Studies in Induced Tetraploids of the Family Apocynaceae

I. *Rauvolfia serpentina* Benth¹

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Introduction

The family Apocynaceae comprises plants of diverse habits, viz., herbs, shrubs and trees, but majority of them are perennial woody climbers, erect shrubs or trees. Quite a good number of genera are important as ornamental, medicinal, and latex yielding plants. Among the medicinal plants of this family, the importance of the genus *Rauvolfia* L. has recently come into great prominence as a source of hypnotic drugs with depressant action of the cardiac system, especially, in reducing the blood pressure. The invention of colchicine as polyploidizing agent has opened a new line of investigation for the improvement of ornamental and medicinal plants. Usually, the polyploid species or varieties are more vigorous with larger flowers and more alkaloid content than their diploid progenitors.

Through tetraploid breeding, it has been possible to increase latex in rubber yielding *Taraxacum*. Warmke (1945) reported that, field grown tetraploid *Taraxacum Kok-saghyz* plants had both significantly larger roots and higher rubber content. In the induced tetraploids of *Lobelia inflata*, Steinegger (1948) observed about 50 per cent alkaloid content. The tetraploid plants of *Datura stramonium* and *D. tatula*, induced by Rowson (1944) possessed greater total amounts of alkaloids, 1-hyocyamine, atropine, and hyoscyne as compared to their diploid progenitors. The tetraploids of *Atropa belladonna* and *Nicotiana* species induced by Kostoff (1943) also showed increased alkaloid content.

Practically no work has been done for breeding better types through induction of tetraploidy in this family, except in *Vinca rosea* L., where tetraploidy was induced by Furusato (1940), Cross and Johnson (1941), Schnell (1941), Eigsti and Schnell (1943), Eigsti and Tenney (1943). Tetraploid breeding offers great potentiality with medicinal plants, through increase in the active principle content and yield. Considering the economic importance

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of this family, a project was undertaken with the expectation of producing superior types of plants by artificial induction of polyploids through colchicine treatment. The observations on *Rauvolfia serpentina* are presented in this paper. The observations on other ornamental and medicinal genera viz., *Ervatamia*, *Thevetia*, *Nerium* etc. would be published in series.

Materials and methods

Technique for raising seedlings. Healthy seeds were ruptured on one side to facilitate water absorption and then soaked in water for 24 hours in Petri-dishes. Three seeds were sown per pot (4 inch size) having well manured soil, but finally the best seedling was kept for colchicine treatment.

Technique for colchicine treatment. In the seedlings after the first pair of leaves were fully opened, exposing the terminal rudimentary bud, small amount of absorbent cotton was placed covering the apical bud. The apical buds thus covered were treated with aqueous solution of colchicine. Usually the treatments were started in the morning between 7.00 a.m. and 9.00 a.m. Two different concentrations i.e. 0.25 per cent and 0.5 per cent colchicine solution were used for 6 hrs. and 9 hrs. duration. During the treatment the seedlings were covered by earthenware pots previously soaked in water. The pots containing the seedlings were also made moist with water, so as to create a humid condition in air inside the space of the two pots. Following this procedure, there was no need of adding fresh solution even for 9 hrs. duration treatment. After the treatment apical buds were thoroughly washed with distilled water. The seedlings thus treated were then transferred to bigger pots, or to nursery beds for better nourishment.

Criteria used to detect induced tetraploid shoots

- a) Stem : Slow growth with shortened internodes ; shorter but sturdier branches.
- b) Leaf : Dark green colour with shorter petiole, malformed or distorted, rough, thick, comparatively shorter but broader, larger but fewer stomata.
- c) Flower : Larger flowers and floral parts, thicker stalk, delayed flowering.
- d) Pollen : Increased sterility, larger and of variable sizes.
- e) Fruits and seeds : Reduced fruit setting, fruits smaller with fewer seeds, seeds larger.
- f) Chromosome count from meiosis in pollen mother cells. Irregularity and multivalent formations in meiosis.

Observations on the colchicoid generation

The following data were taken from the colchicine-treated plants.

- a) Immediate effect of colchicine on the seedlings and shoots.
- b) Change in size and shape of the first formed leaves.

c) Length and width of mature leaves, leaves were collected from the corresponding regions.

d) Leaf thickness : Stomatal measurement and frequency per unit area were taken from approximately the middle of the leaves. The thickness of the midrib was not considered in collecting the data for leaf thickness. Thin hand sections were mounted in 50 per cent glycerine for observation of leaf thickness. Stomatal count and measurement were studied from the peelings of lower epidermis in freshly collected leaves or from the leaves preserved in 70 per cent alcohol. In either case the peelings were mounted in 50 per cent glycerine.

e) Approximate date of flowering and number of flowers per inflorescence.

f) Comparative data of various floral parts.

g) Pollen stainability and size were studied after staining the pollen in 1 per cent aceto-carmin.

h) Fruits and seed setting and their sizes.

i) Meiosis : For the study of meiotic stages, flower buds of suitable sizes were fixed in : acetic-alcohol (1 : 3) with a pinch of ferric acetate, kept overnight and then transferred to 70 per cent alcohol for storage in a refrigerator. Flower buds were smeared in aceto-carmin. Excess cytoplasmic stain was removed by running 45 per cent acetic acid under the cover slip, warming and pressing, both at the time of staining and destaining.

Meiotic chromosomes were studied from temporary preparations. Permanent slides were made in normal butyl alcohol.

The shoots which did not show any morphological change in leaves and flowers, pollen size and stainability were removed to avoid competition among suspected tetraploid and normal shoots. Seeds have been collected for propagation ; both from the diploid and tetraploid shoots to study the next generation.

Observations

The *Rauvolfia* plants are propagated through seeds, and the plants generally flower within six months after germination. So, in this material seedlings were treated instead of the shoots as in the other materials.

Both 0.25 and 0.5 per cent colchicine were effective to induce tetraploidy in these plants even when treated for only 6 hrs. duration. However, the ideal doses to induce tetraploidy in *Rauvolfia* appeared to be 0.5 per cent of 9 hrs. treatment. Almost all the treated seedlings survived. The results with different concentrations and durations are given in Table 1.

The immediate effects of colchicine on the survived seedlings have been described below :

a) Retardation or even cessation of growth of the treated buds, which varied from two to three weeks.

- b) Increased size of cotyledons of the treated seedlings.
- c) First formed leaves smaller and variously deformed.
- d) The commonly occurring malformed leaves include twisting, curling, lobing, cohesion of leaves or bifurcation of one leaf etc.

Table 1. Frequency of tetraploids in different treatments of *Rauvolfia*

Colchicine conc. %	Treatment duration	Seedlings treated	Seedlings survived	Tetraploid plants	% 4n in treated	% 4n in survived
0.25	6 hr. 1 day	3	3	2	66.6	66.6
0.25	9 hr. 1 day	3	3	2	66.6	66.6
0.5	9 hr. 1 day	9	8	7	77.7	87.5

General morphology of the tetraploid plants. Eleven tetraploid plants obtained so far did not show characteristic gigantism, in some of their morphological features. The tetraploid plants were slow growing, reduced in height, with sturdier stems in comparison with the untreated plants. In a few plants, the arrangement of leaves was found to be opposite decussate, instead of whorled arrangement—the usual phyllotaxy in *Rauvolfia* plants. The leaves were smaller but wider (Fig. 1) with increased thickness both in the palisade and spongy tissues. Stomata size increased with lesser number per unit area. Veins were more prominent in tetraploid leaves, having stouter petioles.

Flowering was delayed in all the tetraploid plants. The differences of periods of flower-bud initiation in diploid and tetraploid plants varied from two to three months. Number of inflorescence per plant and number of flowers per inflorescence were seen to be lesser in tetraploid plants. But, the tetraploid plants flowered for longer durations than their diploid progenitors. Flowers were shorter in length with increased diameter of corolla and width of petal-limbs (Fig. 1). Apart from these, their tetraploid features were also manifested through the following morphological characters—larger calyx, larger stamens and shorter but stouter pistils. Petals in tetraploid flowers did not open fully as in diploid flowers.

Pollen grains. In control plants the pollen grains were always triaperturate and triangular almost without any exception. But, the tetraploid pollen grains were found in various shapes and sizes—such as triangular, quadrangular or pentangular or somewhat roundish or irregular in shape, having three, four, or five apertures respectively depending on the shapes of the grains. A significant change in pollen sterility was observed in tetraploid plants, where about 37 per cent of the pollen was found to be unstained or undeveloped as against only 5 per cent in the diploids.

Meiosis. In diploid plants 11 bivalents were seen at diakinesis and first meiotic metaphase. Subsequent meiotic stages were normal. But in a few metaphase I plates univalents even upto 14 per cell and their precocious movement, were observed.

Tetraploid meiosis was studied in four plants. Although, at metaphase I, variable proportion of univalents, bivalents and multivalents were seen, yet the number of univalents, trivalents, pentavalents, hexavalents and octavalents were rather rare. The most common and predominating configurations were the bivalents and tetravalents (Fig. 2).

Figs. 1-6. 1, comparative sizes of the leaf and floral parts (Diploid- left and tetraploid- right). 2, tetraploid metaphase I-multivalent associations. ($\times 900$). 3, tetraploid metaphase I-4 quadrivalents and 14 bivalents. ($\times 430$). 4, tetraploid metaphase I-I hexavalent and a few quadrivalents and bivalents. ($\times 900$). 5, tetraploid anaphase I-equal distribution in two poles (22-22). ($\times 625$). 6, tetraploid anaphase II-unequal distribution (23-23)+(21-21). ($\times 900$)

Among the fifteen cells studied, 11 different types of associations could be observed (Table 3). Quadrivalents were present in all the cells. They were found in various shapes - viz. chains, rings and in other irregular patterns. The number of quadrivalents per cell varied from one to seven. The mean frequency of quadrivalents per cell was 4.86 (Fig 3).

The number of bivalents per cell varied from four to twenty. They were, however, observed in all the cells. The mean number of bivalents

per cell was 9.46. Octavalents were seen in three cells, trivalents in three cells and hexavalents were observed in five cells (Fig. 4). The mean numbers per cell are shown in Table 3.

At anaphase I and II, apart from normal segregation of 22-22 chromo-

Table 2. Comparative data of diploid and tetraploid *Rauvolfia*

Characters	2n	4n
Diameter of stem (cm)	0.317 ± 0.01	0.38 ± 0.01
Leaf size (cm)		
Length	14.32 ± 0.63	13.68 ± 0.38
Width	3.97 ± 0.19	5.46 ± 0.13
Leaf thickness (μ)		
Entire	361.79 ± 25.954	390.39 ± 21.307
Palisade	73.34 ± 1.238	94.24 ± 1.356
Stomata size (μ)		
Length	27.36 ± 0.494	31.35 ± 0.934
Width	16.72 ± 0.463	16.91 ± 0.524
Stomata frequency per unit area	26.5 ± 0.617	24.5 ± 0.76
No. of inflorescence per plant	4.9 ± 0.458	3.0 ± 0.148
No. of flowers per inflorescence	107.6 ± 7.45	41.5 ± 0.76
Length of flower (cm)	2.4 ± 0.024	2.3 ± 0.037
Length of corolla-tube (cm)	1.9 ± 0.022	1.8 ± 0.031
Diameter of corolla-tube (cm)	0.16 ± 0.003	0.19 ± 0.005
Width of petal-limbs (cm)	0.32 ± 0.007	0.37 ± 0.011
Pollen measurement (μ)		
Long axis	87.02 ± 1.318	103.36 ± 6.536
Short axis	85.50 ± 1.292	98.42 ± 4.864
Unstained pollen (per cent)	5.0	37.0

Table 3. Different types of chromosome association at metaphase I in tetraploid *Rauvolfia*

Sl. No.	Type of association							No. of cells
	VIII	VI	V	IV	III	II	I	
1	—	—	—	1	—	20	—	1
2	—	—	—	3	1	14	1	1
3	—	—	—	4	—	14	—	2
4	—	2	—	4	—	8	—	2
5	—	1	—	5	—	9	—	1
6	—	1	—	5	—	8	2	1
7	1	—	—	5	—	8	—	2
8	—	1	—	6	—	7	—	1
9	1	—	—	6	1	4	1	1
10	—	—	—	7	—	8	—	2
11	—	—	1	7	1	4	—	1
Total								15
Frequency Per cell	0.20	0.46	0.06	4.86	0.20	9.46	0.26	

somes to each pole (Fig. 5) cells with irregular separation i.e. 23-21 were also seen (Fig. 6). Lagging chromosomes were not observed. Precocious movement of one univalent was seen in a solitary p.m.c. out of total 14 plates studied at metaphase I.

Besides normal occurrence of four groups of chromosomes, five groups and two free or sometimes even seven groups of chromosomes were seen at telophase II (Table 4).

Table 4. Different types of chromosome distribution at anaphase I and chromosome groupings at telophase II

Sl. No.	Mode of distribution at anaphase I	No. of cells	Chromosome groupings at telophase II	No. of cells
1	22-22	10	4 groups	23
2	21-23	2	5 groups+2 free	1
3			7 groups	1
Total		12		25

Fruits and seeds. In spite of marked increase in pollen sterility fruits were set in all the tetraploid plants, though the number of fruits per plant was comparatively much less. Tetraploid fruits were mostly one-seeded as against the usual 2-seeded diploid fruits. Due to decrease in number of seeds per fruit, the tetraploid fruits were usually smaller in size. Tetraploid seeds were of variable sizes, with a slight change in shape.

Discussion

Aqueous colchicine solution gave satisfactory result. In *Rauwolfia serpentina*, a non-woody herbaceous plant, seedlings were treated, with 0.25 and 0.5 per cent colchicine and it has been found that even 0.25 per cent colchicine for 6 hrs. duration was quite effective to induce tetraploidy. Eigsti and Tenney (1943), while determining the concentration of colchicine in a glycerine base and number of applications required spaced at an interval of 12 hours for *Vinca rosea*, concluded that five applications of 1.0 per cent colchicine in glycerine base was found to be most effective. Eigsti and Schnell (1943), pointed out that a 1.0 per cent colchicine-in-glycerine proved to be more effective for the induction of polyploidy in *Vinca* than 1.0 per cent colchicine-in-water. From the results obtained in this project, it would not be out of place to mention here that aqueous colchicine solution of 0.25 and 0.5 per cent strength, were quite satisfactory for the induction of tetraploidy in non-woody Apocynaceous plants.

The immediate effect of colchicine treatment on the shoot-apices has been the arrest of stem elongation and subsequent enlargement of the treated regions. Swelling of the colchicine affected regions depended to some extent, on the concentration of the solution applied. Cross and Johnson (1941),

investigated the structural features of the shoot apices of diploid and colchicine-induced tetraploid strains of *Vinca rosea* L. with respect to the quantitative relationships of the tunica and corpus, and the shoot apices of diploid and colchicine-induced tetraploid strains of *V. rosea* were seen to be similar. Cellular measurements showed that the increased width of the tetraploid apices was due to increased lateral dimensions of cells in the apical meristem. The vertical dimensions of the cells in the tetraploid did not show a significant increase. Walker (1938), observed that when the stems of *Tradescantia* were cut from the plant and placed in colchicine, the latter stems showed extreme swelling at the node, where leaves were attached. According to him, this nodal enlargement was in every respect comparable to root and hypocotyl tumors. Hawkes (1942), has shown that colchicine causes an isodiametric expansion of cells. He has also pointed out that the volumes of the cells from C-tumors and from untreated roots were about the same. In fact, in all the cases of reported C-tumors, the same conclusion was reached that colchicine altered the growth pattern.

The lower leaves enlarged after the cessation of shoot growth. The enlargement of the lower leaves situated below the apical bud is mostly due to accumulation of food materials as a consequence of arrested growth. After recuperation of growth, the treated buds developed smaller leaves, which were variously deformed and wrinkled. However, the latter effect soon disappeared and almost normal leaves were produced.

In the induced tetraploids, characteristic gigantism was confined only to some plant organs. The tetraploid branches were usually reduced in size, but were more sturdier. Leaves were smaller in length but broader, thicker both in the palisade and spongy tissues. Stomata were larger, but fewer in number per unit area. Corolla was shorter in length, but wider in diameter. Styles were shorter, but had greater diameter. The diameter of stem was greater than that of the diploid. Warmke and Blakeslee (1939), pointed out that the roots of diploid, triploid, and tetraploid *Nicotiana* were built after the same pattern, the number and arrangement of cells in all of them being strikingly the same. According to them, the differences were only quantitative, accompanied by an increase in cross-sectional area of cells as the chromosome number increased. Sen and Vidyabhushan (1960), pointed out that the greater thickness of the tetraploid stem and the branches in *Dolichos biflorus* was due to an increase in size of the constituent cells rather than increase in the number of layers.

In the tetraploid plants the leaves were so distinctly different from those of the respective diploids, that this character was considered as one of the most conspicuous features of tetraploidy. In fact, a close observation of the leaf, provided a fairly reliable information for identifying the tetraploid shoots. The prominent features of the tetraploid leaves were - decreased length but increased width, prominent veins, dark-green colour, increased thickness and

larger stomata with lower frequency per unit area. Leaves were thicker in all the induced-tetraploids. This was due to the increase in thickness of both the palisade and spongy tissues.

An increase in thickness of the tetraploid leaves has been observed by various authors. Johnson and Sass (1944), in sweet clover, reported that both the palisade and spongy tissues contributed equally to the increase in thickness of tetraploid leaves. Newcomer (1941) in cabbage, and Tandon and Bali (1959) in *Iberis amara* and *Linaria maroccana*, considered this increase in thickness as due to increase in cell size.

Stomatal guard cell size (both length and width) was found to increase in all the tetraploid plants. As a consequence of increase in size of the guard cells and other epidermal cells, the frequency (number of stomata) per unit area was reduced in the tetraploid leaves. In detecting the tetraploid shoots, the stomatal measurement and frequency criterion was seen to be quite satisfactory.

The tetraploid plants flowered about two to three months later. Induction of tetraploidy is known to be associated with delayed flowering. Like all other physiological phenomena, delayed flowering has been attributed to the slower rate of metabolic activities in the tetraploid plants.

A significant decrease in number of flowers per inflorescence was found, where it was less than half of the control. The lesser number of branches of the inflorescence axis is being considered as one of the reasons of the fewer flowers per inflorescence. Sen and Chheda (1958) in black gram, also observed fewer flowers per inflorescence, where the inflorescence axis was seen to be shorter.

The flowers and floral parts showed a marked tendency towards decrease in length, but increase in diameter of the corolla-tube. In other words, it has been observed that generally the floral parts were shorter in length, but wider in diameter. Owing to short stature of the tetraploid flowers, they appeared more compact and showy.

Pollen character was found to be a good criterion for detecting polyploid shoots. Change in pollen-sterility has been observed to be invariably associated with induction of tetraploidy. The pollen sterility and size of the pollen grains have considerably increased in the tetraploid plants. The size of the pollen in tetraploids was more variable, Bragdo (1955) in tetraploid red clover, observed much greater variation in pollen size, some of the grains being even smaller than the diploids.

A remarkable change in the morphology of the tetraploid pollen grains was seen in all the colchicine induced plants. The tetraploid pollen grains were triangular, quadrangular, or pentangular or in rare cases irregular with 3, 4, or 5 distinct apertures, as against triangular and triaperturate pollen in diploids.

Several workers have observed increased number of germinal pores in

colchicine-induced tetraploid pollen grains. In tetraploid *Oenothera*, Hecht (1940) observed many pollen with four or more germinal pores, whereas those from the diploid had three. Dermen (1947) in peach varieties, observed change in shape of the polyploid pollen grains. Most of the polyploid pollen grains were four-sided in contrast to the triangular appearance of normal pollen grains. But, Johnson and Sass (1944) reported that in sweet clover the pollen was triangular in tetraploid and oval in diploid. Sen and Ghosh (1960) in green gram, observed the presence of four-germ pored pollen in tetraploids as against triangular and spherical-shaped ones in diploids.

In *Rauvolfia serpentina*, the tetraploid plants set fruits and seeds, though the quantity per plant was considerably reduced. The tetraploid fruits were smaller in size. These fruits were mostly one-seeded as compared to 2 seeded diploid fruits. However, tetraploid seeds were seen to be of variable sizes, which appeared either smaller, equal or bigger in size in comparison with the diploid seeds.

Dermen (1952) in colchicine-induced polyploid apple, observed increased fruit size with flattened shape and irregularity in contour. Pal et al. (1943) in chilli, reported larger fruits in autotetraploids than their diploids. But, Sen and Chheda (1958) in black gram and Sen and Vidyabhushan (1960) in horse gram observed usually shorter pods with fewer seeds in the tetraploids.

Meiotic study in diploid *Rauvolfia* revealed 11 bivalents. Univalents were found in a few cells, but subsequent meiotic stages were normal.

With the duplication of chromosome sets in the tetraploids, a considerable number of quadrivalent associations were seen. The frequency of quadrivalents per cell was 4.8 and maximum number was 7 in a cell. No p.m.c. was found with all quadrivalent or bivalent formation. However, the frequency of bivalents per cell was highest of all types of chromosome associations observed. The high frequency of bivalent formation may be attributed to large number of short chromosomes present in its complement.

The irregularities observed during first and second divisions were early movement of chromosomes at metaphase I and II, unequal separation at first and second anaphase divisions, variable number of laggards and formation of supernumerary groups at telophase II. It is evident that irregularities in chromosomal distribution can be taken into account as one of the causes of partial sterility in this species.

Several investigators have pointed out, the causes for the reduced fertility in the autotetraploids. A number of hypotheses were offered to explain the phenomenon. Darlington (1937) and Kostoff (1940) considered the irregularities of chromosome behaviour at meiosis, as one of the factors responsible for reduced fertility. Müntzing (1936), cited some examples of autotetraploids with small chromosomes and bivalent synapsis that were quite sterile, and many spontaneous autotetraploids in spite of multivalent association which were highly fertile. Thus, it appears, that meiotic irregularities should not

be considered as the principal cause of sterility in all the partial or complete sterile autotetraploids.

However, the more acceptable explanation for the causes of sterility in autotetraploids, has been either genetical or physiological disturbances. This was at first pointed out by Müntzing (1936). Stebbins (1950) considered that the sterility in autotetraploids was mainly due to genetically controlled physiological factors of an unknown nature.

A few seeds that are set are enough to propagate this species which can also be multiplied through cuttings. Its utilization will depend on the increase in the alkaloid content of its root which remains still to be tested.

Summary

1. Tetraploidy was induced in *Rauvolfia serpentina* ($2n=22$)—a plant of considerable medicinal importance.

2. Aqueous colchicine solution of two different concentrations viz. 0.25 and 0.5 per cent were applied on cotton wad around stem tip at seedling stage. Both the concentrations responded well to seedling treatment but 9 hrs. treatment with 0.5 per cent gave maximum tetraploids.

3. Colchicine affected shoots were mostly sectorial chimeras, but some were more or less complete tetraploid for all practical purposes.

4. Immediate effects of colchicine on the treated shoots were arrested stem elongation, leading to the enlargement of the treated regions. Tetraploid branches were slow growing and somewhat shorter but sturdier.

5. Leaves were shorter in length but wider, thicker and darker green. The increased thickness was shared both by the palisade and spongy tissues. Stomata were larger and fewer per unit area.

6. Flowering was delayed in the tetraploid plants. Number of flowers per inflorescence was less in the tetraploids and this criterion was quite significant in *Rauvolfia serpentina*.

7. The flowers and floral parts showed a marked tendency towards decrease in length, but increase in diameter or width.

8. Change in pollen sterility was found to be invariably associated with induction of tetraploidy. Pollen grains were larger in size with remarkable change in their morphology. Number of apertures varied with the shape. Tetraploid pollen grains were mostly quadrangular or pentangular in shape.

9. Number of fruits and seeds in tetraploid *Rauvolfia* plants was significantly reduced.

10. The mean frequency of quadrivalents per cell was 4.8. Irregular distribution, precocious movements, lagging chromosomes and supernumerary spored quartets were seen in *Rauvolfia*.

11. Though most of the parts of *Rauvolfia* exhibited gigas habit the utility of the tetraploids is yet to be determined by growing the progeny of the tetraploids and estimating the alkaloid contents of the roots of mature plants.

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