CYTOGENETIC BEHAVIOR OF FERTILE TETRAPLOID HYBRIDS OF *NICOTIANA RUSTICA* AND *NICOTIANA PANICULATA*[1]*

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INTRODUCTION

The hybrid between *Nicotiana rustica* and *N. paniculata* occupies a unique position among the many species hybrids in the genus Nicotiana. It is the only one of two parents differing in chromosome number that exhibits any fertility whatever upon selfing. There are some 70 species in the genus, 26 of which have been investigated cytologically and have been shown to have the following chromosome numbers: (8) or 9, 10, 12, 16, and 24. Many hybrids have been made between species differing in chromosome number. It is therefore quite surprising that thus far these two species are the only ones shown to be sufficiently compatible to produce F_1 hybrids showing fertility. It is also interesting to note that this hybrid between *N. rustica* and *N. paniculata* was the first to be studied genetically, KÖLREUTER'S work on this cross being the first genetical research on record.

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[1] Submitted to the Faculty of the Bussey Institution of HARVARD UNIVERSITY in partial fulfillment of the requirements for the degree of Doctor of Science, June, 1930. This study is a part of a general genetic survey of the genus Nicotiana being carried on at the Bussey Institution and is a joint contribution from that institution and from the CONNECTICUT AGRICULTURAL EXPERIMENT STATION.

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Such a hybrid should prove to be good material for a study of sterility in relation to chromosome number since it is possible to obtain selfed progeny from the F_1 plants and since the bivalents and univalents of the F_1 hybrid can be distinguished so clearly. There are 12 bivalent and 12 single chromosomes at meiosis in the F_1 hybrid. The 12 bivalents behave regularly at meiosis, 12 going to each pole; while the 12 singles pass to either pole, supposedly at random. Hence, there should be produced F_1 gametes varying in chromosome number from 12 to 24, assuming that the 12 univalent chromosomes assort at random. By expanding the binomial $(a+b)^{12}$ it is possible to obtain the frequency of gametes containing from 0–12 of the univalent chromosomes. By a cytological examination of F_2 plants it should be possible to determine which of the F_1 gametes are viable and in what proportions they occur. Undoubtedly, some combinations are non-viable, since the F_1 generation is so low in fertility.

The hybrid between *Nicotiana rustica* and *N. paniculata* was selected for this study partly because of the advantageous features mentioned above and partly in hope of finding a cytological explanation of unusual types found in a previous study made by EAST (1921) of hybrids between these two species. Many striking types were obtained, some nearly exact duplicates of either parent and some resembling each of the known varieties of *Nicotiana rustica*. Plants were also obtained having characteristics not found in either parent and unlike any of the described varieties of *N. rustica*. They showed remarkable variation without the gradation in size and structural details usually found in varietal crosses or combinations of similar species. All of these types were obtained in a cross using the variety *humilis* of *N. rustica* as the female parent. The F_2 population was decidedly more fertile than F_1 plants, which showed only about 3 percent of the normal set of seeds.

The author wishes to express his indebtedness to Professor E. M. EAST, under whose direction the problem was completed, for helpful suggestions and valuable criticisms of the work.

MATERIALS AND METHODS

The variety of *Nicotiana rustica* used in this investigation was received under the name of *N. rustica humilis* Schrank from Mr. M. CHRISTOFF. It is not certain where he obtained the seed, although it was then thought that the stock was a direct descendant of the same variety used by EAST (1921) in a previous study of this hybrid.

Seed of *N. rustica* was also obtained under the name of *N. rustica pumila* Schrank from Doctor T. H. GOODSPEED at the UNIVERSITY OF CALIFOR-

NIA. Plants from each of these sources have been grown for several generations. They seem to be identical in every respect and are undoubtedly the same variety. COMES (1899) gives *N. rustica pumila* Schrank as a synonym of *N. rustica humilis* Schrank. SETCHELL (1912), however, recognizes the two varieties, describing *N. rustica humilis* as a fairly robust plant of low stature and early blossoming and ripening. The leaf is broad and ovate, being broadly, but slightly cuneate at the base. This description of *N. rustica humilis* Schrank is in accord with the descriptions and illustrations of both EAST and COMES.

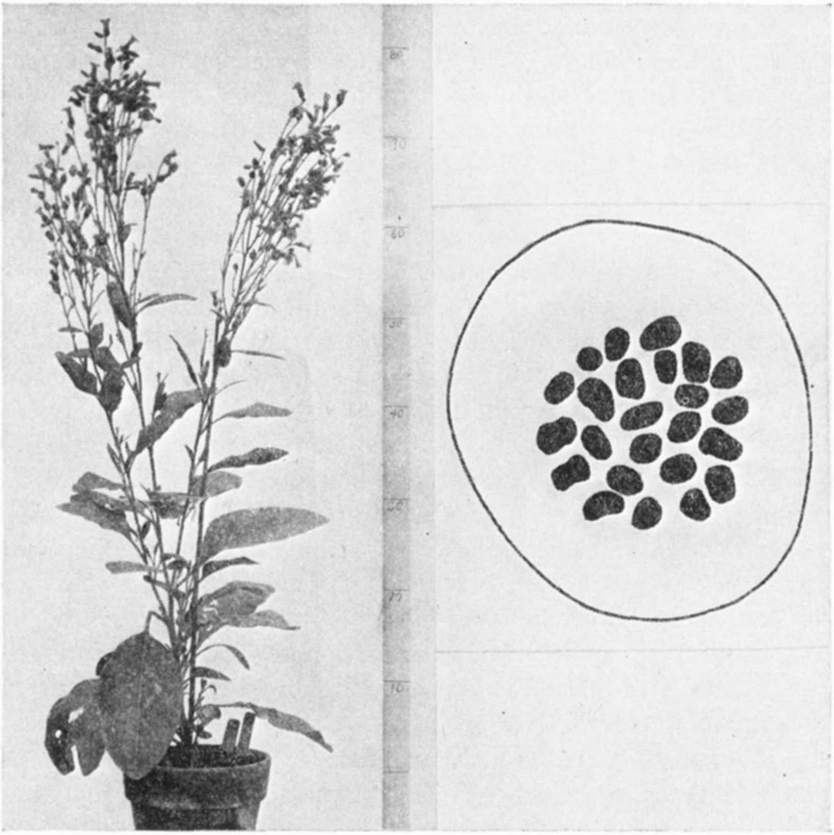


FIGURE 1.—*N. rustica pumila* Schrank. Right, heterotypic metaphase in pollen mother cell; 24 pairs of chromosomes. ×2700.

According to SETCHELL, *N. rustica pumila* Schrank is a low plant, loose in habit, and matures early. The leaves are ovatelanceolate and unequal at the base. The plants we have grown answer very well this description and are more correctly identified as N. *rustica pumila* Schrank than as N. *rustica humilis* Schrank and are different from the plants used by EAST. The average height is 24 to 30 inches whereas the plants grown by SETCHELL averaged 12 to 14 inches in height. This seems to be the only difference and probably can be accounted for by different environmental conditions. The flowers are short, robust, and pale greenish yellow in color. They are produced in an open panicle loosely branching. There are 24 pairs of chromosomes. A fairly typical plant is shown in figure 1.

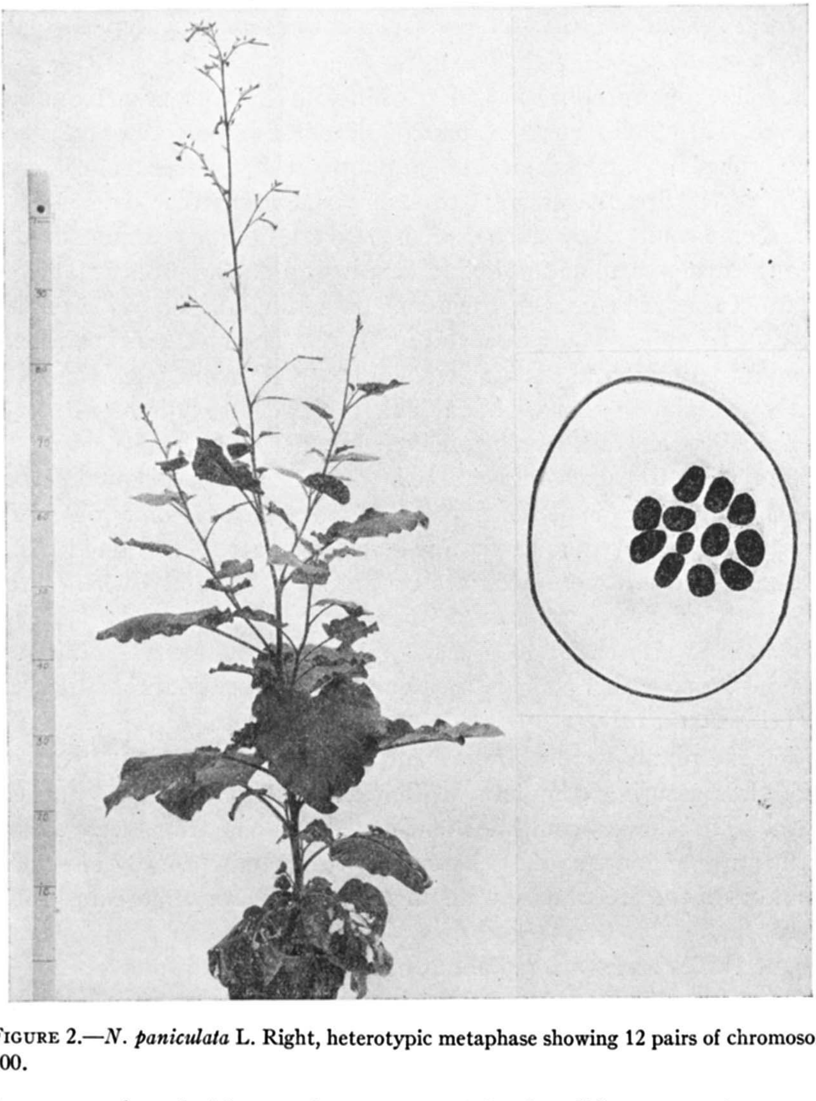


FIGURE 2.—*N. paniculata* L. Right, heterotypic metaphase showing 12 pairs of chromosomes. ×2700.

The other parent, *N. paniculata,* is quite constant in habit of growth wherever grown. It is a tall, dark green, glabrous leaved plant, loosely branching. The leaves are cordate in shape. The long, slender, tubular, green flowers are produced in open, loosely branched panicles. The plant is late maturing, flowering fully a month to six weeks later than *N. rustica pumila. N. paniculata* has 12 pairs of chromosomes. A typical plant is shown in figure 2.

The following procedure was adopted in studying the parents and hybrid material. The plants were examined for chromosome number, the fertility of the plant was obtained, and a photograph was taken of each plant. As soon as the first flowers appeared, material was gathered for obtaining chromosome counts. One anther of the bud studied was examined according to the aceto-carmine method, and if it were in the proper stage of development the bud containing the four remaining anthers was then killed in modified Bouin's solution, B–15 of ALLEN. The material was then embedded in paraffin, and sections were cut 8 to 10 micra in thickness. The slides were stained either with Haidenhain's haematoxylin or with crystal violet. The latter stain seems to have some decided advantages over the haematoxylin. Chromosomes stained with it have a deep violet color while the cytoplasm is perfectly clear. Also the chromosomes out of focus are not as nearly opaque as the jet black haematoxylin-stained chromosomes, and hence a more accurate count can be obtained when chromosomes are lying directly over each other or very close together. The crystal violet stain is also easier to use and requires less time in staining. The method for using this stain is the same as for NEWTON'S gentian violet stain (HUSKINS 1927).

When the plants were examined for fertility, five flowers of each were selfed, thus assuring complete pollination. Every flower was tagged so that the selfed flowers could be identified. The pollinations were all made within the greenhouse so that there was no contamination by insects. The ventilators of the greenhouse were all screened as soon as insects appeared in the spring.

About three weeks after pollination (before the capsules opened) the capsules were all gathered and the calyx removed with a very fine scalpel. Then one-half of the ovary wall was carefully "peeled" and an estimate of the percentage of a full set of seed was determined for each capsule. The average of the individual capsules gave the fertility grade of the plant in question. The plants were grouped into 11 arbitrary classes, from 0 to 10. It should be understood that the classes are not exact and some plants may have been wrongly classified as the number of flowers examined had to be small for each plant. The chance for the greatest error comes in the first class (0). The plants thrown into this class might have shown some fertility if enough flowers had been examined.

In order to have a complete record of each plant for future reference, each was photographed shortly after it began to flower. By this method a record of the height of each plant was also obtained.

Pollen examinations were made by the aceto-carmine method. An anther from a flower newly opened was touched to a drop of aceto-carmine on a clean slide. When all of the loose pollen was then taken up by the solution on the slide, a round cover glass was mounted, the edges of which were ringed with shellac by means of a small hand turn table. Several sealing preparations were used to ring the slides, but none proved as satisfactory as shellac. It makes an air tight cover and it is easily removed when the slide is cleaned. Boiling the slides for a few minutes hardens the shellac and it chips off, leaving the slide and coverglass perfectly clean.

F_1 GENERATION

The F_1 hybrid can be made successfully only one way, by pollinating *N. rustica* with the pollen of *N. paniculata.* Seed thus produced shows almost complete germination. The reciprocal pollination produces a full complement of apparently well developed seeds, most of which fail to germinate. Doctor EAST has obtained germination of this reciprocal hybrid, but with considerable difficulty.

In appearance, the F_1 plants are about intermediate between the two parents, although they resemble the *rustica* parent a little more closely. The flowers are almost exactly intermediate as can be seen in figure 3.

The F_1 plants grown in the field are all very uniform in regard to height, leaf shape, flower shape and other external characters. When grown in the greenhouse, however, there is considerable variation in the shape of leaves on the same plant. All plants behave alike in this respect. The shape varies all the way from lanceolate leaves to those almost as cordate as the leaves of the *paniculata* parent, the older leaves being the most cordate. A typical F_1 plant as grown in the field is shown in figure 4. Figure 5 shows the wide variation of leaves from a single F_1 individual grown in the greenhouse. As the leaves mature they become more cordate. In the *paniculata* parent the young leaves are as cordate as the older ones. Just how to account for this variation is a question. It does emphasize the fact that normal development can be secured only in the field under natural conditions.

The F_1 hybrid is always very vigorous and manifests considerable heterosis. In spite of the fact that an abundance of foliage and flowers is produced, the hybrid is almost sterile. Less than 1 percent of a normal set of seeds is produced, selfed seeds being obtained with considerable difficulty. Because of the difficulty of obtaining selfed seed a large population of 150 F_1 plants was grown in the field and allowed to interpollinate. The F_1 plants grown in the field showed no greater fertility than hand pollinated greenhouse-grown plants. Hand pollination of the plants in the field was no more successful than insect and wind pollination. The plants were watched carefully all summer and any capsules formed were gathered. It is certain that the seed produced was either self or sib-pollinated as no other Nicotiana plants of any sort were grown in the immediate vicinity. Only a fraction of a percent of the flowers produced any capsules whatever and these did not contain more than 1 percent of the normal set of seed. Hence, it is evident that the fertility of the F_1 generation is less than 1 percent and if classed according to the classification of the F_2 population

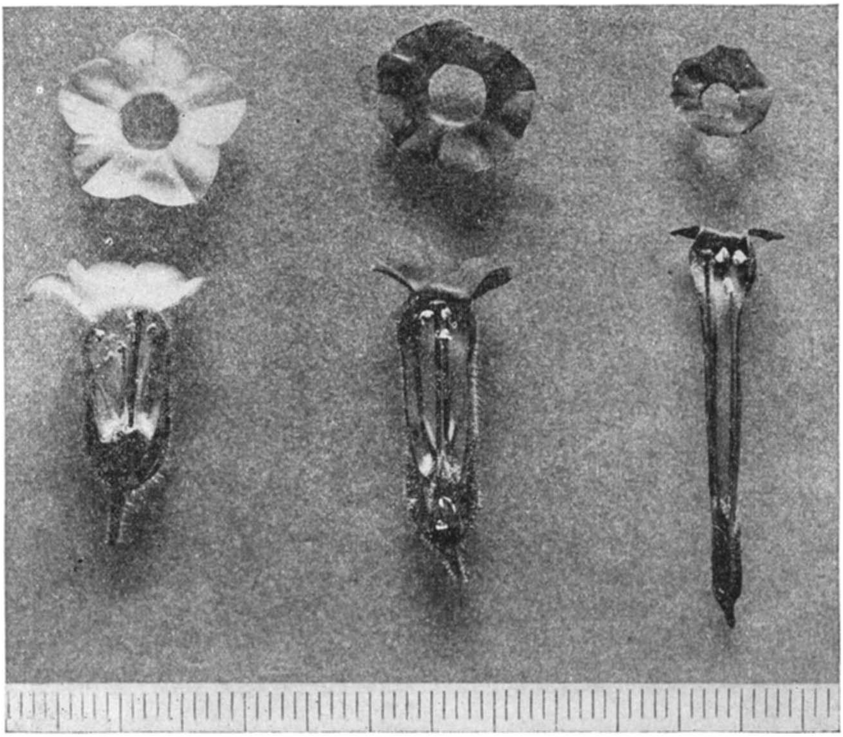


FIGURE 3.—Flower of *N. rustica pumila* (left), *N. paniculata* (right) and F_1 hybrid in center. Magnification 1.6.

would probably be put into class 0, having no fertility, although it does occasionally produce some seeds.

Apparently the ovules are more fertile than the pollen grains. Reciprocal backcrosses to both parents were made with the F_1 plant. The F_1 plant pollinated by *N. rustica pumila* set about 15 percent of the normal amount of seed. According to the fertility classes adopted for the F_2 generation these pollinations would correspond to class 2. There was an average of 24 seeds per capsule. The pollination of the F_1 hybrid by *N. paniculata* showed a little more fertility, having an average of 34 seeds per capsule. It is not certain that the difference in fertility between the *rustica* and *paniculata* pollen is significant, since but 25 flowers were pollinated in each case.

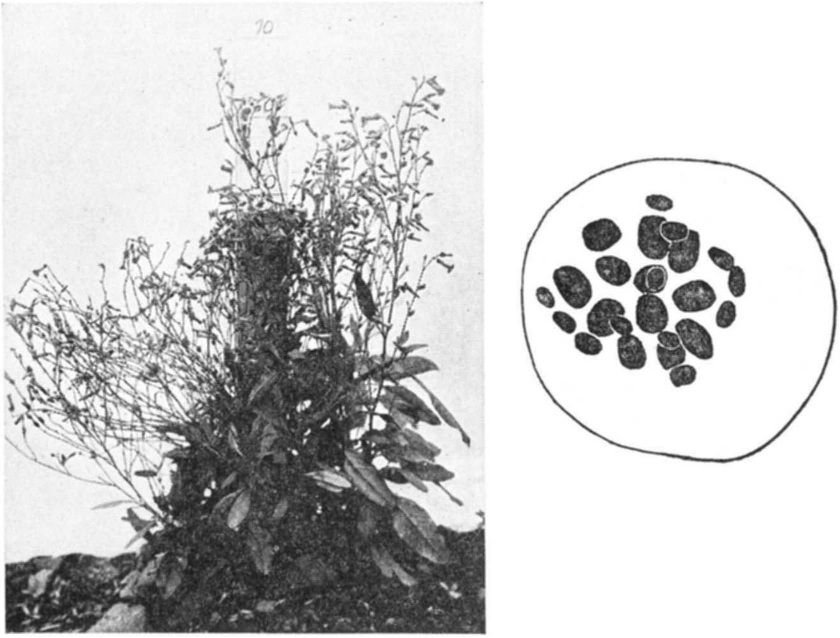


FIGURE 4.—*N. rustica pumila*×*N. paniculata* F_1, grown in the field. Compare with plant grown in greenhouse (figure 9). Right, pollen mother cell showing 12 bivalent and 12 univalent chromosomes. ×2700

The reciprocal pollinations were not so fruitful. *N. rustica pumila*, pollinated by the F_1 pollen, produced only about 2 to 5 percent of the normal seed complement, an average of four seeds per capsule. About half of the flowers pollinated dropped before maturing their capsules, so this would make the fertility lower still. Fifty flowers of *N. paniculata* were pollinated with F_1 pollen and all but two of the flowers dropped. The two capsules produced contained but a few seeds in each. It is safe to say that this pollination is nearly completely sterile.

The pollen mother cells of the F_1 plant at meiosis contain 12 bivalent and 12 single chromosomes (figure 4). It follows the Drosera scheme, the 12 pairs dividing at the first division while the 12 singles pass at random to either pole. The second division is equational. Thus pollen grains are formed each containing twelve chromosomes from the bivalents and 0 to 12 of the single chromosomes. These counts were also obtained by CHRISTOFF (1928), LAMMERTS (1929, 1931) and by GOODSPEED, CLAUSEN and CHIPMAN (1926) who concluded that random assortment of the 12 univalent chromosomes takes place. Their conclusion was based on but 65 counts, hardly enough to be very significant statistically, but surely enough to show that the cells having six or seven univalents are much more frequent than any other.

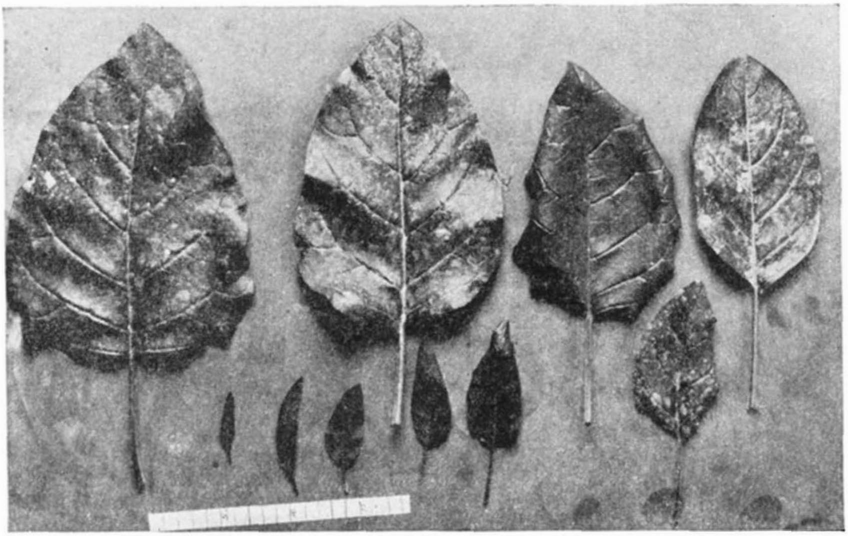


FIGURE 5.—Leaves of a single F_1 plant grown in the greenhouse. Young leaves are lanceolate but become sub-cordate as they reach maturity. Leaf at upper left is almost the shape of *N. paniculata* leaf, while that at upper right resembles leaf of *N. rustica pumila.*

Pollen examinations showed less than 10 percent of apparently viable pollen grains and these varied greatly in size. This point will be discussed later. Figure 6 shows a comparison between the pollen of the two parents and the F_1 hybrid.

F_2 GENERATION

Three separate progenies of F_2 plants were grown. These will be designated A, B and C when they are referred to. The first population (A) was grown in the Bussey greenhouse in Boston in the winter of 1926–27. Only 11 plants reached maturity. The second progeny (B) of 165 F_2 plants was

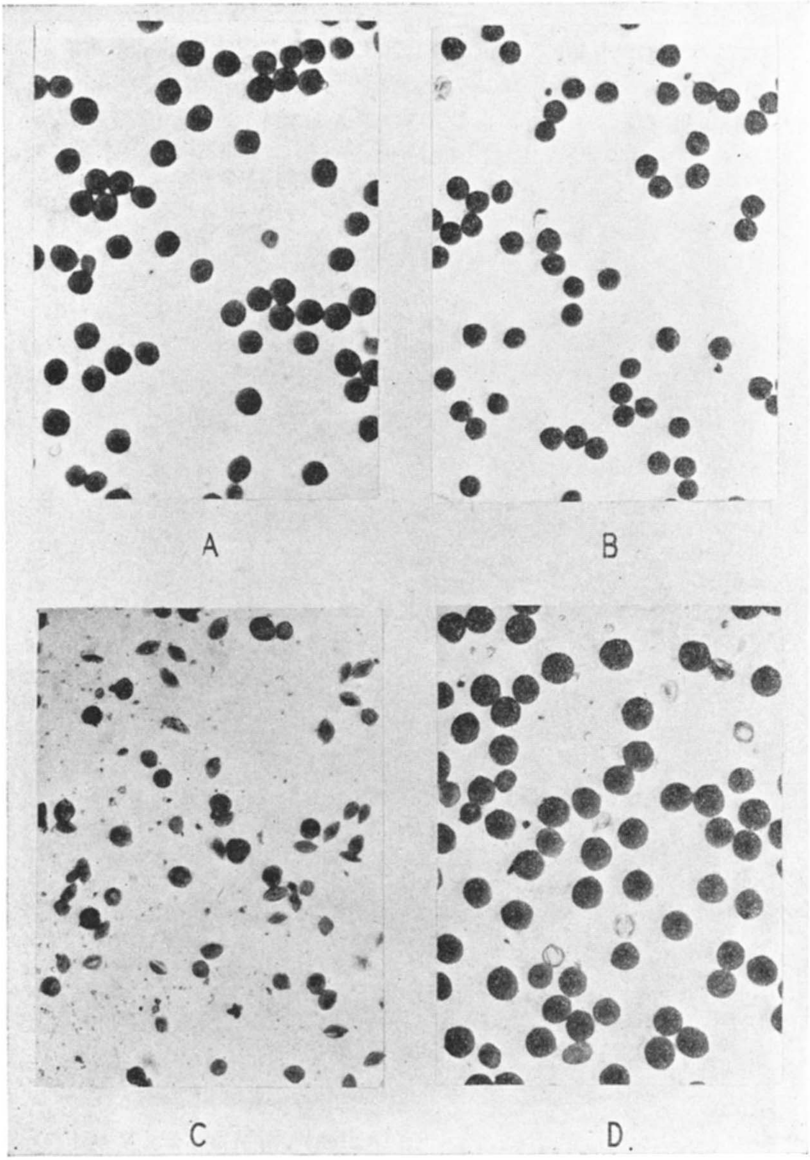


FIGURE 6.—A, pollen of *N. rustica pumila;* B, pollen of *N. paniculata*; C, pollen of F_1 hybrid; D, pollen of tetraploid F_3. Note large percentage of shrunken grains in F_1 pollen. Also note increased size of tetraploid pollen.

produced in the greenhouse of the CONNECTICUT AGRICULTURAL EXPERIMENT STATION in New Haven in the winter of 1927–28. Because of such conflicting results between A and B, a third planting (C) was made of 405 F_2 plants in the field at the CONNECTICUT AGRICULTURAL EXPERIMENT STATION farm in the summer of 1931.

Each plant of progeny A was sterile. Examination of the pollen mother cells showed typical hybrid behavior, each plant containing both bivalents and univalents that were easily distinguished.

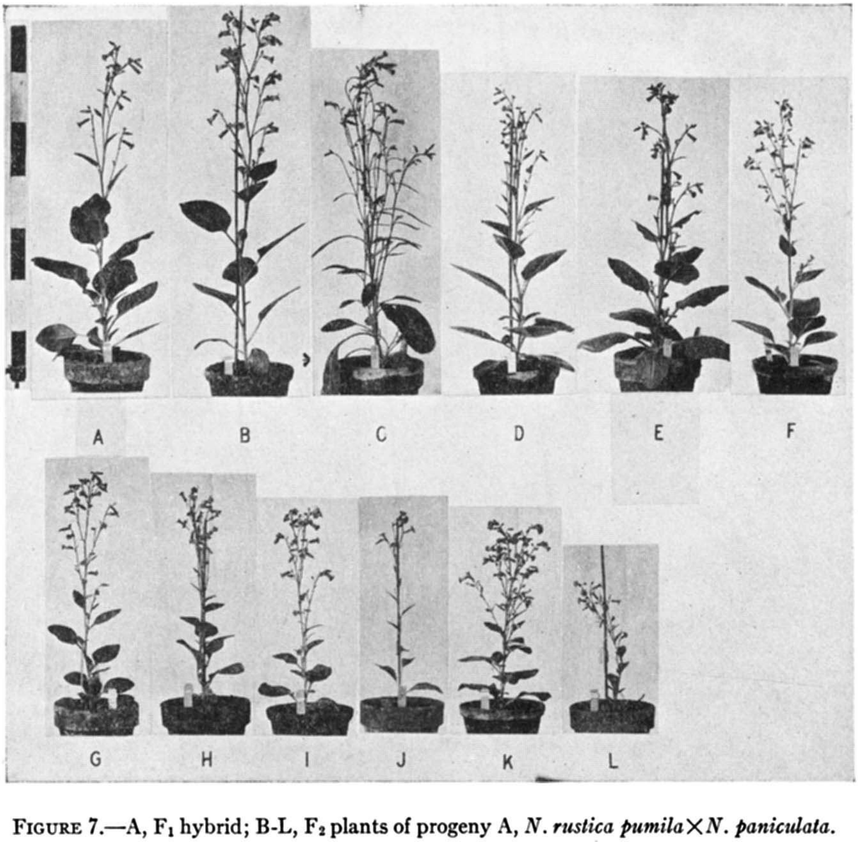


FIGURE 7.—A, F_1 hybrid; B-L, F_2 plants of progeny A, *N. rustica pumila*×*N. paniculata*.

Progeny B showed a great majority of the plants that were high in fertility, had double the chromosomal complement of the F_1 plant, and quite normal cell division as compared to the hybrid behavior of the previous generation. Such F_2 plants with twice the number of chromosomes found in the F_1 plant are called "tetraploids." We realize that the chromosomes at meiosis are for the most part in pairs and to be consistent with haploid and diploid, a tetraploid should have its chromosomes in sets of four. But tetraploid has been used so many times to describe this condition that it seems better to use it than to invent a new term. Some of the terms invented, allotetraploid, autotetraploid and amphidiploid, to us have no advantage over tetraploid and hence tetraploid will be used in referring

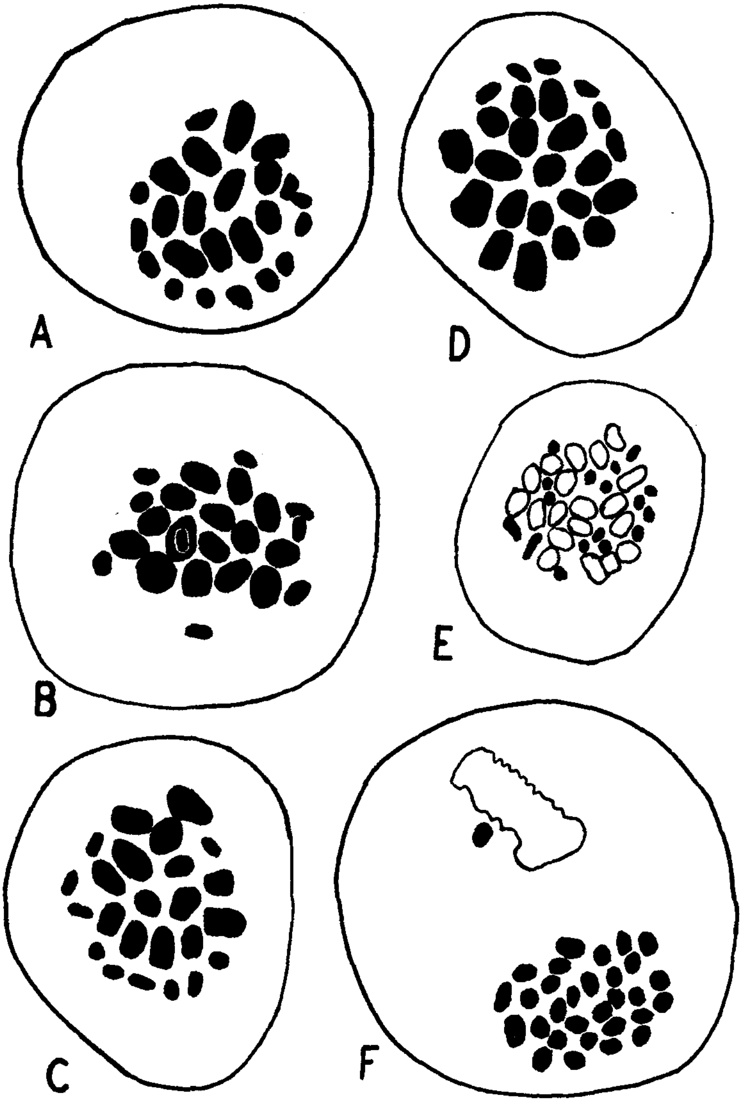


FIGURE 8.—Metaphase plates of plants in figure 7. A, plant 7 A and F 12 bivalents plus 12 univalents; B, plant 7 G 15 bivalents plus 9 univalents; C, plants 7 C, E, K 14 bivalents plus 10 univalents; D, plant 7 J 18 bivalents plus 6 univalents; E, plant 7 B showing 19 bivalents plus 17 univalents; F, plant 7 H second division showing 30 chromosomes. Drawings A, B, C, D, F, ×2700. Drawing E, ×1875.

to the plants with the double chromosome number. Although the tetraploid plants were quite fertile when selfed or intercrossed they were sterile when crossed reciprocally by one parent, *N. paniculata*. Progeny C conformed in general to B.

Progeny A. F_2 generation grown in Boston

All of progeny A are illustrated in figure 7. An F_1 plant is also shown for comparison. There was considerable variation in height, but not so much in flower shape or leaf size and shape. The heights of the F_2 plants varied from 25 to 70 cm with a mean of 45 ± 2.5.

Metaphase plates of the different chromosomal combinations are shown in figure 8. Table 1 gives a summary of the cytological results.

TABLE 1

Summary of chromosomal combinations found in F_2 plants grown at Boston, Massachusetts.

NUMBER PLANTS	CONSTITUTION		TOTAL COMPLEMENT OF CHROMOSOMES
	BIVALENTS	UNIVALENTS	
1	12	12	36
3	14	10	38
1	15	9	39
1	18	6	42
1	19	17	55
1	29–31 chromosomes metaphase second division		58–62

The last two plants were undoubtedly not the products of simple haploid gametes. No first metaphase plates of plant 7 H were obtained but ample second division metaphases were examined. All had from 29 to 31 chromosomes. Apparently we have here a plant formed by the union of two diploid gametes from which some of the chromosomes have been eliminated, or by the union of a diploid and a haploid gamete. In plant 7 B the total number of chromosomes varied from 33 to 36. Some of these were univalents. The most common count obtained was 19 bivalents plus 17 univalents. Occasionally there were as many as three trivalents. Such a plant could be produced by the union of a haploid gamete containing 12 bivalents plus 7 univalents with a diploid gamete containing the full complement of 36 chromosomes. In most hybrids it is quite unusual for diploid gametes to be formed, but in the hybrid between *Nicotiana rustica* and *N. paniculata* this method of gamete formation seems as common as the regular method. More will be said concerning this point when discussing progenies B and C.

The F_2 plants formed by the union of two haploid gametes all have a chromosomal complement of 36 or greater. This would indicate that in the F_1 plants either assortment of the univalents was not wholly at random

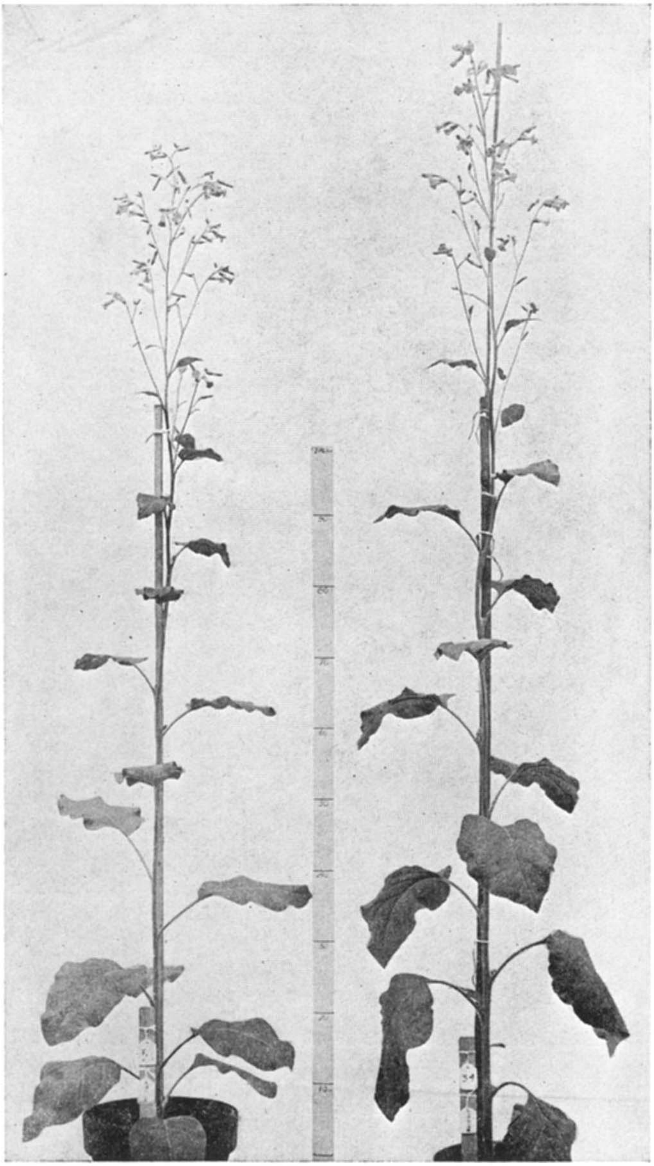


FIGURE 9.—F_1 plant (left) with an F_2 plant (34) representative of a larger share of the second generation. Heights may be determined by meter rule in center.

or else gametes with the greater numbers of univalents were more likely to be included in the zygote.

All of the F_2 plants of this population were entirely sterile according to the tests used. Had a sufficiently large number of flowers been pollinated these plants might have proved no more nearly sterile than the F_1 plant, since with the F_1 it is quite difficult to obtain selfed seed. It is impossible to state definitely that these plants were lower in fertility than the F_1 plants, but they were at least as low.

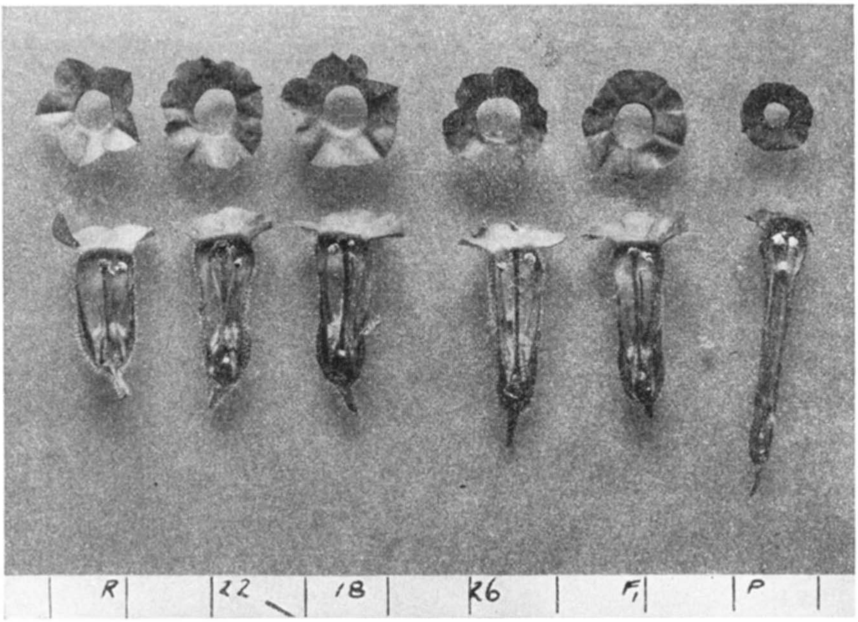


FIGURE 10.—From left to right, flowers of *N. rustica pumila* (R), three different F_3 plants, F_1 hybrid and on the extreme right *N. paniculata* (P). Note that the F_1 flower is intermediate. The F_3 flowers strongly resemble the F_1 except that they produce much more pollen. Most of the F_2 flowers although not shown here are of the same type as the F_3. The markings on the rule at the bottom are in centimeters.

Progeny B. F_2 plants grown in the greenhouse in New Haven

Progeny B consisted of 165 plants of which 146 reached maturity. The plants were remarkably uniform for an F_2 generation of parents differing widely in morphological characters. About 90 percent of the total population was enough alike to be classed as a distinct species, quite different from either parent. They closely resembled the F_1 plants in flower and leaf size and shape (figures 9 and 10). However, they were much more fertile than the F_1 plants. Some were fertile when selfed or intercrossed, but sterile when crossed reciprocally to *N. paniculata*. With *N. rustica* they showed somewhat more fertility. This point will be discussed more fully in another section. All of the plants showing high fertility that were examined cytologically contained about twice the chromosomal complement of the F_1 hybrid. The F_1 hybrid has 12 bivalents and 12 univalents, or a total of 36 somatic chromosomes while the fertile F_2 segregates most commonly have a total of 72 somatic chromosomes.

The chromosome number is best obtained at the second metaphase division of meiosis. Here the numbers most frequently found are 36 and 36 or 35 and 37. In almost all cases the total is 72. Counts can also be obtained at the first metaphase. There were sometimes 36 pairs (figure 11) but more often from 1 to 4 tetravalents and from 0 to 10 univalents. It was a fairly easy matter, aside from actual chromosome counts, to tell whether a plant were tetraploid or diploid, for the reduction divisions of diploid hybrids all show a distinct difference in size between bivalents and univalents and the first meiotic division is always irregular, with univalents scattered along the spindles on either side of the bivalents, which are located on the metaphase plate. In tetraploid plants there was less difference in size between the bivalents and univalents, whenever univalents were present. The univalents in such plants remained closer to the equatorial plate than the univalents of hybrid plants, so that the side view of the first reduction division was much more regular than in the hybrid plants. In some cases trivalent and tetravalent chromosomes occurred in tetraploid plants, although this feature was not constant for all cells in the

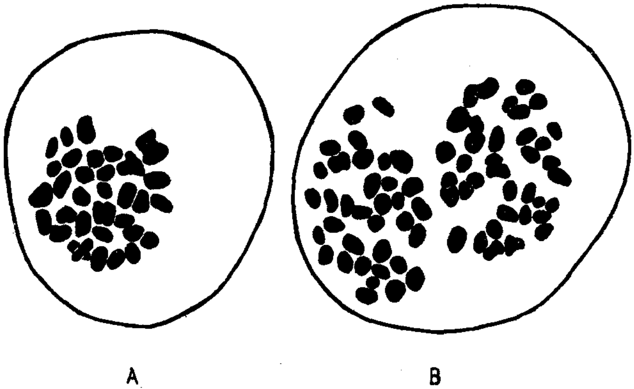


FIGURE 11.—A. First heterotypic metaphase of plant 26 which had regularly 72 somatic chromosomes. In this figure 3 bivalents are joined to form tetravalents, a not unusual occurrence in tetraploid plants. B. Second metaphase of plant 50, showing 35 chromosomes on one side and 37 on the other. ×1350.

same plant. There seemed to be considerable loose pairing of some of the bivalents, which disjoin readily to form univalents and there was also a tendency for more than one bivalent to cling together to form tetravalents.

In spite of some irregularity at the first division, the chromosomal distribution to the daughter cells was very regular. The numbers most common at second metaphase were 36 and 36 or 35 and 37. The high fertility of the tetraploid plants can be attributed to such an even distribution of chromosomes at the first reduction division.

Of a total of 57 plants examined cytologically 53 or 93 percent were quite fertile and had the tetraploid chromosome number. Of the remaining four plants two were the result of the union of two haploid gametes, while the other two resulted from the union of diploid with haploid gametes, and were triploid.

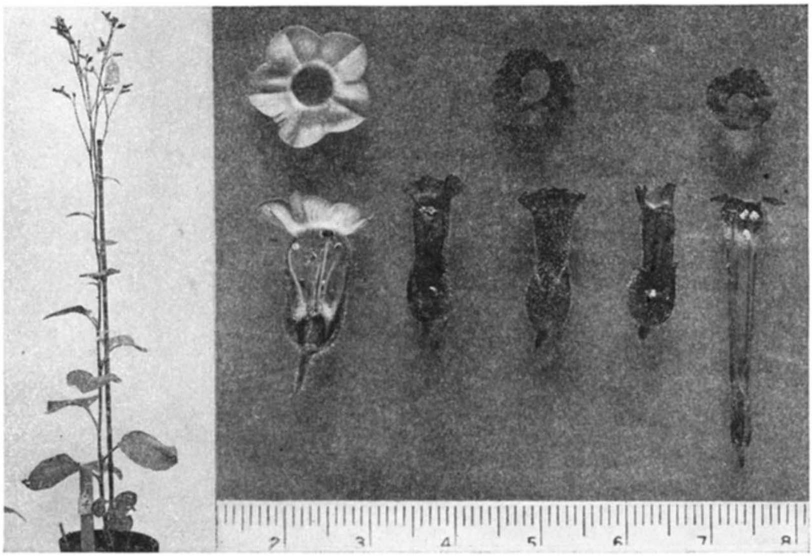


FIGURE 12.—Pistillate plant, and flowers of *N. rustica*, F_2 pistillate plant (3 flowers), and *N. paniculata*. Note complete absence of stamens in pistillate flowers. Plant is 90 cm tall.

Although most of the plants conformed to one general type there were a few exceptional plants. One was found which was completely pistillate, producing what appeared to be a good ovary with a normal pistil, but not a trace of anthers in most of the flowers. Later in the season rudimentary anthers were found at the base of the style, but these never grew normally and were functionless. The stigma was enlarged more than on perfect flowers (figure 12). The plant was entirely sterile to the pollen of *N.*

rustica, *N. paniculata*, and that of F_2 plants. The number of chromosomes was not obtained.

Two extremely short plants were found (figure 13). Both were entirely sterile. Chromosome counts obtained for one of these (115) showed it to have 14 bivalents and 9 univalents, the product of two haploid gametes.

One plant (46) was found having narrow lanceolate leaves and longer flowers than the rest, although not as long as *N. paniculata*. It is shown in figure 14 together with plant 60, which was shorter than the average and had considerably larger leaves. Plant 46 was sterile. The number of bivalents varied from 14 to 17 and there were 14 to 16 univalents. The largest total complement obtained was in one cell showing clearly 16 bivalents plus 16 univalents, a complement of 48. Here again, as in the case of the two plants of progeny A, must be a plant formed by the union of a haploid with a diploid gamete. There is considerable loose pairing of some of the univalents. Some of the chromosomes must have been eliminated in the formation of plant 46 since the total complement is rather low for a haploid-diploid product. Again it is possible some of the chromosomes classed as bivalents were really trivalents, which would increase the total number. This plant could not have arisen through the union of two haploid gametes, and yet it had the hybrid behavior typical of such plants. It was sterile also as were the plants with hybrid behavior of the chromosomes at meiosis. It was sterile not only to its own pollen, but to that of *N. rustica* and *N. paniculata* as well.

FIGURE 13.—The two shortest plants found in the F_2 population. Both were completely sterile. Heights may be determined from centimeter rule in center.

Plant 60 graded 2 in regard to fertility. It had in some cases 36 pairs of chromosomes but in others there were as many as 6 univalents. The lowest number of bivalents found was 33, in which cell there were 6 univalents. The most common number observed at metaphase of the second division was 36. This loose pairing of some of the chromosomes is typical of the behavior of quite a number of the F_2 plants. Evidently a very little unbalanced condition will greatly reduce the fertility, as this plant graded only 2 on a scale of 10.

Plant 187 was another exceptional plant, having rather narrow lanceolate leaves, and flowers slightly longer than usual. The chromosomes at meiosis had typical hybrid behavior and the plant was sterile to its own pollen and also to that of *N. paniculata* and *N. rustica*. It had 17 bivalent and 6 univalent chromosomes (figure 15).

FIGURE 14.—Two unusual F_2 types. Plant 60 (left) graded 2 in fertility while plant 46 was sterile to its own pollen and that of *N. rustica* and *N. paniculata* as well. Plant 60 was tetraploid while 46 was triploid.

Plant 90 had somewhat larger and rougher leaves than most of the F_2 population (figure 16). It had 35 to 36 pairs of chromosomes and was a product of diploid gametes. In fertility it was classified as 8.

Four plants were found that resembled in every respect *N. rustica humilis*. At first it was thought that these plants resembled the variety *brasilia*, the name under which the plants of *humilis* were growing. In a preliminary report of this work (EAST 1928) it was stated that plants resembling *N. rustica brasilia* had been found. It is now evident that this was a mistake due to my unfamiliarity with *N. rustica brasilia* as grown in the greenhouse. One lot of seed grown under the name of *N. rustica brasilia* proved to be *N. rustica humilis*. Of course these four plants may have been volunteer plants of the pure species and not F_2 segregates. In progeny C no plants of this type occurred in a total population of 405 plants.

Summing up we find that out of a population of 145 plants there were only 11 that differed very markedly from the general type resembling the F_1 plant. This is only 7.5 percent of the entire population. The other 92.5 percent were remarkably alike, although there was some variation in height. This variation was much less than would be expected from a second generation hybrid between two species differing as much as *N. rustica* and *N. paniculata*.

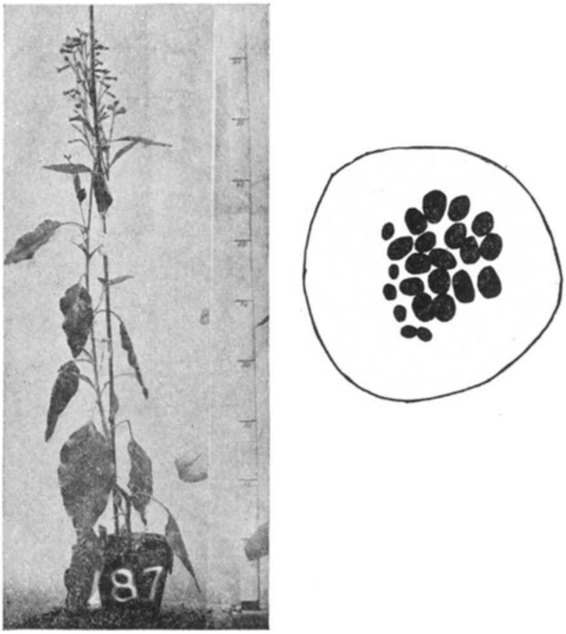


FIGURE 15.—Plant 187, a sterile segregate. Heterotypic metaphase plate (right) shows 17 bivalent and 6 univalent chromosomes. ×1470.

The F_2 plants ranged in height from 20 cm to 170 cm with a mean of 120 ± 1.5 for the 144 plants on which height records were obtained. Under the same conditions *N. rustica pumila* attained a height of 60 to 80 cm, *N. paniculata* 150 to 190 with a mean around 170, and the F_1 varied from

130 to 160 with a mean of about 140. So it is evident that the F_2 plants on the whole were not quite as tall as either the F_1 hybrid or the *paniculata* parent, but much taller than *Nicotiana rustica*. A graphic illustration of the heights may be seen in figure 17.

It has been stated that the F_2 progeny B was quite high in fertility. A graphic representation of the fertility is presented in figure 18. From this graph it is apparent that the fertility of progeny B is much greater than the F_1 which is nearly sterile. Only 8.9 percent of the F_2 population was classed as sterile. The mean was 5.4 ± 0.19. Possibly the 8.9 percent of the F_2 plants classed as sterile were as fertile as the F_1 plants, since the F_1 plants could easily have been placed in class 0 on the basis of 10 flower pollinations, although the F_1 plants are capable of producing some seed. It is uncertain whether the plants classed as sterile might not have produced a few seeds, had enough pollinations been tried.

FIGURE 16.—Plant 90 tetraploid. This plant was about 80 percent fertile. F_3 from this progeny shown at right. It was grown in the field in 1928.

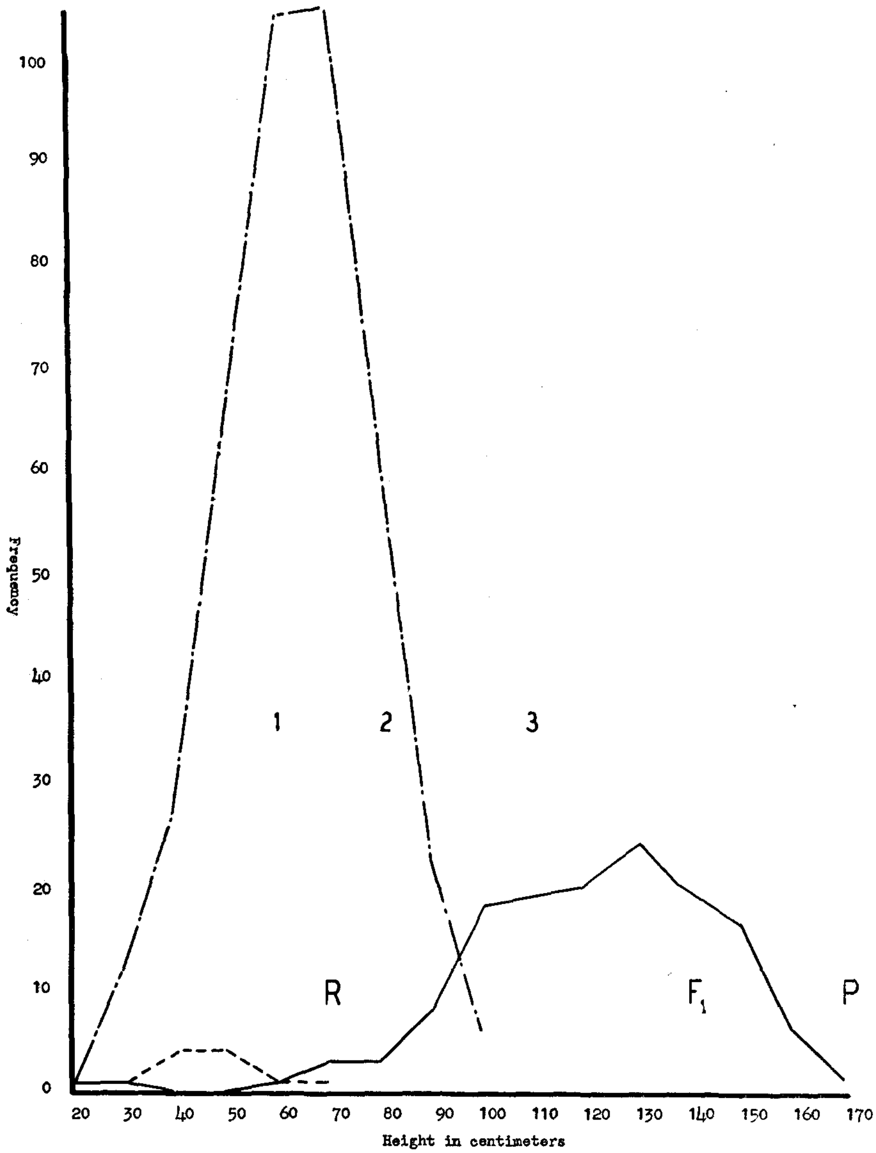


FIGURE 17.—Height in centimeters of 3 F_2 progenies. Regular broken line, progeny A; solid line, progeny B; irregular broken line, progeny C; R, *N. rustica pumila* grown in greenhouse; 1, *N. rustica pumila* grown in field; F_1, F_1 hybrid grown in greenhouse; 2, F_1 hybrid grown in field; P, *N. paniculata* grown in greenhouse; 3, *N. paniculata* grown in field. Note that plants grown in the field are not as high as those raised in the greenhouse. There is also less variation in field grown plants. All the plants of progeny A are comparatively short. They are all diploid or triploid.

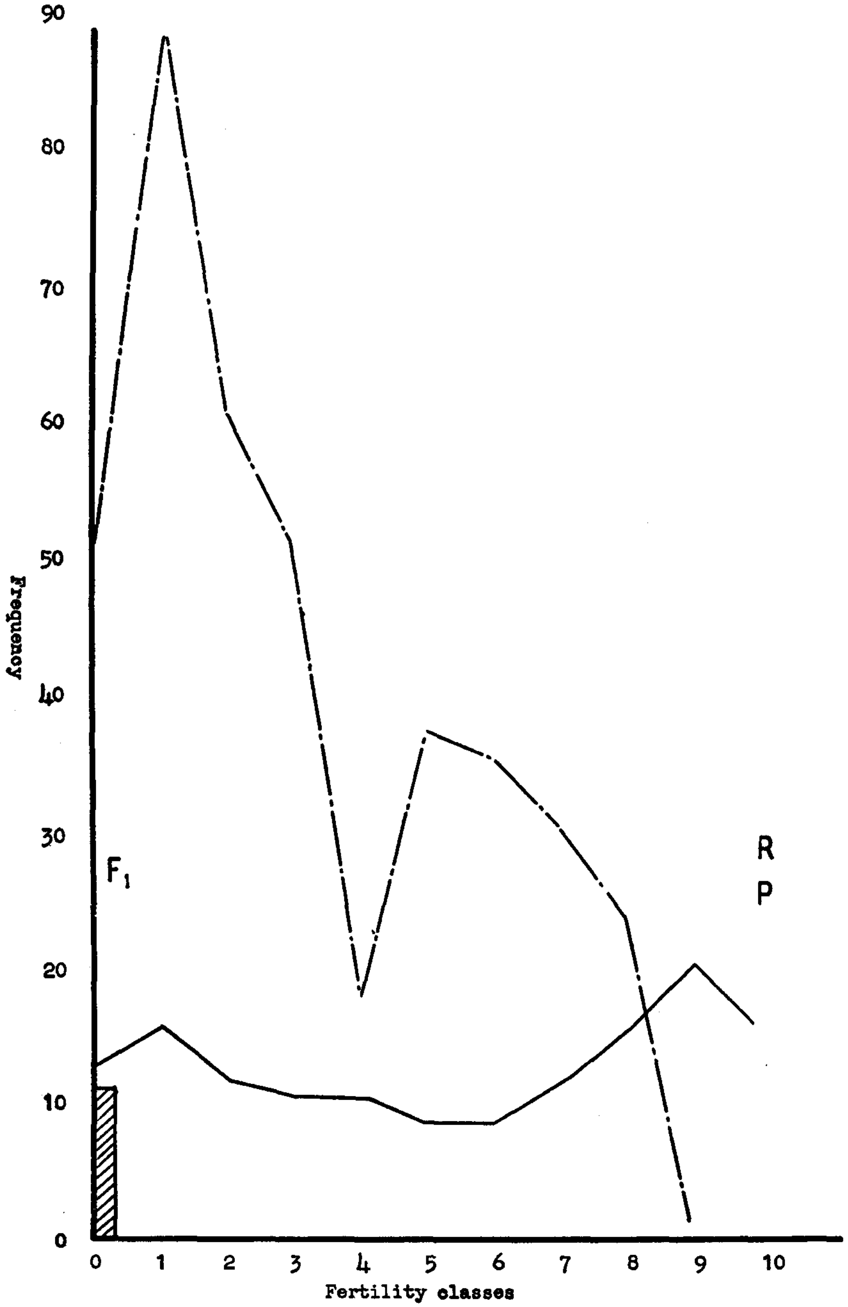


FIGURE 18.—Fertility of 3 progenies. Shaded portion progeny A; solid line, progeny B; broken line, progeny C. Note that A was sterile while B and C were quite fertile.

Progeny C. F_2 plants grown in the field

A third F_2 progeny was grown in the field at the CONNECTICUT AGRICULTURAL EXPERIMENT STATION farm in the summer of 1931. There were 405 plants in this population. Progeny C resembled B very closely in that most of the plants conformed to one general type, similar in appearance and behavior to the dominant type of progeny B (figure 19). More than 85 percent of the plants belonged to this class. Sixty-one plants from this type were examined and found to have just twice the chromosomal complement of the F_1 plants, so it is fairly safe to conclude that all of the plants of this type are tetraploid.

FIGURE 19.—A typical F_2 tetraploid plant of progeny C, grown in the field.

Of a population of 405 plants there were but 59, or 14.6 percent, that were quite different. The 59 plants in general were much shorter, had smaller flowers and leaves and were nearly sterile (figure 20). Eight plants from this group were examined for chromosome number. The numbers found indicate that six were the result of the union of two haploid gametes, one was tetraploid and the remaining plant was the product of diploid and haploid gametes. From this it is evident that most of the unusual plants of progeny C have chromosome numbers indicative of formation by haploid gametes.

The tetraploid plants were much higher in fertility than the diploid. The tetraploid plants averaged 4.2 ± 0.19 for 60 plants, while the mean of the diploid plants was less than 4 percent. Three of the diploid plants were sterile, three showed about 5 percent fertility, one ten percent, and one was dead when classified for fertility.

The average fertility of the triploid plants (formed by haploid and diploid gametes) was 15 percent for 6 plants. Of this group of plants 1 was sterile, 1 graded 5 percent, one 10 percent, one 20 percent and 2 were classified as showing 30 percent fertility.

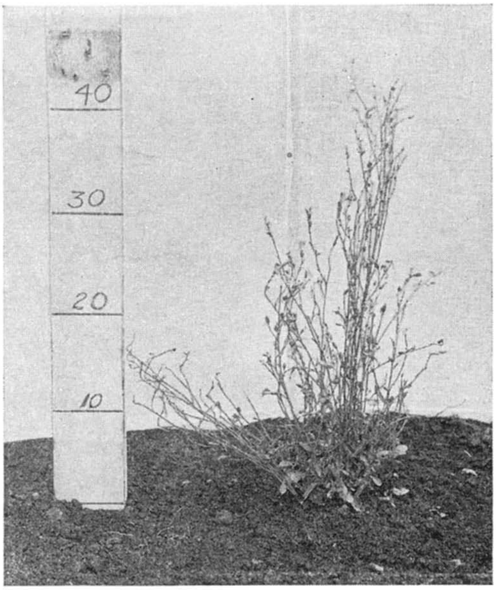


FIGURE 20.—A typical diploid plant of progeny C grown in field.

There seems to be a discrepancy between the fertility of these two groups of plants and similar ones found in previous generations. The plants of progenies A and B, that were formed by two haploid gametes or haploid and diploid gametes, were sterile. Progenies A and B were both grown in the greenhouse and the plants were selfed before classifying for fertility. By this method all diploid and triploid plants were sterile. Progeny C was grown in the field and the plants could have been pollinated by their own or sib pollen, or pollen of either of the parents or the F_1 hybrid. Apparently the plants were capable of setting some seed if pollinated by the proper pollen.

The fertility of the whole group of unusual plants, composed largely of diploid individuals, was 0.3 ± 0.06 or about 3 percent. Most of the plants of this class were sterile. The mean of the whole F_2 population was 3.1 ± 0.21 (figure 18).

The height of the F_2 plants grown in the field varied from 20 to 100 cm with a mean of 63.9 ± 0.09 (figure 17). The 61 tetraploid plants averaged 69 ± 0.20, which is much higher than the average of the diploid, 55.6 ± 0.77 for 8 plants. Individuals formed by diploid-haploid gametes averaged 55 for 6 plants, just about the average of the diploid.

The tetraploid plants on the whole were much taller and more fertile than the diploid. Hence in the whole population we should expect to find a positive correlation between height and fertility. Such was the case. The correlation was 0.45 ± 0.026, which shows a fairly high association.

In general progenies B and C were much alike. In each population more than 85 percent of the plants conformed to a general type closely resembling the F_1 plants except in fertility, in which the F_2 plants were much higher. All of the fertile F_2 plants examined cytologically had the double number of chromosomes. It is safe to conclude that the only F_2 plants high in fertility were tetraploid plants. Nearly all of the diploid plants were sterile. Likewise the plants formed by diploid-haploid gametes showed very little fertility.

Progeny A differed considerably from B and C in that A was composed wholly of diploid plants and plants formed by haploid and diploid gametes. All of the plants of progeny A were sterile. It may be that different strains of *N. rustica pumila* exist and the *rustica* parent of progeny A was different from the *rustica* parent of B and C. Otherwise it is hard to explain the different behavior of progeny A as contrasted with B and C. It is certain that progenies B and C had the same strain of *N. rustica pumila* as one parent.

It is quite remarkable that the F_2 population was composed almost wholly of tetraploid plants containing twice as many chromosomes as the F_1 hybrid. The plants although inter-fertile were sterile when crossed reciprocally by *N. paniculata.*

F_3 GENERATION

F_3 lines from four different F_2 plants of progeny B, 18, 26, 62, and 75, which had the double chromosome number, were grown in the greenhouse in the fall of 1929. These four lines exhibited the high chromosome numbers characteristic of the F_2 plants although there was perhaps a little greater tendency of the chromosomes to form tetravalent groups. All cells in the same plants were not constant but the total somatic number of chromosomes as determined at the second metaphase was around 72. The F_3 plants were like the F_2 in being sterile to the pollen of *N. paniculata*. There was no apparent segregation, since all four lines appeared about the same.

HISTOLOGY OF OVARIES OF TETRAPLOID PLANTS FOLLOWING POLLINATION WITH *N. paniculata*

Since the new tetraploid plants were sterile to the pollen of *N. paniculata*, an attempt was made to find out the reason for this sterility. Several flowers of two F_3 plants having the double chromosome number were pollinated with pollen of *N. paniculata*. Five anthers were used for each pollination, which assured plenty of pollen for each pistil. Then at the end of 12, 24, 36 and 48 hours and 3, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 21 days, 3 to 5 of the flowers were killed, embedded in paraffin, sectioned, and stained for examination. The first lot of developing capsules was fixed in Navaschin's killing agent as used for pollen mother cell study. These were so poorly fixed that a little experimenting was done to find the best fixative for this material. Six different solutions were tried as follows: Bouin's solution, Carnoy's solution, Chrom-acetic (weak), Navaschin's fixative, Mod. Allen B-15, and Flemming's solution.

Bouin's and Carnoy's solutions were most satisfactory and were used in running up the second lot of material for histological examination. Development was found to take place as follows:

1. The pollen grains germinate on the style of the high chromosome plants within 12 hours.
2. The pollen tubes grow down the style at apparently a normal rate of speed as they have reached the ovary at the end of 36 hours.
3. Fertilization occurs and the endosperm development proceeds immediately.
4. At the end of 3 to 5 days a mass of embryonic tissue can be seen at one end of the embryo-sac. This continues to enlarge but does not become differentiated as a normal embryo. It remains just an oval mass of embryonic tissue. Such development proceeds (but more slowly than a normal embryo) for about twelve days. Older sections that were examined showed that the embryo remained stationary while the endosperm deteriorated. Even when the endosperm tissue has almost wholly disappeared the embryonic tissue is still present.
5. When the endosperm has completely disappeared (16 to 18 days after pollination) the embryo gradually disintegrates.

6. At the expiration of 21 days when the seed should be fully developed nothing remains of the embryo and endosperm and the walls of the embryo sac are completely compressed together (plate 1).

These are the facts. The reasons for them may be somewhat more difficult to comprehend. Is it possible that an embryo containing 36 chromosomes from one gamete and 12 from the other is so unbalanced that it is incapable of functioning? We might assume that this is true were it not for the fact that zygotes containing just such a union of gametes were raised to mature plants by LAMMERTS (1929), who found that 32 percent of the viable gametes produced by the F_1 plant were diploid. When backcrossed to *N. paniculata* these 36 chromosome gametes would unite with 12 from *N. paniculata*. Such plants grew to maturity.

We might ask why the endosperm deteriorates first. Is it because of a more unbalanced condition, since the endosperm has 72 parental chromosomes to 12 from *paniculata*? Such an hypothesis seems plausible but here again, for the reason mentioned above, our reasoning breaks down. Some of the plants raised by LAMMERTS had 72 chromosomes from the F_1 plant and 12 *paniculata* chromosomes, and produced not only mature endosperms, but endosperms of viable seeds.

The only reason which seems plausible is that the unbalanced zygote, the embryo of which contains 36 plus 12 and whose endosperm has 72 plus 12 chromosomes, is incapable of developing in a medium that consists wholly of cells having 72 somatic chromosomes.

FUNCTIONAL GAMETES PRODUCED BY F_1 PLANTS

Ordinarily at the reduction division in most plants and animals we expect to have gametes produced that contain just half as many chromosomes as the somatic cells. But in the case of the F_1 hybrid between *N. rustica pumila* and *N. paniculata*, a large majority of the functional gametes produced are diploid. This seems to be the most logical explanation for the tetraploid number of chromosomes in a vast majority of the F_2 plants. The doubling of the chromosomes could take place in rare cases after fertilization, but it is hardly conceivable that this would occur on such wholesale production. Another reason for assuming that the duplication took place in the gametes is that the pollen of the F_1 plants showed several large grains, which undoubtedly contained double the chromosome number. Assuming that the chromosomes double after fertilization, it would be very difficult to explain the chromosome count of plants intermediate between the tetraploid and the diploid. Diploid gamete formation seems to be the only explanation for so large a percentage of F_2 plants

LEGEND FOR PLATE 1

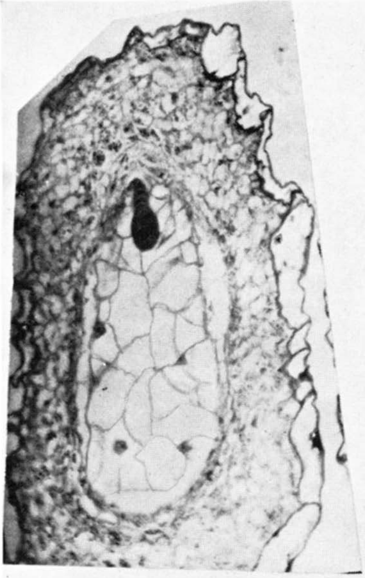
FIGURE 1.—Ovule of *N. rustica pumila* six days after self-pollination, showing well developed endosperm and embryo beginning to develop.

FIGURE 2.—Ovule of tetraploid plant following pollination with *N. paniculata*, 6 days after pollination. Note embryonic tissue, but no well formed embryo.

FIGURE 3.—Twelve days after pollination.

FIGURE 4.—Fourteen days after pollination.

FIGURE 5.—Twenty-one days after pollination.



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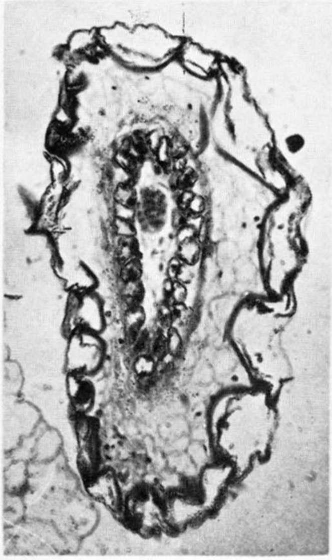

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TABLE 2

Percentage of functional haploid and diploid gametes produced by F_1 plants.

	TOTAL PLANTS	NUMBER EXAMINED CYTOLOGICALLY	TETRAPLOID PLANTS		DIPLOID PLANTS		TRIPLOID PLANTS			TOTAL GAMETES		PERCENT DIPLOID GAMETES
			NUMBER	DIPLOID GAMETES	NUMBER	HAPLOID GAMETES	NUMBER	NUMBER GAMETES		HAPLOID	DIPLOID	
								HAPLOID	DIPLOID			
Progeny A	11	8	0	0	6	12	2	2	2	14	2	12.5
Progeny B	146	57	53	106	2	4	2	2	2	6	108	94.7
Progeny C	405	77	63	126	8	16	6	6	6	22	132	85.7
Total	562	142	116	232	16	32	10	10	10	42	242	85.2
Percentage of plants examined cytologically	..	..	81.7	..	11.3	..	..	7.0	..	..	..	..

with the tetraploid chromosome number. Occasionally the F_1 hybrid behaves in a normal manner, producing haploid gametes, as is evidenced by progeny A. But in most cases diploid gamete formation is quite common. Table 2 shows the percentage of functional haploid and diploid gametes for the different F_2 progenies.

In progenies B and C the percentages of functional diploid gametes were 94.7 and 85.7 respectively. It is safe to say that at least 85 percent of all functional gametes were diploid, a quite unusual occurrence.

LAMMERTS (1929) in backcrosses of the F_1 hybrid of *N. rustica-paniculata* to both parents found that 32 percent of the viable female gametes contained the diploid chromosome number. An analysis of an F_2 population of 37 plants grown by LAMMERTS (1931) shows that 85.2 percent of the functional gametes contained the somatic number of chromosomes. This is almost the same percentage of diploid gametes produced by progenies B and C. Such a close agreement of two independent researches on the same material is indeed remarkable.

In table 3 we have calculated the percentage of plants in each of the three types, diploid, tetraploid and the triploid. The expected percentages were also calculated on the assumption that 30 percent of male and female gametes are diploid. We do not know that the percentage of functional male gametes is the same as the female but have used this figure (found by LAMMERTS to be the female percentage) for both male and female gametes.

TABLE 3

Types of plants found in different F_2 progenies, and expected percentages in each class assuming 30 percent and 90 percent diploid gametes.

	NUMBER OF PLANTS	DIPLOID PERCENT	TRIPLOID PERCENT	TETRAPLOID PERCENT
Expected percentage assuming 30 percent diploid gametes	. .	49	42	9
Progeny A	8	75	25	0
Progeny B	57	3.5	3.5	93
Progeny C	77	10.4	7.8	81.8
LAMMERTS data (1931)	37	10.8	8.1	81.0
Expected percentage assuming 90 percent diploid gametes	. .	1	18	81

The expected number of plants for each class is also calculated assuming 90 percent of functional diploid male and female gametes. (If there were no selective mating of gametes or zygotic elimination it would require 90 percent of diploid gametes to give 81 percent of tetraploid plants.)

Several significant facts can be deduced from a study of tables 2 and 3. The most outstanding are as follows:

A great many more tetraploid plants were found than expected. Even if we should assume 100 percent male diploid gamete formation with 30 percent of female gametes the number of tetraploid plants should not exceed 30 percent. The 3 larger progenies all had more than 80 percent. This indicates that:

A. Either there were about 90 percent of functional diploid male and female gametes or

B. The tetraploid zygotes formed had a very much higher survival rate.

The second of these two suppositions (B) seems more probable. With 90 percent of diploid gametes and no selective mating or zygotic elimination more plants should be in the class formed by haploid and diploid gametes. The proportions of this type of plants to the diploid plants are about the same as would be expected from 30 percent of diploid gamete formation in both males and females. Apparently there is little selective mating among the gametes but there is a zygotic elimination of diploid and triploid individuals. With approximately 30 percent of diploid male and female gametes, 9 percent of the plants should be tetraploid. Instead we find more than 80 percent. This difference of 71 percent must be accounted for by zygotic elimination of diploid and triploid individuals.

In a preliminary report of this work (EAST 1928, SINGLETON 1928) it was stated that plants had been found with 36 pairs of chromosomes, just twice the number of the F_1 hybrid. At that time it was not known just how common is the occurrence of tetraploid individuals. It is now evident that more than 80 percent of the F_2 individuals were tetraploid. Such a large proportion is due partially to a fair percentage of diploid gametes, but the major factor causing such a large percentage of tetraploid individuals was undoubtedly the zygotic elimination of all other than tetraploids.

DISCUSSION

The production through hybridization of plants and animals having chromosome numbers greater than either parent is a phenomenon that is now quite common and familiar to all. Such cases are so often cited that it hardly seems necessary to review them here.

Most instances of chromosomal duplication are the result of chance happenings and only one or a few individuals have the increased number of chromosomes. However, in the case of the hybrid *Nicotiana rustica pumila* × *N. paniculata*, duplication of chromosomes seems to be the rule.

Great zygotic elimination of diploid and triploid individuals takes place after fertilization, so the majority of the F_2 population consists of plants with the increased number of chromosomes. In only two other hybrids do we get a majority of the F_2 population containing mostly plants with the increased chromosome number. These two examples are the well known hybrids *Raphanus sativus*×*Brassica oleraceae* (KARPECHENKO 1927a) and *Digitalis purpurea*×*D. ambigua* (BUXTON and NEWTON 1928).

In general the hybrids we have studied parallel very closely the Brassica-Raphanus hybrids of KARPECHENKO and the Digitalis hybrids of BUXTON and NEWTON. All three hybrids produced F_2 progenies which were composed largely of tetraploid plants. The tetraploid Nicotiana plants were sterile when crossed with *N. paniculata*. With *N. rustica* there was nearly a full set of seeds, which showed only about 50 percent germination. The tetraploid plants raised by KARPECHENKO were almost completely sterile with either parent. The fertility was not stated for the tetraploid Digitalis plants backcrossed to their parents.

In each of these three cases the F_2 tetraploid hybrids resembled the F_1 plants and were fairly constant. Genetically and cytologically they behaved as new species with increased numbers of chromosomes. Such cases certainly lend support to the theory that plants with the larger numbers of chromosomes have arisen through chromosomal duplication.

The F_2 populations we grew were much more constant and uniform than the plants grown by EAST (1921). However, the regularity with which diploid gametes were produced by the F_1 hybrids, and the higher survival rate of tetraploid zygotes leads us to believe that diploid gamete formation and zygotic elimination were in a large part responsible for the plants studied by EAST.

At the beginning of this problem we had hoped to make a correlation between fertility and the proportion of bivalents and univalents. This was found to be impossible because the only place where fertility was manifest was in the tetraploid plants. The diploid plants with their varying numbers of bivalents and univalents all were sterile.

SUMMARY

1. Three separate F_2 progenies of the hybrid *N. rustica pumila*×*N. paniculata* were studied for fertility and cytological behavior. In all 562 plants were classified for fertility. Of these 142 plants were examined for number of chromosomes.

2. Two kinds of gametes were produced, haploid and diploid. More than 85 percent of the gametes that united to form the F_2 plants of progenies B and C were diploid. The F_2 plants of progeny A contained but

12.5 percent of diploid gametes. Progeny A consisted of only 11 plants. The difference in behavior between progeny A and progenies B and C can probably be explained by the fact that slightly different strains of *N. rustica* may have been used as one parent. It is certain *N. rustica pumila* was one parent of progenies B and C and the behavior of these hybrids is typical for progenies of *pumila.*

3. When haploid gametes united to form a zygote there was either selective mating favoring the gametes with the higher chromosome numbers or else a differential viability of zygotes. Plants formed by haploid gametes all had more chromosomes than would be expected with a random distribution of univalents.

4. In the F_1 plants some selective mating probably took place among the diploid gametes. There was a great differential viability of the zygotes, so that more than 80 percent of the F_2 population was composed of tetraploid plants.

5. Plants composed of two haploid gametes or a haploid and diploid gamete were sterile.

6. Tetraploid plants varied from 0 to 100 percent in fertility. Most of them set seed readily. Only tetraploid plants were fertile.

7. Tetraploid plants showed no fertility when crossed reciprocally by *N. paniculata.* Fertilization occurred and the embryo and endosperm started to develop but degenerated before maturity.

8. The tetraploid plants contained just twice as many chromosomes as the F_1 plant. Counts were best obtained at second metaphase where the number most frequently found was 72. At first metaphase there were frequently 36 pairs, but more often from 0 to 4 tetravalents, from 0 to 10 univalents and the remaining number bivalents.

9. The tetraploid plants resembled the F_1 hybrid very closely, except that they were much more fertile. There was marked uniformity among the F_2 plants, much more than expected.

10. The F_3 plants showed the same fertility and high chromosome number as the F_2 generation.

11. The tetraploid plants could be classified morphologically and physiologically as a new species of the genus Nicotiana, with a chromosomal constitution (72 somatic) higher than any wild species of the genus.

12. Our results parallel very closely the work of LAMMERTS on the same hybrid.

13. No complete cytological explanation was found for the unusual types obtained by EAST (1921) since none of the unusual types could be duplicated. The regularity with which the F_1 hybrid produces somatic

gametes, however, leads us to believe that diploid gamete formation played a part in securing the fertility of the F_2 populations grown by EAST. This one factor alone is probably not responsible for his results.

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