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MEGASPOROGENESIS AND MEGAGAMETOGENESIS IN SEVERAL *TRIPSACUM* SPECIES (POACEAE)[1]

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The *Tripsacum* agamic complex ($x = 18$) will provide valuable characters for maize breeding, provided that apomixis can be manipulated. Apomixis in *Tripsacum* was first reported 40 years ago, but its prevalence in the genus has not been established. Reproductive development was determined for eight Mexican and two South American *Tripsacum* species by microscopic analysis of ovaries cleared in a benzyl benzoate-dibutyl phthalate solution using interference contrast optics. The occurrence and distribution of callose deposition during megasporogenesis were determined by fluorescence microscopy of ovaries optically cleared in an aqueous sucrose solution containing aniline blue. Diploid genotypes were sexual. Polyploid forms reproduced apomictically following the Antennaria type (complete meiosis abortion) of diplospory. The Taraxacum type (unreduced megaspore production through meiotic restitution nuclei) of diplospory also occurred but rarely. The walls of diplosporic megasporocytes lacked callose whereas the walls of sexual megasporocytes contained a normal complement of callose. The absence of callose suggests that the diplosporic forms of reproduction result from mutations affecting the normal meiotic process. Apomixis in the *Tripsacum* genus is facultative, and the production of new polyploid genotypes through genetic exchanges involving both apomictic and sexual genotypes is possible.

The *Tripsacum* Agamic Complex is widespread in the American continents (from 42° N to 24° S). *Tripsacum* is portrayed in numerous systematic studies as a complex genus generally containing 16 species with chromosome numbers ranging from $2n = 2x = 36$ to $2n = 6x \approx 108$ (Randolph, 1970; de Wet, Gray, and Harlan, 1976; de Wet et al., 1981). The majority of species are found in Mexico, which is considered the center of *Tripsacum* diversity (Berthaud and Savidan, 1989). It is a member of the secondary gene pool of maize (Harlan and de Wet, 1971) and is thus a potential source of valuable genes for this crop. In addition to the character of apomixis, accessions of *T. bravum* Gray may also be resistant to *Striga* ssp., a major parasitic weed of tropical cereals (Dr. D. Berner, International Institute for Tropical Agriculture, Ibadan, Nigeria, personal communication). A better understanding of the reproductive mechanisms occurring in the entire genus is required for efficient transfer of genes from *Tripsacum* to maize.

Apomixis was first described in polyploid strains of *T. dactyloides* (L.) L. by Farquharson (1955) who reported polyembryony and facultative apomixis without describing the cytoembryological mechanisms. Brown and Emery (1958) provided a fragmentary description of diplosporous megasporogenesis in a *T. dactyloides* clone, while de Wet et al. (1973) reported apospory in maize × *T. dactyloides* hybrids. Diplospory in *Tripsacum* was clearly documented by Burson et al. (1990) using sectioned and stained ovaries from two triploid and one tetraploid *T. dactyloides* accessions from the United States. Diplosporic megasporogenesis in their materials was of the Antennaria type characterized by total meiosis abortion and direct development of the megasporocyte into megagametophyte through mitoses (Nogler, 1984).

The *Tripsacum* germplasm analyzed to date (reviewed above) represents a narrow sampling of the total genetic diversity in this genus. The lack of more thorough investigations may in part be due to 1) the absence of extensive collections especially for Mexican accessions and 2) difficulties in cytologically studying diplospory in very young sectioned ovules (Bashaw, 1980; Hanna, 1991). However, optical clearing techniques to observe megasporogenesis cytologically in entire pistils have recently been developed (Crane and Carman, 1987). Furthermore, an abnormal absence or severe reduction of callose within the cell walls of megasporocytes is tightly correlated with diplospory in *Elymus rectisetus* (Nees in Lehm.) Löve et Connor (Carman, Crane, and Riera-Lizarazu, 1991), and techniques to observe this abnormality in optically cleared ovaries have been developed (Peel, 1993). These techniques greatly increase the ease with which cytological observations of diplospory are made, especially with species that do not produce extremely large seeds. In the present study, we used these techniques to characterize cytologically both apomictic and sexual megasporogenesis and megagametogenesis in a geographically diverse sampling of *Tripsacum*.

MATERIALS AND METHODS

Plant materials—Accessions examined in this study were obtained from 1) the CIMMYT collection (eight of 80 accessions), which was assembled by Randolph and Gutièrez in the 1970s; 2) a collection of Central and South

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TABLE 1. *Tripsacum* germplasm analyzed.

Species	Accession number[a]	2n[b]	Origin[c]
T. bravum	6-552	72	Rancho Neuvo, Guerrero, Mexico
	10-597	72	Zuluapan, Mexico, Mexico
	39-1583, 38-1627	72	Tequila, Jalisco, Mexico
	57-632	36	Valle de Bravo, Mexico, Mexico
T. dactyloides dactyloides	65-1234*	72	Everglades, Florida, USA
T. dactyloides mexicanum	39-1614, 39-1617	72	Tequila, Jalisco, Mexico
	52-497	72	Acahuizotla, Guerrero, Mexico
T. dactyloides hispidum	15-53	108	Juchatengo, Oaxaca, Mexico
	16-63	72	Jachatengo, Oaxaca, Mexico
	55-607	72	Santo Tomas, Mexico, Mexico
	152-2357	36	Pozo Largo, Guerrero, Mexico
	154-2426	36	El Peral, Guerrero, Mexico
	7122-6*	72	Mazatlan, Sinaloa, Mexico
	7130-5*	72	Amacuzac, Jalisco, Mexico
T. dactyloides meridionale	579-5145**	36	Las Cuevas, Trujillo, Venezuela
	590-5177**	36	Quebrada La Calderera, Santander Norte, Colombia
T. intermedium	100-1130	72	La Trinitaria, Chiapas, Mexico
	104-1183	90	Ixtapa, Chiapas, Mexico
	150-2331	54	Agua de Obispo, Guerrero, Mexico
	7158-1*	72	El Sumidero, Chiapas, Mexico
	7160-6*	72	—, Chiapas, Mexico
T. pilosum	20-94	72	Chicahuxtla, Oaxaca, Mexico
	34-246	72	21 de Noviembre, Jalisco, Mexico
	39-1586, 39-1653	36	Tequila, Jalisco, Mexico
	47-419	36	Independencia, Jalisco, Mexico
T. maizar	29-183	72	El Tigre, Jalisco, Mexico
	7149-1*	54	La Herradural, Nayarit, Mexico
MZ-PL[d]	28-163	72	Puerto Los Mazos, Jalisco, Mexico
T. zopilotense	49-1382, 50-1410, 51-470	36	Zopilote Cañon, Guerrero, Mexico
	49-439, 7002-1*	72	Zopilote Cañon, Guerrero, Mexico
	49-1395	54	Zopilote Cañon, Guerrero, Mexico
T. lanceolatum	60-664	72	Ahuacatlan, Querertaro, Mexico
	72-815*	72	Sycamore Cañon, Arizona, USA
T. latifolium	77-1011	36	San Mateo Yetla, Oaxaca, Mexico
T. cundinamarse	613-5245**	36	Tamarindo, Viota, Colombia

[a] * Accessions from the CIMMYT collection; ** accessions from Dr. D. H. Timothy collection; others from the ORSTOM-CIMMYT collection.
[b] Chromosome numbers were counted from root tip squashes.
[c] Origin: locality, state, country.
[d] Intermediate form between *T. maizar* and *T. pilosum*.

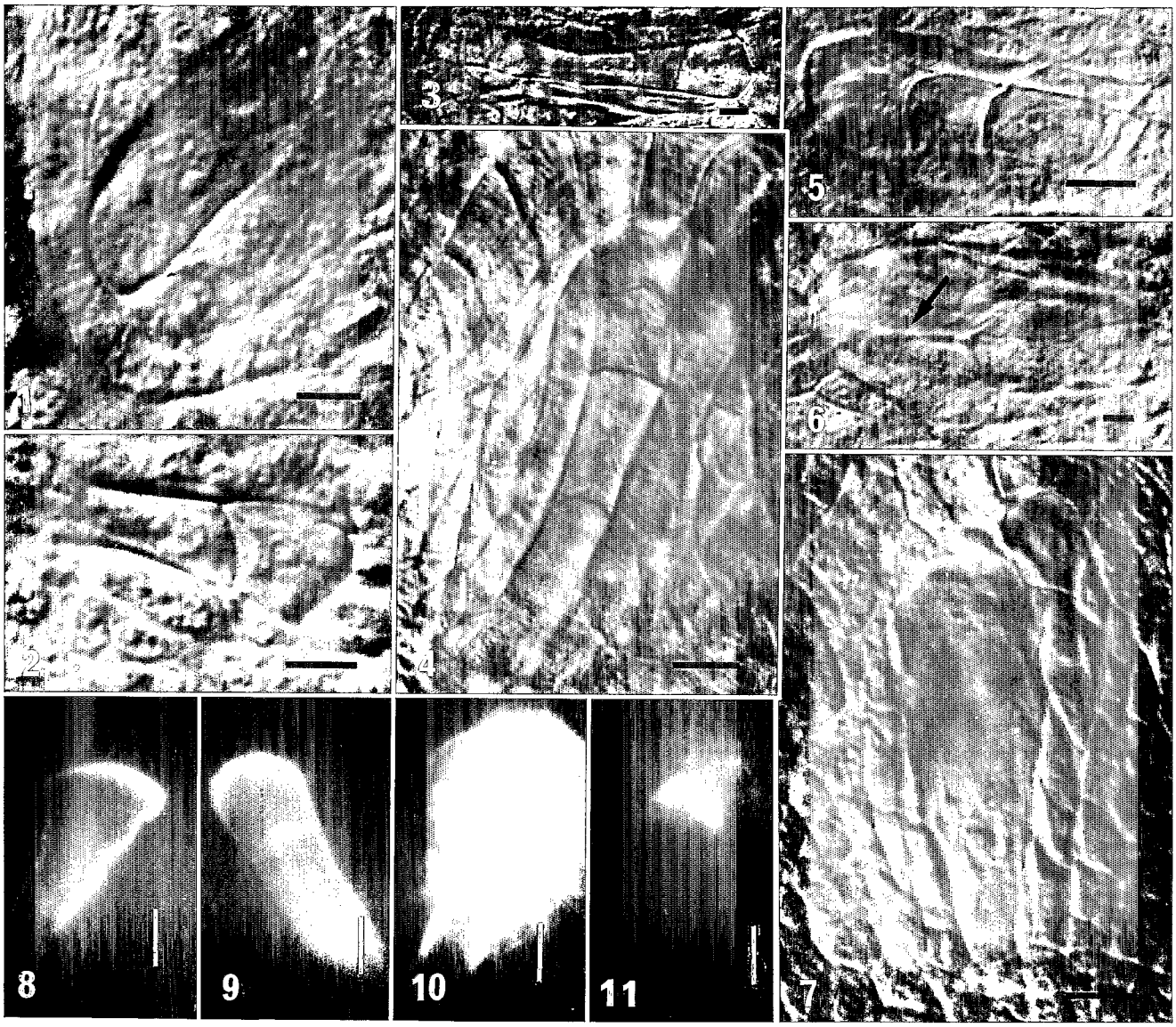
American accessions (three of 117 accessions); and 3) the ORSTOM-CIMMYT collection (30 of 1,481 accessions from 156 populations), which was recently collected from Mexico. These collections are maintained at the CIMMYT Experimental Station at Tlaltizapan, Morelos State, Mexico. The accessions analyzed in the present study were chosen according to species, origin, and chromosome number (Table 1), and most species and all ploidy levels ($2n = 36, 54, 72, 90$, and 108) found in wild populations (Farquharson, 1955; Berthaud and Savidan, 1989) are represented. A total of 1,317 pistils was analyzed; 944 from polyploids and the rest from diploids.

Clearing procedures—Young inflorescences were fixed in Carnoy's 6:3:1 (v:v:v) 95% ethanol : chloroform : acetic acid for 24 hr and stored in 70% ethanol at 4 C. After dissection, pistils were stored either in 70% ethanol or directly cleared according to Crane and Carman (1987), except that the final clearing solution was adjusted to 2.1:1 benzyl benzoate : dibutyl phthalate. Pistils were mounted on a slide in sagittal section between two doubled #1 coverslips. Air spaces were filled with clearing solution and a third coverslip applied. Observations were made with differential interference-contrast optics (Leitz Aristoplan equipped with a universal condenser UKO or Olympus BH-2 equipped with a Nomarski condenser).

Callose analysis—Pistils stored in 70% ethanol were hydrated using standard procedures and successively transferred to one-third, two-thirds, and full strength clearing solution (136 μM aniline blue, 2.46 M sucrose, pH adjusted to 9.5 with 1 M sodium bicarbonate). Pistils were kept in each clearing series solution for at least 30 min and were mounted as above. Observations were made by UV vertical epifluorescence microscopy (Leitz Aristoplan, BP 355-425 exciter filter, RKP 455 dichroic mirror, LP 460 barrier filter; or Olympus BH-2, UG-1 exciter filter, Y-455 dichroic mirror, L-435 barrier filter).

RESULTS

All diploid accessions ($2n = 36$, Table 1) were sexual. The megasporocytes in these accessions arose by direct enlargement of a hypodermal archesporial cell. Young sexual megasporocytes were frequently free of vacuoles or poorly vacuolate, were rectangular in shape, and had

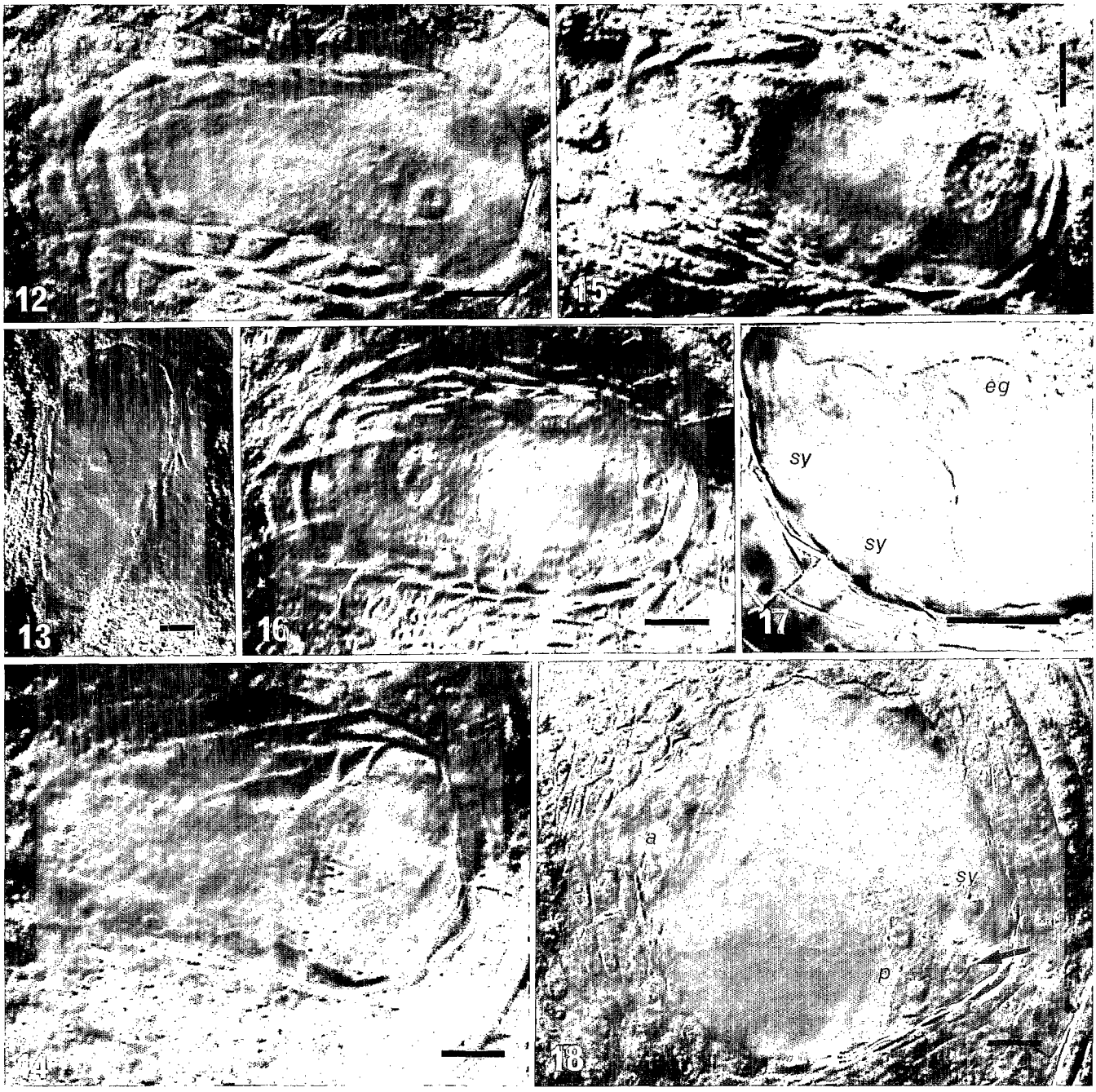


Figs. 1–11. Sexual development (Figs. 1–7) and callose deposition during meiosis observed under UV illumination (Figs. 8–11) in *Tripsacum*. **1.** Meiotic megasporocyte surrounded by callose. Note prominent cell walls and abundant callose deposition at the micropylar end (see also Fig. 8). **2, 3.** Dyads surrounded by callose. Note elongated chalazal megaspore in Fig. 3. **4.** Linear tetrad of megaspores surrounded by callose. **5.** T-tetrad of megaspores. **6.** Remaining chalazal megaspore after tetrad degeneration. The arrow points to the remnants of the three degenerated micropylar megaspores. **7.** Young binucleate embryo sac. **8.** Megasporocyte. Note absence of callose at the chalazal end. **9.** Dyad. **10.** Linear tetrad. **11.** Micropylar callose cap in a tetrad: callose is restricted to the micropylar wall of the micropylar megaspore, indicating termination of callose degradation. The three micropylar megaspores will soon degenerate. Bars = 50 μm.

thick cell walls especially at the micropylar end (Fig. 1). Prophase nuclei were dense, centrally located in the micropylar region of the megasporocytes, and exhibited one single nucleolus.

Metaphase I occurred when the enlarging integuments had encased about 50% of the nucellus. Dyads with two square to rectangular megaspores were common (Fig. 2). However, dyads with an elongated chalazal megaspore were occasionally observed (Fig. 3). The dyad stage was short-lived. Meiosis yielded both linear and T-tetrads (Figs. 4, 5), but the linear type was more common (80% of the tetrads observed). The three micropylar members of each tetrad degenerated (Fig. 6) while the chalazal member developed into a binucleate megagametophyte, which at first was elongate, slightly vacuolate, and had almost spherical nuclei (Fig. 7). Megasporocytes, dyads, and tetrads had thick cell walls that fluoresced brightly when stained for callose (Figs. 8–10). Callose was degraded rapidly from the chalazal megaspore but more gradually from the three micropylar members. The residual tissues of degenerate megaspores, which were compressed against the micropyle, retained callose fluorescence during the early stages of megagametophyte development (Fig. 11).

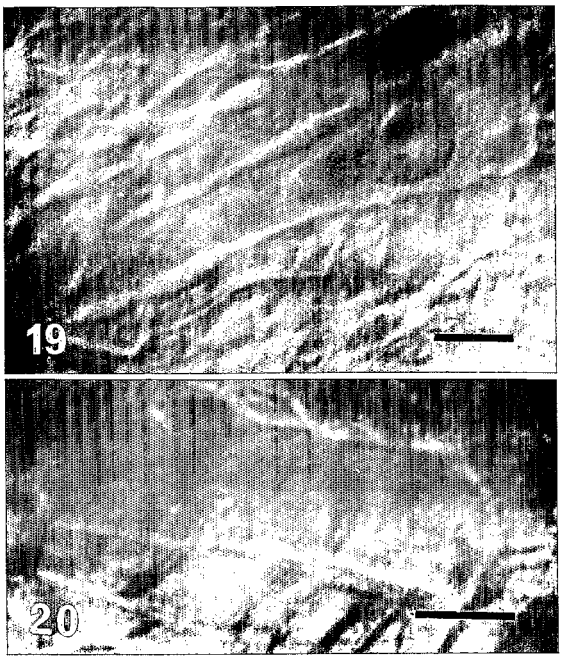
The integuments expanded during meiosis to encase about 75% of the nucellus, which left three to six epidermal nucellar cells exposed at the end of meiosis. Mature Po-



Figs. 12–18. Apomictic development in *Tripsacum*: Antennaria type of diplospory. **12, 13.** Diplosporous megasporocytes. **14.** Metaphase of the first mitotic division. **15, 16.** Vacuolate binucleate embryo sacs with compressed nuclei. **17, 18.** Eight-nucleate embryo sacs. Note detail of the micropylar apparatus in Fig. 17. eg = egg cell nucleus, sy = synergids, arrow = egg cell, p = polar nuclei, a = antipodal cells. Bars = 20 μm.

lygonum type megagametophytes developed through vacuolation, and three mitotic divisions followed by cytokinesis. One exception to the sexual pathway was observed in one pistil of the diploid accession 579-5145 (*T. dactyloides meridionnale*): a large slightly tear-shaped megasporocyte was observed that had thin cell walls. However, its nucleus was diffuse and the entire megasporocyte may have been degenerating.

All polyploid *Tripsacum* accessions were diplosporic with megasporogenesis being primarily of the Antennaria type. No evidence of polyembryony or apospory was observed. Within each ovary, a single diplosporic megasporocyte usually formed from a hypodermal nucellar cell as occurred in sexual accessions, but some of them were positioned chalazal to that typical of sexual megasporocytes. Young diplosporic megasporocytes had thin cell walls that lacked callose fluorescence, were elongated, swollen at the micropyle, and generally lacked vacuoles. Each megasporocyte contained relatively small nucleus located in the central rather than in the micropylar region,



Figs. 19, 20. Reproductive behavior other than sexuality and Antennaria type diplospory. **19.** Elongated tear-shaped megasporocyte in accession 7122-6. **20.** Unreduced dyad in accession 39-1583. Typical of Taraxacum-type diplospory. Bars = 50 μm.

and had a single nucleolus. They became vacuolate and enlarged as the integuments closed in upon the micropyle. By the first mitotic division, the micropylar regions were extremely dilated, whereas the chalazal regions generally remained narrow (Figs. 12, 13) producing a club-shaped megasporocyte. The large nuclei often assumed a spherical or bean shape.

The first mitotic division was perpendicular to the long axis of the megasporocyte (Fig. 14). The two nuclei of the resulting megagametophyte migrated and became separated by a large, central vacuole (Figs. 15, 16). They were often found compressed by vacuoles into half moon (micropylar end) or angular (chalazal end) shapes (Fig. 16). Two nucleoli per nucleus were common at this stage, particularly on the micropylar side. Two or more additional mitotic divisions followed by cytokinesis produce mature diplosporic Polygonum type megagametophytes, each one containing one egg cell and two synergids on the micropylar end, two polar nuclei closer to micropylar than the chalazal end, and three or more antipodals at the chalazal end (Figs. 17, 18).

A few exceptions to Antennaria type megasporocytes were encountered. Elongated nondilated tear-shaped megasporocytes, as described by Burson et al. (1990), were only rarely observed (Table 2). Each contained a few small micropylar and central vacuoles and a slightly elongated nucleus located near the micropylar end (Fig. 19). One or two dense nucleoli per nucleus were observed, which suggests that these cells were not degenerative. Extremely elongated nuclei, such as occurs in diplosporous *E. rectisetus,* which produces unreduced dyads (Crane and Carman, 1987), were not observed.

The Taraxacum type of diplospory (the megagametophyte forms from the chalazal unreduced megaspore of an unreduced dyad that results from meiotic restitution at metaphase I of the first meiotic division; Nogler, 1984) infrequently occurred in a few accessions (Table 2). Dyads (Fig. 20) but never tetrads were observed. These generally formed at an earlier stage than in sexual meiocytes. Their cell walls were thin, and callose fluorescence was not observed. Also, their shape differed from that of meiotic dyads, which were square to rectangular. The putative Taraxacum type dyads had square micropylar members but triangular chalazal members (the chalazal side being narrower). Nuclei in these dyads were larger than in meiotic dyads, and each possessed a dense nucleolus.

Meiotic dyads and tetrads were observed among the diplosporous accessions at an average frequency of 2.1% (Table 2). Concomitant development of nucellar cells into embryo sacs (apospory) was not observed, which indicates that these dyads and tetrads were sexual and that apomixis is facultative at least in some of the *Tripsacum* accessions we surveyed.

TABLE 2. Reproductive forms other than Antennaria type diplospory among apomictic *Tripsacum.*

Apomictic accessions	*N*[a]	Sexual development[b]	*Taraxacum* type of diplospory	Elongated tear-shaped megasporocytes
29-183	24	2	—	—
34-246	25	—	—	2
39-1583	41	—	8	4
39-1614	37	1	—	—
39-1617	41	3	—	—
49-439	32	1	3	—
55-607	33	2	—	—
65-1234	21	—	2	—
100-1130	27	1	—	—
7002-1	56	4	—	—
7122-6	62	4	11	3
7130-5	45	—	—	1
7149-1	47	—	—	1
7160-6	34	2	—	—
Others	419	—	—	—
Total	944	20	24	11
%	—	2.10	2.55	1.15

[a] *N,* total number observed.
[b] Sexual stages observed were dyads and tetrads. Callose synthesis was checked by UV fluorescence in 39-1617, 7160-6, 29-183, and 7122-6.

DISCUSSION

Apomixis in Tripsacum — As in other agamic complexes (de Wet and Harlan, 1970; Savidan and Pernès, 1982), apomixis in *Tripsacum* is restricted to polyploids. *Tripsacum* polyploids are highly heterozygous (J. Berthaud, ORSTOM-CIMMYT, Mexico, unpublished data), and the low levels of sexuality within them (Table 2) should permit genetic exchange with diploids and other polyploids. New genotypes may also arise through fertilization of unreduced gametes (Harlan and de Wet, 1975; Bashaw, Hussey, and Hignight, 1992). Thus, facultative apomixis in *Tripsacum* is not viewed as the blind alley of evolution envisioned by Darlington (1939). In contrast, numerous intermediate forms and extensive natural diversity exist (J. Berthaud, unpublished data).

Diplospory was the only form of apomixis observed in the 41 *Tripsacum* accessions we analyzed. In most cases, diplosporic megasporocytes were of the Antennaria type. Taraxacum type megasporocytes were only rarely observed and were restricted to three accessions that produced primarily Antennaria type megasporocytes. These two types of diplospory are believed to be similar, and the shift from one form to the other may be influenced by environment (Gustafsson, 1947; Nogler, 1984).

The Antennaria type of diplospory in our *Tripsacum* materials was characterized by 1) enlargement of the megasporocyte through vacuolation, 2) total meiotic failure, and 3) direct development of the meiocyte into megagametophyte through mitoses. In these respects it closely resembles diplospory in the warm season grass *Eragrostis curvula* Schrad. (Voigt and Bashaw, 1972). In contrast, Burson et al. (1990) described elongated and tear-shaped megasporocytes in two triploid (1249-Santa Claus, IN, and 1459-La Grange, TX) and one tetraploid (1008-Baird, TX) *T. dactyloides* accession. Such elongated megasporocytes and nuclei are frequently observed in *Chondrilla juncea* (Bergman, 1950) and *E. rectisetus* (Crane and Carman, 1987), which commonly undergo meiotic restitution to produce unreduced dyads (Taraxacum type of diplospory). Elongated meiocytes were rarely observed in our study (34-246, 39-1583, 7122-6, 7130-5 and 7149-1, Table 2), and these were associated with the formation of unreduced dyads in two cases (39-1583 and 7122-6, Table 2).

Cytological peculiarities of diplospory—A major characteristic of diplospory is abortion or total absence of meiosis. In *Tripsacum*, diplosporic megasporocytes of the Antennaria type enlarge to more than double the size of sexual megasporocytes. They do not exhibit any typical meiocytic behavior and once vacuolation starts, development is clearly along gametophytic rather than meiotic. Distinction between megasporocyte and megagametophyte therefore is blurred, the megasporocyte functioning as an unreduced megaspore. Judging from integument growth, which varies greatly among pistils, the first megagametophyte mitosis occurs at about the same time as the end of meiosis in sexual megasporocytes. Young sexual binucleate megagametophytes are not strongly vacuolate at this stage, and their nuclei are spherical. In contrast, large vacuoles in diplosporic binucleate megagametophytes compress the nuclei into flattened shapes against the cell wall (Fig. 16).

In the present study, total absence of callose fluorescence in and around the walls of female megasporocytes distinguished diplosporic from sexual developments. Callose is typically synthesized in meiocyte cell walls of angiosperms that produce sexual monosporic or bisporic megagametophytes, and is degraded rapidly following meiosis (Rodkiewicz, 1970). Absence of callose in megasporocyte cell walls during megasporogenesis is also observed in diplosporic *E. rectisetus* (Carman, Crane, and Riera-Lizarazu, 1991), *E. curvula* (Peel, 1993), and *Poa nemoralis* L. (Naumova, den Nijs, and Willemse, 1993). In diplosporic *E. rectisetus*, unusual patterns of callose deposition in ovular tissues were infrequently observed and included deposits in the micropylar wall of megasporocyte and sporadically in nucellar cell walls. Such deposits were not observed in sexual meiocytes; thus they might represent a more general lesion in cell wall synthesis (Carman, Crane, and Riera-Lizarazu, 1991).

Developmental differences between diplospory and apospory—Meiosis often occurs successfully in aposporous apomicts, and both aposporic megagametophytes (from nucellar cells) and a sexual megagametophyte (from the surviving megaspore) may form within the same ovary (Nogler, 1984). The surviving sexual megaspore often is eliminated after meiosis, which suggests, as noted by Harlan et al. (1964), that the genetic lesions responsible for apospory do not affect meiosis. Furthermore, normal levels of callose deposition in female meiocytes (Peel, 1993; Naumova, den Nijs, and Willemse, 1993) have been observed in aposporous species. In contrast, diplospory appears to be caused by a different set of genetic lesions that prevent callose deposition and grossly alter meiosis. Expression of such lesions varies among the diplosporous materials we analyzed, which permits residual sexuality (up to 8%) within the polyploid genetic pool.

Absence of megasporocyte callose may be responsible for meiotic failure in diplosporic species (Carman, Crane, and Riera-Lizarazu, 1991). However, absence of callose in megasporocytes of angiosperms that produce tetrasporic megasporocytes does not interfere with meiosis, and normal meiotic divisions occur in the absence of callose deposition (Rodkiewicz, 1970). Heslop-Harrison and Mackenzie (1967) assumed that during microsporogenesis callose acts as a molecular barrier for RNAs and proteins, avoiding their incorporation in meiocytes and allowing the insulation of the differentiating meiocytes from the maternal tissues. The relationship, if any, between meiosis and megasporocyte isolation by callose requires further investigation.

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