating *Brassica* species and allied genera.

The present study of seed coat patterns revealed clear groupings, interrelationships, and variability between the examined rapid-cycling *Brassica*. The mature RCBr seeds showed a number of morphological characters, some of which conformed to the features noted for some *Brassica* species (Mulligan and Bailey, 1976; Buth and Roshan, 1983; Takahata and Hinata, 1992). The surface characters of RCBr seeds are here described in more detail, and they have micromorphological features that have not been reported before.

Our study indicated that seed coat morphology in RCBr was remarkably similar to that observed in six cultivated *Brassica* species (Zeng et al., 2004; Karcz, unpubl. data), but some variations were noted. Generally, the seeds were ovate in shape, but the color varied from light brown to dark brown or grey, except for the yellow seeds of RC *B. carinata*.

SEM observations of seed surface sculpture were focused on the arrangement of epidermal testa cells, the curvature of the outer periclinal and anticlinal cell walls (primary sculpture) and the fine cuticular ornamentation of the cell walls (secondary sculpture). These SEM features are summarized in Table 2. Seeds in diploid and allotetraploid RCBr forms had primary sculpture that appeared reticulate in RC *B. rapa* and RC *B. juncea* (Figs. 2, 3, 11, 12), reticulate-foveolate in RC *B. nigra* (Figs. 5, 6), randomly reticulate in RC *B. oleracea* and RC *B. napus* (Figs. 8, 9, 14, 15), and



Figs. 11–19. SEM micrographs of allotetraploid rapid-cycling (RC) *Brassica* seeds. **Figs. 11–13.** RC *B. juncea*. **Fig. 11.** Mature seeds. **Fig. 12.** Reticulate seed coat pattern showing isodiametric polygonal testa cells. **Fig. 13.** Polygonal pits of outer periclinal cell wall. **Figs. 14–16.** RC *B. napus.* **Fig. 14.** Mature seeds. **Fig. 15.** Irregularly reticulate seed coat sculpturing with less developed outer anticlinal cell walls. **Fig. 16.** Detail of testa cell with pitted outer periclinal cell wall. **Figs. 17–19.** RC *B. carinata*. **Fig. 17.** Mature seed. **Fig. 18.** Reticulate-rugose seed coat pattern with differently shaped testa cells. **Fig. 19**. Outer periclinal cell wall with irregularly formed pits. Bars = 500 µm in Figs. 11, 14, 17; 50 µm in Figs. 12, 15, 18; 5 µm in Figs. 13, 16, 19.

reticulate-rugose in RC *B. carinata* (Figs. 17, 18). Seeds of RC *B. carinata* had a very irregular testa surface topography, with differently exposed epidermal cell walls. The situation was similar in cultivated *Brassica* species, where the major seed coat patterns are reticulate, followed by reticulate-foveolate and rugose types (Koul et al., 2000). The seed surface pattern in RCBr seeds was less protruded than in cultivated brassicas, and moderately reticulated (Karcz, unpubl. data). The anticlinal walls were also irregularly raised, with weakly striated secondary ornamentation.

The surface sculpture was made up of irregular polygons with more or less collapsed outer periclinal cell walls delimited by raised anticlinal boundaries. Most anticlinal walls were unevenly thickened, undulated, sometimes pitted, and with smooth or striated secondary sculpture (Figs. 3, 6, 9, 12, 15, 18). A network of raised anticlinal cell walls was a common feature in the reticulate type, but the curvature and cuticular relief of the outer periclinal walls varied in the RCBr forms. Seed coat cells of RC *B. rapa* (Fig. 4), RC *B. oleracea* (Fig. 10), RC *B. juncea* (Fig. 13) and RC *B. napus* (Fig. 16) possessed distinctive pits. Shallow depressions characterized the testa cells of RC *B. nigra* (Fig. 7), whereas the most irregular depressions of periclinal walls occurred in RC *B. carinata* (Fig. 19).

According to Barthlott (1981, 1990), microcharacters connected with the structure of the anticlinal and periclinal epidermal cell walls are usually of high systematic significance at various taxa levels. Thus, the micromorphological characteristics of the *Brassica* seed coat surface, especially those of the anticlinal and periclinal walls, are species-specific and may be valuable taxonomical criteria for RCBr delimitation.

Reticulate-type primary sculpturing is fairly common in both diploid and allotetraploid RCBr forms; thus, there is a strong tendency for similar seed coat patterns to occur in groups of apparently closely related species. However, in all seed accessions of diploid RC *B. rapa* and RC *B. oleracea* and allotetraploid RC *B. napus*, the reticulate seed coat pattern differed intraspecifically. The variation in their surface pattern was often subtle. In RC *B. nigra*, all seed accessions were similar intraspecifically. Moreover, in all accessions of allotetraploids, except those of RC *B. juncea*, the reticulate seed coat pattern was more variable than in diploid seeds. Recently, micromorphological diversity of seed coat reticulation was also described for allotetraploid seeds and their putative parents in cultivated *Brassica* species (Zeng et al., 2004). In these species, the main differences in seed surface topography were connected with the relief of the outer epidermal cell walls. Our SEM observations also showed variation in the surface reticulation of RCBr seeds, both in diploids and in allotetraploids.

PHYLOGENETIC IMPLICATIONS OF SEED MICROCHARACTERS

In this study, seed microcharacters were considered in tracing the relationships between rapid-cycling *Brassica*. SEM observations of seed surface topography revealed that in diploids the seed coat patterns of all accessions of RC *B. rapa* and RC *B. oleracea* resembled each other (Figs. 3, 9), but varied significantly from RC *B. nigra* (Fig. 6). In allotetraploids, the seed coat pattern in RC *B. napus* (Fig. 15) and RC *B. carinata* (Fig. 18) were intermediate between the putative parents, suggesting their hybrid nature, whereas RC *B. juncea* (Fig. 12) had a surface pattern resembling one of the parental diploids, RC *B. rapa* (Fig. 3). However, the reticulate type of seed surface varied slightly in the nature of the reticulum, especially in the prominence of the outer anticlinal cell walls. In an earlier study on cultivated brassicas, Mulligan and Bailey (1976) also confirmed the presence of an intermediate seed coat sculpture in *B. napus* and *B. carinata*. In contrast, Buth and Roshan (1983) showed that seed samples of cultivated allotetraploid *Brassica* forms from the India collection had a surface sculpture resembling one of the ancestral progenitors, and also an intermediate seed coat pattern.

Our description of different types of reticulate surface pattern in these forms is consistent with the observations of Koul et al. (2000) and Zeng et al. (2004), who examined seed microsculpture in cultivated brassicas and allied genera collected from China populations. Chloroplast, mitochondrial and nuclear DNA analyses (Delseny et al., 1990; Warwick and Black, 1993; Song and Osborn, 1992; Snowdon et al., 2002; Yang et al., 2002; Schelfhout et al., 2004) also indicated the close relationship of *B. rapa* to *B. oleracea*, their distinctness from *B. nigra*, and the possible ancestry and evolution of their allotetraploid species.

The present study supported the use of seed coat pattern as an additional parameter for identification of rapid-cycling *Brassica*. Seed characters such as reticulate-type seed surface ornamentation and the structure of the anticlinal and periclinal walls of testa cells appeared to be phylogenetically informative. However, in RCBr forms the seed coat surface had less exposed testa cell walls than in the cultivated brassicas. The addition of this suite of micromorphological features to other morphological, biochemical and molecular characters should provide more robust information about phylogenetic affinities between these species.

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