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MORPHOLOGICAL, CYTOLOGICAL AND EMBRYOLOGICAL CHARACTERIZATION OF F_1 *A. CEPA* × *A. ROYLEI* HYBRIDS

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In the previous study we obtained a population of interspecific F_1 *A. cepa* × *A. roylei* hybrids. In this study, in comparison to the parental species: *A. cepa* and *A. roylei*, the F_1 hybrids were evaluated in terms of plant morphology, pollen viability, microsporogenesis and female gametophyte. Most of the morphological characters of the F_1 hybrids were intermediate as compared to those of both parental accessions. In pollen mother cells (PMCs) of the F_1 hybrids abnormalities were observed in meiosis as well as at the tetrad stage. Pollen viability of F_1 *A. cepa* × *A. roylei* hybrids was reduced to 30.1%. In the F_1 hybrids, 45.8% of the analyzed ovules showed developmental disturbances, whereas in 26.7% of the ovules necrotic processes were observed.

Key words: *Allium cepa*, *A. roylei*, embryo sac, interspecific hybrids, microsporogenesis, morphological characters, pollen viability

INTRODUCTION

Interspecific hybridization has always been considered an important tool for broadening the genetic variation of cultivated *Allium* species (Kik, 2002; Chuda and Adamus, 2009). For most of edible *Allium* species, crossing with wild relatives is especially important, as genetic resources of onion, shallot, leek and garlic are very limited (Shigyo and Kik, 2008). Improvement of *Allium* crops is currently accomplished by introgression breeding, with particular emphasis on disease and pest resistance, bulb quality and cytoplasmic male sterility (Kik, 2002). However, due to several pre- and post-fertilization barriers, incorporation of valuable traits into cultivated *Allium* species through classical breeding can be problematic (Kik, 2002; Odeny and Narina, 2011).

The overriding goal of *Allium cepa* breeders is to obtain pollen- and egg-fertile introgressants, which as a new variety would combine bulb-type onions with desirable traits derived from wild relatives (Peffley and Hou, 2000). According to Kik (2002), the attempts of genetic improvement of the bulb onion concentrate mainly on hybridization with other species that belong to section *Cepa*: *Allium fistulosum*, *A. roylei* and *A. galanthum* (Gurushidze et al., 2007). The first sexual hybridization between *Allium cepa* and *A. fistulosum* was reported by Emsweller and Jones (1935). However, backcrossing and successful introgression of desirable traits such as resistance to pink root and onion leaf blight, earliness, winter-hardiness, etc. have proved problematic over the years (Doležel et al., 1980; van der Valk et al., 1991a, b; van Raamsdonk et al., 1992; Khrustaleva and Kik, 1998; Peffley and Hou, 2000; Mangum and Peffley, 2005). The difficulties with introgression of resistance to downy mildew, leaf blight and anthracnose through backcrossing of reciprocal *A. cepa* × *A. roylei* hybrids have been reported independently by van der Meer and de Vries (1990), Kofoet et al. (1990), Kofoet and Zinkernagel (1990), van Heusden et al. (2000a, b) and Scholten et al. (2007). Problems with successful introgression of *A. galanthum* cytoplasm into the nuclear background of shallot (*A. cepa* L. Aggregatum group) have been mentioned by Yamashita and Tashiro (1999).

The attempts to broaden the narrow onion gene pool are limited due to a high level of male and female sterility of the interspecific hybrids (Emsweller and Jones, 1938; van der Meer and de Vries, 1990; van Raamsdonk et al., 1992; Keller et al., 1996; Peterka et al., 1997; Song et al., 1997; Vu

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TABLE 1. Morphological characters of interspecific F_1 *A. cepa* × *A. roylei* hybrids, *A. cepa* and *A. roylei* plants.

Examined character	Accession		
	F_1 *A. cepa* x *A. roylei* hybrids (mean ± SD)	*A. cepa* (mean ± SD)	*A. roylei* (mean ± SD)
No. of bulbs	2.5 ± 0.04 b*	1.0 ± 0.00 c	5.2 ±0.16 a
No. of leaves per plant	9.5 ± 0.15 b	13.8 ± 0.57 a	9.2 ±0.74 b
Length of first three leaves (cm)	35.3 ± 0.31 b	43.1 ± 0.98 a	35.0 ±0.66 b
Diameter of first three leaves (cm)	0.4 ± 0.01 b	1.5 ± 0.03 a	0.3 ±0.01 c
Length of stem (cm)	8.9 ± 0.10 b	12.1 ± 0.47 a	9.0 ±0.20 b
Diameter of stem (cm)	0.7 ± 0.01 b	1.6 ± 0.03 a	0.6 ±0.01 c
No. of flower stems per plant	1.8 ± 0.04 b	2.0 ± 0.11 b	2.6 ±0.18 a
Length of flower stem (cm)	62.0 ± 0.76 a	67.0 ± 1.79 a	28.3 ±0.82 b
Length of inflorescence (cm)	2.2 ± 0.04 b	3.5 ± 0.10 a	1.9 ±0.04 c
Diameter of inflorescence (cm)	2.4 ± 0.07 b	5.0 ± 0.19 a	1.4 ±0.11 c

*Different superscripts (a–c) within each column indicate significantly different mean values according to the Tukey's HSD test ($p \leq 0.05$)

et al., 2012). According to Mangum and Peffley (2005), the reduced pollen viability and abnormalities in embryo sac development of the hybrids are usually a consequence of the incompatibility and incongruity between the cytoplasm and nuclear genome of the parental species. As these mechanisms can limit backcrossing, determination of the hybrids' fertility barriers seems to be crucial. Usually, in order to identify any potential irregularities in male gametophyte development, observation of microsporogenesis and pollen viability tests are conducted (Saini and Davis, 1969; van der Meer and de Vries, 1990; van Raamsdonk et al., 1992; Yamashita and Tashiro, 1999; Yamashita et al., 1999; Mráz and Paule, 2006; Bureš et al., 2010). It is worth emphasizing that the reduced pollen fertility does not limit fertilization of the egg cell, as in contrast to egg cells, pollen grains are produced abundantly. Unlike male gametophyte, where pollen grains are easily observed, the female gametophyte is a few-celled structure hidden in a small ovule (Jones and Emsweller, 1936; Ohsumi et al., 1993). However, difficult to study the issue is, embryological analysis of megagametophyte development is necessary, as the female gametophyte plays a fundamental role in every step of reproductive process, including pollen tube guidance, fertilization, the induction of seed development upon fertilization, and maternal control of seed development after fertilization (Johri et al., 1992; Yadegari and Drews, 2004).

In this paper we report the results from morphological, cytological and embryological characterization of F_1 *A. cepa* × *A. roylei* hybrids that were obtained previously (Chuda and Adamus, 2012). As successful backcrossing requires production of viable pollen and female gametophyte with functional egg cell, synergids and antipodals, we investigated pollen viability, microsporogenesis and mature embryo sacs of the F_1 hybrids. To our knowledge this is the first report of the use of the clearing method for observing embryo sacs of the interspecific F_1 *A. cepa* × *A. roylei* hybrids.

MATERIALS AND METHODS

ANALYSIS OF PLANT MORPHOLOGY

Seven hundred and forty two F_1 *A. cepa* × *A. roylei* hybrids, 100 *A. cepa* and 100 *A. roylei* plants were examined in terms of 10 morphological characters. At the vegetative stage, the number of bulbs, as well as the number, length and diameter of the first three leaves and length and diameter of stems were evaluated. In the generative phase, the number and length of flower stems and length and diameter of inflorescences were estimated. The collected data were statistically evaluated by one way analysis of variance (ANOVA). The level of significance was set at 5% ($p \leq 0.05$). The differences between the means of the analyzed plant populations were compared using Tukey's HSD test (Statsoft Statistica v. 10 package).

POLLEN VIABILITY TEST

Pollen viability of 507 flowering F_1 *A. cepa* × *A. roylei* hybrids, 20 *A. cepa* and 20 *A. roylei* plants was examined using a smear method according to Alexander's procedure (Dafni, 1992). The fertile pollen grains were stained red while the sterile ones – green. The mean percentage of fertile pollen for

each plant was evaluated from the morphology and stainability of a minimum 300 pollen grains that were observed under a Nikon Eclipse E600 light microscope and photographed using a Panasonic GP-KR222E CCD camera.

OBSERVATION OF MICROSPOROGENESIS

Flower buds of 110 F_1 *A. cepa* × *A. roylei* hybrids, 10 *A. cepa* and 10 *A. roylei* plants at different developmental stages were fixed in a mixture of acetic acid and ethyl alcohol (1:3, v/v) for 24 h. The microspores were stained with 1% acetocarmine using a smear method. For each plant the mean percentage of abnormalities both in meiosis and at the tetrad stage was estimated from at least 300 PMCs and 300 tetrads that were observed under a Nikon Eclipse E600 light microscope. Cells with abnormalities were photographed using a Panasonic GP-KR222E CCD camera.

OBSERVATION OF EMBRYO SACS

A total number of 535 ovules from 100 F_1 *A. cepa* × *A. roylei* hybrids, 120 ovules from 20 *A. cepa* and 102 ovules from 20 *A. roylei* plants were examined. Inflorescences were fixed in FAA (40% formalin : glacial acetic acid : 50% ethanol, 5:5:90, v/v/v) for 24 h and stored in 70% ethanol at 4°C until used. Isolated ovules were dehydrated in 70%, 80%, 96% (one change) and 100% ethanol (three changes) and cleared in methyl salicylate series: 1.5 h in one change of ethanol/methyl salicylate (1:1), one change of ethanol/methyl salicylate (1:3), and two changes of 100 % methyl salicylate (Young et al., 1979, Mól et al., 1988). Cleared ovules were examined using an AxioImager.M2 microscope equipped with Nomarski differential interference contrast (DIC) optics.

RESULTS

ANALYSIS OF MORPHOLOGICAL CHARACTERS

Onion (*A. cepa* L.) is a biennial plant growing from one round bulb, while *A. roylei* is a perennial plant with a few to several characteristic ovoid bulbs. The analyzed F_1 *A. cepa* × *A. roylei* hybrids were perennial, grew like *A. roylei* plants and formed on average three ovoid bulbs (Tab. 1). In the study we also revealed that in terms of the diameter of the first three leaves and stems as well as the size of the inflorescences, the morphology of F_1 *A. cepa* × *A. roylei* hybrids was intermediate as compared to the parental species. Through the statistical analysis we also showed that in terms of the number of leaves per plant, the length of the first three leaves and stems the F_1 hybrids resembled *A. roylei* plants.

TABLE 2. Pollen fertility of interspecific F_1 *A. cepa* × *A. roylei* hybrids, *A. cepa* and *A. roylei* plants.

Accession	Pollen fertility (%)	Range of variation (%)
F_1 *A. cepa* x *A. roylei* hybrids	30.1	0.0–97.6
A. cepa	96.1	89.0–98.0
A. roylei	96.4	85.0–99.0

Only in terms of the number and length of flower stems, the F_1 hybrids were similar to *A. cepa* plants.

POLLEN VIABILITY TEST

The frequency of fertile pollen grains in F_1 *A. cepa* × *A. roylei* hybrids was 30.1% (Tab. 2). Within the examined population, 0.4% of the F_1 hybrids with sterile pollen as well as 3.2% of plants with pollen fertility over 90% were found. On the other hand, pollen fertility of both *A. cepa* and *A. roylei* accessions was very high and equaled to 96.1% and 96.4%, respectively.

OBSERVATION OF MICROSPOROGENESIS

The studies of PMCs of the analyzed accessions revealed that abnormalities in meiosis occurred only within the F_1 *A. cepa* × *A. roylei* hybrid population with a mean frequency of 1.7% (Tab. 3). We observed laggard and eliminated chromosomes as well as chromosome bridges in 1.2% and 0.5% of the examined PMCs, respectively (Fig. 1a, b). At the tetrad stage, among 10.5% of irregularities, configurations other than tetrads (0.1%, Fig. 1c, d), tetrads with microcytes (0.2%, Fig. 1e) and degeneration of tetrads (10.2%, Fig. 1f) were identified in the F_1 hybrids. Tetrads of *A. cepa* and *A. roylei* plants showed only degeneration, with the mean frequency of 3.6% and 5.0%, respectively.

OBSERVATION OF EMBRYO SACS

Ovules of *A. cepa*, *A. roylei* and F_1 *A. cepa* × *A. roylei* hybrids were investigated embryologically in detail (Fig. 2a–f). In the parental plants, most of the examined ovules showed structure typical of embryo sacs (ES), (Fig. 2a, b). The most common developmental irregularity observed in *A. roylei* was the presence of doubled embryo sacs. Necrotic processes were detected only in 2.5% of *A. cepa* embryo sacs, whereas in *A. roylei* degeneration was more frequent and referred to 11.8% of the studied ovules (Tab. 4). In the population of F_1 *A. cepa* × *A. roylei* hybrids, among 535 analyzed ovules, only 27.5% contained typical embryo sacs, while 45.8% and 26.7% showed signs of abnormalities and necrosis, respectively.

TABLE 3. Abnormalities in microsporogenesis of interspecific F$_1$ *A. cepa* × *A. roylei* hybrids, *A. cepa* and *A. roylei* plants.

Abnormalities (%)	Accession		
	F$_1$ *A. cepa* x *A. roylei* hybrids	*A. cepa*	*A. roylei*
in meiosis:	1.7	0.0	0.0
laggard and eliminated chromosomes	1.2	0.0	0.0
chromosome bridges	0.5	0.0	0.0
in the tetrad stage:	10.5	3.6	5.0
configurations other than tetrads	0.1	0.0	0.0
tetrads with microcytes	0.2	0.0	0.0
degeneration of tetrads	10.2	3.6	5.0

Irregularities concerning egg apparatus cells included delays in cellularisation, abnormal shape of cells and atypical vacuolization (Fig. 2c). Embryo sacs with improper polarization (Fig. 2d) as well as multiple ES were also observed. The most frequent irregularities included abnormal number of nuclei or cells in ES (Fig. 2d). Necrosis concerned mainly egg apparatus cells (Fig. 2e) or nuclei in the central cell. However, in a considerable number of ovules, ES were collapsing (Fig. 2f) or there was no embryo sac lumen present and the developmental stage was very difficult to recognize due to an advanced stage of degeneration.

DISCUSSION

To our knowledge the first successful interspecific hybridization within the genus *Allium* was reported by Emsweller and Jones (1935). With the aim of introgression of pink root, smut and thrips resistance into onion they conducted hand pollinations between *Allium cepa* and *A. fistulosum*. The obtained hybrids were slightly bulbing and similarly to the paternal species grew as perennials. By reciprocal crossing of *Allium cepa* and *A. roylei* plants, van der Meer and de Vries (1990) made the preliminary attempt to introgress downy mildew resistance into onion germplasm. The obtained *A. roylei* × *A. cepa* plant was considered a hybrid as it showed intermediate appearance between both parental species. In this study we proved that the morphology of the obtained F$_1$ hybrids was intermediate as compared to *A. cepa* and *A. roylei* plants. The observations revealed that similarly to the paternal species, the F$_1$ hybrids grew as perennials, but formed a higher number of bulbs as compared to *A. cepa*. Moreover, we proved that at the vegetative stage some of the examined characters of the F$_1$ hybrids were similar to those of the seed parent while in the generative phase in terms of some of the characters the hybrids resembled onion. The intermediate appearance of F$_1$ hybrids was also reported after crossing *Allium galanthum* and shallot (Yamashita and Tashiro, 1999), *A. galanthum* and *A. fistulosum* (Yamashita et al., 1999), *A. cepa* and *A. sativum* (Ohsumi et al., 1993), *A. cepa* and *A. ampeloprasum* (Peterka et al., 1997) and *A. ampeloprasum* and *A. sativum* (Yanagino et al., 2003).

High levels of pollen sterility were reported for interspecific hybrids between *A. cepa* and *A. fistulosum* (Emsweller and Jones, 1935), *A. fistulosum* and *A. cepa* (van der Valk et al., 1991a), *A. roylei* and *A. cepa* (van der Meer and de Vries, 1990), *A. cepa* and *A. sativum* (Ohsumi et al., 1993) and *A. galanthum* and shallot (Yamashita and Tashiro, 1999). According to van Raamsdonk et al. (1992), the reduced pollen viability of the hybrid plants is closely related to low crossability between the parental species. Strong crossability barriers were found between *A. oschaninii* and *A. cepa*, *A. oschaninii* and *A. vavilovii* and *A. galanthum* and *A. pskmense* (van Raamsdonk et al., 1992). According to Kik (2002), the closest known relative of bulb onion is *A. vavilovii*. Both these species are quite similar in appearance and completely interfertile. This was proved by van Raamsdonk et al. (1992), who reported that pollen fertility of the reciprocal *A. cepa* × *A. vavilovii* hybrids was very high and varied from 85% to even 97%. In this study we revealed that pollen fertility of the obtained F$_1$ *A. cepa* × *A. roylei* hybrids was reduced to 30.1%. Similar results (pollen fertility on the level of 55%) for the same hybrids were reported by van Raamsdonk et al. (1992). According to Kik (2002), *A. cepa* is classified into section *Cepa*, while *A. roylei* has a hybrid origin as its nuclear profile is related to members of section *Cepa* and its chloroplast DNA to the section *Schoenoprasum*. This taxonomic distance between both parental species probably results in reduced pollen fertility of F$_1$ *A. cepa* × *A. roylei* hybrids.

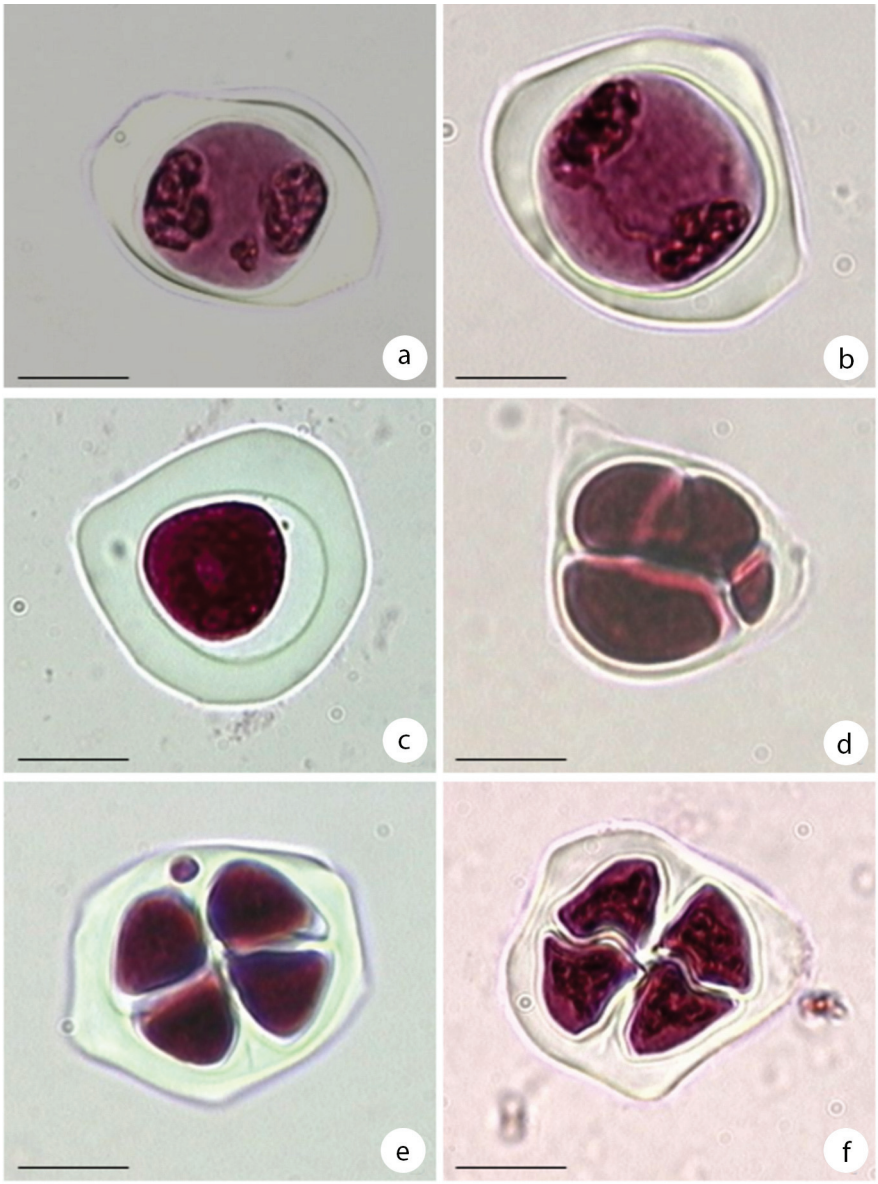

Fig. 1. Abnormalities in meiosis of PMCs and at tetrad stage of F_1 *A. cepa* × *A. roylei* hybrids. (**a**) Eliminated chromosomes, (**b**) Chromosome bridge, (**c**) Monad, (**d**) Polyad, (**e**) Tetrad with a microcyte, (**f**) Degeneration of tetrads. Bars = 10 μm

As expected, the reduced pollen fertility was related to the abnormalities that occurred during microsporogenesis in PMCs of the F_1 *A. cepa* × *A. roylei* hybrids. We know only a few reports on detailed analysis of PMCs' development of the interspecific *Allium* hybrids. Through cytological analysis we proved the presence of fragments such as laggard and eliminated chromosomes (1.2%) as well as chromosome bridges (0.5%) in meiotic cells of the F_1 *A. cepa* × *A. roylei* hybrids. The same types of irregularities in the meiocytes of F_1 *A. cepa* × *A. fistulosum* hybrids were mentioned by Emsweller and Jones (1938) and identified by Peffley (1986) with the frequency of 10% and 28%, respectively. The presence of micronuclei in 51% and bridges in 5% of the analyzed PMCs of F_1 *A. fistulosum* × *A. cepa* hybrids was reported by Ulloa-G et al. (1995). In this study, as a result of microsporogenesis in PMCs of the F_1 *A. cepa* × *A. roylei* hybrids, a large number of normal tetrads were observed. The identified abnormalities concerned mainly degeneration of protoplasm, which occurred in about 10% of the analyzed microspores. Degeneration of microspores was also reported in plants of backcross progenies of (*Allium galanthum* × *shallot*) × *shallot* (Yamashita

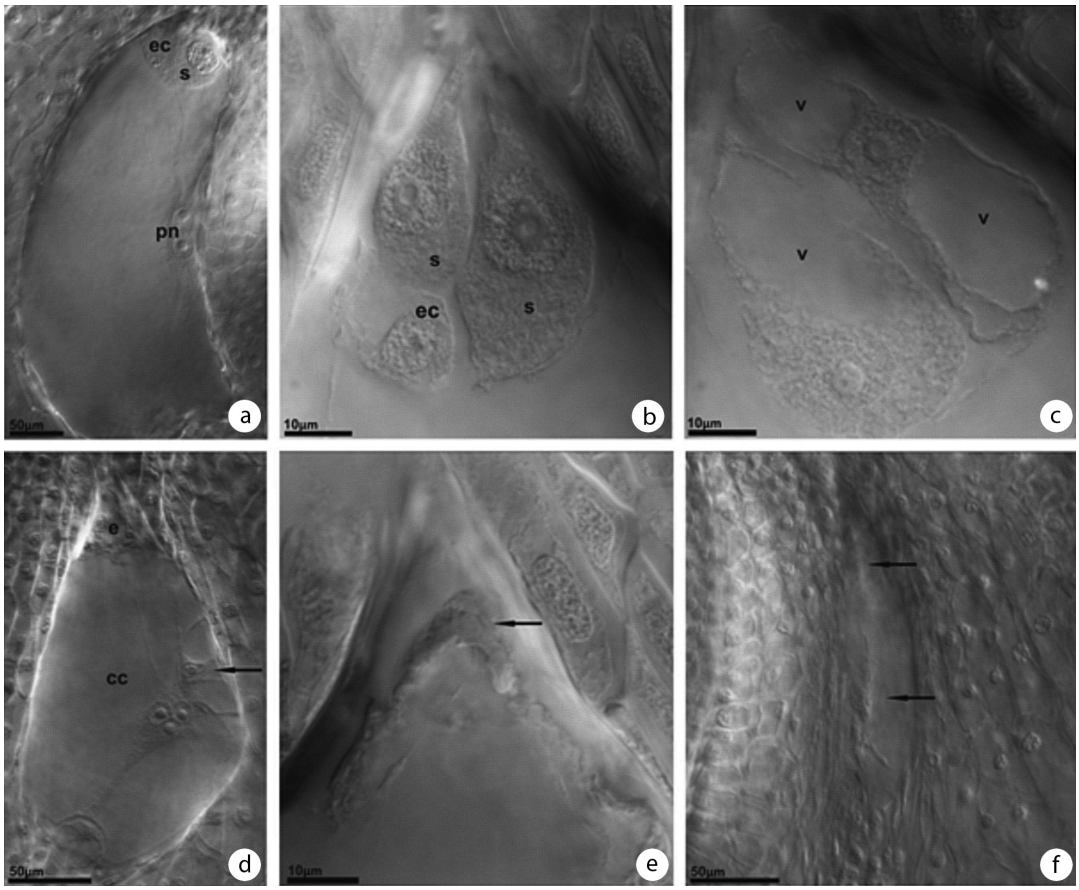

Fig. 2. Images obtained from cleared material using Nomarski DIC optics. (**a**) Mature female gametophyte of *A. cepa*, (**b**) Egg apparatus cells in *A. cepa* embryo sac, (**c**) Atypical vacuolization of egg apparatus cells in F$_1$ *A. cepa* × *A. roylei* hybrid embryo sac, (**d**) Mature F$_1$ hybrid embryo sac with atypical cellularisation (arrow) and three nuclei in central cell, (**e**) Degenerated egg apparatus cells in mature F$_1$ hybrid embryo sac, (**f**) Ovule of F$_1$ hybrid with a narrow cavity of degenerating embryo sac (arrows). cc – central cell; e – egg apparatus cells; ec – egg cell; pn – polar nuclei in central cell; s – synergid cell; v – vacuole

and Tashiro, 1999) and (*A. galanthum* × *A. fistulosum*) × *A. fistulosum* (Yamashita et al., 1999).

In our study developmental irregularities and degeneration also concerned the female gametophyte of the F$_1$ *A. cepa* × *A. roylei* hybrids. The abnormalities included mainly abnormal number of nuclei or cells in developed embryo sacs. Atypical embryo sacs were reported in *A. cepa* (Nadirashvili et al., 2005), where variations in the number of nuclei and cells as well as structural transformations of the components of female gametophyte were observed. The authors suggested that the increase in the number of egg cells might increase the chances of the ovule to produce a seed, whereas additional number of hypertrophic cells might improve nutrition of the developing embryo. The number of studies on female gametes and gametophytes in interspecific hybrids is relatively small. Abnormalities that occur during megasporogenesis and megagametogenesis in the plants of F$_1$ generation are the most likely result of incongruity and defects which occur in developmental processes (Winiarczyk, 1999; Mangum and Peffley, 2005). In embryological studies of indica/japonica hybrids in rice irregularities both in megasporocytes and embryo sacs were observed (Zeng et al., 2009). The abnormalities that occurred from the early stage of the eight-nucleate embryo sac development to the mature embryo sac stage included smaller in size and wrinkled antipodals, asynchronous nuclear migra-

TABLE 4. Irregularities and necrosis in female gametophyte of interspecific F$_1$ *A. cepa* × *A. roylei* hybrids, *A. cepa* and *A. roylei* plants.

Accession	Irregularities (%)	Necrosis (%)
F$_1$ *A. cepa* x *A. roylei* hybrids	45.8	26.7
A. cepa	0.8	2.5
A. roylei	15.7	11.8

tion, abnormal positioning of nucleus and degeneration of egg apparatus. In our study advanced stages of degeneration in F_1 *A. cepa* × *A. roylei* hybrids were difficult to determine because they probably began at the stage of embryo sac mother cells or during megagametogenesis. The degeneration of the entire embryo sac preceded by atypical megasporocyte was described in the indica/japonica hybrid. Although no irregularities were observed during the division of the megasporocyte, most of them occurred after meiosis, when some abnormal megaspores failed to form typical mononucleate embryo sacs (Liu et al., 2004; Zeng et al., 2009). On the other hand, investigation of female meiosis in *A. roylei* (Sharma and Gohil, 2011) resulted in identification of additional chromosomes and multivalents in embryo sac mother cells that, as the authors suggest, may adversely affect the reproductive capacity and lead to production of genetically imbalanced gametes. This indicates that atypical female gametophytes and degeneration of embryo sacs in the analyzed F_1 *A. cepa* × *A. roylei* hybrids may possibly result from meiotic irregularities.

As mentioned above, problems with subsequent backcrosses of reciprocal F_1 *A. cepa* × *A. roylei* hybrids were reported independently by several authors. Van der Meer and de Vries (1990) proved that the effectiveness of obtaining BC1 (*A. roylei* × *A. cepa*) × *A. cepa* plants was fifty times lower as compared to control (*A. cepa* × *A. cepa* pollinations). Similarly, our preliminary attempts of developing BC_1 (*A. roylei* × *A. cepa*) × *A. cepa* generation resulted in obtaining only one plant (Chuda et al., 2012). The main difficulty with successful introgression of *Allium roylei* traits into onion germplasm is a high level of hybrids' sterility, especially when used as seed parents. In this study, through embryological analysis we identified a large number of abnormalities which occur in the ovules of F_1 *A. cepa* × *A. roylei* hybrids. The identified and described for the first time irregularities in embryo sac development might be helpful in explaining the low crossability of the F_1 hybrids.

To gain a comprehensive understanding of the reasons for unsuccessful backcrossing of the F_1 *A. cepa* × *A. roylei* hybrids, the process of fertilization as well as post-fertilization development of the embryo and endosperm should be studied. That is why the next step of our investigation will include identification of irregularities in embryogenesis and endosperm development of BC_1 plants.

AUTHORS' CONTRIBUTIONS

AA and CA formulated the original idea of the experiment; CA and KK designed and performed the experiments and wrote the manuscript; AA critically read and revised the manuscript. The authors declare that they have no conflicts of interest.

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