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Full Length Research Paper

Haploid embryos of lettuce (*Lactuca sativa*) induced by alien pollen or chemical factors

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In order to work out an effective haploidization method in lettuce (*Lactuca sativa*), we attempted to induce the development of haploid embryos by chemical treatment of pistils or through wide crossing of lettuce with 25 species (mostly of the Asteraceae family). The highest frequency of embryos was achieved after crosses with *Helianthus annuus* (16%) or *H. tuberosum* (19%). All the embryos, developed up to the globular or heart stage, were haploid (*n* = 9). Stigma treatment with seven of the eight tested chemical inductors resulted in the development of only several celled embryos, of which the most effective were: 0.05% Dicamba (15%), 0.05% Picloram (14%), and 0.05% 2, 4-D (13%). Embryo haploidy was verified by: counting of chromosomes in dividing cells and comparison of nucleus perimeter. The processes of pollination and embryogenesis were analysed under light and fluorescence microscopes.

Key words: Intergeneric crossing, lettuce, parthenogenesis, chemical induction, haploidization.

INTRODUCTION

Plant improvement programmes need more effective breeding tools. Among these, tools, haploidization is of great interest for producing pure lines. The first haploid plant of *Datura stramonium* L. (formed spontaneously in natural conditions) was described in 1922 (Blakeslee et al., 1922). In 1963, as many as 71 species (of 39 genera and 16 families) were known to produce haploid plants spontaneously (Kimber and Riley, 1963). In 1964, the first effective haploidization method was developed in *Datura inoxia* Mill through anther culture *in vitro* (Guha and Maheshwari, 1964). An innovative method of haploidization was introduced in 1976, when an *in vitro* culture of unpollinated ovaries (gynogenesis) resulted in fully-developed haploid plants of *Hordeum vulgare* L. (San Noeum, 1976). This method was later used successfully in species such as *Allium cepa* L. (Musiał et al., 2001), *Gerbera jamesonii* L. (Meynet and Sibi, 1984) or *Beta vulgaris* L. (Lux et al., 1990).

The first haploid plant produced by the *bulbosum* method (as a result of the elimination of chromosomes of one of crossing components) was *Hordeum vulgare* L., after wide crossing with *Hordeum bulbosum* L. (Kasha and Kao, 1970). Several years later, using the same method, haploids of *Triticum aestivum* L. was produced (Zenkteler and Straub, 1979). Parthenogenesis was first induced experimentally through wide hybridization. The resultant haploid embryos (pseudogamy) developed concomitantly with hybrid endosperm. By applying this method, haploid embryos in *Cichorium intybus* L. were obtained by crossing with *Cicerbita alpina* Walbr (Dore et al., 1996) or in *Solanum tuberosum* L. sp. *Tuberosum* by crossing with *Solanum phureja* Juz and Bukasov (Maine, 2003). Parthenogenesis was also induced by physical factors, such as high/low temperature, scarring (Kasha, 1974) or treatment of stigmas or whole flower parts with chemical substances such as: colchicine (induction of haploids in *Solanum chacoense* Bitt. (Hermsen, 1969) or toluidine blue (haploidization in *Populus tremula* L. (Ilies, 1974). Another procedure for producing parthenogenetic embryos was pollination of the stigma with pollen of the same species which had been inactivated by physical (X-rays, gamma radiation, UV radiation) or chemical factors. The development of haploid embryos in *Cucurbita moshata* Duch was induced by pollination of stigmas with

Table 1. Haploidization of *Lactuca sativa* induced by pollination with alien pollen.

Pollen donor	The number of chromosomes of pollinators	Pollen germination on stigma	Number of pollinated inflorescences	Frequency of haploid embryo (%)
Asteraceae				
Anthemis arvensis L.	18	+	114	12
Coreopsis lanceolata L.	24	+	185	no embryos
Gailardia aristata Pursh	34	+	57	no embryos
Helianthus annuus L.	34	+	4950	16
Helianthus decapetalus L.	34	+	93	no embryos
Helianthus giganteus L.	34	+	253	11
Helianthus grosseratus M.Mart.	32	+	64	no embryos
Helianthus lactiforus Pers.	102	+	85	no embryos
Helianthus mollis Lam.	34	+	65	no embryos
Helianthus nuttalli L.	34	+	58	9
Helianthus tuberosus L.	102	+	5300	19
Heliopsis helianthoides L.	28	+	67	no embryos
Inula helenium L.	20	+	80	8
Matricaria maritima L.	18	+	124	9
Rudbeckia fulgida Aiton	38	+	63	12
Rudbeckia grandiflora L.	38	+	3800	14
Senecio vernalis Waldst&Kit.	20	+	90	6
Silphium perfoliatum L.	14	+	127	10
Tragopogon pratensis L.	12	+	237	11
Verbesina helianthoides Michx.	34	+	54	7
Asparaginaceae				
Anthericum liliago L.	30	-	800	5
Chenopodiaceae				
Chenopodium album L.	14	-	550	3
Oenotheraceae				
Oenothera biennis L.	14	-	1300	15
Salicaceae				
Populus tremula L.	38	-	650	1
Araceae				
Spathiphyllum wallisii Regel	30	+	1350	15

pollen inactivated by gamma radiation (Kurtat et al., 2009).

In lettuce (*Lactuca sativa* L.), a popular leafy vegetable (Jones, 1927; De Vries, 1997), no effective haploidization method has so far been reported. This study aimed to produce haploid embryos of lettuce as preliminary experiments on this topic (not published) revealed that *L.sativa* embryos may develop after wide crossing, particularly by the application of pollen grains of various species of the Asteraceae family or by the chemical induction of stigmas.

MATERIALS AND METHODS

In the wide crossing and chemical induction experiments, we used male-sterile forms of *Lactuca sativa* L. (obtained from Rijk Zwaan Company, Fijnaart) and as pollen donors fertile plants (growing in natural locations), mostly of the Asteraceae family were used (Table 1). Male sterile lettuce plants were cultured in a culture room with a 16 h photoperiod (the light was put on at 6.00 a.m till 10.00 p.m), photosynthetic photon flux density 250 of 300 μmol m^{-2} s^{-1}, humidity of 40%, and adequate irrigation. For control pollination, fresh pollen was collected from fertile lettuce. Male-sterile plants of lettuce were separated from pollen donors to avoid uncontrolled wide crossing.

Table 2. Effects of the tested chemicals on embryo formation.

Chemical inductor	(%)	Number of treated inflorescences	Frequency of haploid embryos (%)
2,4-D : (2,4-dichlorophenoxy)acetic acid	0.05	208	13
Dicamba : 3,6-dichloro-2-methoxybenzoic acid	0.05	209	15
Picloram : 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid	0.05	219	14
*Confidor ®200 SL : imidachlopryd1-(6-chloro-3-pirydylometylo)-*N*-nitro(imidazolydin-2-ylideno)amina	0.5	197	1
Acetylsalicylic acid : 2-acetoxybenzoic acid	0.5	185	0
Hexanoid acid (caproic acid): $C_5H_{11}COOH$	0.05	170	0.5
Colchicine: N-[(7*S*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide	0.05	190	0.5
Tolonium chloride (toluidyne blue): (7-amino-8-methyl-phenothiazin-3-ylidene)- dimethyl-ammonium	0.05	195	0.5

*Fungicide.

Pistils of *L. sativa* with receptive stigmas were pollinated *in vivo* and *in vitro* with pollen grains of various species (Table 1). *In vitro*, in order to overcome prezygotic barriers, pollen was placed on an opened ovary (style cut off) or directly on ovules. However, the main experiments were conducted *in vivo* because of the higher efficiency of this technique. The most efficient pollen donors were: *Helianthus annuus* (4950 pollinated lettuce inflorescences), *Helianthus tuberosus* (5300), *Rudbeckia grandiflora* (3800), *Oenothera biennis* (1300), and *Spathiphyllum wallisii* (1350). A single inflorescence of *L.sativa* contained 14 to 15 flowers with unilocular ovaries, each with one ovule. Aqueous solutions of 8 chemical substances, at selected concentrations (Table 2) were placed with a fine brush on receptive lettuce stigmas. The applied chemical substances were selected on the basis of published data, while their concentrations were optimized during this study according to our earlier experiments.

Pollen germination on lettuce stigmas was observed under a fluorescent microscope with the use of aniline blue (1 g per 100 ml H_2O, pH=7.2) on freshly squashed pollinated stigmas 1 to 24 h after pollination. The ratio of the number of germinating pollen grains of the donor species to the total number of pollen grains on the whole stigma was expressed as a percentage (%). The development of embryos resulting from wide crossing and chemical induction was compared to the control embryos. We recorded the number of embryos cells, the size of the nuclei, as well as the presence and structure of suspensors. To analyse the development of embryos and endosperm, ovules were isolated 6, 24 h to six days after pollination (DAP). A similar procedure was conducted after the chemical treatment of stigmas. For embryological analysis, ovules and ovaries were fixed in FAA, embedded in Paraplast, sectioned (12 µm thick) and stained with iron haematoxylin (3 g of haematoxylin per 500 ml of 80% alcohol) and counterstained with fast green FCF (0.3 g fast green FCF per 100 ml of clove oil).The ratio of the number of haploid embryos produced as a result of wide crossing or chemical treatment to the total number of analysed ovules was expressed as a percentage (%).

The ploidy of embryo cells was assessed by squashing whole ovules or isolated embryo sacs (surrounded by endothelium) and staining them with aceto-carmine (1% solution of carmine in 45% glacial acetic acid). The perimeters of the nuclei of control embryos and of embryos resulting from wide cross-pollination (haploidization) were compared by using AxioVision software. Mean nucleus perimeters were calculated on the basis of 15 cells for 5 control embryos and for 5 embryos of the selected combination of wide crossing at the stage of globular proembryos (2 DAP) as well as after chemical treatment. Pollen germination and the course of embryogenesis were observed under a light microscope (Zeiss Axioscope A1, Jena, Germany). Micrographs were recorded by using AxioVision software and a multimedia digital camera.

RESULTS

Embryogenesis in the control material

Lettuce stigmas reached their optimum developmental stage for pollination late in the morning; about 10 to 11 a.m. Elongated papillae were localized only on the inner side of the stigma (wet type) where pollen grains germinated. Pollen tubes of lettuce germinated on receptive stigma within 1 h after pollination. Long tubes of *L.sativa* were observed growing through the styles around 3 h after pollination. Embryological analysis of the control pistils after selfing, showed that male gametes were present in the embryo sac as early as 4 h after pollination (Figure 1a), while fertilization took place about 2 h later. The first, transverse division of the zygote took place about 1 h after fertilization. The resultant 2-celled embryo was composed of a terminal cell and the basal one. At the same time, the secondary nucleus divided. The terminal cell of the embryo divided transversely, while the basal one longitudinally, giving rise to a 4-celled embryo (12 to 13 h after pollination). The 2 cells resulting from the division of the terminal cell, divided longitudinally, while the cells derived from the basal one divided transversely, and thus formed an 8-celled embryo

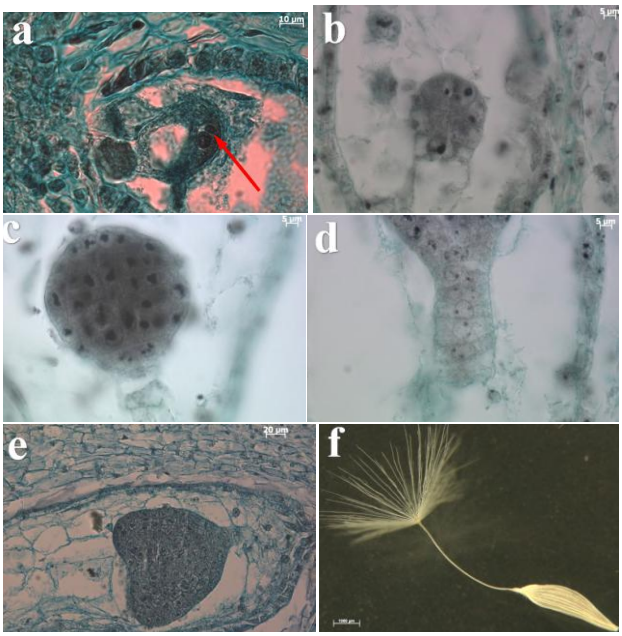

Figure 1. Diploid embryos of *L. sativa* (control)**.** (a) Male gamete (arrow) inside the nucleus of the egg cell, 4 h after pollination. **(b)** 8-celled globular embryo, 1 DAP. **(c)** ~32-celled embryo, 2 DAP. **(d)** Several-celled suspensor of globular embryo, 2 DAP. **(e)** Heart-stage embryo, 3 DAP. **(f)** Mature fruit 15 DAP.

(20 h after pollination) (Figure 1b). A 16-celled embryo was formed approximately 25 h after pollination, and a 32-celled embryo 5 h later (Figure 1c). The suspensor resulted from the transverse divisions of cells derived from the basal cell (Figure 1d). About 24 h after pollination, cellular endosperm was seen to be developing. In the suspensor, 6 to 8 cells were arranged in a row, 3 days after planting (DAP) (Figure 1e). An embryo with well-developed cotyledons was formed 5 DAP and the fruit was mature 13 to 14 DAP (Figure 1f).

Haploidization through wide crossing

The wide crossing experiments showed that pollen of 21 species of the 25 used in the experiments (Table 1) germinated on receptive lettuce stigma within 1 h after pollination. Most frequent germination was observed for pollen of *H. annuus*, *H. tuberosus*, and *S. wallisii* (species with 2-celled pollen grains). Embryo frequency was positively correlated with the frequency of pollen germination in the donor species and depended on the season. When pollen germinated most frequently, the frequency of embryos was also the highest. Abounded germination occurred between June and August while in May, September and October, pollination efficiency was reduced or pollen did not germinate at all. What needs to be stressed is that, globular embryos were observed even in those ovules which were isolated from pollinated pistils, but with no signs of pollen germination.The highest number of embryos was produced after wide crossing in the summer months (a similar relationship was also seen to occurafter chemical induction, embryos were most numerous in July and August). This suggests that pollination efficiency (reflected in the number of produced embryos) did not depend directly on the frequency of pollen germination of alien species, but rather on the physiological state of the lettuce during the individual months of cultivation.

In 19 of the 21 tested donor species, pollen tubes were short and did not penetrate the styles. Only in the combinations with *H. annuus* and *H. tuberosus* were long pollen tubes seen to be growing through the styles (Figures 2a and b). The pollen tubes did not show any symptoms of pre-fertilization incompatibility (incorrect growth, bursting, or swelling of the apical part). Male gametes of *H. annuus* were observed at the micropyle region of the embryo sac of *L. sativa* (Figure 2c), but never inside the egg cells. In the enlarged ovules, globular embryos were noticed after crossing with pollen representing 18 donor species (Table 1). Parthenogenetic globular embryos (Figures 2d, e and f) and also heart-shaped embryos (occasionally appearing in the combination *L. sativa* × *H. annuus*) did not develop further. The most efficient crossing components for induction of the development of haploid embryos proved to be *H. annuus* and *H. tuberosus*, where the frequency of production of haploid embryos reached up to 16 and 19%, respectively (Table 1).

When lettuce was crossed with *H.annuus* or *H. tuberosus*, embryo development was often accompanied by cellular endosperm formation. However, in endosperm cells caryological disturbances such as nuclei 2-3-fold larger than in the control, and multinucleoli (Figures 3d and e), were often distinguished. In combination *L. sativa* × *H. tuberosus* in endosperm, the nuclei chromosome number was: $3n = 18+51$ (*L. sativa* 2n=18, *H.tuberosus* 2n=102=6x) (Figure 3f). In all of the analysed embryos, the chromosome number was haploid $n = 9$ (Figures 3a and b), while diploid in the control embryos was $2n = 18$ (Figure 3c). Mean values of nuclei perimeter in globular proembryos 2 DAP were nearly 2-fold lower in the cross with *H.annuus* (Figure 4b) than in the control (Figure 4a).

Chemical induction

In the attempts to induce haploidization by chemical treatment, the effectiveness of seven of the eight tested inductors was confirmed (Table 2). In embryo sacs isolated 2 to 7 days after the chemical treatment (DAT) of stigmas, several-celled haploid embryos (n=9) were found (Figure 5b), but they degenerated soon (Figure 5a). After the application of 0.05% Dicamba solution, 2 embryos appeared in one embryo sac (Figures 5c and d). Presumably, the second embryo developed from a

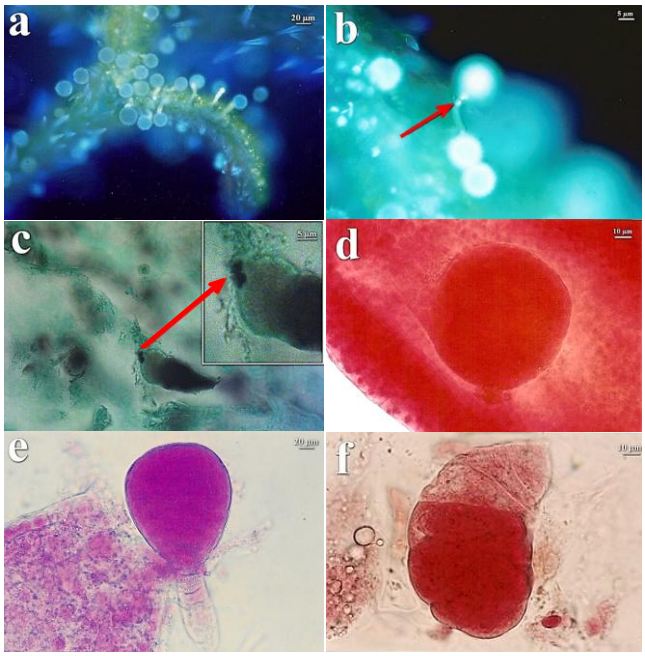

Figure 2. Embryos of *L.sativa* developed after cross pollination. **(a)** Germination of pollen grains of *H. annuus* on the stigma of *L. sativa* 1h after pollination. **(b)** Two sperm cells (arrow) of *H. annuus* inside the pollen tube on the stigma of *L. sativa*. **(c)** Two sperm cells of *H. annuus* (arrow) in the apical part of the pollen tube in the micropyle region of the embryo sac of *Lactuca sativa* 6h AP. **(d)** Globular embryo of *L. sativa* x *H. annuus* (suspensor not visible), 6 DAP. **(e)** Globular embryo of *L. sativa* x *H. annuus* with a well developed suspensor 6 DAP. **(f)** With a 3-celled, malformed suspensor, 5 DAP.

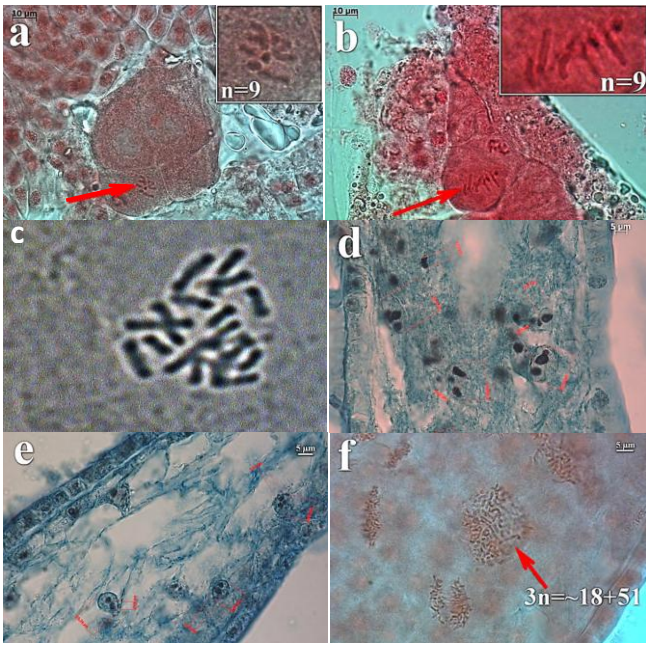

Figure 3. Ploidy level of embryos obtained after cross pollination. **(a)** Haploid number of chromosomes (n=9) of several-celled globular embryo of *Lactuca sativa* x *Oenothera biennis* 6 DAP (arrow). **(b)** Globular embryo of *L. sativa* x *S. wallissi*, haploid number of chromosomes n=9 (arrow) 6 DAP. **(c)** Diploid metaphase plate 2n=18 in the globular embryo of *L. sativa* (control). **(d)** Structural abnormalities of cellular endosperm of *L. sativa* x *H. annuus* 3 DAP. **(e)** Cellular endosperm of *L. sativa* (control) 3 DAP. **(f)** In cellular endosperm of *L. sativa* x *H. tuberosus* probably the hybrid number of chromosomes 3n=~18+51, 8 DAP.

dividing synergid. No cellular endosperm was formed under the influence of any of the tested chemical inducers. Haploidization was induced most effectively by Dicamba (15%), Picloram (14%), and 2, 4-D (13%) (Table 2).

Embryogenesis in the experimental material - the structure of proembryos

As a result of pollination of lettuce with pollen of alien species, parthenogenetic globular embryos were produced; composed of several to about a dozen cells, but they did not develop any further. The early stages of embryogenesis (several hours after pollination) proceeded just as in the control. About 7 to 8 h after pollination, the first transverse division of the egg cell took place (in analogy to the transverse division of the zygote in the control). The next divisions (longitudinal and transverse) of embryo cells were observed several hours later. In many embryos, the cells size and their arrangements were preserved in the embryo and suspensor comparably to those as in control embryos (Figure 6e). Globular embryos composed of 16 to 18 cells were recorded 1 DAP, and 25-30-celled embryos one day later (Figure 6c). Globular embryos ceased at various stages of embryogenesis. Some embryos stopped developing at the stage of four to six cells (12 h after pollination), while others as late as at the stage of heart-shaped embryos (observed sporadically 3-4 DAP).

In well developing embryos, suspensors were present (Figures 6a and b) but their cells were highly vacuolized and degenerated soon. Suspensors were not present in those globular embryos which from the onset developed abnormally. If the embryos developed concomitantly with cellular endosperm, their suspensors were composed of a row of five to eight cells with central situated nuclei. Some other embryos possessed short suspensors consisting of two to three cells, varying widely in size and shape (triangular, strongly elongated, or flattened, isodiametric), often highly vacuolized, with their structure disturbed, so that their axis deviated from the main axis of the embryo (Figures 6a and b). After the chemical treatment of lettuce stigmas, only small several-celled embryos were formed. Many embryos stopped developing at the stage of 3 to 5 cells (about 12 to 14 h after chemical treatment (Figures 6d and f). Endosperm was absent, and embryos had only 2 to 3 celled suspensors (their cells were highly vacuolized, with degenerated nuclei and rudimentary cytoplasm). However, in most embryos, no suspensors were present

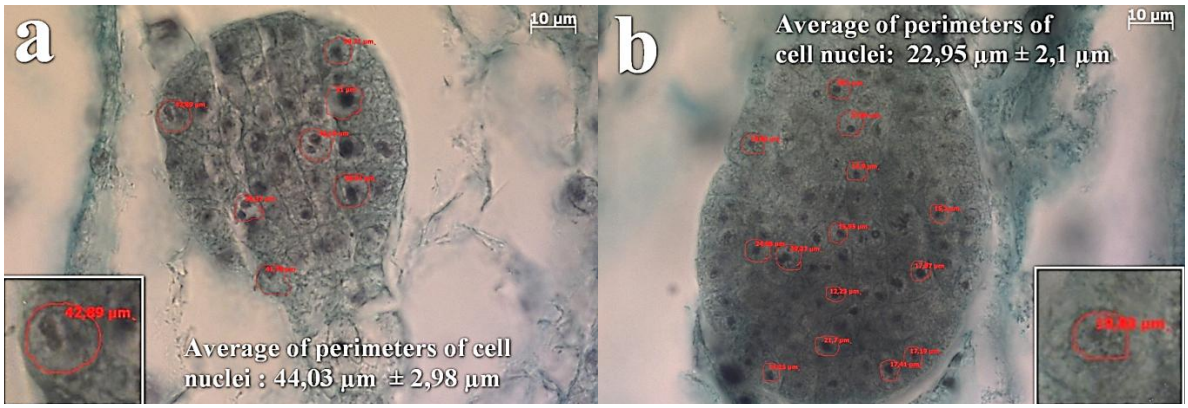

Figure 4. Comparison of perimeters of nuclei in (a) diploid *Lactuca x Lactuca* 2 DAP (control) and (b) haploid embryos obtained after cross pollination *L. sativa* x *H. annuus,* 2 DAP.

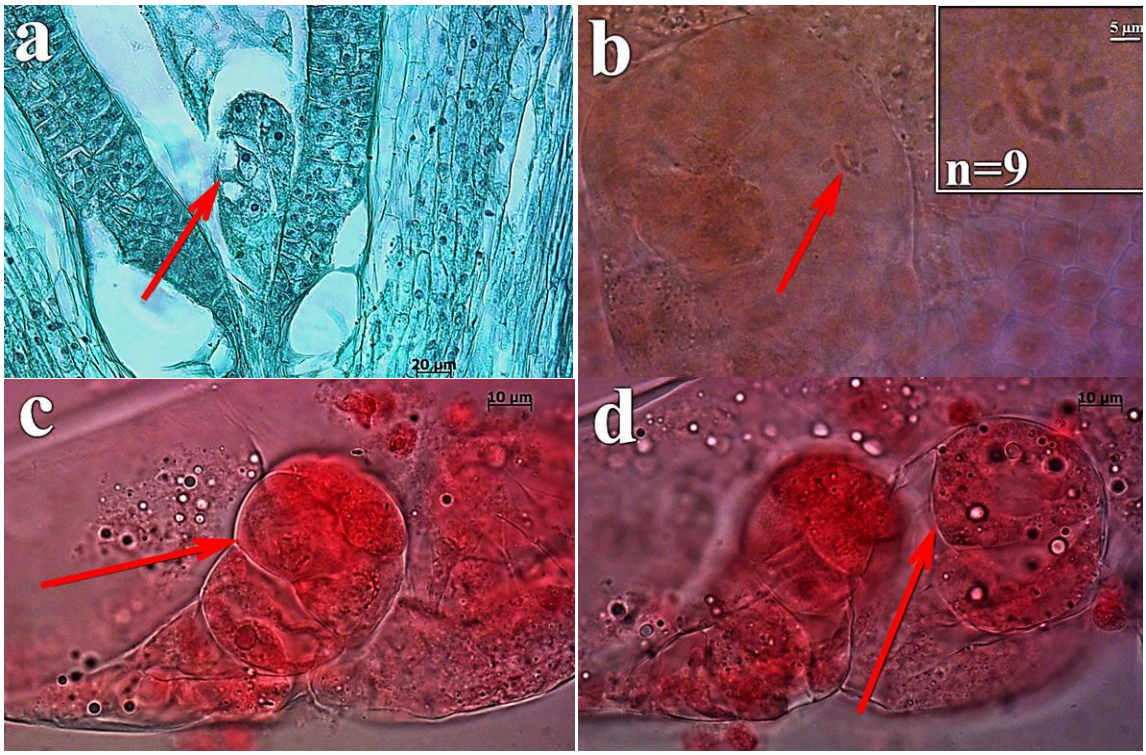

Figure 5. Embryos of *L. sativa* developed after chemical induction. **(a)** Globular embryos (arrow) 6 DAT with 0.05% 2,4-D; **(b)** Globular embryo with a haploid number of chromosomes n=9 (arrow) 6 DAT with 0.05% 2,4-D. **(c-d)** Two several-celled embryos in the same embryo sac 6 DAT with 0.05% Dicamba.

(Figure 6f). Cells of some embryos divided properly (in relation to the planes of division of cells of thecontrol embryos) up to the 4 celled stage (Figure 6d). The lack of suspensors and the absence of cellular endosperm led to their degeneration during the early stages of development, that is, as early as several hours after chemical treatment. After wide crossing or chemical induction, considerable disturbances were often observed in the cell arrangement of embryos. As they divided, they resembled intensively growing embryo-like structures, with enlarged, irregularly shaped cells in the apical or peripheral parts of the embryo (Figures 6g and h). Irregular arrangement of cells, diversification of their size and shape made it difficult to assess the stage of their embryogenesis (Figure 6i).

DISCUSSION

Wide crossing of plants by conventional methods (pollination of the stigma with alien pollen) rarely leads to the production of a hybrid generation. There are many pre-

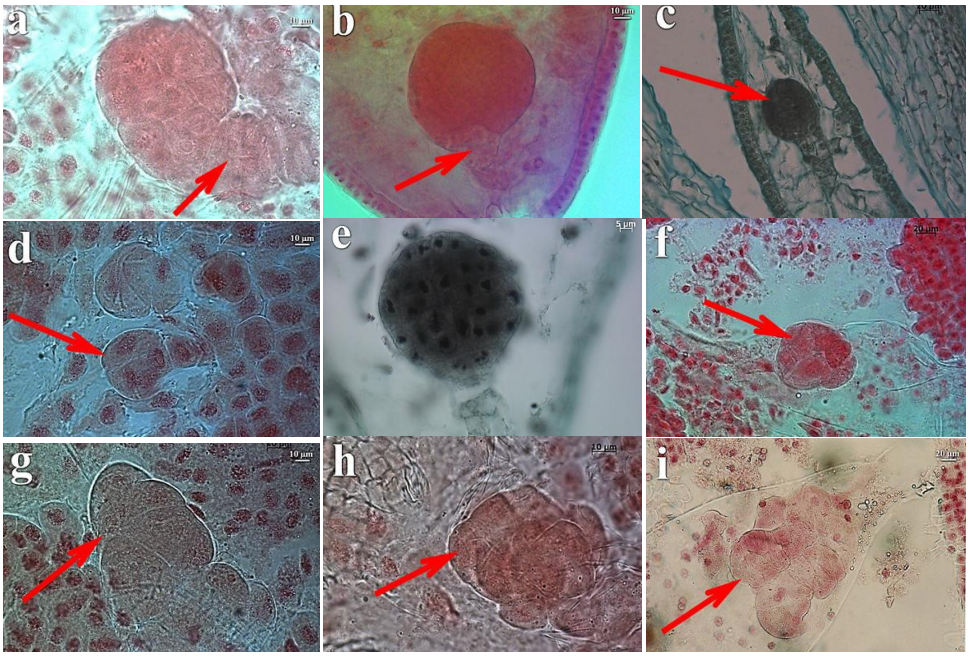

Figure 6. Comparison of the development of diploid (e) and haploid embryos of *Lactuca sativa*. **(a)** Globular embryo of *L.sativa* x *O. biennis* with a well developed suspensor, 6 DAP. (**b)** Globular embryo of *L.sativa* x *H. annuus* with vacuolated cells and a degenerated suspensor, 6 DAP. **(c)** well developed globular embryo of *L.sativa* x *H.annuus* with a suspensor, 7 DAP. The structure of the embryo is similar to the control. **(d)** Three-celled globular embryo with a 2-celled suspensor, 6 DAT with 2,4-D 0,05%. **(e)** Globular diploid embryo of *L.sativa* 2 DAP (control). **(f)** Several-celled embryo without suspensor, 6 DAT with 0,05% Picloram. **(g)** Several-celled globular embryo with irregularly arranged cells without suspensor, 6 DAT with 2,4-D 0,05%. **(h)** Several-celled globular embryo of *L.sativa* x *H.annuus*, 7 DAP. **(i)** Several-celled embryo of *L.sativa* x *H.annuus*, 5 DAP.

and post-fertilization barriers that effectively limit the development of hybrids. Several methods have been devised to overcome these barriers such as placing the alien pollen at the opened ovaries or directly into isolated ovules *in vitro* (Zenkteler, 1999; Popielarska, 2005) or by applying the embryo rescue technique. These methods have proved to be successful; for hybrid embryos of *Salix viminalis* × *Populus* sp. (Bagniewska-Zadworna et al., 2011), for hybrids *Lilium longiflorum* × *L. dauricum* (Van Tuyl et al., 1991) or hybrids *Camelia azalea* × *Camelia japonica* (Lattier et al., 2008). In our experiments, in most of the tested cross combinations, the barriers preventing fertilization appeared as early as at the stage of germination of alien pollen. Pollen of 21 plant species (20 of Asteraceae, 1 of Araceae) out of the 25 tested species (Table 1) germinated on lettuce stigma, but pollen tubes of only 2 species (*H. annuus* and *H. tuberosus*) were observed in the style and near the micropyle of the embryo sac. Despite the entrance of male gametes of sunflower in the embryo sac of lettuce, fertilization of egg cells did not occur. However, abnormally developed endosperm around the embryos resulting from the crossing as well as the hybrid number of the chromosomes of endosperm cells might indicate that single fertilization of the central cell could have occured. Published data show that, hybrid plants of *L. sativa* have been produced as a result of interspecific hybridization within the genus (*Lactuca*) (D'Andrea et al., 2008; De Vries, 1990). However, no hybrids have been produced through intergeneric crossing, as in the present study.

Haploid embryos of lettuce were formed in the present study as a result of wide crossing with 18 pollen donor species. It is likely that the embryos resulted from induction initiated by the pollen-stigma interaction. This assumption is confirmed by the fact that haploid embryos were also produced in those crosses (combinations) when pollen did not germinate. Many published reports indicate that apomictic embryos can be produced under the influence of alien pollen on the stigma, e.g. in *Cichorium intybus* (Dore et al., 1996) or *Hypericum perforatum* (Asker and Jerling, 1992; Koltunow, 1993). However, no information exists on the signal that may trigger the division of the egg cell (or other cells of the embryo sac) despite the lack of fertilization. It is supposed that the pollen-stigma interaction initiates this process. In some species (in *Zea mays* L.), pollination with pollen of the same species accelerates maturation and changes in the structure of egg cells (Mól et al., 2000). Moreover, during compatible or incompatible pollination, pollen tubes (and papillae on the stigma sur-

face) release a higher concentration of calcium ions Ca^{2+}, which allows their penetration through the transmission tissue into the style (Śnieżko and Winiarczyk, 1993; Lenartowska et al., 2001). Probably, the signals produced at that time induce the development of apomictic embryos.

Conclusions

The results of this study show that all the produced embryos were of parthenogenetic origin and all were haploid. Additionally, the frequency of embryo development as a result of wide crossing (up to 19%) or chemical induction (up to 15%) indicates that haploid embryos are formed easily in *L. sativa* under the influence of various induction methods. The use of male-sterile forms of lettuce in this study proved to be extremely important due to cleistogamy; any experiment on wide crossing would be very difficult with fertile male plants (Jones, 1927). Lettuce is a good model for research on parthenogenesis *in vivo* under the influence of distant pollen. The development of globular embryos, even very rarely heart-shaped embryos show that there is a chance to optimize the efficiency of the embryo rescue technique to produce homozygotic lettuce plants.

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