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Cytology of parthenogenesis of five *Meloidogyne* species

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Summary – Oogenesis was investigated using a fluorescent staining technique with Hoechst 33258 in several isolates of facultative meiotic parthenogenetic -*Meloidogyne hapla* race A, *M. chitwoodi, M. fallax* – and obligate ameiotic (mitotic) parthenogenetic – *M. hapla* race B, *M. javanica* – and isolate Xa of an unidentified species. Without fertilisation, the somatic chromosome number was restored by fusion of pronucleus and second polar body in *M. hapla* race A, *M. chitwoodi,* and *M. fallax* isolates. In oocytes of isolate Xa, a second meiotic division occurred in a few cases while in all other cases the somatic chromosome number was restored by duplication of the chromosomes of the egg nucleus after the first division. In one mono-female line of Xa, embryonic development occurred within the body of the female and was genetically controlled. In *M. hapla* race B and *M. javanica,* maturation of the oocytes consisted of one mitotic division. This study confirmed that a large variation of the mode of reproduction exists in *Meloidogyne* spp., which may be a reason for their world-wide success. In one *M. hapla* race A isolate, only 12 % of the oocytes of inseminated females contained sperm, and prophase I instead of prometaphase I oocytes were found posterior to the spermatheca. © Orstom/Elsevier, Paris.

Résumé – ***Étude cytologique de la parthénogenèse chez cinq espèces de* Meloidogyne** – La présente étude a porté sur l'ovogenèse de plusieurs isolats à parthénogenèse méiotique facultative – *Meloidogyne hapla* race A, *M. chitwoodi*, *M. fallax* – et à parthénogenèse améiotique (mitotique) – *M. hapla* race B, *M. javanica* et l'isolat Xa appartenant à une espèce non identifiée – et ce au moyen d'une technique de coloration fluorescente au Hoechst 33258. En l'absence de fécondation, le nombre de chromosomes somatiques est rétabli par fusion du pronucleus et du second corps polaire chez les isolats de *M. hapla* race A, de *M. chitwoodi* et de *M. fallax.* Dans les oocytes de l'isolat Xa une deuxième division méiotique, exceptionnelle, se produit ; dans tous les autres cas la réduction chromosomique est compensée par la duplication des chromosomes dans le noyau de l'œuf après la première division. Chez l'une des lignées mono-femelles de l'isolat Xa le développement embryonnaire, génétiquement contrôlé, se produit à l'intérieur de la femelle. Chez *M. hapla* race B et *M. javanica* la maturation des oocytes consiste en une division mitotique. Cette étude confirme la grande variabilité des modes de reproduction des espèces de *Meloidogyne* ce qui peut expliquer leur succès mondial. Dans un isolat de *M. hapla* race A, seuls 12 % des oocytes des femelles fécondées contiennent des spermatozoïdes et ce sont les oocytes en prophase I - au lieu de la prométaphase I - qui sont observés en position postérieure à la spermathèque. © Orstom/Elsevier, Paris.

Key-words : meiosis, *Meloidogyne arenaria*, *M. fallax*, *M. hapla*, *M. javanica*, parthenogenesis, root-knot nematode.

Root-knot nematodes (*Meloidogyne* spp.) are widely distributed plant pathogens with a very wide host-range. They cause more economic damage to food crops than any other group of plant-parasitic nematodes (Lawrence Apple, 1985). Several species reproduce by either facultative meiotic or obligate ameiotic (or mitotic) parthenogenesis. The apomicts are geographically more widespread, ecologically less restricted, and more polyphagous than their amphimictic relatives (Triantaphyllou, 1985*a*), a phenomenon also described in other animals (Schultz, 1977; Lokki, 1983). Besides parthenogenesis, these nematodes have undergone extensive cytogenetic diversification, with chromosome numbers varying from 2n = 14 in the amphimictic *Meloidogyne spartinae* Whitehead, 1968, to 2n = 51-56 in the apomictic *M. arenaria* Chitwood, 1949 (Triantaphyllou, 1985*a*; Eisenback & Hirschmann-Triantaphyllou, 1991). Males play a facultative role in reproduction of meiotic parthenogenetic populations. In ameiotic populations, males may be produced, but their function is still unclear.

An in-depth knowledge of cytogenetics is a prerequisite for studying the genetic variation in parthenogenetic *Meloidogyne* spp. The oogenesis of ameiotic *M. arenaria*, *M. hapla* race B Chitwood, 1949, *M. incognita* Chitwood, 1949, and *M. javanica* Chitwood, 1949 and of meiotic *M. hapla* race A has been studied earlier (Triantaphyllou, 1962,1963, 1966, 1981; Dalmasso, 1973; Dalmasso & Bergé, 1975). We have used Hoechst 33258 and fluorescent UV microscopy to investigate the oogenesis of several *Meloidogyne* populations. The results are presented and their relevance to the reproduction of *Meloidogyne* spp. is discussed.

Materials and methods

NEMATODE ISOLATES

Several isolates of *M. chitwoodi*, *M. fallax* Karssen, 1996, *M. hapla* races A and B, *M. javanica*, and isolate Xa of an unidentified *Meloidogyne* species were used (Table 1). According to morphology and malate dehydrogenase pattern, isolate Xa is related to the *M. arenaria* group (Van der Beek, 1997).

Almost all isolates were collected from various sites in the Netherlands (Table 1). All isolates were maintained on *Lycopersicon esculentum* cv. Moneymaker. Eggs and females were obtained from plants inoculated with one to three second-stage juveniles (J2) per ml silver sand. The plants were maintained in a growth cabinet at 20 °C and 70 % relative humidity with 16/8h light/dark. The isolates were found to be true to species by isozyme pattern (Esbenshade & Triantaphyllou, 1990) and by Internal Transcribed Spacer (ITS) region of rDNA (Zijlstra *et al.*, 1995).

Chromosome numbers were determined for the following isolates: *M. hapla* race A isolate Hj ($2n = 34$),

Table 1. *Origin of the isolates of* Meloidogyne *spp.*

Isolate	Location*	Final host	Obtained from**
M. chitwoodi			
Ca	NL	Maize	PD
Cb	NL	Wheat	PD
Cj	NL	Unknown	PD
Co	NL	Black salsify	PAV
Caq	NL	Potato	CPRO
M. fallax			
Fb	NL	Beet	PD
M. hapla race A			
Ha	NL	Peony	PD
Hb	NL	Astilbe	PD
Hc	NL	Aconitum	PD
He	NL	Potato	PAV
Hf	NL	Carrot	PAV
Hi	NL	Immortelle	PAV
Hj	NL	Carrot	PAV
Hk	NL	Chicory	PAV
Hak	NL	Carrot	HLB
Haw	France	Grape	INRA
M. hapla race B			
Hh	NL	Fallow (weed)	PAV
Han	South Korea	Hosta	PD
Hbt	NL	Unknown	BLGG
M. javanica			
Ja	China	Bonsai	PD
Unidentified *Meloidogyne* sp.			
Xa	Unknown	Unknown	PD

* NL = The Netherlands

** PD = Plant Protection Service, Wageningen, The Netherlands; CPRO = Centre for Plant Breeding and Reproductional Research, Wageningen, The Netherlands; PAV = Applied Research and Field Production of Vegetables, Lelystad, The Netherlands; HLB = Hilbrands Laboratory for soilborne Diseases and Pests, Assen, The Netherlands; INRA = Institut National de Recherche Agronomique, Montpellier, France; BLGG = Laboratory for Soil and Crop Testing, Oosterbeek, The Netherlands.

M. hapla race B isolate Han (2n *ca* 42), *M. chitwoodi* isolate Ca (2n = 36), *M. fallax* isolate Fa (2n = 36), *M. javanica* isolate Ja (2n *ca* 46), and isolate Xa of a *Meloidogyne* sp. (2n *ca* 36).

MONO-FEMALE ISOLATES

To study the parthenogenetic nature of isolate Xa, 29 J2 were inoculated singly on root tips of *Solanum tuberosum* cv. Eigenheimer, grown on 2 % water agar in 29 Petri-dishes kept at 20 °C in the dark. Seven weeks after inoculation, egg-masses of two selected females, forming two so-called mono-female isolates, were hatched in tap water at 20 °C, and 60 J2 were inoculated in the same manner as above on root tips in Petri-dishes. Seven females were studied from this culture, together with the two original females. J2 from the two mono-female isolates were also inoculated on tomato plants in a climate chamber. Young egg-producing females were collected 7 weeks after inoculation and used for isozyme electrophoresis.

PREPARATION OF MICROSCOPIC SLIDES OF FEMALES AND EGGS

Seven weeks after inoculation, infected roots were put into a Petridish with a 0.9 % NaCl solution, and white undamaged females seen on a dark background were collected under the binocular microscope (6.5 ×). Females were prepared on slides according to the method of Triantaphyllou (1985*b*).

Eggs were collected as follows. Egg masses were removed from the roots and vigorously shaken for 3 min in a solution of 5 % NaOCl to remove the matrix. The egg suspension was poured onto a 20 µm mesh micro-sieve, rinsed several times with tap water, and washed into a small container. With a micropipette, eggs were transferred to a slide, dried in ambient air, and preserved or immediately used for fixation. Only intact eggs were used for our observations.

FIXATION AND STAINING OF FEMALES AND EGGS

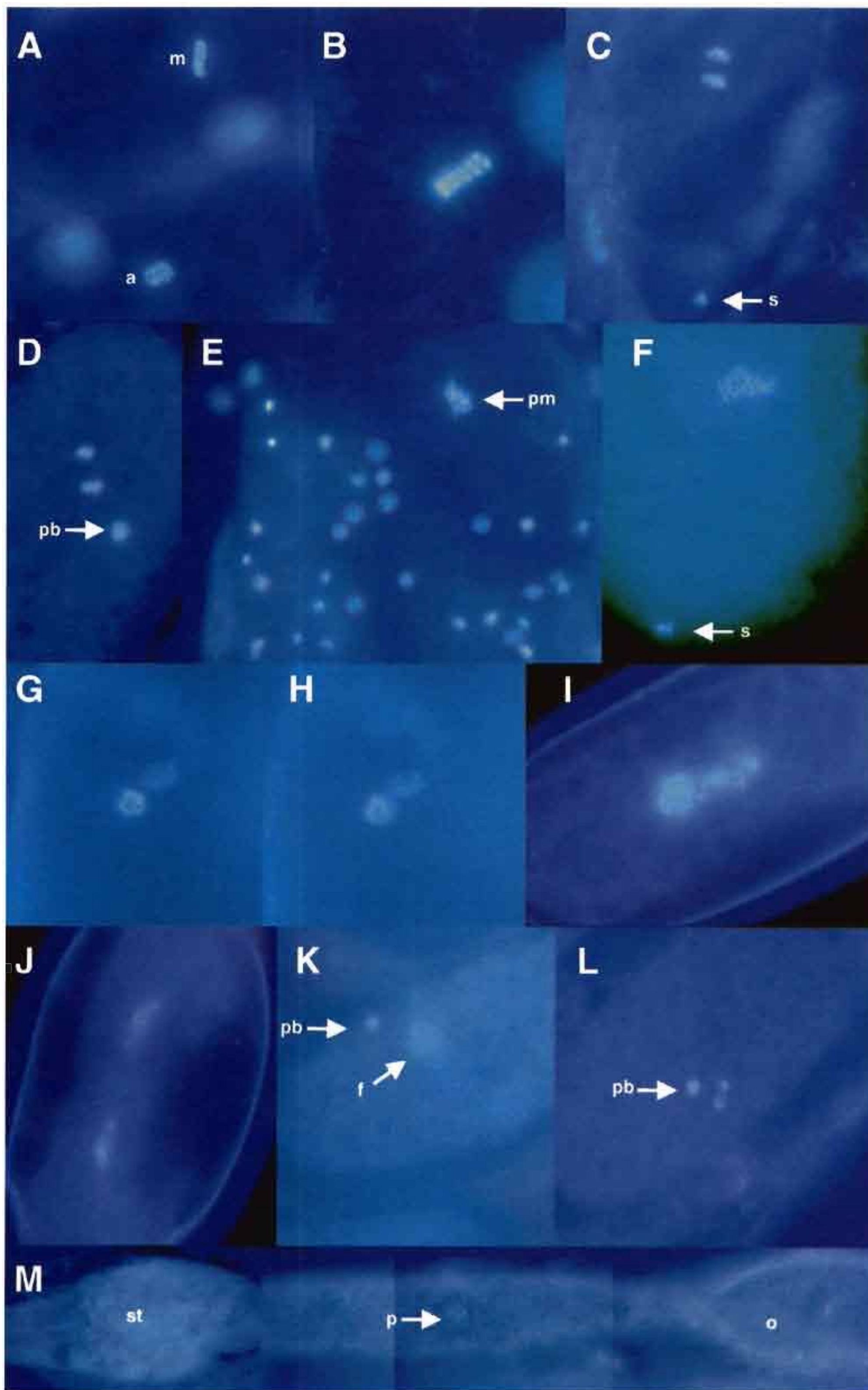
Triantaphyllou (1985*b*) stained the females of *Meloidogyne* spp. with propionic-orcein stain. We used Hoechst 33258, which was used for the first time for nematode cytology by Albertson *et al.* (1978) and which gives a better contrast between cytoplasm and chromosomes. The following fixation and staining procedure is a modified combination of the methods of Triantaphyllou (1985*b*) and Albertson *et al.* (1978). Smears of females or eggs were hydrolysed in 1 N HCl for 10 min at room temperature to extract RNA. The slides were then fixed in Carnoy's solution (3 glacial acetic acid: 1 ethanol 100 %) for 30 min, and dried to ambient air. The slides were soaked in 0.1 M PBS at pH 7.2 for 5 min, immediately stained in 1 µg/ml Hoechst 33258 in 0.1 M PBS at pH 7.2 for 5 min, and finally rinsed in either tap water or 0.1 M PBS at pH 7.2 for 1 min. After drying, the smears were embedded in 50 % glycerine and the coverslips sealed with nail polish. Stored in a dark place, the slides kept their quality for at least one year.

The slides were examined under a Zeiss Axioplan UV microscope with excitation filter G365, beam splitter FT395, and barrier filter LP420.

Results

OOGENESIS IN *M. HAPLA* RACE A, *M. CHITWOODI*, AND *M. FALLAX*

Oogenesis of the *M. hapla* race A isolates Ha, Hb, Hc, Hf, Hi, Hk, Hj, Hak, and Haw was found to be exactly as described earlier (Triantaphyllou, 1966; Dalmasso, 1973; Dalmasso & Bergé, 1975) for the isolates studied by these authors, *i.e.*, typically with two meiotic divisions. In short, in the gonad, the sequential stages of oogenesis in caudal direction are as follows: the apical epithelial cell (called the cap cell), oogonia that are characterized by mitotic divisions, and primary oocytes in synapsis during which chromatin can be condensed. The first maturation division (Fig. 1A, B) takes place in the centre of the oocyte, with a spindle oriented lengthwise in the oval oocyte. The haploid number of elements with four chromatids, especially distinct during diakinesis and prometaphase I, indicates a normal pairing of the tiny chromosomes. Metaphase I occurs when the oocyte passes through the spermatheca and enters the uterus. Anaphase I begins in the uterus and a haploid number of elements, consisting of two parallel chromatids, separate towards each pole, oriented perpendicularly to the equatorial plane (Fig. 1C). These configurations are determined by the holokinetic nature of the chromosomes (Goldstein & Triantaphyllou, 1978; Van der Beek, 1997). One of the sets becomes the first polar body. It is expelled and disintegrates after oviposition and before the second cleavage division. The second maturation division (Fig. 1D) generally takes place perpendicularly to the cell surface. The fate of the resulting haploid second polar body and pronucleus, which takes an interphase structure, depends on the presence of a spermatozoon. In metaphase I oocytes of inseminated females, a spermatozoon almost always enters at the rostral end when the oocyte passes through the spermatheca. This spermatozoon stays far from the bivalents (Fig. 1C) and does not change position and morphology until oviposition. Then it migrates towards the oocyte centre where it takes an interphase structure. A fusion of the female and male pronuclei takes place, for a single nucleus is observed before the first cleavage division. The second polar body is expelled shortly after oviposition. If the oocyte is not fertilised, normally because of absence of sperm in the spermatheca, the two inter-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phase nuclei resulting from the second maturation division fuse in the centre of the egg after oviposition.

M. chitwoodi isolates Cb, Cj, Ck, Co, and Caq and *M. fallax* isolate Fb have a similar oogenesis, with or without sperm, which also demonstrates a facultative and meiotic parthenogenetic type of reproduction.

In the seventeen inseminated females of *M. hapla* isolate He, 33 of the 379 oocytes studied did not contain sperm, ranging from 50 to 100 % in individual females. These oocytes were observed in the uterus directly posterior to the spermatheca. They were often found to be in late prophase I whereas they should have been at least in metaphase I (Fig. 1M). Typically, sperm was observed not only in the spermatheca but also in the lumen of that region of the uterus.

OOGENESIS IN *M. HAPLA* RACE B AND *M. JAVANICA*

Oogenesis of *M. hapla* race B (Fig. 2) and *M. javanica* isolates was also identical to what was described by Triantaphyllou (1962). Only univalents were observed at the end of prophase I and during prometaphase I, which indicates absence of chiasmata formation and of genetic cross-over. The single maturation division is mitotic and produces two sets of chromosomes, each with the somatic number of one-chromatid chromosomes (Fig. 1G, H). The orientation and position of the spindle is as described above. One set of chromosomes migrates towards the cell surface, where it disintegrates as the first polar body after oviposition. The other set takes an interphase structure (Fig. 1I), then starts embryonic development after deposition (Fig. 1J). In inseminated females, a spermatozoon enters the metaphase I oocyte as described above, but it does not interfere with the maturation of the oocyte and probably degenerates without any migration (Fig. 1F). This demonstrates that obligate ameiotic parthenogenesis occurs also in the Dutch *M. hapla* race B populations and in the Chinese *M. javanica*.

OOGENESIS IN ISOLATE XA

In isolate Xa, oogenesis follows a different pattern (Fig. 2). Bivalents are formed and, at anaphase I, the reduced number of two-chromatid elements, oriented parallel to the spindle, goes to each pole. At telophase, one set of elements takes a flocculated appearance, while the other set remains contracted and becomes the first polar body, which moves towards the surface (Fig. 1K), then degenerates after oviposition.

The flocculated set enters mitosis of the first cleavage division early after oviposition. Females may become inseminated by males and the spermatozoon enters the oocyte normally when in prometaphase I. However, in inseminated females no spermatozoon could be found in oocytes in stages beyond metaphase I.

In three oocytes out of more than 100, a second maturation division was observed similar to that in *M. hapla* race A (Fig. 1L). It concerned two oocytes from an inseminated female and one oocyte from a non-inseminated female, neither of them containing a spermatozoon. This type of meiosis occurred rarely in two subsequent parthenogenetic generations of mono-female lines in which parthenogenetic reproduction could be inferred from the production of offspring by single females.

Although cleavage divisions normally start after oviposition, all eggs of one female started embryonic development within the body of this female. Apparently, all the parthenogenetic mono-female progeny of this female shared this trait.

Discussion

These studies on meiotic divisions of various isolates of meiotic parthenogenetic *M. hapla* race A and of ameiotic parthenogenetic *M. hapla* race B and *M. javanica* confirm earlier work on the cytology and oogenesis of these *Meloidogyne* spp. (Triantaphyllou, 1962,1963, 1966, 1981; Dalmasso, 1973; Dalmasso & Bergé, 1975). Oogenesis of *M. chitwoodi*, *M. fallax*, and, in a few cases, isolate Xa, seemed to be similar to that of the other investigated meiotic parthenogenetic species.

The various meiotic systems occur in populations of seven species from various sites world-wide (Eisen-

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Fig. 1. *Oogenesis in* Meloidogyne *spp. A: Metaphase I (m) and early anaphase I (a) in* M. hapla *race A isolate Hi; B: Metaphase I in* M. hapla *race A isolate Hj; C: Early telophase I with penetrated sperm (s) in* M. hapla *race A isolate Hj; D: Telophase II with the first polar body (pb), in* M. chitwoodi *isolate Cz; E: Prometaphase I (pm) in* M. hapla *race B isolate Hh, passing through the spermatheca in which sperm is visible; F: Metaphase I in* M. hapla *race B isolate Hh with penetrated sperm (s); G, H: Interkinese in* M. hapla *race B isolate Hh using different focusses; I: Egg outside the female body just prior to first cleavage division in* M. hapla *race B isolate Hh; J: First cleavage division in* M. hapla *race B isolate Hh; K: Oocyte after the first maturation division in isolate Xa of unidentified* Meloidogyne *sp., showing first polar body (pb) and the second set chromosomes in flocculated appearance (f); L: A rare late anaphase II in isolate Xa of unkown* Meloidogyne *sp. (pb = first polar body)); M: Spermatheca and the adjacent anterior part of the uterus in* M. hapla *race A isolate He showing successively a spermatheca (st) filled with sperm, two oocytes in prophase I, of which one clearly visible (p) and an oocyte in metaphase I (o) without sperm in the oocytes (A 100× objective was used for all illustrations, except for B: 200× objective.)*

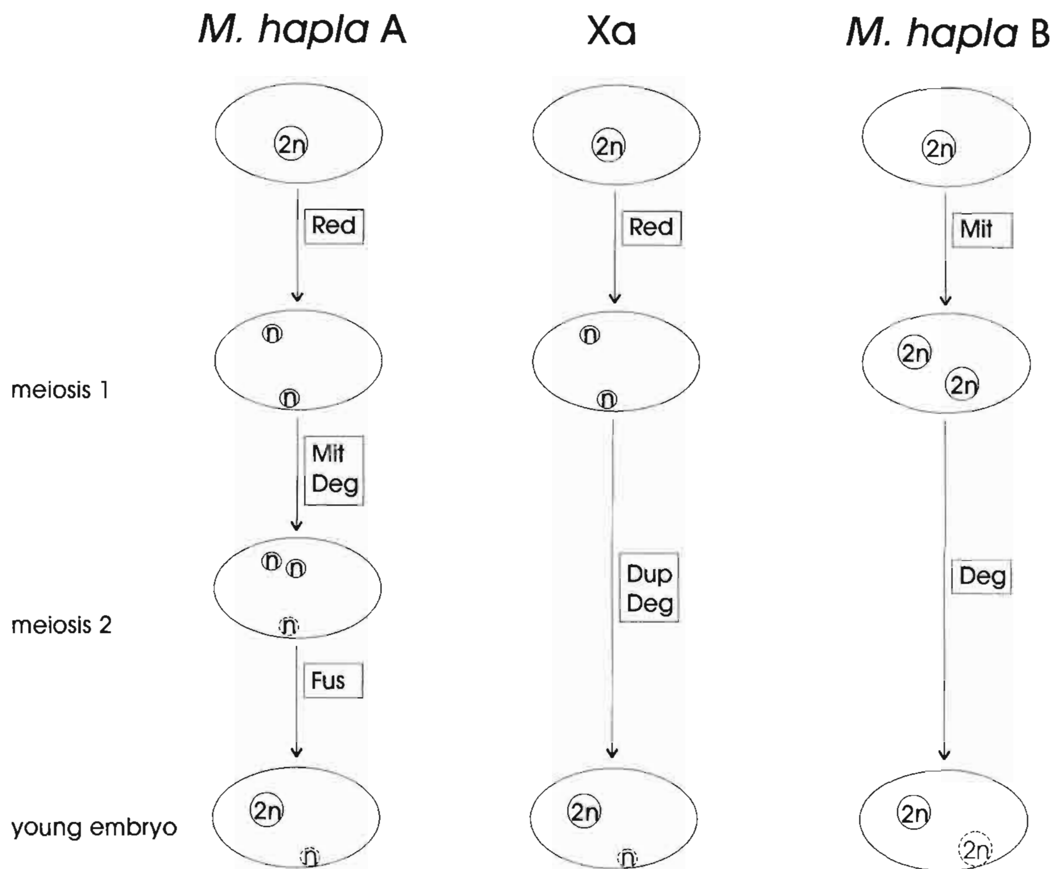

Fig. 2. *Schematic representation of parthenogenetic pathways in meiotic and ameiotic parthenogenesis of* Meloidogyne hapla *race A and race B, respectively, compared to that of isolate Xa (Abbreviations: 2n = set of chromosomes with the somatic number; n = set of chromosomes with the haploid number; Deg = degeneration of the first polar body; Dup = chromosome duplication; Fus = fusion of second polar body with the egg pronucleus; Mit = mitotic division; Red = reductional division).*

back & Hirschmann-Triantaphyllou, 1991), which seems to indicate that they have arisen several times.

That reproduction in this genus is still open to changes is indicated by some odd observations. Firstly, in *M. hapla* race A isolate He, oocytes in prophase were observed in the anterior part of the uterus, in contrast to all other *M. hapla* isolates studied. A comparable behaviour has been reported for oocytes of *M. incognita* (Triantaphyllou, 1981). However, in *M. incognita*, most oocytes in inseminated females were reported with sperm nuclei, whereas in most of our *M. hapla* isolate He, no sperm entered the oocytes. A possible explanation for the aberrant behaviour of these oocytes may be that the egg shell and/ or the cell membrane and the yolk are not yet morphologically and functionally prepared to receive male gametes. This "bad timing" of oocyte development and sperm penetration may trigger obligatory parthenogenesis in an otherwise meiotic population. The low percentage of oocytes with penetrated sperm in isolate He implies only limited possibilities for sexual recombination. Consequently, the genetic composition of this isolate will be more homogeneous, but not necessarily more homozygous, as post-reduction during meiosis will cause conservation of heterozygosis in meiotic parthenogenetic *Meloidogyne* (Van der Beek, 1997).

Secondly, in isolate Xa, most oocytes had only one reduction-maturation division. The chromosome number is probably maintained by chromosome duplication after the first nuclear division, as indicated by the cytology of one set of chromosomes. A similar obligatory parthenogenesis with release of only one polar body has already been reported in the nematode *Xiphinema index*, in which the diploid chromosome number is restored by fusion of the egg nucleus with

the first polar body (Dalmasso, 1974). In addition, this type of meiosis seems to be similar to that found in the tardigrade *Hysibius dujardini* (Ammermann, 1967). Genetically, this aberrant type of meiosis has the same genetic consequences as the meiosis of parthenogenetic *Meloidogyne* spp. with a fusion of the second polar body with the egg pronucleus. A few oocytes were observed with a second maturation division, which points to the ability of completing a normal meiosis and sexual reproduction.

The occurrence of embryonic development within the body of females of isolate Xa appeared to be genetically controlled. The phenomenon had not been recorded before in *Meloidogyne*, though it is known in cyst-forming nematodes (see *i. al.* Golden, 1986). Whether this premature embryonic development is directly caused by the omission of the second meiotic division and the early presence of a diploid nucleus is not known.

This study shows that a large flexibility in meiotic systems exists in *Meloidogyne* spp. This may contribute to a great extent to the global successful spread and establishment of these plant-parasitic nematodes.

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