STUDY OF CHROMOSOMES AND SOLUBLE PROTEINS OF *RHAZYA STRICTA* DECASINE. AND *NERIUM OLEANDER* L.

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Abstract

Electrophoresis of proteins, especially seed proteins, and karyological studies are useful techniques for determining species and solving the problems of modern taxonomy. The present study was carried out on two species of the Apocynaceae family, *Rhazya stricta* Decasine. and *Nerium oleander*.L. Seed samples of these two genera were taken from the south-east of Iran and SDS-PAGE analysis of their proteins showed 35-88% and 40-80% similarities between different populations of each species respectively. In the protein groups from a selected population of each one of these two species and *Vinca rosea* L., another species of the Apocynaceae family, 47-55% similarity was observed. However, for identification of these genera, the possession of 2-5 protein groups which were present in excessive quantities could be used. Studies on mitotic and meiotic chromosomes in the various populations of *Rhazya* and *Nerium* showed that the basic chromosome number in both species was 11, (2n=2x=22) but the small size of the chromosomes made karyotyping problematic. In spite of this difficulty, the relatively larger chromosomes of one of the *Nerium* populations were prepared.

Introduction

Rhazya stricta Decasine. is a member of the tribe lstonieae belonging to the subfamily Plumerioideae of ıe family Apocynaceae. It is found in the north-west ;gions of India and Pakistan and the south-east area of an [10,11]. The plant is employed in the indigenous 'stem of medicine, as a curative for chronic rheumatism. ifty different indole alkaloids of *Rhazya stricta* have been lentified. Some of these compounds have been shown to hibit anticancer activity e.g. tetrahydrosecomines and llesiachotamine [7,9,11].

Nerium oleander L. also belongs to the family Apocynaceae. This plant is widely cultivated in the Caucasus and in the southern part of Soviet Central Asia, growing wild along the Mediterranean seaboard (in Europe, Africa and Asia) and in Iran [15,16]. Some compounds of this plant, especially glycosides, are used in the treatment of cardiac disease, leprosy and inflammation [15,16].

Seed protein electrophoresis is utilized as an additional approach for species identification, as a useful tool for phylogenetic and evolutionary studies of various groups of plants and as a significant approach for resolving specific taxonomic problems [6]. In addition, seed protein electrophoresis is a unique and powerful tool in studies concerning the origin of polyploidy and the evolution of cultivated plants. The composition of seed proteins is

Keywords: Karyology; Mixoploidy; Phylogeny; Similarity ıdex

highly stable and is affected only slightly by environmental conditions or seasonal fluctuations. In addition, intrinsic changes in the plant such as chromosomal rearrangements or even a doubling of chromosome numbers have little effect on the seed protein profiles [6,14].

Karyological information on *Rhazya* and *Nerium* is restricted mostly to chromosome counting. Chromosomal studies showed that in both species the basic chromosome number was 11 (2n=2x=22).

In the present research, a study on proteins and chromosomes was carried out on plants which were collected from different areas in the south-east of Iran and on one sample from Tehran.

Materials and Methods

Plant Material

Seeds of *Rhazya* and *Nerium* were collected from different parts of Iran as indicated in Tables 1 and 2.

In addition to the above genera, protein analysis was carried out on *Vinca rosea* L. another genus of the Apocynaceae family. In this study, commercial seeds of this plant were used.

Electrophoresis Preparations

Protein extraction was carried out following the same method as that described by Smith [17] and Richwood *et al.* [12]. Protein extracts were obtained by grinding 0.5 g seeds for each sample at cold-room and mixing with 2.5 ml 0.08 M tris-HCl buffer containing 10% glycerol at pH=8.3. The resulting mixture was centrifuged at 15000 g for 65 minutes at 4°C and the supernatant containing total proteins was collected.

The soluble protein concentration was determined for each seed preparation using the simplified Lowry method [2]. Bovin serum albumin was used in order to establish the standard curve. To determine total protein concentration, a Shimadzu spectrophotometer uv-100, λ= 660 nm was used.

Protein Fractionation

The crude protein preparations were fractionated after extraction by the vertical SDS-PAGE method in discontinuous buffer system on two concentrations of acrylamide (12.5 and 15%). Electrophoresis was carried out using balanced quantities of crude protein (calculated as equation I) from all specimens and was loaded into wells of an equilibrated slab gel with a 10 µl microsyringe. The gel was charged at 75 mA until the samples began to migrate into separating gel. Electrophoresis was then carried out for 4-5 hours at 65 mA constant current with the surrounding buffer temperature maintained at 4°C until the marker dye, bromophenol blue, had reached the gel end. The gels were fixed by 0.025% coomassie brilliant blue (R-250) in methanol and 10% acetic acid.

Destaining and clearing of peptide bands was performed using a solution of 96% ethanol and 5% acetic acid until

Table 1. Localities of the collected seed of eight groups of *Rhazya stricta* species in Iran. Abbreviation: R=*Rhazya stricta*.

Population of *Rhazya* species:	Localities
1) R_1	60 km away from Bam towards Iranshahr, Bam-Iranshahr road
2) R_2	150 km towards Iranshahr, Bam-Iranshahr road
3) R_3	Bezman region of Iranshahr
4) R_7	15 km away from Sarbaz river towards Chabahar
5) R_8	Sarbaz region
6) R_{12}	Espakae-Bampour region of Iranshahr
7) R_{13}	50 km away from Iranshahr towards Khash, Iranshahr-Khash road
8)R_{115}	115 km away from Bam towards Iranshahr

Table 2. Localities of the collected seed of five groups of *Nerium oleander* species in Iran. Abbreviation: N=*Nerium oleander*.

Population of *Nerium* species:	Localities
1) N_5	Sarbaz region of Iranshahr
2) N_{10}	Chabahar
3) N_{15}	Kerman
4) N_{16}	Ardecan
5) N_{17}	Tehran

Table 3. Chromosomal number of two species: *Rhazya stricta* (R) and *Nerium oleander* (symbol designations for each population are listed in Tables 1 and 2).

Percent	Chromosomal number (2n)	Locality	Percent	Chromosomal number (2n)	Locality
3	12	N_{15}	5	16	R_{12}
8.5	16		5	20	
8.5	18		90	22	
6	20				
72	22		5	16	R_{13}
2	36		5	20	
			85	22	
12	16	N_1	5	24	
8	18		8	18	R_{115}
10	20		8	20	
70	22		84	22	
8	16	N_{17}	4	12	N_5
6	18		6	18	
6	20		2	20	
76	22		84	22	
4	24		4	44	

background stain was removed. All banding patterns were scored on a light table for position and intensity. The distance of each band center from the top surface of the gels was noted.

$$\text{Volume of interest} = \frac{\text{Total protein concentration of the sample with minimum concentration}}{\text{Total protein concentration of interest}} \times 100 \quad \text{(Eq. I)}$$

In order to determine polypeptide bands, in particular their position and intensity in the profile, optical density was carried out using a Shimadzu Scanning DR (CRT) model densitometer by transmission mode DR-13 computer and using Deuterium (D) lamp in $\lambda = 540^{nm}$.

$$SI = \frac{\text{No. of pairs of similar bands}}{\text{No. of pairs of different bands + No. of pairs of similar bands}} \times 100 \quad \text{(Eq. II)}$$

Similarity index was calculated as Equation II and a computer program was used to construct the dendrogram of clustering patterns. The approximate relative molecular weights (Mrs) of polypeptide bands were estimated by SDS-PAGE using the following standard proteins: α- lactalbumin (14.2 kd), trypsin inhibitor (20.1 kd), trypsinogen (24 kd), carbonic anhydrase (29 kd), glyceraldhyde 3-phosphate dehydrogenase (36 kd), egg-albumin (45 kd), bovin-albumin (66 kd) run in the same gel.

Chromosome Preparations

Mitotic Chromosome Preparations

Preparations were made from root tips of seedlings. After treatment for 3-4 h in 0.002 M 8-hydroxyquinoline solution, and fixation with Carnoy's (3:1) solution, root tips were stored in 70% ethanol under refrigeration at 4°C until use.

Chromosome staining using 1% acetocarmine and chromosome observation were carried out following the same method as that of Tsuchiya [see 8]. The samples were studied using a Vanex-S-AH_2 light and phase contrast microscope.

Meiotic Preparations

Flower buds were collected from plants in the middle of March and November and immediately fixed in Pineare solution for 24 h at 4°C. After washing with water three times, the buds were stored in 70% ethanol under refrigeration at 4°C until use.

Pollen mother cells were stained with 1% acetocarmine. The tissue was squash and chromosome observation was carried out in the same manner as that described for mitotic preparation.

Results and Discussion

Since there is no report on the electrophoretic separation of *Rhazya* and *Nerium* seed protein in the literature, methods

developed for seeds of other plants were used.

Protein analysis showed that among four ratios of seed in extraction buffer ($\frac{1}{5}$, $\frac{1}{10}$ (a), $\frac{1}{15}$ (b), $\frac{1}{20}$ (c), g/ml), the optimum composition was 1/10 (g/ml) and protein fractionation was performed better when 12.5% rather than 15% of polyacrylamide was used (Figs. 1 and 2).

There was no difference between seeds of *Nerium* with pappi (N_{16b}) and without pappi (N_{16a}) (Fig. 3). Furthermore, the populations of the same species, which were collected from areas close to each other were very similar. Consequently they were not included in subsequent experiments.

To increase the accuracy of electrophoresis, optimum preparation and equal conditions, electrophoresis was carried out on the remaining samples on the same gel slab (Fig. 4)

The Mrs of polypetide bands were estimated by SDS-PAGE using the standard proteins as described earlier. At first glance, the electrophoretic bands of proteins of different specimens of the same species appeared to enjoy great similarity, densitometric analysis, however, showed 35-88% and 40-80% similarity between different populations of *Rhazya* and *Nerium* respectively (Figs. 5 and 6).

Great similarity was observed between R_1 and R_{115} and between R_{12} and R_{13} populations of *Rhazya* (Fig. 5). Strong similarity was also found among N_5, N_{15} and N_{16}. However N_{17} is distinguished by its lower similarity than the other three

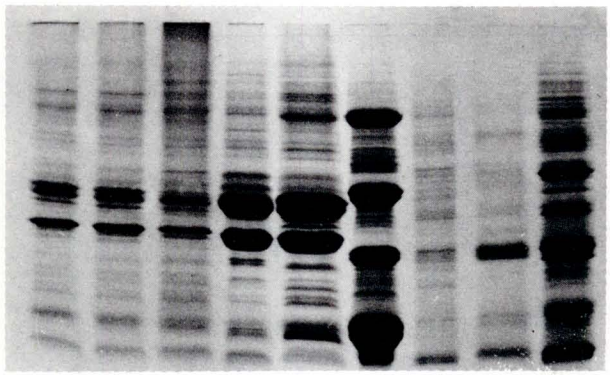


Figure 1. Electrophoregram of seed soluble proteins using SDS-PAGE. Samples from left to right are: R_{115}, R_8, R_7, V, N_{17}, N_{16}, N_{15}, N_{10}, N_5.

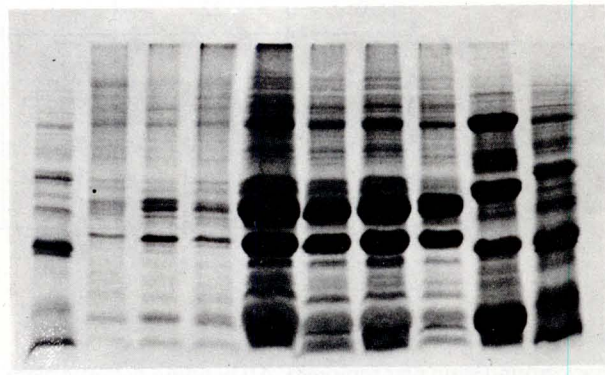


Figure 3. SDS-PAGE of seed soluble proteins. Samples from left to right are: R_{115}, V, N_{17}, N_{16B}, N_{16A}, N_{15}, N_{10}, N_{5a}, N_5, R_{13}.

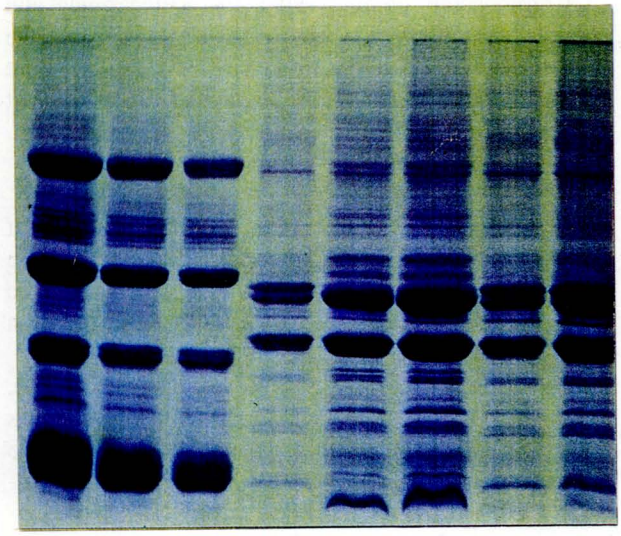


Figure 2. Electrophoregram of seed soluble proteins using SDS-PAGE. Samples from left to right are: N_{17a}, N_{17b}, N_{16b}, N_{16c}, N_{17c}, V_c, V_b, V_a

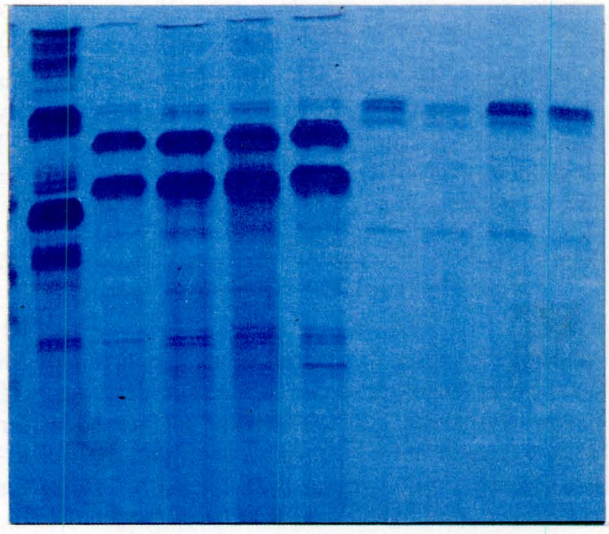
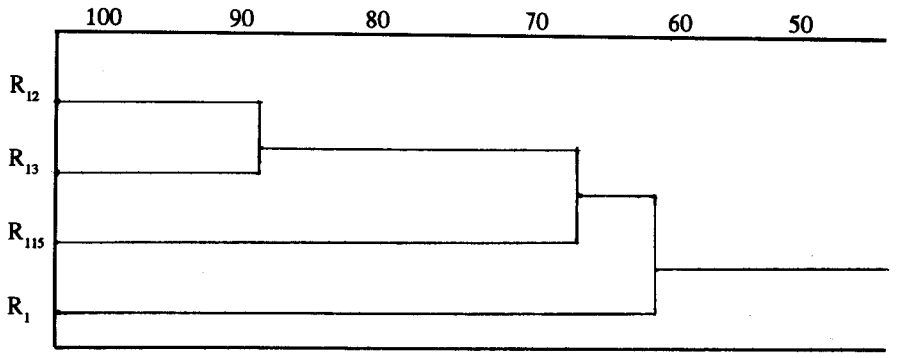
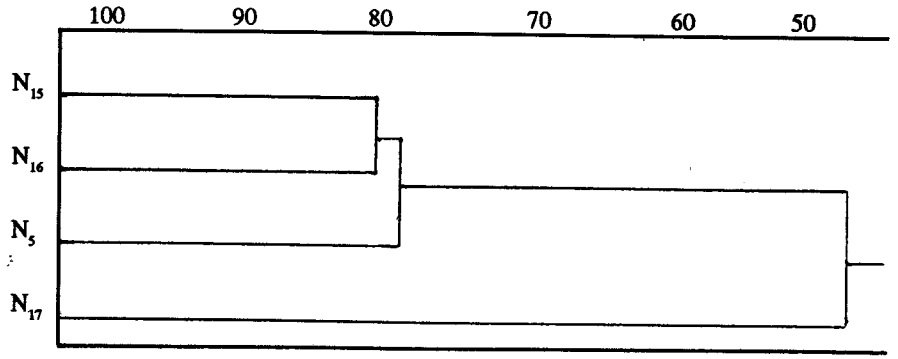


Figure 4. Electrophoregram of seed soluble proteins using SDS-PAGE. Samples from left to right are : R_{12}, R_{13}, R_{14}, R_{115}, N_{17}, N_5, N_{16}, N_{15}, V and standard proteins.



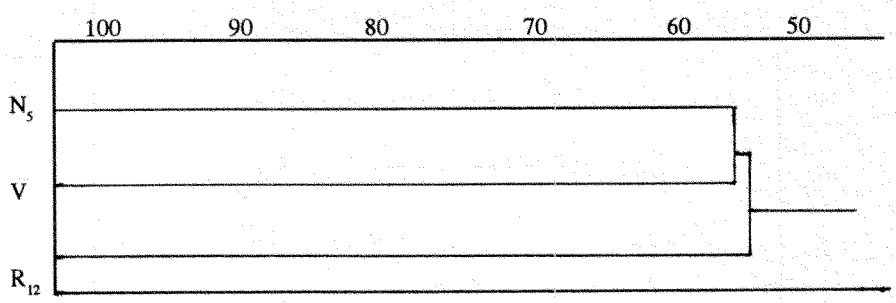
	R_1	R_{12}	R_{13}	R_{115}
R_1	100.0	51.0	60.0	35.0
R_{12}		100.0	88.0	65.0
R_{13}			100.0	55.0
R_{115}				100.0

Figure 5. Dendrogram of four populations of *Rhazya* species based on similarity indices



	N_5	N_{15}	N_{16}	N_{17}
N_5	100.0	64.0	78.0	40.0
N_{15}		100.0	80.0	44.0
N_{16}			100.0	47.0
N_{17}				100.0

Figure 6. Dendrogram of populations of *Nerium* based on similarity indices.



	R_{12}	N_5	V
R_{12}	100.0	47.0	54.0
N_5		100.0	55.0
V			100.0

Figure 7. Dendrogram of three species: *Nerium oleander* (N_5), *Rhazya stricta* (R_{12}), *Vinca rosea* (V), based on similarity indices.

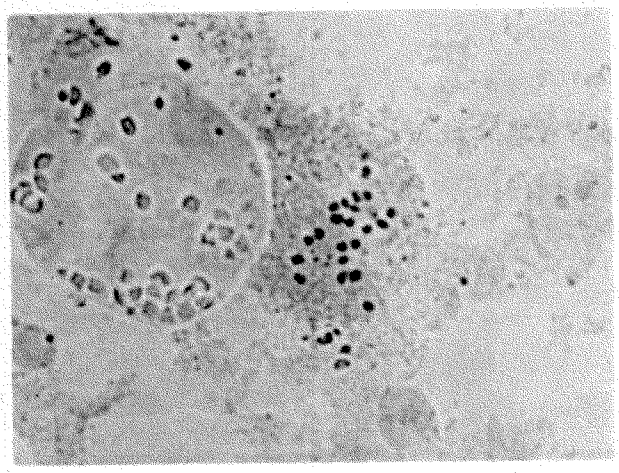


Figure 8. Photograph of R_{12} chromosomes during early metaphase. 1400x, 2n = 22.

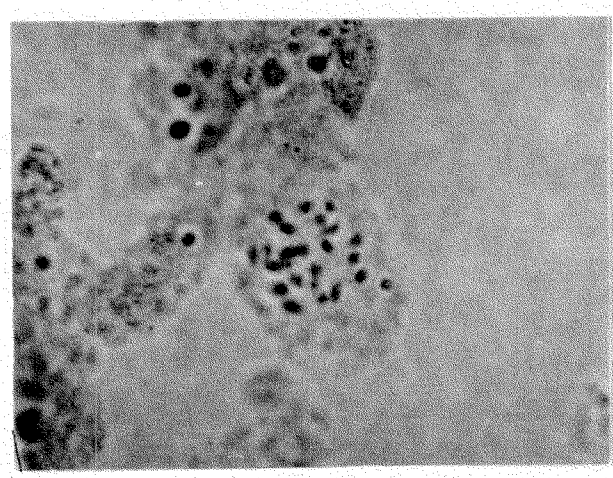


Figure 9. Photograph of R_{13} chromosomes during early metaphase 1400x, 2n = 22.

populations (Figure 6).

A comparsion of selected populations of each genus (based on high similarity with other populations) indicated 47-55% similarity between R_{12}, N_5 and *Vinca rosea* (Fig. 7). However, this similarity may only be extrinsic and structural differences can exist between the same bands. In addition, the intensity of the same bands of three genera showed significant differences which results in each genus being distinguished from the other two by 2-5 protein bands, which seem to be characterstic of the species, and by the electrophoretic pattern which is markedly different in different genera.

A mitotic chromosomal study was carried out on three populations of *Rhazya*: R_{12}, R_{13}, R_{115} (Figs. 8-11), and a meiotic chromosomal study on only one population: R_{12}. Based on mitotic and meiotic studies, the basic chromosome number 11 was obtained and slight mixoploidy was also observed (Table1).

A mitotic chromosomal study was carried out on four populations of *Nerium*: N_5, N_{15}, N_{16}, N_{17} (Figs. 12-15), while a meiotic chromosomal study was restricted to N_5 (Fig. 16). In this genus, the basic chromosome number 11 was also obtained and mixoploidy observed (Table 1). The small size

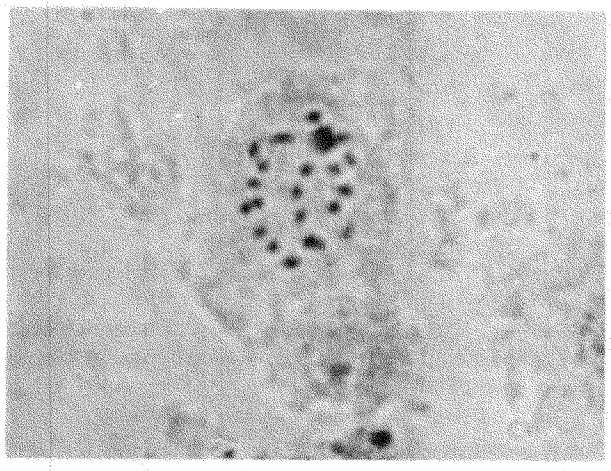


Figure 10. Photograph of R_{115} mitotic chromosomes during early metaphase. 2000x, 2n=22

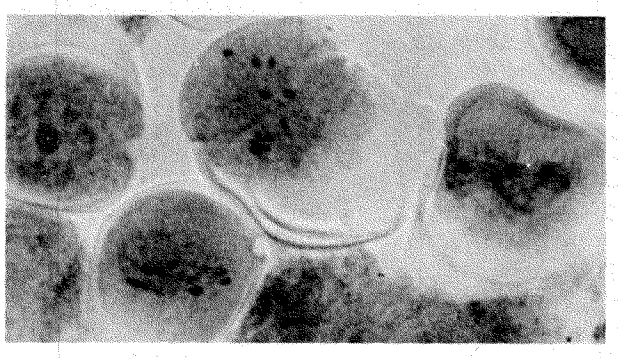


Figure 11. Photograph of meiotic chromosomes of R_{12} pollen mother cells during metaphase II. n=11

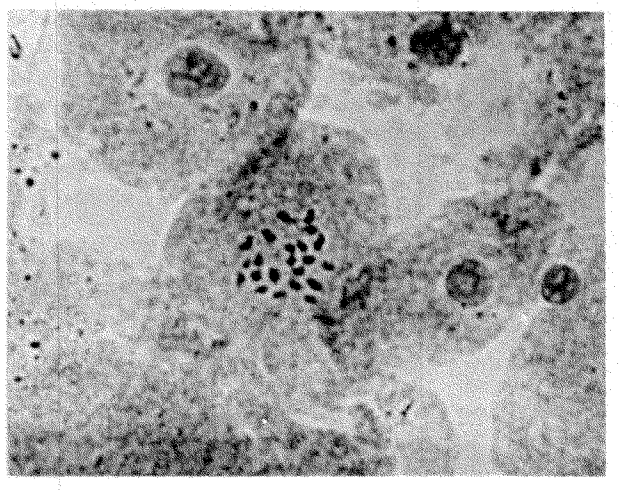


Figure 12. Photograph of N_5 mitotic chromosomes during early metaphase. 2000x, 2n=22

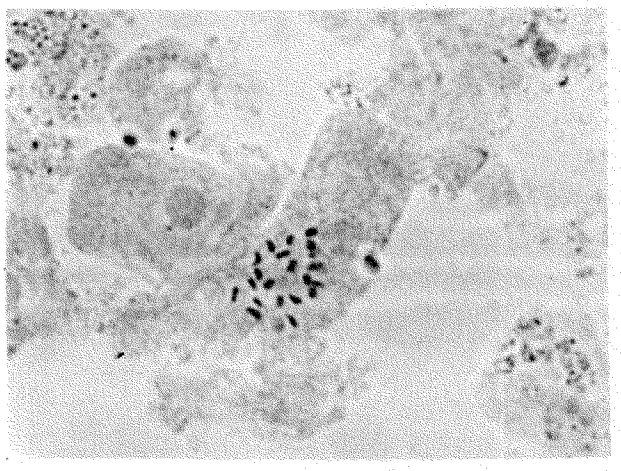


Figure 13. Photograph of N_{15} mitotic chromosomes during early metaphase. 1400x, 2n=22

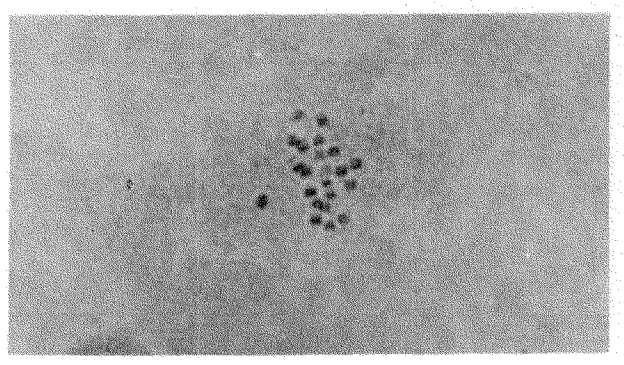


Figure 14. Photograph of N_{16} chromosomes during early metaphase. 1400x, 2n=22

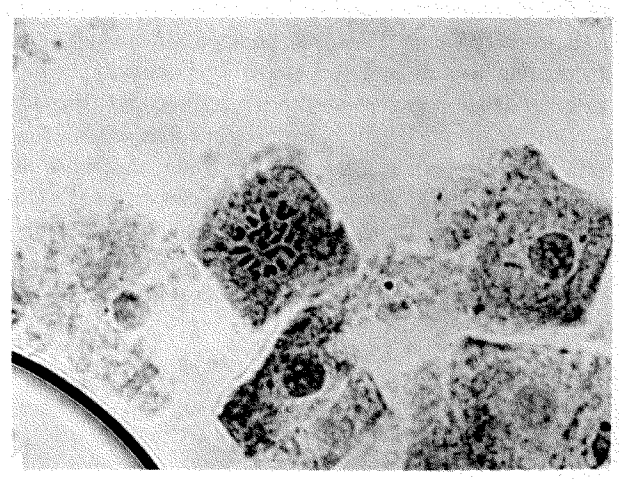


Figure 15. Photograph of N_{17} chromosomes during early metaphase. 1600x, 2n=22

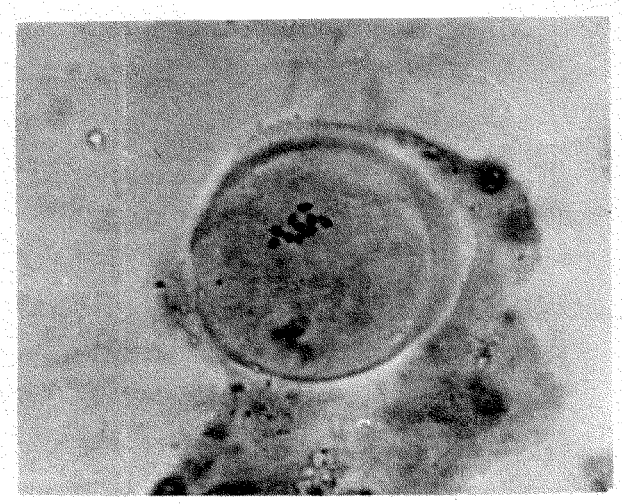


Figure 16. Photograph of meiotic chromosomes of N_{17} pollen mother cells. n=11

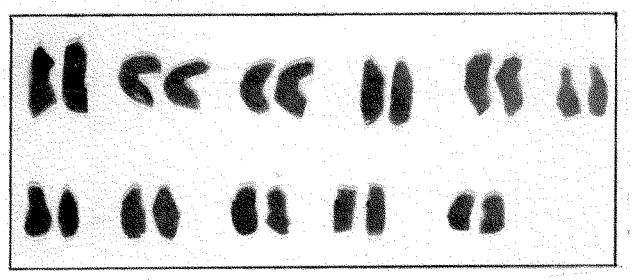


Figure 17. Approximate karyotype of *Nerium oleander* (N_{17}); chromosomes are arranged according to size and presumed homologies. 6400x, 2n = 22.

of the chromosomes renders karyotyping problematic in *Rhazya* and *Nerium*. However, an approximate karyotype (according to size and shape) of N_{16} population, which possessed the larger chromosomes, was prepared. (Fig. 17).

In conclusion, despite the small size of chromosomes and mixoploidy in both species, we were able to determine the basic chromosome number of this species as x=11. This chromosome number agrees with the findings of Baquar [1] for *Rhazya stricta* and of Gadella [3] and Hill [4] for *Nerium oleander*. The basic chromosome number of *Vinca rosea* was reported 2n = 2x=11 [5, 13].

Acknowledgements

We are grateful to the National Research Center for Genetic Engineering and Biotechnology for financial support during the course of this research.

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