Rev. Brasil. Genet. 12, 4, 803-817 (1989)
(Brazil. J. Genetics)

EMBRYOLOGY AND CYTOGENETICS OF APOMICTIC HEXAPLOID *Eupatorium odoratum* L. (COMPOSITAE)

James R. Coleman

ABSTRACT

The embryology of *Eupatorium odoratum* indicates it to have Antennaria type diplospory with autonomous development of the endosperm and pronounced precocious embryony. The embryo sac is of the Polygonum type and the polar nuclei do not fuse. Both polar nuclei apparently contribute to the formation of the endosperm. It is estimated that about 20% of the ovules reach anthesis with the egg undivided and available for sexual reproduction. Pollen production is extremely reduced as a consequence of the degeneration of the sporogenous tissue prior to meiosis. Microsporocytes at diakinesis showed univalents, bivalents and trivalents, although in some cells pairing was highly reduced. *E. odoratum* is demonstrated to be an autohexaploid with a basic set of ten chromosomes, each represented six times. It is suggested that apomictic hexaploid *E. odoratum* originated from hybridization between facultatively apomictic triploid populations which were previously isolated and adaptively differentiated, in accordance with Stebbins secondary contact hypothesis.

INTRODUCTION

The occurrence of apomixis in the genus *Eupatorium* sens. lat. was first demonstrated by Holmgren (1919). Until recently, apomixis had been conclusively documented for only a few additional northern hemisphere species (Sparvoli, 1958, 1961; Sullivan, 1976), although indirect evidence suggested a more wide spread occurrence. This evidence consists primarily of the determination of triploid species or races and the observation of irregularities during meiosis of microsporogenesis (Grant,

Departamento de Botânica, Instituto de Biociências, Letras e Ciências Exatas, UNESP, 15055 São José do Rio Preto, SP, Brasil.

1953; Fryxell, 1957; Turner and Irwin, 1960; Sullivan, 1976; Strothers, 1983). Apomixis has lately been demonstrated in four South American species of the genus, three of which are Brazilian (Coleman and Coleman, 1984, 1988) and one of which is Argentine (Rozenblum *et al.*, 1988). The existence of apomixis in species of *Eupatorium* native to both the northern and southern hemispheres, as well as its distribution in several taxonomic sections, compel the conclusion that apomixis has been a fundamental evolutionary process in the genus. An appreciation of the true extent and significance of apomixis in *Eupatorium* will require detailed studies of many additional species and, especially, species groups representing different taxonomic areas and ploidy levels within the genus. The objetive of the present paper is to report on the embryology and cytogenetics of apomictic hexaploid ($2n = 6X = 60$) Brazilian material of *E. odoratum* L. (= *Chromolaena odorata* (L.) R.M. King and H. Robinson).

MATERIAL AND METHODS

Material of *E. odoratum* used in this study was collected within a radius of 15 km of the city of São José do Rio Preto, State of São Paulo, Brazil. Voucher specimens are deposited in the Herbarium of the Jardim Botânico de Rio de Janeiro (RJ). Buds for embryological studies were fixed in FAA. Ovules were dissected from the ovaries, cleared in $4\frac{1}{2}$ clearing solution (Herr, 1971, 1972) and mounted directly in Hoyer's medium (Alexopoulos and Benke, 1952). Ovule content was analyzed in three floral stages: initial stages (florets judged to be more than 24 hours from anthesis), pre-anthesis (florets judged to be within 24 hours of anthesis) and anthesis. Buds for the study of microsporogenesis were fixed in 1:3 acetic acid-ethanol. Staining was done with acetocarmine and the slides made permanent with Hoyer's medium. Karyological studies were done using root tips of seedlings obtained by germinating achenes on humid filter paper in petri dishes. The root tips were pre-treated in 0.002M 8-hydroxyquinoline during 7 hours at 12^{o} to 18^{o}C and fixed in 1:3 acetic acid-ethanol. Hydrolysis was done in 6% HCl for five minutes at 60^{o}C. After staining with acetocarmine, the apices of the root tips were squashed directly in Hoyer's medium. Selected cells were photographed and prints for analysis made at 3750X; metaphase chromosomes of five cells were measured. A Zeiss Standard WL photomicroscope equipped with phase contrast was used for all microscopical work. Germination tests were made on humid filter paper in petri dishes under prevailing conditions of light and temperature and terminated after 7 weeks.

RESULTS AND DISCUSSION

E. odoratum is a weedy, perennial shrub native to tropical America but widely established in the Old World tropics where it is often a major weed (King and

Robinson, 1975; Holm *et al.*, 1977). Reproduction is exclusively by seeds, and the species is a prolific seed producer. An analysis of 1200 achenes from 12 plants (100 achenes per plant) revealed that 74.4% (893) contained an embryo. Values for individual plants ranged from 60% to 87%. Germination tests (50 achenes from each of the 12 plants) showed that 70% (420) of the achenes germinated, values for individual plants varying from 20% to 98%.

The analysis of the contents of clarified ovules showed a total of 196 megasporocytes and 191 two-nucleate embryo sacs (Table I), but no megaspore production was observed. Instead, the megasporocyte (Figure 1a) functions directly as an unreduced megaspore, the nucleus of which divides to form a two-nucleate embryo sac (Figure 1b) whose nuclei migrate to the extremities of the elongating cell (Figure 1c). A second division produces a four-nucleate embryo sac (Figure 1d), and a third division results in the formation of a mature embryo sac of the Polygonum type (Figure 2a). The polar nuclei remain unfused, and Figure 2b suggests that they divide simultaneously to initiate the endosperm, although this may not always be the case. These observations demonstrate the material studied to have Antennaria type diplospory.

Autonomous endosperm development regularly precedes the division of the egg, and precocious embryony results in embryos in more than 70% of the ovules prior to anthesis (Table I). It is probable that about 20% of the florets reach anthesis with the egg still undivided and available for sexual reproduction.

E. odoratum is the third species of section Osmia (or the genus *Chromolaena*) to be studied embryologically, the other two being *E. callilepis* and *E. squalidum*, both of which are triploid (Coleman and Coleman, 1984, 1988). These three species are similar in having Antennaria type diplospory with autonomous endosperm development and pronounced precocious embryony. Unlike *E. odoratum*, the polar nuclei of *E. squalidum* fuse, but the resulting central nucleus retards its division until after the embryo is initiated. *E. callilepis* is similar to *E. odoratum* in that the polar nuclei apparently do not fuse; however, whether the endosperm or embryo is first to initiate its development is not known for that species. Interestingly, no sexual species or populations have yet been detected in section Osmia.

An examination of about twenty plants of *E. odoratum* revealed extremely reduced pollen production, most anthers producing no pollen (Figure 3a,b) in consequence of microsporocyte degeneration before the onset of meiosis. Although this turned a quantitative study of microsporogenesis infeasible, some observations can be made.

Cells observed at diakinesis regularly showed univalents, bivalents and trivalents (Figure 3c), although in some cells pairing was extremely restricted (Figure 3d). Lost univalents resulted in the formation of micronuclei at first and second divisions and sporads typically revealed their presence (Figure 4a). In *E. squalidum* restriction of chromosome pairing was determined to be associated with the production of

Table I - Contents of ovules of *Eupatorium odoratum* at different stages of floral development.

Floral stage	Number of plants examined	Number of ovules analyzed	Aborted embryo sacs		Megasporocytes		Two-nucleate embryo sacs	
			Number	Percent	Number	Percent	Number	Percent
Initial stages	12	1187	67	5.6	191	16.1	184	15.5
Pre-anthesis	12	600	36	6.0	4	0.7	7	1.2
Anthesis	10	521	43	8.3	1	0.2	0	0

Continued

Four-nucleate embryo sacs		Egg and polar nuclei		Egg and endosperm		Embryo and endosperm	
Number	Percent	Number	Percent	Number	Percent	Number	Percent
94	7.9	391	32.9	92	7.8	168	14.2
6	1.0	57	9.5	66	11.0	424	70.7
2	0.4	18	3.5	9	1.7	448	86.0

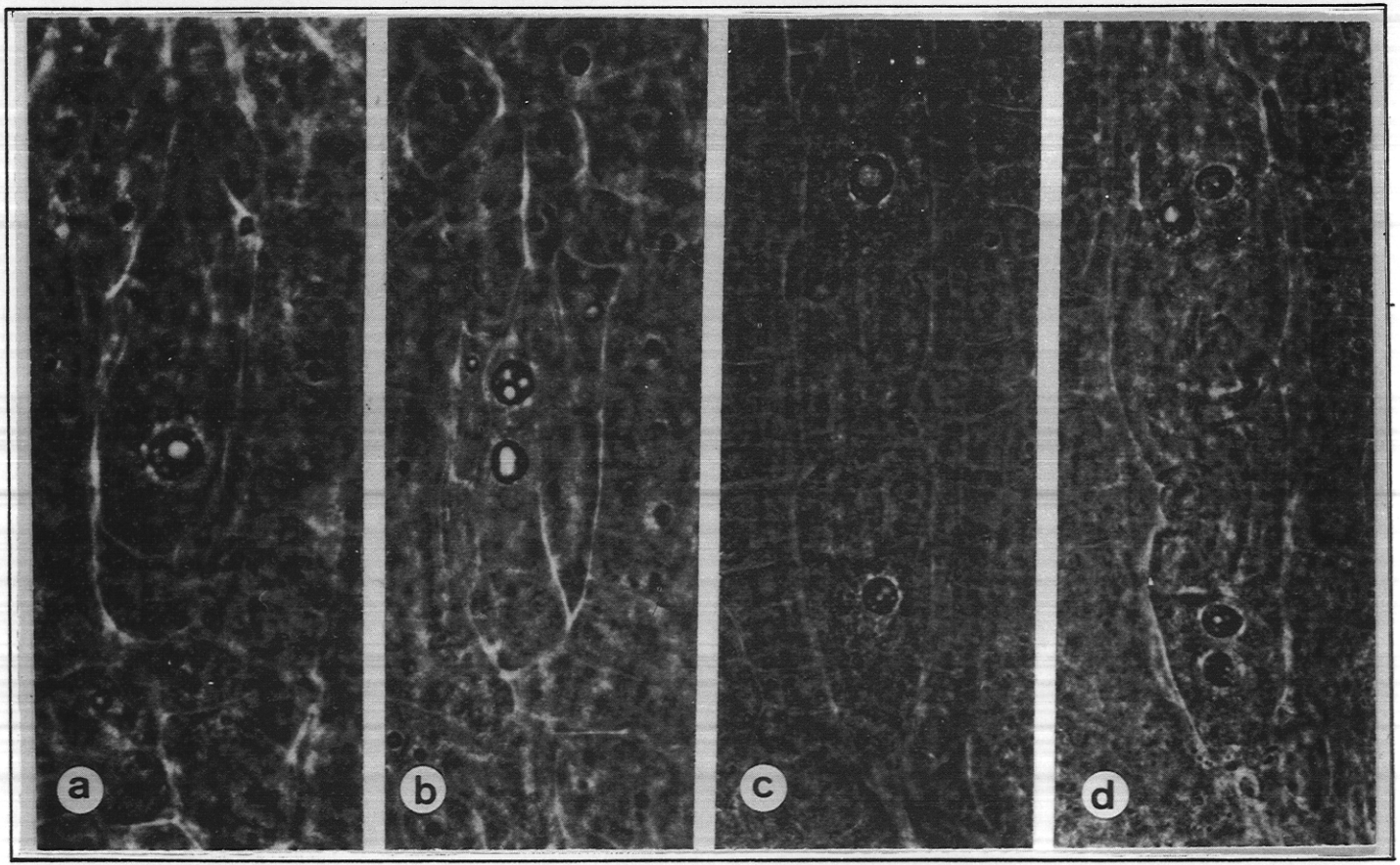

Figure 1 - Embryo sac formation in *Eupatorium odoratum*. a, Megasporocyte; b, early stage of 2-nucleate embryo sac; c, late stage of 2-nucleate embryo sac; d, 4-nucleate embryo sac. a, 1125X; b, 1050X; c, 750X; d, 825X.

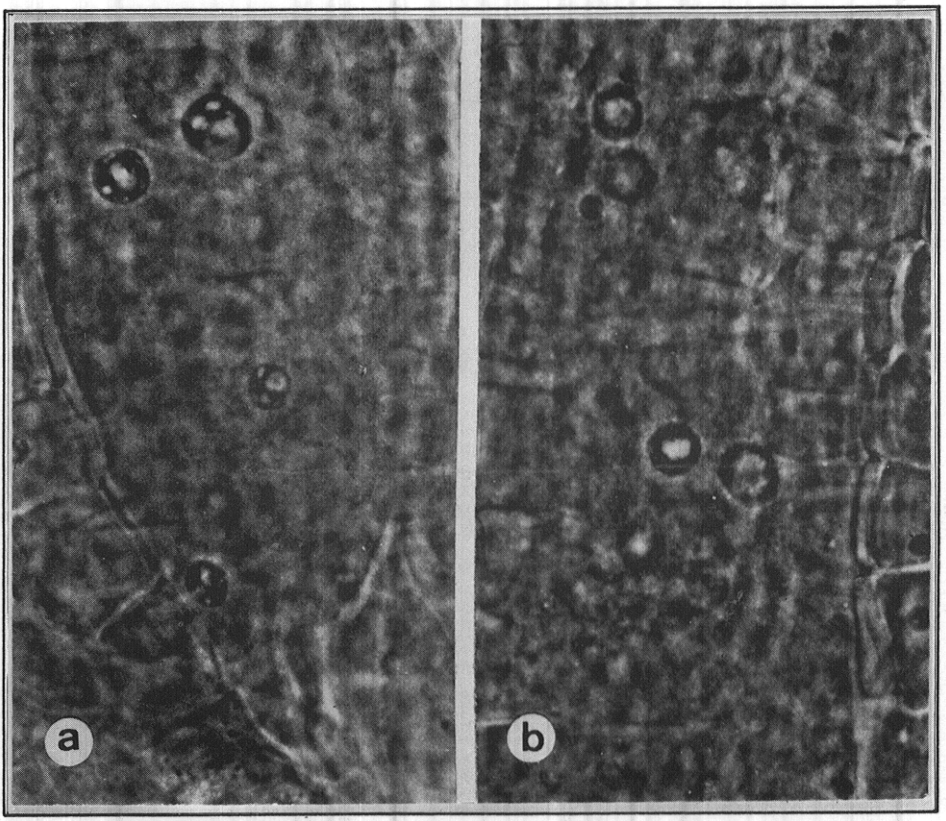

Figure 2 - Mature embryo sac structure and edosperm initiation in *Eupatorium odoratum*. a, Mature embryo sac showing synergids, egg and polar nuclei; b, embryo sac showing egg and divided polar nuclei originating endosperm. a-b, 1125X.

dyads of unreduced microspores from restitution nuclei (Coleman and Coleman, 1988). Restitution nuclei were not encountered in *E. odoratum*, but the observation of dyads of microspores (Figure 4b) strongly suggests their existence. Large microspores with two and four nuclei were observed (Figure 4c) and probably resulted from irregularities of cytokinesis, such that entire microsporocytes or half microsporocytes were transformed into microspores following meiosis. The gross irregularities observed, especially the degeneration of the sporogenous tissue, indicate a very high degree of male sterility in the material studied. Possibly only the very occasional, unreduced pollen grains could be expected to be functional.

Infrequent pollination would be an expected consequence of the low pollen production observed. This expectation was confirmed since only 14 (9.3%) of 150 pistils examined showed the presence of pollen grains (Table II). The mean number of

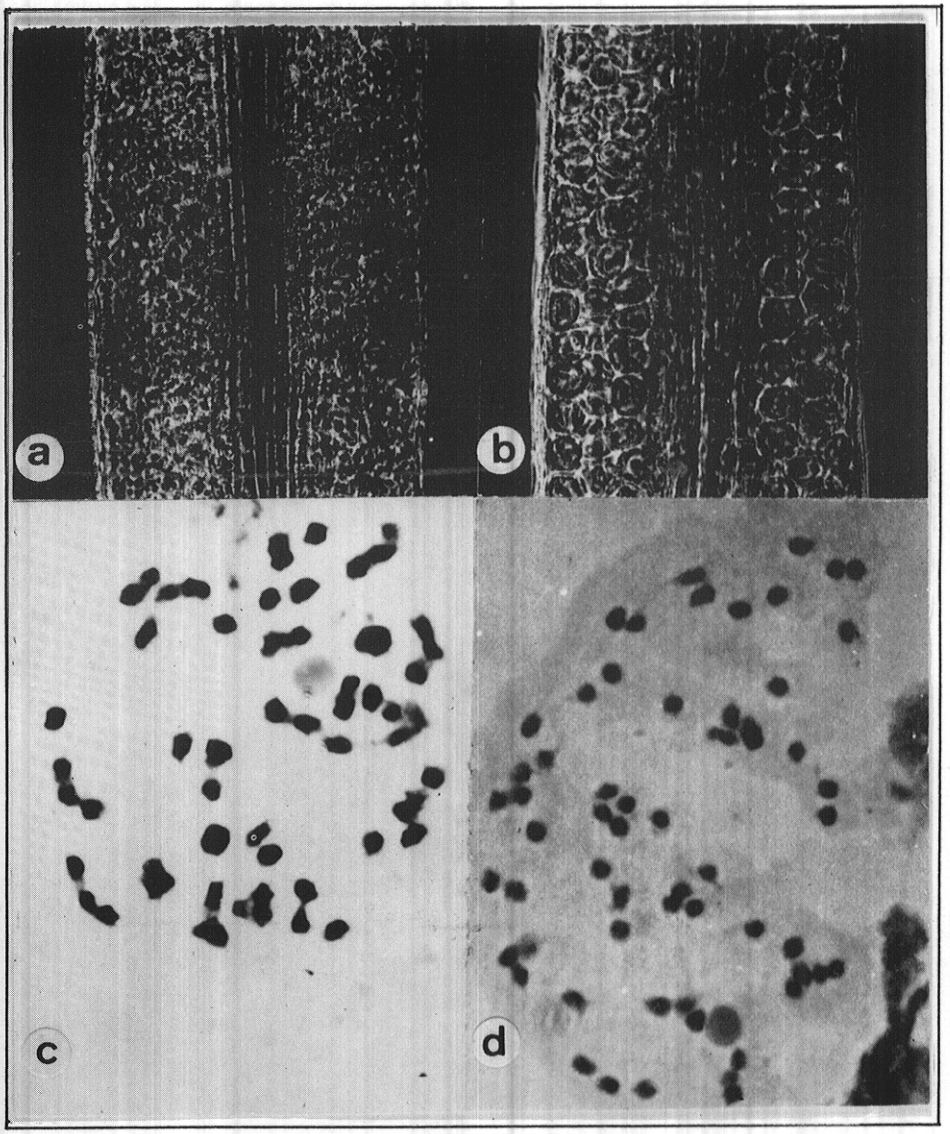

Figure 3 - Microsporogenesis in *Eupatorium odoratum*. Optical sections through cleared, undehisced anthers of, a, *E. odoratum* indicating absence of pollen grains and, b, *E. squalidum* with pollen grains. The latter species shown for purpose of comparison; c, diakinesis showing univalents, bivalents and trivalents; d, diakinesis with mostly univalents. a-b, 360X; c, 1800X; d, 1525X.

grains on these pistils was 4.6 and nine had three or fewer grains. Although it is probable that many of these grains were not germinated, the germination of at least a

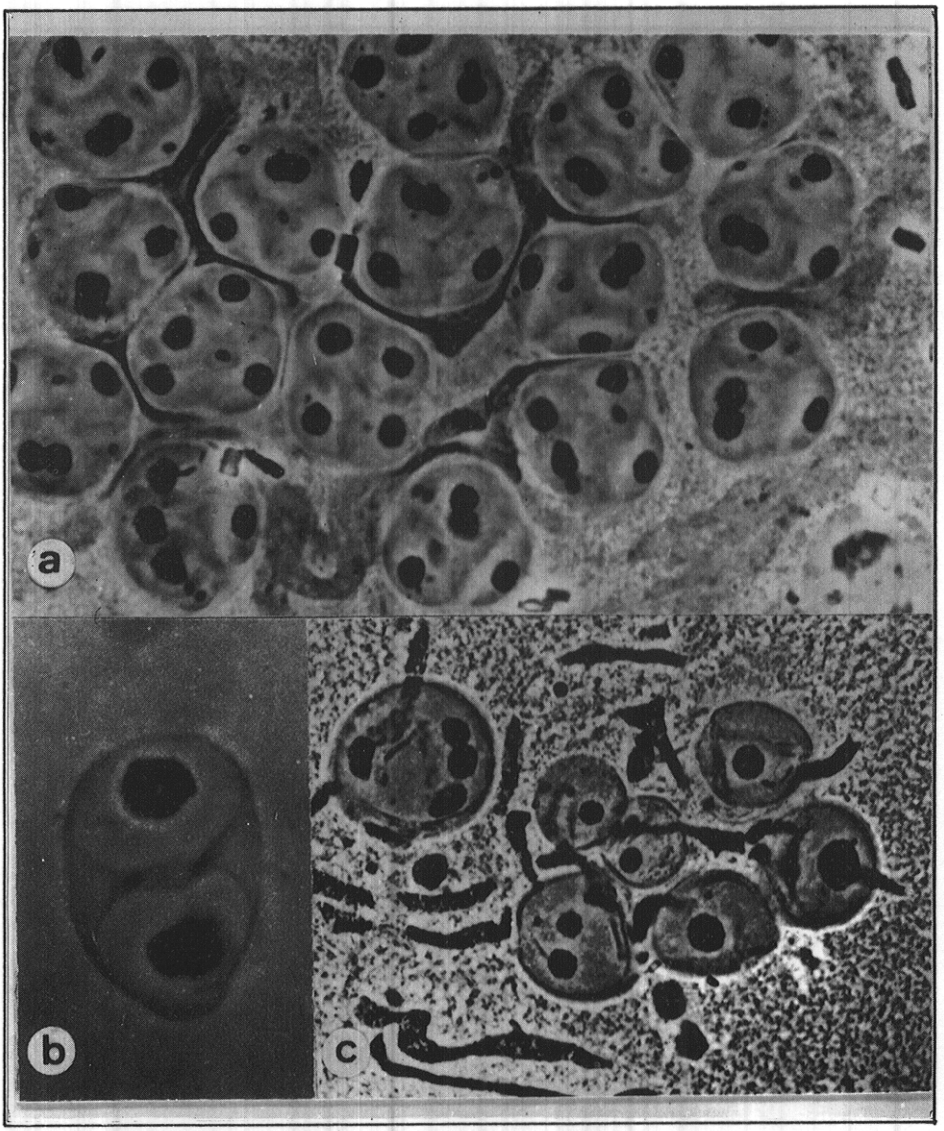

Figure 4 - Microsporogenesis in *Eupatorium odoratum*. a, Tetrads showing micronuclei; b, dyad of microspores; c, large microspores with one, two and four nuclei. a, 620X; b, 600X; c, 500X.

minority is certain since pollen tubes were seen. These figures compare with 80.7% of the pistils with pollen and a mean of 12.4 grains per pistil observed in a similar study of *E. squalidum* (Coleman and Coleman, 1988). It is possible that some of the pollen grains observed on pistils of *E. odoratum* were grains of *E. squalidum* since these two

closely related species generally occur associated and frequently form mixed pollinations in the study area. This could possibly explain the high number of pollen grains, 16 and 18, observed on two pistils of a single plant of *E. odoratum* (plant 6). The consequences of such possible pollinations are not known at this time. The infrequency of pollination and the low production of functional pollen indicate that sexual reproduction must be rare in the material studied, even though some ovules with undivided eggs are available at anthesis. Despite low pollen production and infrequent pollination, pollinator visits were observed to be frequent, presumably with the purpose of collecting nectar.

Table II - Pollen grains observed on open-pollinated stigmas of *Eupatorium odoratum*.

Plant number	Number of pistils examined	Pistils with pollen grains		Pollen grains per pistil	
		Number	Percent	Range	Mean[a]
1	15	0	0	0	0
2	15	0	0	0	0
3	15	5	33.3	0-3	1.8
4	15	0	0	0	0
5	15	0	0	0	0
6	15	7	46.7	0-18	7.7
7	15	1	6.7	0-1	1.0
8	15	0	0	0	0
9	15	1	6.7	0-1	1.0
10	15	0	0	0	0
Totals:					
10	150	14	9.3	0-18	4.6

[a]Mean based only on those pistils with pollen grains.

The chromosome complement of *E. odoratum* is formed of ten basic chromosomes, each represented six times (Figure 5). Although no cell examined showed more than two SAT-chromosomes, it is probable that six are present in the complement, one for each basic set. Evidence to this respect is presented by a survey of clarified root tip cells which, while mostly showing one or two nucleoli, exceptionally showed up to five (Figure 6).

The karyotype of *E. odoratum* indicates the species to be an autohexaploid; however, trivalents were the highest pairing association observed during meiosis of

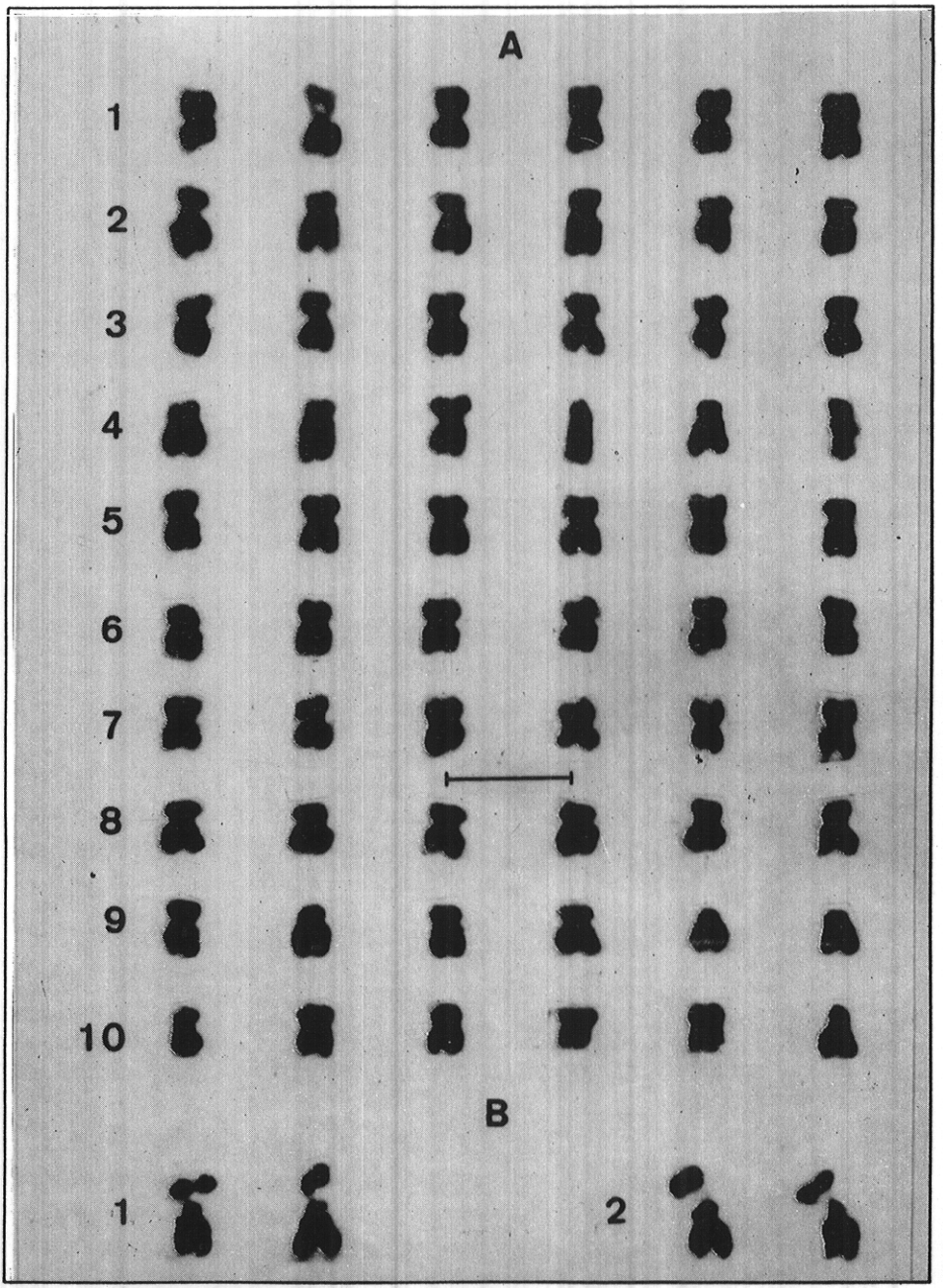

Figure 5 - Mitotic metaphase chromosomes of *Eupatorium odoratum*. a, Karyotype indicating the presence of ten basic chromosomes, each represented six times; b, two pairs of SAT-chromosomes observed in separate cells. Bar represents 5 μ.

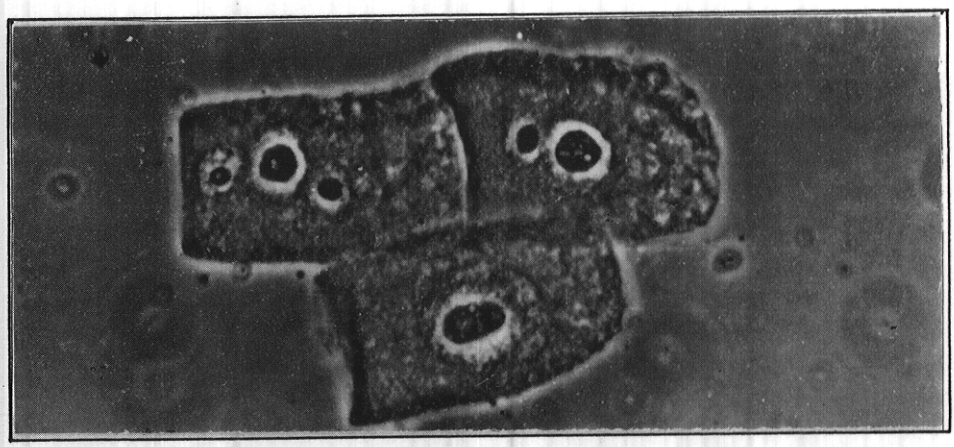

Figure 6 - Optical section through clarified root tip cells of *Eupatorium odoratum* with one, two and four nucleoli. 1375X.

microsporogenesis. Although pairing relations can admittedly be misleading in diplosporous species, this situation suggests the possibility that there may in fact be two basic sets in the complement of *E. odoratum* which, while being morphologically undifferentiated, are nevertheless sufficiently modified in their homologies to result in preferential pairing within two triploid sets. This possibility seems all the more likely since strict autopolyploids of non-hybrid origen are generally not successful colonizers (Stebbins, 1971, 1985), whereas *E. odoratum* is successful and agressive. If the complement of *E. odoratum* is in fact composed of two basic sets, apomictic hexaploid *E. odoratum* would very probably have originated from hybridization between facultatively apomictic triploid populations in consequence of the fertilization of triploid eggs by triploid male nuclei produced by unreduced pollen grains.

Although formulated specifically to apply to sexual species, Stebbins' secondary contact hypothesis (1985) may well be applicable to *E. odoratum*. In part, this hypothesis states that hybridization between previously isolated and adaptively differentiated populations, including interfertile races of a single species, can result in favorable heterozygous gene combinations which impart agressivity. In sexual species heterozygosity would be buffered by tetrasomic inheritance and preferential pairing of fully homozygous chromosomes. However, an even stronger buffering effect would be acheived by apomixis since gene segregation does not occur and the genotype is passed essentially unmodified through the generations. In an apomictic species preferential pairing is of interest in that it probably indicates a fairly high degree of chromosomal differentiation, but it could be expected to be adaptively neutral when occurring during microsporogenesis.

E. odoratum is a plant of open, sunny areas or forest margens. Before the

wide spread destruction of forests, it is highly probable that numerous, well isolated areas of this type existed in eastern Brazil. Adaptively differentiated populations of the triploid progenitor of hexaploid *E. odoratum* could be expected to have evolved in response to this patchy distribution pattern. The destruction of the forests and the concomitant expansion of open, sunny areas would have permitted numerous opportunities for contact and hybridization between these populations. It can be expected that some of the resulting hybrids would have had favorable heterozygous gene combinations for agressivity which would have been transmitted intact by apomixis. Clearly, secondary contact could have been initially occasioned by natural events before the arrival of Europeans in South America. Secondary contact could also have been an important factor in the evolution of the apomictic triploid species of *Eupatorium* which occur in Brazil, such as the agressive *E. squalidum.*

The characterization of the basic chromosome set of *E. odoratum* (Table III) demonstrates it to be essentially identical to that of apomictic triploid *E. squalidum* (Figure 7). Since *E. squalidum* frequently occurs together with *E. odoratum* and has been shown regularly to produce seedlings with 60 chromosomes by sexual reproduction (Coleman and Coleman, 1988), it is appealing to suppose that *E. odoratum* is the autohexaploid of *E. squalidum.* This possibility is being investigated and will be reported on in a later paper.

Table III - Mean arm length values with standard errors (μ) and percent of total length (27.43 ± 1.17) for the basic chromosome set of *Eupatorium odoratum.*

Chromosome number	Long arm	Short arm	*Total chromosome* length	Arm index	*% total length* of set
1	1.62 ±0.03	1.44 ±0.03	3.06 ±0.06	1.13	11.18 ±0.12
2	2.08 ±0.06	0.94 ±0.03	3.02 ±0.09	2.21	10.95 ±0.22
3	1.79 ±0.04	1.10 ±0.02	2.89 ±0.06	1.63	10.55 ±0.25
4	1.66 ±0.03	1.20 ±0.02	2.86 ±0.05	1.38	10.48 ±0.33
5	1.70 ±0.06	1.15 ±0.03	2.85 ±0.08	1.48	10.33 ±0.37
6	1.52 ±0.04	1.17 ±0.02	2.68 ±0.05	1.30	9.76 ±0.22
7	1.70 ±0.03	0.96 ±0.01	2.66 ±0.04	1.77	9.74 ±0.31
8	1.33 ±0.03	1.17 ±0.02	2.50 ±0.05	1.14	9.16 ±0.14
9	1.67 ±0.04	0.78 ±0.02	2.45 ±0.05	2.14	8.94 ±0.18
10	1.44 ±0.04	1.00 ±0.02	2.45 ±0.06	1.44	8.91 ±0.14

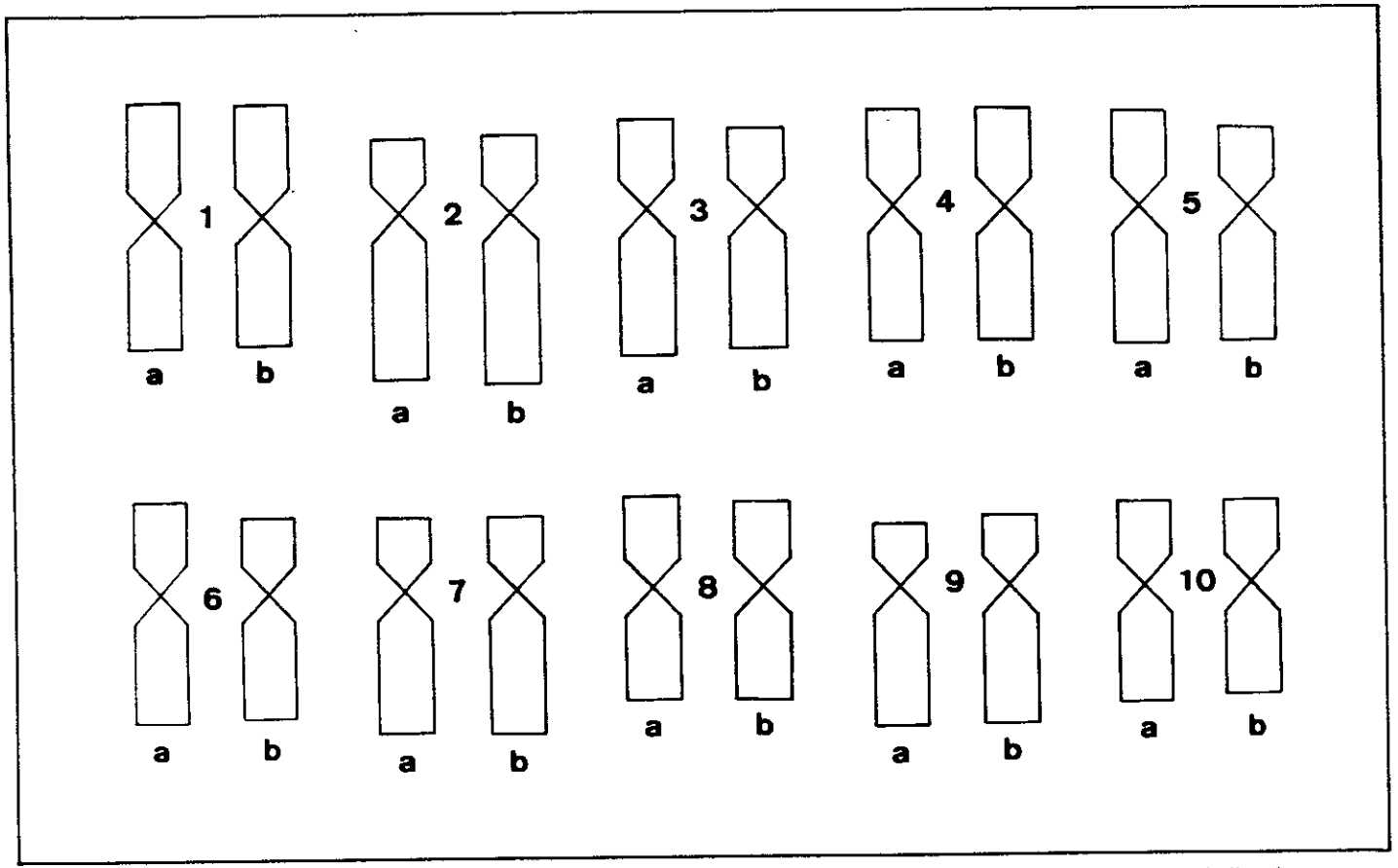

Figure 7 - Ideograms which compare basic chromosome sets of, a, *Eupatorium odoratum* and, b, *E. squalidum*, with most similar chromosomes matched. Ideograms of *E. squalidum* based on Coleman and Coleman, 1988.

ACKNOWLEDGMENTS

The initial stages of this investigation received financial support from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the later stages from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Sincere appreciation is expressed to both these agencies. Thanks is also expressed to Maria Helena Carabolante for technical assistance.

RESUMO

Estudos embriológicos indicam que *Eupatorium odoratum* é caracterizada por diplosporia do tipo Antennaria, desenvolvimento autônomo do endosperma e desenvolvimento precoce pronunciado dos embriões. O saco embrionário é do tipo Polygonum e os núcleos polares não se fundem; ambos aparentemente participam na formação do endosperma. Estima-se que cerca de 20% dos óvulos alcançam ântese com a oosfera não dividida e disponível para a reprodução sexuada. A produção de pólen é extremamente reduzida em conseqüencia da degeneração do tecido esporógeno antes de ocorrer meiose. Microsporócitos na fase de diacinese mostraram-se univalentes, bivalentes e trivalentes, embora em algumas células o pareamento foi bastante reduzido. É demonstrado que *E. odoratum* é autohexaploide, havendo um grupo básico de dez cromossomos repetido seis vezes. Sugere-se que *E. odoratum* apomítica e hexaploide originou-se da hibridização entre populações facultativamente apomíticas e triplóides que foram previamente isoladas e diferenciadas adaptativamente, de acordo com a hipótese de contacto secundário formulado por Stebbins.

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(Received April 5, 1989)