Apocynaceae III. Investigations on the role of chromosomal aberrations and polyploidy in evolution of varieties in *Tabernaemontana divaricata*

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During the course of our programme of investigations of chromosomal basis of evolution and induced polyploids in family Apocynaceae (Raghuvanshi and Chauhan 1968) study of *Tabernaemontana divaricata* was undertaken. This species is represented by single and double white flowering varieties. They are ever green hedge ornamentals found throughout India (Duthie 1960, Bailey 1949). Being sterile, these varieties are propagated vegetatively in plains of Uttar Pradesh, but seed setting in single variety has been reported from hilly tracts of Himalayas. Careful studies indicated certain marked differences between these two well known varieties which made it apparent that a single evolutionary step could not explain the origin of double from single type. Intensive screening of different populations of *Tabernaemontana* were amply rewarded by discovery of two more varieties (var. nos. 2 and 3) which provided intergrading series between single and double varieties. The detailed description of all the four varieties (Fig. 1A) is as follows:

Var. 1. Single flowering (pointed corolla lobes). This is a variety on which species *T. divaricata* is based. It is a shrub, leaves glossy, rather coraceous green, margins wavy, apex tapering. Flowers pure white, corolla lobes obliquely ovate, margins curved. Ovaries two with single style and stigma.

Var. 2. Single flowering (rounded corolla lobes). It differs from previous variety in comparatively smaller leaves, lamina slightly curved between veins. Flowers differ only in having more broad corolla lobes with rounded tips.

Var. 3. Single large flowering. Plants more vigorous and sturdy than first two varieties, stem thicker, leaves larger, broader, thicker and lamina has distinct curves between veins. Flowers are large with overlapping petal lobes.

Var. 4. Double flowering. Morphological characters of plants resemble var. 3 but it differs mainly in its floral characters. The flowers had ten petals arranged in two whorls, petal lobes rounded, ovaries two showing all stages from free to fused condition.

The variety no. 2 differs from no. 1 mostly in shape of petal lobes which are more rounded while variety no. 3 is indistinguishable from no. 4 in its

vegetative characters, and the distinction lies in the flowers only.

Materials and methods

For study of karyotypes of different varieties cutting were rooted. Para-dichlorobenzene, 8-hydroxy-quinoline and aesculine were tried for pretreatment at about 12 to 15°C for 2½ hours. Best results were obtained in aesculine treatment. After pretreatment root tips were fixed in 1:3 acetic alcohol saturated with iron, for 24 hours. For staining, the root

tips were placed in small tubes of acetocarmine at 60°C for 3 hours. Then deeply stained tip portions were squashed in a drop of acetocarmine under cover glass by tapping it with blunt end of a pencil. Sufficient pressure was applied to obtain uniform scattering of chromosomes. The slides were sealed and studied. These were later made permanent by ethanol-butanol schedule. The study of morphology of chromosomes is based on the position of centromere. The percentage of short arm length over total length of chromosome gives the F% (form percentage). This F% was determined on the basis of study of 10 somatic cells in each variety. Besides the mean F% the chromosomes were classified into three categories subterminal, submedian and median

Figs. 1A-1B. 1A, flowering shoots of var. nos. 1, 2, 3 and 4 of *T. divaricata*. 1B, shoot of double flowering variety showing a single flower.

when the F% values fell within 0-30, 30-45 and 45-50 respectively. For meiotic study anthers were fixed in 3:1 acetic alcohol for 24 hours and squashed in acetocarmine in the usual way and later slides were made permanent.

Observations

Morphological: The stomata of vars. 1 and 2 are smaller than vars. 3 and 4. There was frequent instability of various floral characters in double flowering variety. In this variety the number of petals ranged from 10 to 11 which were arranged in two whorls. Careful study revealed the instability of presence of inner whorl. In some cases different stages of suppression of inner whorl were clearly noted so much so that in extreme cases only one whorl of 5 large over-lapping petals was present (Fig. 1B). All the other varieties have

Figs. 2-6. 2, 3, 4, 5, somatic chromosomes of vars. 1, 2, 3, 4 and 5 respectively. 6, total chromosome complements of different varieties arranged according to size and numbered from 1 to 11.

two ovaries with single style and stigma characteristic of the family. In double variety all the different stages from two separate ovaries with separate style and stigma to condition where there was one ovary with single style and stigma were present (Figs. 11-14). Various combinations of long, short and reduced style and stigma were metwith. In some cases one carpel was reduced. The length of the carpel also varied. The details of this aspect shall be presented in a later publication.

The pollen sterility was found to be 98.4%, 58.4%, 85% and 97.5% in the variety nos. 1, 2, 3 and 4 respectively.

Karyosomatic studies

Var. 1. The chromosome number is $2n=22$ (Pathak *et al.* 1949). The somatic chromosomes (Fig. 2) show gradual variation from 1.626μ to 3.017μ . In Figure 6 total somatic chromosome complements of all the four varieties have been presented for direct comparison. The chromosome have been arranged according to their length and numbers 1 to 11 have been used to show chromosomes in decreasing length series, being the largest as number 1. The chromosome nos. 1 to 4 have secondary constrictions. The F% of chromosomes 1 to 11 is 39.2, 40.2, 38.8, 42.7, 41.4, 44.8, 40.8, 48.9, 39.1, 37.09 and 41.1 respectively. It is apparent that only one pair (chromosome no. 8) has median constriction while rest have submedian constrictions (Table 1, Fig. 6).

Var. 2. Chromosome number is $2n=22$ (Figs. 3, 25). Chromosome nos. 1-4 have secondary constrictions. The F% of chromosomes 1-11 is 34.8, 38.8, 39.9, 38.1, 35.2, 38.7, 38.3, 47.8, 37.3, 38.1 and 37.03, respectively. In this variety also only chromosome no. 8 has median constriction while rest have submedian constrictions (Table 1, Fig. 6). The karyotype is similar to var. 1.

Table. 1. Average length and F% of chromosomes of different varieties of *Tabernaemontana divaricata*

Name of variety	Chromosome length		No. of chromosomes with F%.			Mean F%
	Mean	Range	0-30.	30-45.	45-50	
Var. 1	2.24	1.626-3.017	0	20	2	41.35
Var. 2	2.655	1.979-3.392	0	20	2	38.5
Var. 3	2.658	1.951-3.383	0	30	3	39.2
Var. 4	1.961	1.605-2.448	0	30	3	41.7

Var. 3. This variety is triploid with $2n=33$ (Fig. 4) and every chromosome is represented three times. The F% of chromosomes 1-11 is 39.2, 34.8, 38.1, 39.9, 39.3, 37.8, 37.7, 46.5, 38.5, 39.2 and 40.3 respectively. Chromosome nos. 1-4 have secondary constrictions while only chromosome no. 8 has median constriction, rest have submedian constrictions (Table 1, Fig. 6).

Var. 4. This variety is also triploid with $2n=33$ (Fig. 5). The F% of chromosomes 1-11 is 39.5, 39.6, 39.9, 39.9, 39.8, 44.6, 42.1, 48.8, 40.3, 41.6 and 39.3 respectively. Chromosome nos. 1-4 have secondary constrictions. Only chromosome no. 8 has median constriction while rest of the chromosomes have sub-median constrictions. The chromosome complements of this variety are

similar to var. 3.

Meiosis

Var. 1. The meiosis was very much irregular with high frequency of univalents (Table 1, Figs. 15, 16, 17). Rarely compact metaphase plates were formed. In some cases univalents appeared to lie in separate groups but this did not give any idea of formation of multiple spindle. Division of 1-8

Figs. 7-10. 7, more than 4 nuclei at T. II (Var. 2). 8, laggards at telophase I (Var. 1). 9, 8-dividing univalents at A. I (Var. 1). 10, diagram showing evolution of four varieties of *T. divaricata*.

univalents was observed (Fig. 9). The average chiasma frequency per cell was 2.8 with variation of 0-5.

At A.I spindle broke down in 71% of the cells. The frequency of unequal distribution into 9/13, 10/14 was 7.46% and 17.9% respectively. The half univalents generally moved to the opposite poles, however, in certain cells they were observed lying in haphazard manner. Tripolar separation of chromo-

somes was also observed.

Anomalies at T.I were presence of micronuclei (36.6%), 2-3 laggards (1.3%, Fig. 8), dividing univalents (2.8%), while 57.7% cells were normal. These observations indicated that in fairly high proportions the univalents and half univalents were able to reach the poles in time to be included in telophasic nuclei.

Stray chromosomes and dividing univalents were present at M.II. In some cases break down of one spindle was noted. At sporad stage number of spores varied from 3 to 7 (Table 3).

Var. 2. Though diploid meiotic studies revealed the presence of ring of four chromosomes in 11 out of 60 cells studied (Figs. 18, 19, 20). It is apparent from Table 2 that chromosomal synapsis was quite regular in this variety which is in sharp contrast to the behaviour of var. 1. Generally only one quadrivalent was present except in one cell where one ring and another chain quadrivalent were

Figs. 11-14. Different types of ovary in var. 4. 11, normal type. 12, one carpel very much swollen. 13, carpels fused and style absent. 14, carpels fused and one style reduced.

Table 2. Average per cell of different configurations of chromosomes at M.I in different varieties of *T. divaricata*

Var.	Level of ploidy	Quadrivalents			Bivalents				Univalents	
		Ring	Chain	Variation	Ring	Variation	Rod	Variation	Univ.	Variation
Var. 1	2n	—	—	—	.024	0-1	2.7	0-5	16.0	12-20
Var. 2	2n	.183	.166	0-2	2.65	0-6	8.5	3-11	—	—
Var. 3	3n				7.2	3-11	3.34	0-7	11.3	11-15
Var. 4	3n				6.08	2-11	4.6	0-9	11.44	11-15-one 19

Table 3. Frequency of varying number of spores in different varieties at sporad stage

Var.	Level of ploidy	2-cells	3-cells	4-cells	4-cells unequal	5-cells	6-cells	7-cells
Var. 1	2n	—	6.3	10.9	19.48	34.79	24.6	4.24
Var. 2	2n	—	—	97.3	—	1.53	1.14	—
Var. 3	3n	—	1.7	70.6	5.8	19.6	2.3	.58
Var. 4	3n	.002	.004	78.9	—	16.09	2.82	.007

Figs. 15-26. 15 to 17, (var. 1) PMC's showing high univalents frequency. 18 to 20, (var. 2) 18, diakinesis (1 ring quadrivalent+9 bivalents). 19, M.I (1 ring quadrivalent+9 bivalents). 20, (1 ring+1 chain quadrivalent+7 bivalents). Fig. 21 to 24, var. 4. 21, M.I (10 bivalents +13 univalents). 22, A.I (unequal separation 14/19). 23, A.I (dividing univalents). 24, A.I (break-down of spindle). 25, somatic chromosomes of var. 2. 26, cytomixis in var. 1.

observed in the same cell (Fig. 18). The orientation of these quadrivalents on spindle was non-disjunctional. The average chiasma frequency per cell was 14.19 with variation of 11-19.

A.I was normal. The presence of quadrivalent did not effect the balanced separation of chromosomes. In rare cases 1 to 2 laggards were present at T.I.

The presence of 5 to 6 nuclei at T.II resulted in variable number of spores at tetrad stage. (Table 3, Fig. 7).

Var. 3. This vareity is single flowering triploid. The meiosis was very much disturbed as expected. The number of univalents at M.I varied from 11 to 15 (Table 2). The univalents were found scattered on the metaphase plate. They also underwent division in some cases. No trivalents were observed. The chiasma frequency was found to be 18.03 per cell, with variation from 13 to 22. The other division stages were also disturbed.

Var. 4. This is a double flowering triploid variety. The number of univalents at M.I varied from 11 to 15 (Fig. 21) except in one case where 19 univalents were observed. In some cells 1 to 8 univalents underwent division precociously. The chiasma frequency was 16.7 per cell, with variation from 18 to 22.

Due to unequal separation at A.I the number of chromosomes in different groups varied from 12 to 19 (Fig. 22). Complete failure of spindle was also noted (Fig. 24). The dividing univalents in some cases were left in the cytoplasm and they along with stray chromosomes formed micronuclei. The number of chromosomes at M.II was variable along with the presence of stray chromosomes. At A.II stray, laggards, break down of spindle was observed which resulted in 1-7 nuclei at T.II. The maximum number of laggards observed at A.II or T.II was 14. The number of spores in a sporad varied from 2-7 (Table 3).

Cytomixis was observed in all the varieties (Fig. 26).

Discussion

It is apparent from Fig. 6 that chromosomal complements of these varieties show great similarity. Chromosome nos. 1 to 4 have secondary constriction in all the varieties. Chromosome no. 8 in all the cases has median constriction and rest of the chromosomes have sub-median constrictions. Non of the chromosomes in any of the varieties have subterminal constrictions. The first two varieties are diploids while the other two are at triploid level. They appear to be autotriploids because every chromosome is represented three times. Thus same chromosomes from 1 to 11 appear to be present in all these varieties. Obviously chromosomal repatterning on an extensive scale has not occurred during varietal differentiation. That chromosomal aberrations are not altogether absent is revealed by translocation in var. 2. But it must be emphasized that whatsoever chromosomal aberrations may have occurred they

did not drastically effect the location of the centromere in any of the chromosomes.

The meiotic behaviour of var. 1 is extremely anomalous. Despite presence of pairs of homologous chromosomes as revealed by karyological studies, the pairing appears to be very much effected resulting in high univalent frequency ranging from 12 to 20 with an average of 16 per cell. The behaviour of var. 2, another diploid is in sharp contrast with complete absence of univalents. In vars. 3 and 4, which are triploids, univalent frequency is comparatively lower with an average of 11.3 and 11.4 per cell respectively. This frequency may not be unexpected considering the unbalanced nature of these cytotypes. But if they are to be considered autotriploids then complete absence of trivalents is rather marked. Comparatively small size of chromosomes could to a certain extent explain their low frequency as reported in certain polyploids of Umbelliferae (Raghuvanshi and Joshi 1967) and *Catharanthus roseus* (Raghuvanshi and Chauhan 1968). Very low multivalent frequency was observed in artificially induced tetraploids of six varieties of *Catharanthus roseus*, another member of family Apocynaceae. The total absence of trivalents in these triploids is unusual because the chromosomes after all are not very small. Obviously some mechanism hindering higher association appears to be at work. As var. 1 is the one on which species is based such high frequency of univalents is peculiar. Besides lack of homology a whole series of environmental and genetical factors might effect the conjugation of chromosomes (Gomen 1928, Beadle 1930, Berger *et al.* 1934, Katayama 1931). The high univalents frequency in hybrid derivative of *Avena* has been found to be controlled by single gene (H. Thomas and T. Rajhathy 1966). The somatic chromosomes deny any possibility of marked chromosomal alteration between homologous chromosomes. Though no doubt minute changes may not be perceptible.

The meiotic anomalies in varieties I, III and IV are the result of mostly the aberrant behaviour of univalents which show precocious division at M.I and consequently no division and lagging at A.II. Spindle function is also effected resulting in restitution nuclei after first or second divisions. Multiple spindle has also been observed. The presence of micronuclei indicates that strays and laggards are not lost in cytoplasm. The varying number of spores at spored stage is out come of irregularities observed at earlier stage (Table 3).

The var. 2 showed ring of four chromosomes in 18.33% cells. In very rare cases two-quadrivalents in a single cell have been observed. These rings of four chromosomes in a diploid plant is indication of reciprocal translocations. As the individual chromosome cannot be distinguished it is not possible to say as to which chromosomes had undergone translocations. In any case fairly long chromosomal arms appear to be involved because quadrivalent formation shows fairly high frequency. Despite translocations meiosis is regular when the other diploid variety is so very meiotically disturbed.

Chiasma frequency in different varieties is presented in Fig. 27. Both chiasma frequency per cell and per half-bivalent have been calculated for comparison. Though no doubt when these varieties are at different levels of ploidy chiasma frequency per half-bivalent gives the real picture for an accurate comparison. But because vars. 3 and 4 are suspected autotriploids even then they do not show any trivalents and further the number of univalents is never less than 11, which is the haploid number indicating thereby that at least one haploid set is always left unpaired. Thus the maximum pairing even in autotriploids is between two haploid sets. Comparison of chiasma frequency per cell in this light shows that values in triploids are higher than diploids. The extreme low chiasma frequency in var. 1 indicates more the possibility of some mechanism which obstructs the chromosomal pairing despite presence of two normal homologous sets.

Pollen sterility in different varieties appears to be correlated with univalent frequency (Fig. 28) except in var. 2 in which though univalents are absent 58.4% pollen grains are sterile. Non-disjunctional separation of quadrivalents has been

Fig. 27. Chiasma frequency per cell and chiasma per half-bivalent.

observed in this translocation heterozygote. This leads to deficiency and duplication of certain chromosomal segments in two nuclei. However, further work on possible cause of univalent frequency and pollen sterility in different varieties is underway. The frequency of univalents and pollen sterility in different seasons all the year round is being investigated. The results obtained will indicate whether pollen sterility is due to high univalent frequency or is genic in nature.

Evolution of varieties. On the basis of morphological and chromosomal studies of these four varieties of *T. divaricata* certain interesting facts have been brought to light. The species is based on var. 1 which is diploid. It is suggested that certain mutation in this variety effected the flower shape,

the lobes of which became more rounded (Fig. 10). The leaf characters were also effected resulting in decrease in its size and slight curving of lamina between the veins. These differences are certainly slight because curving of lamina between veins is present in most of the older leaves and some younger ones of var. 1 but in var. 2 this character is uniformly marked in the leaves of different sizes. It is suggested that var. 3 originated from var. 2 or 1 by simple change in level of ploidy. This could have been brought about by unreduced gamete combining with normal gamete resulting in triploid indi-

Fig. 28. Graph showing univalent frequency and percentage of pollen sterility.

vidual or dispermy, or some somatic disturbance resulting into triploid shoot which may later have been propagated vegetatively. The comparatively vigorous plants of var. 3 with thicker stem, thicker and broader leaves, larger stomata, pollen and flower in comparison to diploid varieties clearly indicates the normal effect of triploidy. The curves between veins under such circumstances will naturally become more exaggerated. It is from this variety that double flowering type arose by a mutation for double flowering because both vars. 3 and 4 are triploids and have similar vegetative characters and karyotypes. The difference between these two varieties is only in their flower. Evidence in favour of such an evolution was obtained by an extensive screening of flowering behaviour of natural population of double type which resulted in presence of rare single flowers on

double variety plants. No doubt the spontaneous appearance of single flowers is very rare and so far we have not seen any double flower on var. 3. But the only puzzling point left to be explained is the unstable character of the ovary in double type, which shows all stages from free to fused condition along with other anomalies. In this particular character var. 4 stands perfectly apart from rest of the three which have two ovaries with single style and stigma-characteristic of the family. The syncarpous gynaecium is found in genus *Carissa* of family Apocynaceae. But what has resulted in this instability in gynaecium? Could it be the mutation for double flowering which

has also effected the gynaecium character or this variation is independent one. The possibility of pleotropic effect of gene which has resulted in double flowering certainly cannot be denied. It may be further mentioned that the distribution of flowers having normal and anomalous gynaecia on the plant is random and same inflorescence has both types of flowers.

Summary

Investigations on evolution of double variety (var. 4) from single (var. 1) of *Tabernaemontana divaricata* lead to the discovery of 2 more varieties (vars. 2 and 3), which provided intergrading series between the two well known varieties. Var. 1 is diploid with highly disturbed meiosis; var. 2 is diploid translocation heterozygote and differs from var. 1 in its flower shape; var. 3 is single flowered triploid; var. 4 is double flowered triploid and resembles var. 3 in morphology and karyotype. The pollen sterility shows direct correlation with univalents frequency in all the varieties except in var. 2 where it appears to be the outcome of translocation heterozygosity. Further work on this aspect is underway. Karyosomatic studies revealed similar chromosome types in all the varieties indicating thereby that chromosomal repatterning on intensive scale was not involved in varietal differentiation. It is suggested that var. 2 arose from var. 1 by mutation which effected flower and leaf shape. The var. 3 arose from var. 2 or 1 by simple change in level of ploidy and var. 3 gave rise to var. 4 by mutation for double flowering. Explanation of gynaecium instability in var. 4 has been suggested.

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