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Further Studies on the Role of Polyploidy in the Evolution of *Meloidogyne*[1]

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Abstract: Two tetraploid isolates of *Meloidogyne hapla*, 86P and E289P, with haploid chromosome numbers of 34 and 28, respectively, were studied cytogenetically and biologically in relation to the diploid populations, 86-Va (n = 17) and E289-Taiwan (n = 14), from which they had been originally isolated. Both isolates were quite stable, converting to diploidy at the low rate of about 2.5%. The tetraploid isolate 86P maintained itself in competition with its diploid counterpart in mixed cultures, although an initial frequency of 50% polyploidy was reduced to about 9% at the end of the sixth generation. Both tetraploid isolates could maintain themselves in greenhouse cultures without artificial selection for at least 2 years. Crosses between diploid females and tetraploid males resulted in a few triploid females that produced mostly nonviable eggs, suggesting partial reproductive isolation between the two ploidy forms. Ten generations of propagation of only polyploid females of isolate 86P that were associated with males failed to yield an obligatorily amphimictic isolate that would not convert at all to diploidy. If one accepts a previous assumption that the present day amphimictic root-knot nematodes are tetraploids derived from diploid ancestors, results of the present study are not inconsistent with an evolutionary trend toward an even higher level of ploidy in *Meloidogyne*, presumably octaploidy.

Key words: cytogenetics, cytology, evolution, *Meloidogyne hapla*, parthenogenesis, polyploidy, northern root-knot nematode.

In a survey of *Meloidogyne hapla* from 16 countries, polyploidy was detected in 4 of 21 populations (2). Extensive study of polyploid isolate E24P from South Korea revealed that tetraploid females (n = 34) produced 78% tetraploid and 22% diploid (n = 17) progeny by meiotic parthenogenesis. Thus, there was a high rate of conversion to diploidy, and this feature substantially reduced the survival capabilities of this tetraploid isolate. Selection and propagation of tetraploid progeny in nearly every generation were required in order to maintain the tetraploid form in continuous greenhouse culture. Nevertheless, this early study suggested that polyploidy may have played an important role in the evolution of *Meloidogyne* (2).

Examination of other tetraploid isolates discovered in the same survey indicated that tetraploidy in two of them was more stable. Unlike E24P, isolate 86P maintained its tetraploid nature following propagation for several generations without selection. Tetraploid isolate E289P was not as stable, but its rate of conversion to diploidy diminished following consecutive cycles of selection during which only tetraploid juveniles were propagated. Because of these features, isolates 86P and E289P were employed in the present study to further explore the role of polyploidy in the evolution of the genus *Meloidogyne*. Four specific questions were investigated: 1. What is the conversion rate from tetraploidy to diploidy in these isolates? 2. How successful is each tetraploid form in competing with its diploid counterpart? 3. Is it possible to obtain an obligatorily amphimictic, tetraploid form by continuous selection of tetraploid individuals that reproduce by cross-fertilization? 4. Are there viable triploid progeny resulting from crosses between diploid and tetraploid parents?

MATERIALS AND METHODS

The polyploid isolate 86P was established in 1982 by inoculating a tomato seedling with 40 large juveniles obtained from a single egg mass of population 86-Va from Virginia. Cytological preparations (3) indicated that all seven females recovered in the first generation were tetraploid, with n = 34 chromosomes. In the absence of males, reproduction was by meiotic parthenogenesis. In subsequent gen-

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erations a small number of diploid progeny (n = 17) were recovered, suggesting that conversion to diploidy was occurring in this polyploid isolate. The polyploid isolate E289P (n = 28) was originally established in 1982 as an egg-mass isolate from the diploid population E289 (n = 13–14) from Taiwan. This original isolate was subsequently lost, and a new isolate was established in 1983 by inoculating a tomato seedling with seven large juveniles found among tens of thousands of juveniles of the original population E289.

Studies were conducted, in parallel, with both isolates, 86P and E289P. However, because of the similarity of the results, studies with E289P will be reported only in a summary form.

Rate of conversion to diploidy: Five tomato seedlings in 15-cm-d pots were each inoculated with a single polyploid egg mass (i.e., one with large juveniles). The ploidy status of the females developing on these plants was determined 50 days later by making cytological preparations (3) and determining their chromosome numbers (diploids of isolates 86 and E289 had n = 17 and 14, tetraploids had n = 34 and 28, respectively).

In a second test, 30 tomato seedlings were inoculated with a single polyploid egg mass each. Fifty days later, three egg masses were isolated from the roots of each plant and placed in water on small dishes. The state of ploidy of the juveniles that hatched from each egg mass was evaluated according to body length (2) (tetraploid juveniles are 25% longer than diploid juveniles and can easily be recognized at a 25× magnification in a stereo microscope).

To test whether large juveniles can develop to diploid females, 20 tomato seedlings were inoculated with 200 large juveniles each. Fifty days later, the egg masses of females that developed on these plants were placed in water in small dishes. The state of ploidy of each female was determined by body length of the juveniles that hatched from its egg mass.

Competition between polyploids and diploids: Competition between polyploid isolate 86P and its diploid counterpart was evaluated as follows: Two sets of five tomato seedlings were inoculated with 1,000 second-stage juveniles (J2) of the diploid or the polyploid isolate per seedling. Another five seedlings were inoculated each with a mixture of 500 J2 of the diploid and 500 J2 of the tetraploid isolate. Adult females with egg masses in the roots of the plants inoculated with either the diploid or the tetraploid isolate were counted 50 days after inoculation to determine the relative infectivity and rate of development of the first-generation J2 of the two isolates. Eighty days after inoculation, eggs and J2 were NaOCl extracted (1) from the roots of the plants inoculated with the mixture of diploid and polyploid J2. Aliquots of the suspensions containing about 5,000 eggs and J2 were then used to inoculate another five tomato seedlings for establishment of the third generation. Eighty days later, white egg masses of the fourth-generation females present in the roots of the five plants were individually placed in dishes with a small quantity of water. The ploidy of the J2 hatched from the eggs was determined by body length, and the percentage of tetraploid females in each plant was estimated. The egg masses isolated from each plant were subsequently treated with 0.5% NaOCl for 10 minutes to free the eggs, and an aliquot of the egg suspension containing about 5,000 eggs and J2 was used to inoculate another tomato seedling. About 80 days later, the percentage of sixth-generation polyploid females was estimated following the same procedure as described for the fourth generation.

Fate of triploid progeny: Crosses were conducted between diploid females and tetraploid males in order to study the role triploidy may play in a natural system where diploids and tetraploids coexist. Tomato seedlings were inoculated with 50 J2 of the diploid form. Twenty days later, about 200 males of the polyploid form were added to the soil. The plants were washed free of soil 45 days after inoculation, and individual egg masses of females associated with males (i.e., females with one or more males

located between the female and its egg mass) were used to inoculate new tomato seedlings. About 42–48 days later, these plants were washed and all females that had produced egg masses were studied cytologically (3) to determine their degree of ploidy. Egg masses of triploid females were propagated for another generation on tomato, and these plants were examined 50 days later for the presence of adult females.

Selection for obligate amphimixis: In an attempt to obtain a polyploid isolate that would reproduce obligatorily by cross-fertilization, five tomato seedlings were each inoculated with 10 polyploid egg masses of isolate 86P obtained from females associated with males. Fifty days later, 100 females from each plant were examined for association with males and for state of ploidy of their egg masses. Ten polyploid egg masses of females associated with males were used to establish the next generation on a tomato seedling. This selective propagation was continued, separately on five tomato plants, for 10 consecutive generations. One hundred females per plant were examined at the end of the 10th generation to determine the state of ploidy of each female, the frequency of females associated with males, and the presence or absence of egg masses in females not associated with males.

Results

Rate of conversion to diploidy: Of a total of 100 egg-laying females obtained from the roots of five tomato plants (20 females per plant) that had been inoculated each with a single polyploid egg mass of isolate 86P, 98 were polyploid (n = 34) and two (from two different plants) were diploid (n = 17), indicating an overall conversion rate of 2%. In a similar test, the conversion rate of isolate E289P was 1.6%.

In a second test, all juveniles that hatched from each of 900 egg masses of isolate 86P (30 egg masses from each of 30 plants) were either polyploid or diploid. No egg mass gave a mixture of polyploid and diploid juveniles. In some cases, a few juveniles of a polyploid egg mass were slightly smaller than regular polyploid juveniles, but morphological examination revealed that such juveniles had a large stylet, equivalent to that of polyploid juveniles (2). Of the 30 plants examined, 10 had only polyploid egg masses, 15 had 1 diploid and 29 polyploid, and the remaining 5 plants had 2 diploid and 28 polyploid egg masses. The overall rate of conversion to diploidy in this test was about 2.8%. The equivalent test with isolate E289P resulted in a 1.8% conversion rate to diploidy (14 plants with only polyploid egg masses and 16 plants with 1 diploid and 29 polyploid egg masses).

From a total of 535 egg masses of isolate 86P isolated from the 20 tomato plants that had been inoculated with 200 large juveniles 50 days earlier, 14 were diploid and the remainder polyploid. The distribution of the diploid egg masses was relatively uniform among the 20 plants. Thus, a single diploid egg mass was found in 10 of the 20 plants and two diploid egg masses in another two plants. Overall, 2.6% of the large juveniles developed to diploid females that produced diploid egg masses. In a similar test with isolate E289P, 2% of the large juveniles developed to diploid females (10 diploid egg masses found among 489 egg masses checked).

Competition between polyploid and diploid isolates: Infectivity of the second-stage juveniles and the rate of their development to egg-producing females were approximately the same in the diploid and polyploid forms of isolate 86 when propagated separately (Table 1). Thus, approximately 550 egg masses were recovered from the roots of tomato plants inoculated with 1,000 juveniles of either the diploid or the tetraploid isolate.

At the end of the fourth generation of cultures originally established with inoculum consisting of 500 diploid and 500 polyploid J2, only 9.1% of the egg masses scored were polyploid; the rest were diploid (Table 1). This percentage did not change appreciably at the end of the sixth generation, when polyploid egg masses were about 8.7% of the total scored.

The fate of triploid progeny: Crosses be-

TABLE 1. Reproduction† of polyploid isolate 86P and diploid isolate 86D of *Meloidogyne hapla* alone and in competition with each other.

			86D + 86P			
			4th generation		6th generation	
	1st generation		Egg masses		Egg masses	
Plant number	86D	86P	scored	Polyploids (%)	scored	Polyploids (%)
1	589	576	145	8.3	74	8.8
2	563	649	160	7.5	82	9.3
3	487	541	115	8.7	59	10.9
4	510	405	132	11.4	79	8.2
5	673	586	122	9.8	70	6.1
Mean	564	551		9.1		8.7
CV	13%	16%		16%		20%

Each plant was inoculated with 1,000 second-stage juveniles for starting the first-generation single isolate cultures; a mixture of 500 diploid and 500 polyploid juveniles was used for dual isolate experiments (86D + 86P).
† Number of egg-producing females recovered per plant.

tween diploid females and tetraploid males of isolate 86P yielded nine triploid females with haploid chromosome numbers varying from 23 to 28. Each of these females produced a normal-size egg mass which was used to inoculate a new tomato seedling. However, none of the inoculated plants showed any infection 45 days after inoculation. In a supplementary crossing test, egg masses from another three triploid females were incubated for 12 days at 28 C. Only three or four J2 hatched from each egg mass. The rest of the eggs, most of which were at an advanced state of embryonation, did not hatch and were deteriorating. In a similar test, no J2 hatched from eggs produced by 15 triploid females of isolate E289.

Selection for a polyploid, obligatorily amphimictic isolate: Ten consecutive generations of propagation of only polyploid females of isolate 86P that were associated with males neither decreased the rate of conversion to diploidy nor resulted in an obligatorily amphimictic isolate. Thus the conversion rates to diploidy of the original isolate and the isolate that resulted after 10 generations of selection were approximately the same, ranging among individual cultures between 1 and 5%. Similarly, approximately 10–20% of the females of the first and the tenth generations were associated with males and, therefore, had a chance to reproduce by cross-fertilization; the remaining 80–90% apparently reproduced by meiotic parthenogenesis. There were no females that did not produce eggs. The presence of such females would have indicated that they were obligatorily amphimictic, requiring fertilization before initiation of egg production.

DISCUSSION

Three tests in this study indicated that tetraploid females of *M. hapla* produced a small number of diploid, in addition to their normal tetraploid, progeny. The cytogenetic mechanism of conversion from tetraploidy to diploidy could not be studied directly because conversion occurred at a very low rate (ca. 2.5%). However, the observation that a few large juveniles do develop to diploid females suggests that conversion to diploidy in isolates 86P and E289P may follow the same cytological pathway as described for population E24P of *M. hapla* (2). In that population, an occasional oocyte of a tetraploid female (large-size oocyte) may undergo two complete maturation divisions that reduce its chromosome number to the diploid state. Such an oocyte may develop without fertilization to a large juvenile, similar in size to, and consequently indistinguishable from, the polyploid juveniles of the same female. This juvenile will develop into a diploid female, which will produce only diploid juveniles (complete conversion to diploidy). Similarly,

limited cytological observations have indicated that the state of tetraploidy of the rest of the progeny of a tetraploid female is maintained by regular meiotic parthenogenesis or by cross-fertilization, as described for populations E24P (2).

The infectivity and rate of development of the diploid and tetraploid juveniles used in the competitiveness test was very similar when 1,000 juveniles of either isolate were used to inoculate individual plants. When 500 diploid and 500 polyploid juveniles were used as inoculum and serially subcultured, the polyploid isolate comprised only 9.1% of the recovered egg masses by the fourth generation. This low frequency, indicating moderate or poor competitiveness of the polyploids, was maintained at approximately the same level through the sixth generation. Additional observations from various greenhouse cultures during these studies indicated that both tetraploid isolates could maintain themselves in competition with their diploid counterparts without artificial selection for at least 2 years. The low rate of conversion to diploidy and, possibly, their higher competitiveness distinguish these tetraploid isolates from isolate E24P, which has a high conversion rate to diploidy and can only be maintained in greenhouse cultures by selection of polyploid egg masses practically in every generation.

Crosses between diploid females and tetraploid males yielded a few triploid females which produced normal-size egg masses. When used as inoculum, such egg masses failed to produce infection on tomato seedlings and gave only a small number of viable juveniles in hatching tests. These limited observations indicate at least a partial reproductive isolation of the tetraploid isolates from the parental diploid forms. Such reproductive isolation may provide stability to the tetraploid isolates and better chances for their successful establishment in nature.

Ten generations of selection of tetraploid, cross-fertilizing females of isolate 86P failed to yield a tetraploid isolate that would not convert to diploidy and would reproduce obligatorily by amphimixis. Nevertheless, this failure does not exclude the possibility that such an evolutionary change could occur in nature. It should be recognized that evolution involves complex, long-range processes that cannot easily be imitated in laboratory experiments of short duration.

It has been postulated that the present amphimictic forms of *Meloidogyne* with a haploid number of 18 chromosomes may not be true diploids but may represent tetraploid forms that have evolved as a result of polyploidization of earlier diploid forms (with n = 9) during a parthenogenetic phase, followed by more recent conversion to obligatory amphimixis (2). If one accepts this assumption, the stability of the 86P and E289P isolates of the present study further suggests that perhaps a higher degree of ploidy, presumably octaploidy, is in the process of being established in meiotically parthenogenetic forms of *Meloidogyne*. The first presumably octaploid isolate (E24P) studied was very unstable, converting to presumed tetraploidy at the high rate of 22% and being unable to maintain itself in continuous culture without artificial selection (2). The present isolates, 86P and E289P, may represent a more advanced step in the evolution toward stable octaploidy with facultative amphimixis, accompanied by reproductive isolation from the tetraploid forms from which they have derived. They have a low rate of conversion to tetraploidy and can maintain themselves for many generations in competition with their tetraploid counterparts.

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