CYTOMORPHOLOGICAL AND PHYSIOLOGICAL INVESTIGATIONS ON INDUCED AUTOTETRAPLOIDS OF TROPICAL KUDZU [PUERARIA PHASEOLOIES (Roxb.) Benth.] A COVER CROP IN RUBBER PLANTATIONS

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by

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C E R T I F I C A T E

This is to certify that the thesis entitled **'Cytomorphological and physiological investigations on induced autotetraploids of 'Tropical kudzu' [Pueraria phaseoloides (Roxb.) Benth]. A cover crop in rubber plantations'** is an authentic record of original research work carried out by Kum.Meenakumari T at the Rubber Research Institute of India, Kottayam-686 009 under our joint supervision and guidance during the period July 1989 to March 1995, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science, Mahatma Gandhi University. The work presented in this thesis has not been submitted for the award of any other degree or diploma earlier. It is also certified that Kum.Meenakumari.T has fulfilled the course requirements and passed the qualifying examination for the Ph.D. Degree of Mahatma Gandhi University.

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DECLARATION

I, Meenakumari .T, hereby declare that the thesis is entitled 'Cytomorphological and physiological investigations on induced autotetraploids of 'Tropical kudzu' [Pueraria phaseoloides (Roxb.) Benth] A cover crop in rubber plantations' is a bonafide record of the research work done by me at the Rubber Research Institute of India, Kottayam-686 009 and that no part thereof has been presented earlier for any degree or diploma of any other University.

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Dedicated to
my parents and brother

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INTRODUCTION

INTRODUCTION

One of the most crucial and major factors for sustainable crop production in any agricultural system is soil conservation. In the case of plantation crops like rubber, oilpalm, coconut and cocoa soil management practices assume greater significance for several reasons. Utilisation of undulating to steep terrains for cultivation, coupled with the intense rainfall regime experienced in the tropics leads to severe loss of top soil and thereby depletion of soil reserves. An estimated 430 million hectares of land has been irreversibly destroyed through accelerated soil erosion in different countries (Lal, 1990). The practice of growing leguminous ground covers in the plantations not only guard against soil erosion by providing a dense vegetation, but also improves the soil nitrogen profile through symbiotic nitrogen fixation (Smith and Gallon, 1993; Lal, 1994). Nitrogen is the most limiting nutrient in crop production in the tropics. Requirement of N is mainly met through fertilizer inputs, which contribute substantially to ground water and atmospheric pollution (Bohlool *et al.*, 1992). The escalating cost of fertilizers makes it unaffordable to a vast majority of small farmers in developing countries. Studies have shown that green manure legumes can often accumulate 100-200 kg N

ha^{-1} in 100-150 days (Giller and Wilson, 1991).

Beneficial effects of ground covers in rubber plantations have been emphasised time and again (Watson 1957, Watson et al., 1964, Lock, 1977, Potty et al., 1980, Kuan, 1982). Rubber has a long immaturity period of six to seven years before the tree attains tappable girth. The better growth of trees in the legume cover area facilitates early commencement of tapping, thereby reducing this period by six months to one year (Mainstone, 1960; Yogaratnam et al., 1984; Dissanayake and Waidhyanatha, 1987) and also provide significant savings in the use of nitrogenous fertilizers (Pushparajah, 1977; Sivanadhyan, 1983; Mathew et al., 1986). Eventhough the covers die out after about four years when the canopy closes, their net residual effect on the trees extends well into the 15th year of tapping or upto the 20th year of planting. In an experiment conducted at the Rubber Research Institute of India the natural cover area received, on an average, 270 kg ha^{-1} of extra nitrogen to obtain comparable yields to that of legume cover area during nine years of tapping (Punnoose et al., 1993).

An ideal cover should satisfy certain conditions like fast growth, shade tolerance, non-competition with rubber, high nitrogen fixing capacity, drought tolerance and freedom from pests and diseases (Potty et al., 1980). From among several members of the Leguminosae screened for the purpose, two most popular covers currently in wide use are Pueraria phaseoloides and Mucuna bracteata, of which the former has several advantages over the other ground cover species.

The genus Pueraria belongs to the Phaseoleae tribe of Leguminosae and comprises of 25 species (Lackey, 1980). Pueraria phaseoloides popularly known as 'Tropical kudzu' is a short duration plant with a long juvenile phase (Ison and Hopkinson, 1985) and is widely used for soil conservation (Bogdan, 1977). It can be propagated by seeds, vine cuttings and terminal shoots called 'crowns', is comparatively easier to establish, grows faster covering the soil in a very short time and is only mildly selective in its rhizobium requirements (Bogdan, 1977). In India the major portion of the rubber growing area under ground cover is occupied by P. phaseoloides alone and in Sri Lanka 90 per cent of the new planting and replanting area comes under P. phaseoloides (Jayasinghe et al., 1990). Its total nitrogen content, nodulation and nutrient constitution is also comparable with that of other legumes (Oke, 1967). On the other hand P. phaseoloides does have certain disadvantages also. The plant shows a shallow rooting system (Chandapillai, 1967), produces lesser amount of dry matter when compared to M. bracteata (Kothandaraman et al., 1989) and dies out in peak summer.

In India, the annual rubber production for the year 1993-94 was 435,160 tonnes (Rubber Board, 1994). Against this, consumption of natural rubber within the country has been 450,480 tonnes showing a gap of 15,320 tonnes. It is projected that the production of natural rubber in India during 1999-2000 will be 638,000 tonnes while the demand is estimated at 643,000 tonnes (Menon and Unni, 1992). This situation calls for substantial increase in natural rubber production.

Two methods, very relevant to increase the total production, are (1) improvement in productivity through adoption of scientific plantation management procedures including fertilizer application and (2) bringing more areas under rubber plantations. In either case considerable increase in the requirement of nitrogenous fertilizers become inevitable. Hence any attempt towards improving the present performance of the crop is worth considering. In general two approaches can be followed towards augmenting the beneficial effects of ground covers, one of which is the selective introduction of microsymbiont (bradyrhizobium) into the soil for incrased N_2 fixation and associated traits. Work on this line is already in progress. The second and more important method seems to be the manipulation of the macrosymbiont (host) for overall growth performance, as a ground cover notwithstanding N_2 fixation per se, and induction of polyploidy is one significant tool towards achieving this goal. A perusal of literature on induced polyploidy in related crop species further substantiate the scope for improvement in P. phaseoloides by adopting this technique.

Induced Polyploidy

Polyploids are abundant in nature. Grant (1981) has estimated that 47 per cent of all angiosperms are probably of polyploid origin. Polyploidy can also be induced artificially, and colchicine technique is the widely accepted method for chromosome doubling (Eigsti, 1955). The incidence of natural polyploidy in the Leguminosae - representing the third largest family of flowering plants - is very low (Bir and Sidhu, 1967). Levan (1942) has put forth three conditions which decide

the suitability of a crop to polyploidy induction viz., (i) low chromosome number, (ii) allogamy and (iii) vegetative plant parts contributing to economic yield. The two most consistant effects associated with induced polyploidy are an increase in cell size and a reduction in fertility. Hence crop species in which an increase in cell size is advantageous, and are least affected by a reduction in fertility are best suited for induction of polyploidy (Dewey, 1980) and in certain fodder and green manure crops including clover and alfalfa, the polyploids were reported to surpass the diploids in vegetative yield (Burnham, 1962; Hagberg and Akerberg, 1962). In the present context, autopolyploids further attract considerable attention in view of the following reports:

1. Leaf models based on leaflets size and tissue density in tetraploid chickpea, showed that the tetraploid plants had greater productivity potential per unit of leaflet surface area than that of the diploids (Fagerberg et al., 1990).

2. Cytopalynological studies relating pollen characteristics to cytology and genetics showed that pollen grain size, morphology, stainability, production and physiology of pollen have a direct bearing on induced polyploidy (Khoshoo, 1978; Nair and Ravikumar, 1984).

3. Ploidy variations were also reported to be associated with an increase in size of stomata, number of chloroplasts in guard cells, mesophyll thickness and chlorophyll content in various crop species (Cukrova and Avratovscukova, 1968; Dornhoff and

Shibles, 1976a, Joseph et al., 1980). Photosynthesis - the primary plant process governing crop production - was found to be closely associated with volume of different cell types (Dornhoff and Shibles, 1976a, Jellings and Leech, 1984) and thereby influenced by variations in ploidy. Further more, photosynthesis alone accounts for the bulk of the dry matter production in any crop species (Sarkar, 1991).

4. According to Bliss (1992) responses such as increased root growth and branching may lead to greater nutrient uptake and more nitrogen accumulation irrespective of greater fixation potential.

5. Induction of polyploidy enhanced the frequency of root hair infection in Trifolium (Wier, 1961) and Phaseolus species (Kabi and Bhaduri, 1976) and increased nodule size and total nodular volume in berseem and senji (Bhaskaran and Swaminathan, 1958). A number of workers have also emphasised the association of nodule number, nodule mass, nodulation score and total plant nitrogen in several plant species, with increased N_2 fixation (Duhigg et al., 1978; Barnes et al., 1984; Hoffmann and Melton, 1981; Rosas and Bliss, 1986) and with the ploidy level (Leps et al., 1980).

Above all, polyploidy is one of the methods of species formation and induced autopolyploidy provides greater opportunity in the area of crop improvement (Eigsti, 1955) and for cytogenetic stock development

of species (Lewis, et al., 1980).

Information on induced polyploidy in P. phaseoloides is lacking. In this context, the present study was taken up with the following objectives:

(1) To test the effectiveness of colchicine application technique on chromosome doubling in P. phaseoloides and (2) to analyse and evaluate the resultant tetraploids for the following parameters, to have a better insight into those characters contributing to productivity.

i) Morphology and cytology
ii) Palynology
iii) Foliar anatomy
iv) Physiological traits and biomass production
v) Uptake of nutrients and
vi) Nodulation and nitrogenase activity

The pattern of behaviour of the autotetraploids obtained, with respect to the above traits, is discussed in comparison with their diploid counterparts.

MATERIALS AND METHODS

MATERIALS AND METHODS

The field trials and pot culture experiments were conducted at the Rubber Research Institute of India (RRII) situated at 9° 32'N, 76° 36'E in Kottayam District of Kerala State. Seeds of Pueraria phaseoloides (Roxb.) Benth were obtained from the Experiment Station of the RRII.

Percentage germination of seeds was assessed by two popular presowing treatments viz., hot water and acid treatment. For each treatment 100 seeds were used in three replicates. In the former, seeds were soaked in hot water (60-80°C), kept as such for 4 h and germinated in petriplates lined with moist filter paper. In the latter, seeds were soaked in conc. H_2SO_4 for 10 min. washed thoroughly in water and kept for germination.

Cytological studies in the diploids

(i) Mitosis

Mitotic chromosome counts were made from actively dividing root tip cells. Scarified seeds were kept for germination and root tips (3-5 mm) were pretreated with a saturated aqueous solution of pDB for 1 h at 4°C. The specimens were then washed and fixed in

acetic : alcohol (1:3). After overnight fixation, they were were hydrolysed in NHCl for 10 min. at 60°C, mordanted in 4 per cent iron alum (Ferric ammonium sulphate) for 10-15 min., stained in 0.5 per cent Heidenhain's haematoxylin and squashed in a drop of 45 per cent acetic acid.

(ii) Meiosis

Flower buds at appropriate stages were collected and fixed in the morning between 10 to 11 a.m. in 1:3 acetic alcohol, kept overnight and then preserved in 70 per cent alcohol in the refrigerator. Anthers were smeared in a drop of 2 per cent acetocarmine stain. Slight heating and pressing gave better results. All observations were recorded from fresh preparations.

Induction of polyploidy

Seeds and young seedlings were treated with colchicine solution as described below for inducing polyploidy. Aqueous colchicine at different concentrations was prepared in double distilled water without any buffer and preserved at 8-10°C in the refrigerator.

(i) Seed

Full seeds, after pretreatment, was selected and excess water adhering to the surface blotted off. Seeds were submerged in 0.2, 0.3, 0.4, 0.5, 0.6, 0.75 and 1 per cent colchicine solution each for 6, 9, 12 and 15 h. For each treatment 200 seeds were used. After the treatment the seeds were washed well and kept for germination in petriplates lined with moist filter paper.

(ii) Seedling

Seeds were germinated in well washed river sand in trays. At the two-leaf stage cotton wads were placed on the apical vegetative buds and colchicine solution was provided by a dropper. The concentrations tried were 0.5, 0.75 and 1.0 per cent. The duration of treatment under each concentration was 4 h continuous, 6 h continuous and 8 h (4 h for two consecutive days). For every treatment 100 seedlings were used. All the treatments were carried out at room temperature (28 $\pm$ 2°C). Colchicine solution was renewed after every 1 h and constantly stirred to allow aeration. At the end of the treatment the shoot tips were rinsed thoroughly with water.

(iii) Planting of treated seeds and seedlings

Sprouted seeds and seedlings were raised in polythene bags (15 x 10 cm) filled with potting mixture. Top soil upto 15 cm from rubber growing field was collected, sieved and mixed with sand and cowdung in the ratio 1:1:1 to prepare the mixture. They were maintained in the glass house till establishment and then transplanted to the experimental field, during June-July 1990, when rains were available. Square planting was adopted, with a spacing of 90 cm. Diploid side branches, wherever emerging, were identified based on external morphology and clipped off to allow the main shoot to grow. As P. phaseoloides is a vigorous creeper, individual plants were supported on stakes, to prevent inter-twining and to facilitate plant to plant observation. Other usual agronomic practices have also been followed.

Simultaneously, the respective controls (untreated) were maintained.

Detection, isolation and evaluation of autotetraploids

An increase in stomatal size and pollen grain diameter was considered for the preliminary screening of the polyploids in Co-generation. The variants were tagged for detailed chromosome studies. Only those plants with the tetraploid chromosome number ($2n = 4x = 44$) were carried forward to the next generation.

Autopolyploids and the corresponding diploids were studied with respect to the following characters.

(i) Morphology and cytology

(a) Germination and seedling height

(b) General morphology

(c) Stomatal guard cells: Terminal leaflets of the three youngest mature leaves per each sample plant were selected to obtain lower epidermal peels by Jeffrey's method (Purvis et al., 1966). 100 stomata were randomly observed from each plant for the following characteristics:

i) Length and width of guard cell

ii) Frequency of stomata per unit area

iii) Stomatal index (Salisbury, 1927)

$$SI = \frac{S}{E + S} \times 100$$

Where E = Number of epidermal cells

S = Number of stomata in the field and

SI = Stomatal Index

(d) Internodal length: Length of the internodal region between the first and second flowering nodes was measured.

(e) Floral characters:

i) Flower size - length from the thalamus to the tip of the standard petal

ii) Days for flowering

iii) Number of inflorescence per plant

iv) Number of flowers per inflorescence

(f) Cytological studies: Flower buds at appropriate stage from the suspected polyploids were collected, fixed and stained following the laboratory procedure described earlier. Chromosome behaviour at diakinesis and metaphase-I and anaphase-I abnormalities were studied from well spread pollen mother cells (PMCs).

Chiasma frequency per cell was determined from the relative position and frequency of chiasmata in all possible configurations at metaphase-I.

(g) Pollen stainability: Flower buds were fixed in 70 per cent alcohol and mature anthers stained in a drop of iodine - potassium iodide solution (2 g iodine and 4 g potassium iodide dissolved in 100 ml 45 per cent glycerol). Full and uniformly stained pollen grains were taken as fertile. Shrivelled and yellow stained were considered sterile. (Pollen morphological variations in the induced tetraploids and the reliability of pollen grain diameter in the preliminary screening of polyploids are dealt with, in a separate section, described elsewhere).

(h) Ovule sterility: Computed as percentage of fully developed seeds per pod.

(i) Pod length

(ii) Palynological Studies

Pollen from diploid and induced tetraploid plants were studied under light (LM) and scanning electron microscope (SEM). Mature buds were fixed in 70 per cent alcohol and LM observations were made following two methods viz., the 'acetolysis' method by Erdtman (1952) and 'alcohol' method by Nair (1960). In the former, the pollen grains were treated with the acetolysis mixture (acetic anhydride and sulphuric acid 9:1) while in the latter, the grains were stained with iodine-potassium iodide. The acetolysed materials were finally mounted in glycerine jelly on glass microscopic slides.

Morphological characters including polar diameter, equatorial diameter, ora diameter and exine thickness were computed from 100 mature grains randomly selected per plant, from all the tetraploids and their corresponding diploids, with the help of an ocular micro-meter. Pollen size index was calculated according to Tseng and Ting (1964) as $\sqrt{P \times E}$, where P and E are the lengths of the polar and equatorial axes respectively. Pollen shape was worked out according to the formula $\frac{P}{E} \times 100$ (Erdtman, 1952) and the per cent occurrence of various pollen shapes in either ploidy determined.

For SEM studies, the acetolysed grains were fixed on to specimen stubs with double glow discharge and samples vacuum coated with metallic gold to a thickness of 100 A°. Observations were made with the help of an HUS-5B, S-450 Stereo scan (Hitachi, Japan) at a magnification of 3000 x.

Palynogram was drawn from camera lucida sketches.

The 'raw' tetraploids were propagated vegetatively by vine cuttings, as seed set was totally affected in all the tetraploids. Even the two plants, which produced a few unhealthy pods, contained only aborted/non-viable seeds. Three rooted cuttings per plant were maintained in earthern pots (30 x 30 cm) along with that of the control.

The following observations were made in the first vegetative generation.

(iii) Foliar Anatomy

Youngest fully expanded leaves of the tetraploids and diploids were sampled for the purpose. Approximately 1 sq. cm. of leaf tissue was removed from the midlaminar region of leaves s and preserved in FAA solution (70 per cent ethanol : glacial acetic acid : formaldehyde (90:5:5). Transverse sections were prepared according to the conventional techniques (Johansen, 1940), stained in 1 per cent aqueous safranin and mounted in DPX. Ten random observations were recorded from three leaf samples per plant, on total leaf thickness, palisade thickness, palisade cell number per unit leaf area, thickness of spongy mesophyll and

epidermal cell size by means of an ocular micrometer. Measurements on leaf blade thickness and interveinal distance were made at equal distances from either side of the mid rib.

(iv) Physiological traits and biomass production

Measurements on gas exchange properties were made in situ using a Portable Photosynthetic System (LICOR Inc., Model 6200), which is a closed system consisting of an infrared gas analyser (IRGA) and a leaf chamber. Observations on carbondioxide exchange rate (CER), transpiration rate (E) and stomatal resistance r_s were made in triplicate from single intact mature middle leaflet of each potted plant at 8 am. In addition, the other observations recorded are as follows:

a) Single leaf area : measured using a portable leaf area meter (Delta-T devices Ltd., England) from ten randomly selected leaves per pot.

b) Total leaf area: calculated from the total number of leaves per plant and the mean leaf area per trifoliate leaf.

c) Canopy photosynthesis: (Photosynthesis for the entire plant) - worked out from carbondioxide exchange rate and the total leaf area per plant.

d) Fresh weight and dry weight per leaf: Leaves after determination of leaf area were weighed fresh and then dried to constant weight at 80°C for subsequent weighing.

e) Specific leaf weight (SLW) following Pearce et al. (1969).

$$SLW = \frac{\text{dry weight of leaves (mg)}}{\text{Leaf area (sq. cm.)}}$$

f) Quantitative data on biomass production was obtained by destructive sampling of the diploid and tetraploid plants. All the potted plants at maturity were removed and sorted separately into individual plant parts (Leaf, stem, root and nodule) during the fast growing phase. The below ground parts were gently washed over a 40 mesh seive, with a fine jet of water to remove the soil particles. All the above samples were weighed fresh and then oven dried to constant weight, for subsequent weighing.

(v) Estimation of nutrients

In order to determine, the level of major nutrients in either cytotypes aliquots of dried material were homogenised to powder and used for standard chemical analysis.

Total N was determined by microkjeldahl method, from 50 mg of the samples (previously dried at 105°C for six hours). For the rest of the nutrients, 0.5 g of the dried samples were ashed in a muffle furnace at 500-550°C, digested with 5 ml of 1:1 hydrochloric acid, cooled and the contents made upto 100 ml in a standard flask, for further analysis. Phosphorus and potassium levels were determined in autoanalyser (Technicon Model), whereas calcium and magnesium concentrations were read in a GBC double beam atomic absorption spectrophotometer (Model 902) as outlined by

Karthikakutty Amma (1989). The nutrient contents were expressed as percentage. Total nutrient uptake was determined from the nutrient content and dry weight of plant parts.

(vi) Nodulation and nitrogenase activity

Individual nodules were harvested from each plant and the following observations were recorded, before analysing for nutrient content:-

a) Nodule number

b) Nodule fresh weight and dry weight

c) Nodule score following Mytton and Jones, (1971)

Nodules from each plant were graded according to nodule size as follows:-

Grade	Nodule size	Points awarded
1	Small (<2.0 mm diameter)	1
2	Medium (2.0-4.0 mm diameter)	3
3	Large (4.0-6.0 mm diameter)	6
4	Very large (>6.0 mm diameter	10

Nitrogenase activity:- Nitrogenase enzyme action was estimated by measuring the acetylene (C_2H_2) reduction activity (ARA) according to the in situ method suggested by Wani (1988).

Rooted cuttings of the diploid and tetraploid plants, (second vegetative generation) were raised in polythene bags in the glass house. Six weeks after the establishment, the sample plants were transported to the laboratory for pot assay. Each potted plant was enclosed in air tight containers (measuring 2 l) and sealed. A

siliane rubber tube with a rubber stopper was inserted and tied firmly to the base of the container.

200 ml of acetylene gas was then injected into the chamber using a hypodermic syringe, after withdrawing an equal quantity of air. The system was incubated at room temperature (28 $\pm$ 2°C) for 1 h. A 0.5 ml gas sample was withdrawn after flushing twice and fed into a Schimadze 9A gas chromatograph with a flame ionization detector and equipped with a stainless steel column of 80-100 mesh porapak N (Column temp. 75°C; Oven temp. 100°C). Three measurements were recorded per plant at intervals of 30, 60 and 90 min. after the exposure time. Nitrogen was used as carrier gas. The acetylene reduction activity was calculated by measuring ethylene peaks. Correction for endogenous ethylene production and admixture of any ethylene in the acetylene gas was suitably applied and the results are expressed as follows:-

$$\text{nmoles } C_2H_4 \text{ plant}^{-1} \text{ hour}^{-1} =$$

$$\left[\frac{\text{sample ethylene after 1 h.}}{\text{ethylene after 1h.}} \times \frac{\text{acetylene at 0 time}}{\text{acetylene after 1 h.}}\right] -$$

$$\left[\frac{\text{sample ethylene at 0 time}}{\text{ethylene at 0 time}} \times \frac{GV\ (ml) - VCF \times VPM}{22.4\ (T_1 - T_o)\ h.}\right]$$

where GV = gas volume at container

VCF = vacutainer correction factor

VPM = ethylene concentration (standard sample)

T_1-T_o = difference in sampling intervals

Manuring

The polybag plants were given 1.5 g mixture of rock phosphate and muriate of potash in the ratio 3:1, 30 days after establishment.

In the case of pot grown plants and plants grown in the fields, 30 g of fertilizer of the same combination was applied in two instalments after 30 and 60 days of planting.

Statistical analysis

Mean, range and standard error were estimated following the conventional statistical procedures. Comparison of means between the two cytotypes was made by the 2-tailed paired 't' test. ANOVA and simple correlation studies were done following Panse and Sukhatme (1967).

Photography

Photomicrographs were taken employing a Leitz orthopan microscope. Cell plates were exposed using ORWO 35 mm roll film.

RESULTS AND DISCUSSION

1. MORPHOLOGY AND CYTOLOGY

RESULTS

Pueraria phaseoloides is a trailing shrub with rusty brown hairs covering the stem and leaf surfaces. Leaves are large, trifoliate with entire leaflets. Flowers are typical of Papilionaceae, born on long peduncled racemes. The plant sets seeds profusely. Pods are linear with an average of 18 seeds per pod (Figs. 1, 6 and 7).

Germination studies showed that in the case of seeds subjected to acid treatment there was 78 per cent germination as against 49 per cent in those presoaked in hot water. So also, the bulk of the seeds scarified with conc. H_2SO_4 germinated in the first few days, whereas in the other case, germination was slow and continued for several days (Table 1). Hence acid scarification was followed to break seed dormancy in the present investigations.

1.1. Cytology of the diploid

Cytological studies in the root tip cells of the diploids revealed the somatic chromosome number of P. phaseoloides as $2n = 2x = 22$ (Fig. 2). The small size of the chromosomes and lack of proper staining rendered difficulty in obtaining good mitotic preparations by the usual methods. Hydroxyquinoline pretreatment followed by staining in acetocarmine and propionocarmine were tried with less success. However, haematoxylin squash technique was well suited to obtain well spread chromosomes at metaphase.

Pollen mother cells at diakinesis (Fig. 3) and metaphase-I (Fig. 4) exhibited eleven bivalents. One to two bivalents were usually found attached to the nucleolus. Chromosome pairing and disjunction at anaphase-I was regular (Fig. 5).

1.2. Effectiveness of colchicine treatment

1.2.1. Seed

Effect of colchicine on seed germination and survival are given in Table 2. The lower concentrations (less than 0.5 per cent) virtually did not have any effect on both germination and survival. With increase in concentration and duration of treatment, germination was delayed. The first pair of trifoliate leaves emerged in 10-15 days. A few seedlings with swollen radicle and plumule were obtained at concentrations above 0.5 per cent, but they did not survive. In the rest of the seedlings, the effect of colchicine was not persistent in inducing variations in ploidy, and the resultant plants turned out to be normal at maturity.

1.2.2. Seedling

In the case of seedling treatments, the frequency of polyploids varied with the concentration of treatment solution and duration of application (Table 3). Administration of 0.75 per cent colchicine for 4 h was the most effective treatment with respect to the maximum number of morphotypes and tetraploids produced. Application of 0.5 per cent and 1 per cent colchicine solution also gave reasonably good success. Treatment for 8 h in two split

doses of 4 h in two consecutive days was more effective, than continuous application for 6 h. With increase in concentration and duration, there was an increased rate of mortality in the induced polyploids. Altogether ten tetraploid plants were obtained. Of these 4 resulted from 0.75 per cent treatment for 4 h, two from 1 per cent treatment for 4 h and one each from the rest of the treatments.

1.3. Immediate effect of colchicine

Growth of the treated seedlings was totally arrested for about 7-10 days, after which they registered a slow growth rate (Fig. 8). Bulbous swelling of the shoot tip was a characteristic feature, of the treated seedlings. The seedlings resumed normal growth after about 3-4 weeks (Fig. 9). Some of the plants were distinctly short statured with distorted shoot and wrinkled leaves (Fig. 10). Such dwarf plants however, failed to survive till maturity.

1.4. Identification of polyploids

The polyploid plants could be identified based on morphology, stomatal size, and pollen diameter.

The polyploids had fewer leaves than the diploids during the initial period. The first formed leaves were thicker, small in size, and deformed with intensified colour. However, mature leaves were vigorous with increased leaf size, coarser trichomes, long and thick petioles and swollen pulvinus. Few mixoploid plants with diploid and polyploid shoots which appeared in the population grew

like the diploids towards maturity. Tetraploid plants (ultimately confirmed from chromosome counts) were altogether distinct from the respective diploids. This distinction was more pronounced towards later stages of development. A characteristic feature of some of the tetraploids was the production of polyphyllous leaves (Fig. 11) with more than three folioles. Bifoliate to octa-foliate leaves were observed with variation in size, shape, number and insertion of the individual leaflets. So also branched inflorescence (Fig. 12), not observed in the diploids, was a distinctive feature of one of the tetraploids, adding to the novelty of the plant. Other distinguishable features of the tetraploids included increase in size of guardcells (Figs. 13 a, b) and trichomes, reduction in internodal length, pollen stainability (Figs. 14 a, b), flowering and podset (Table 4).

Mean guard cell length for the diploids and tetraploids were 12.45 μm and 25.30 μm respectively. At the same time, there was a considerable reduction in the number of stomata per unit leaf area in the latter. The surrounding epidermal cells also showed a proportionate increase in size with that of the guard cells. As a result the stomatal index was not found to vary significantly in the two groups. The trichome length for the tetraploids was more than that of the diploids. This difference in trichome length also provided a supplementary parameter in distinguishing the ploidy level.

The tetraploids differed significantly from the diploids in flowering and seedset. A few of the tetraploids came to flowering

simultaneously with the diploids, while in the others flowering was delayed by 10 to 15 days. However, the duration of blooming was prolonged in all the tetraploids alike. So also the 4x plants had fewer number of inflorescence per plant. But the racemes were longer with an increased output of flowers per raceme than those of the diploids.

1.5. Cytology of induced tetraploids

Cytologically the induced tetraploids exhibited 2n = 4x = 44 at diakinesis (Fig. 15) and metaphase-I (Fig. 21). Cells at early and mid-diakinesis showed quadrivalents, trivalents, bivalents and univalents at varying frequencies (Figs. 16-20). Apart from IIs , the most common association observed was IVs . The maximum number of chromosome pairs attached to the nucleolus was two (Fig. 17). Pollen mother cells at metaphase-I also exhibited a similar trend in chromosome association (Table 5, Figs. 21-34). The frequency of quadrivalents per cell was relatively high. Mean chromosome association per cell was found to be 6.40 IV + 0.27 III + 8.36 II + 0.58 I s. The percentage of chromosomes involved in trivalents and univalents were low. From a total of 58 cells analysed, the most common association was 8 IV and 6 II s which accounted for 17 per cent of the total. 3.40 per cent of the cells studied, showed complete formation of 11 quadrivalents, the maximum possible number in a cell (Fig. 21). The configuration of the different types of quadrivalents observed showed that open or

closed rings and zig-zag or cross shaped quadrivalents were more prevalent. A tendency for secondary association of chromosomes was observed in some of the pollen mother cells (Figs. 27 and 28).

Chiasma frequency in the autotetraploid was related to the number of ring bivalents, chain and ring quadrivalents, trivalents and univalents. Chiasma frequency per cell in the diploids ranged from 16.00 to 19.00 whereas in the tetraploids the corresponding scores were 24.00 to 38.00 (Table 6).

Varying degrees of stickiness was a common feature in anaphasic cells (Figs. 35,36). Hence unequal separation of chromosomes, wherever occurred, could not be made with accuracy. Majority of the cells showed regular to near normal segregation (Figs. 37, 38). Other anaphasic abnormalities included varying number of laggards (Figs. 39, 40) and sticky bridges (Figs. 41, 42), relatively at lower frequencies (Table 7). Cells at the second division also followed a similar trend. However, tripolar orientation of chromosomes (Fig. 43) and sticky bridge with laggards at the 2nd anaphase (Fig. 44) was also observed.

Autotetraploids were characterised by deformed pollen grains, with considerable variation in grain size, morphology (described elsewhere) and stainability. Stainability varied from 35.42 to 65.60 per cent with a mean of 46.20 per cent stainable pollen as against 90.50 per cent in the diploids (Table 4). Pod set was also drastically reduced in the 4x plants whereas the normal plants among the colchicine treated population, showed profuse seedset,

almost similar to that of the diploids. Only two out of the ten tetraploids produced 3-4 weak pods, with reduced pod length as compared to those of the diploids. Each pod contained only 4-5 seeds as against a mean number 18 seeds per pod in the diploids. All the seeds were shrivelled and non-viable. Some of the developing pods abscised, during early stages of development.

DISCUSSION

Seeds of Pueraria phaseoloides contain very hard seeds, the proportion ranging from 80 to 95 per cent (Aya, 1973). The hard seedcoat reduces germination percentage (Rubber Research Institute of Malaysia, 1982). Presowing treatments are therefore warranted to ensure uniformity and higher percentage of germination as well as in reducing the toxic compounds present in the seed coat, which in turn inhibit nodule forming bacteria. Dipping in conc. H_2SO_4 and heating to 40°C were tried in the laboratory while in the field trials a combination of heating and soaking was attempted which was reported to be superior to sulphuric acid treatment. This gave a germination of 41-42 per cent (Aya, 1973). Under plantation practices pretreatment of Pueraria seeds either with acid or with hot water is usually adopted (Potty et al., 1980). In the present study, therefore only these two treatments were compared.

Both the treatments gave a higher germination percentage than that reported by Aya (1973). However, acid scarification had the advantage of effecting early and higher germination rate. While seed germination started the very next day after both the

presowing treatments, 50 per cent germination was observed after six days in the former, as against 10 days in the seeds soaked in hot water. The viable seeds alone, were selected for colchicine treatment, to ensure better penetration of the chemical.

Cytology of the diploid

Cytologically, the genus Pueraria has received very little attention so far. Information available on the chromosome number of P. phaseoloides is scanty and contradictory (Lackey, 1980). Some of the earlier workers reported 2n = 24 as the somatic chromosome number of P. phaseoloides (Fraham-Leliveled, 1953). Later Hardas and Joshi (1954) and Kumar and Hymowitz (1988) recorded the chromosome count as 2n = 22. The present study is in conformity to this number.

Microsporogenesis was normal, with the microcytes showing 11 bivalents at metaphase-I with a predominance of ring bivalents, accompanied by regular anaphasic separation and very high pollen stainability and ovule fertility (Meenakumari et al., 1993). Bir and Siddu (1967) reported that the genus Pueraria is dibasic with x = 10, 11. They also mentioned that in Phaseoleae, out of 75 species studied cytologically, 50 species have the basic number x = 11 and its multiples. P. phaseoloides also exhibits the basic number n = x = 11.

Induction of Polyploidy

In general, successful induction of polyploidy in members of Leguminosae, is much more difficult, than in majority of other

families (Gottschalk, 1978).

Although seeds, due to their ease of application are the best suited for colchicine treatment, seedling treatment has been found to be more effective than treatment of seeds. In P. phaseoloides seed treatment failed to produce any polyploids, despite the wide range of concentrations and treatment durations tried. Germination was reduced and recuperation of sprouted seedlings to normalcy was observed in due course. This is indicative that colchicine did produce some effect, but the required quantum of mutagen was not available to the growing meristem to induce any variation in the ploidy level. Failure of seed treatment in inducing polyploidy has also been reported earlier in Phaseolus mungo (Sen and Chedda, 1958) Cyamopsis psoraloides and Glycine max (Biswas and Bhattacharyya, 1971, 1972) and Trifolium riograndense (Schifino et al., 1987).

Seedling treatment invariably was more effective to induce polyploidy and for better establishment of plants. Various concentrations were successful in producing polyploids. Nevertheless, low concentrations were found to be more effective. In fact, high concentration of colchicine and long treatment duration showed pronounced lethal effect as judged from a reduction in germination and rate of survival. Moribund growth coupled with deformities and mottling of leaves, at the initial stages were characteristic of the treated plants. However, these results do not always predict the successful induction of polyploidy. For eg., in

flax, Dirks et al. (1956) found unexpected morphological variation in plants with no chromosome doubling after colchicine treatment. Reduced growth rate in the initial months of growth of the treated seedlings might be attributed to the physiological disturbance induced soon after colchicine application as reported by Swanson (1957).

Polyphylly, with an occurrence of even upto eight folioles per leaf was a remarkable feature of some, but not all, of the tetraploids. Such phenotypic modifications could be a manifestation of the double dosage of genes in the tetraploids. The induced tetraploids of Phaseolus (Biswas and Bhattacharyya, 1976) and Trifolium (Schifino et al., 1987) are also reported to produce multiple leaflets. In the case of tetraploid soybean, extra leaflets developed due to branching of lateral leaflets at the bases (Sen and Vidhyabushan, 1960). The variation in leaf number and size observed in the tetraploids provides scope for selection for improving the total biomass.

Gigantism and slow growth in the autotetraploids are reported to be associated with reduced rate of cell division (Stebbins, 1950), smaller amount of growth hormones (Gustaffson, 1944), and slower rate of metabolic activities (Noggle, 1946, Tal, 1980). In the present study, gigas nature of plant parts was observed only with respect to certain characters. On the other hand, some of the plants were distinctly dwarf statured. Gunckel (1957) attributed dwarfing of plants to the physiological factors

associated with chromosome variations. Dwarfing of induced polyploids was also reported by other workers (Biswas and Bhattacharyya, 1971).

An increase in cell size due to polyploidisation was well manifested in the size of stomatal guard cells as well as pollen grains. A selection strategy based on these parameters will be more advantageous in crops with relatively small chromosomes, encountering difficulties in chromosome studies. It also helps in culling out a large number of unaffected plants soon after the treatment. In P. phaseoloides with a seasonal flowering habit, stomatal guard cell enables in the preliminary screening of polyploids. Many earlier workers have also resorted to this method as an indicator of the level of ploidy in other plant species (Evans, 1955; Speckman et al., 1969; Tan and Dunn, 1972; Borrino and Powell, 1988; Davis et al., 1990).

There was also significant variation in pubescence of diploid and polyploid cytotypes. Trichomes were longer and coarser in the latter. Dark green leaves with enhanced leaf thickness and prominent trichomes might have resulted from an increase in cell size and a corresponding increase in palisade and spongy cells. The polyploid vines were thicker with a reduction in length of the internode. An increase in basal circumference due to an increase in the size of constituent cells and wider medullary activity was reported earlier in tetraploid soybean (Sen and Vidyabushan, 1960).

Delayed flowering is characteristic of many autopolyploids (Biswas and Bhattacharyya, 1971). In P. phaseoloides the onset of flowering was not much delayed in the tetraploids. However, the prolonged duration of flowering, when compared to the diploids might presumably be due to a longer growth cycle. Dnyansagar and Nadkarni (1983) suggested this phenomenon as a contrivance to obtain at least a few fertile seeds to perpetuate the progeny, since induced polyploidy is associated with very high sterility. Simultaneous flowering of 2x and 4x plants in the field was observed in Trifolium (Schifino et al., 1987).

The salient morphological features of the autotetraploids of P. phaseoloides is thus, similar to those observed in general in induced polyploids (Burnham, 1962; Hagberg and Akerberg, 1962).

Cytology of the induced tetraploids

Autotetraploids are characterised by the presence of four homologous chromosomes. Chromosome paring in general and in the autotetraploids is reported to be a function of homology and genetically controlled phenomenon of chiasma formation, among other factors (Durrant, 1960; Stebbins, 1971). Venkateswaralu (1975) has broadly classified pairing in autopolyploids as pachytene pairing and metaphase-I pairing. Other workers like Henderson (1969) considers only zygotene and pachytene pairing in that, if chiasmata had taken place during prophase, the homologous chromosomes are held together in subsequent stages till the end of metaphase-I. However, the exact pairing pattern of homologues at zygotene and pachytene is difficult to ascertain in most crop species as also in

the present case. Hence pairing configurations in P. phaseoloides were determined at diakinesis and metaphase-I, the latter being more ideal. The percentage of chromosomes involved in quadrivalent and bivalent formation were 58.00 and 38.00 per cent respectively. Morrison and Rajhathy (1960) observed in several autopolyploids that approximately, 2/3rd of the chromosomes are involved in quadrivalent formation irrespective of chromosome size. According to Canderon (1986) the percentage of chromosomes associated as quadrivalents, usually ranges between 30 and 60 per cent. In general, there are several factors which determine the frequency of multivalents in a crop species:-

(a) For a quadrivalent formation, chiasmata must be present on either side of the point of partner exchange. The high incidence of quadrivalents at metaphase in the present study is indicative of the persistance of chiasmata towards later stages of meiosis as reported by Swanson (1957) and is suggestive of greater affinity within the genome. Such a positive correlation between quadrivalent formation and chiasma frequency has been reported earlier by other workers (Roseweir and Rees, 1962; Swami and Thomas, 1968; Bhan et al., 1990). On the other hand the reverse situation was reported in tetraploid tomato (Riley, 1967) and teosinte (Sybenga, 1972) where quadrivalents were present in the pachytene, but localisation of chiasmata resulted in complete formation of bivalents at metaphase-I.

(b) Chiasma formation is genotypically controlled (Jackson and Casey, 1980). In P., phaseoloides, the mean chiasma formation in the diploids and tetraploids were 17.50 and 31.75 respectively.

According to Gupta and Sinha (1983) the predominance of ring bivalents with interstitial chiasma in the diploids as presently observed, indicates greater homogeneity during synapsis. Therefore an increase in chiasmata in the autotetraploids of P. phaseoloides, is suggestive of the influence of genes governing high chiasma frequency in the diploids.

(c) A third factor determining the production of multivalents /alents is karyotype symmetry. P. phaseoloides is characterised by a symmetrical karyotype showing very little of intrakaryotypic size difference between chromosomes, with a preponderance of metacentrics or submetacentrics. This condition will again increase the pairing effeciency of homologous chromosomes. In such a situation the plant would exhibit more than the expected mean number of quadrivalents and may even go upto the theoritical maximum possibility of 11 IVs as encountered in a few cells. In autotetraploid Avena (Zadoo, 1989) a reduction in IVfrequency has been attributed to asymmetric chromosome morphology, among other factors.

Another distinct feature of P. phaseoloides was the occurrence of secondary association between multivalents. Secondary association of chromosomes, at various ploidy levels, in many angiosperms have been postulated to be indicative of ancestral homology of the existing apparently diploid constitutions. For eg: in Rubiaceae, most of the genera have x = 11 constitution, but in some members like Ophiorhiza with n = 11, manifestations of secondary association of bivalents at the diploid state point out the

possibility of x = 11 being a derived basic number from a still lower constitution (Phillip and Mathew, 1987).

Literature shows that the relationship between chromosome behaviour and fertility in autopolyploids is primarily a matter of the species concerned. In general, high fertility is associated with preferential selection for bivalent formation (Stebbins, 1950; Gottschalk, 1978; Riley, 1967; Sybenga, 1972; Gupta and Koak, 1976; Lavania, 1991). On the other hand multivalents undergoing normal disjunction has been reported in autotetraploid rye (Ellerstrom and Sjodin, 1963) sorghum (Siddiqui, 1967) fescue (Simonsen, 1975) and maize (Mastenbroek et al., 1982). Accordingly, zig-zag, ring or ngor chain quadrivalents have a better chance to disjoin regularly at anaphase-I.

Chromosome doubling had significant but variable effects on fertility in P. phaseoloides. The shape of the quadrivalents at metaphase could be clearly distinguished only from a limited number of cells in which a trend towards higher occurrence of symmetric quadrivalents was evident. As regards anaphase, chromosome stickiness hindered the estimation of cells showing regular segregation to a certain extent. This in addition to segregational imbalance due to the formation of univalents, sticky bridges and tripolar orientation of chromosomes, collectively contribute to a reduction in pollen fertility in the autopolyploids as reported by Rana and Swaminathan (1968).

The mean pollen stainability in the tetraploids was 46.2 per cent as against 90.5 per cent in the diploids. Eighteen per cent

of the tetraploid pollen were found to germinate in situ, 24 h after dehiscence. However when stigma from flower buds of tetraploids were stained in cotton blue, pollen adherance and tube growth was negligible. Wherever the pollen tube was formed, the tube length was shorter with thicker walls. As a result, all the plants except two were completely sterile. Seeds obtained were however shrivelled and nonviable. In the present study, therefore, the presence of multivalents at meiosis although lowers the production of viable pollen, is not the only factor involved in a drastic reduction in podset and seed viability and other genetic factors might be involved. Gupta and Gill (1985) observed complete formation of nonviable achenes in induced tetraploids of Chrysanthemum with 83 per cent pollen fertility and attributed it to the functional inviability of gametes through chromosome addition. Phadnis and Narkhede (1972) observed three categories viz., sterile, partially fertile and fertile plants in the advanced generations of autotetraploid Cicer arietinum. Among the induced tetraploids of Zinnia (Gupta and Koak, 1976) one of the plants was completely male sterile, whereas the other was normal with 96 per cent pollen fertility and good seedset. According to Parthasarathy and Rajan (1953) fertility is governed by a system of polygenes that are in a balanced state in the diploids and that, the disturbed balance in the tetraploids can be restored by adopting suitable selection procedures. From these observations, a fertilization barrier appears to be operating at the tetraploid level in P. phaseoloides. The possibility of vegetative multiplication in this crop is however advantageous in propagating any of the induced tetraploids even at commercial level.

Table 1. Germination of seeds of Pueraria phaseoloides under acid and hot water treatments.

No. of days after sowing	Germination (Per cent)	
	Acid treatment	Hot water treatment
2	31	8
4	20	4
6	-	-
8	16	11
10	8	18
12	3	6
14	-	2
Total	78	49

Table 2. Effect of colchicine on seed germination and survival in *P. phaseoloides*

Conc. of colchicine (Per cent)	Duration (h.)	Germination (Per cent)	Mean plant height at 60 days(cm)	Survival (Per cent)
0.20	6	81	125.75	76
0.30	"	81	88.50	75
0.40	"	77	89.85	77
0.50	"	73	90.01	72
0.60	"	83	84.37	81
0.75	"	80	93.88	75
1.00	"	76	87.71	72
0.50	9	74	79.85	70
0.75	"	74	86.55	71
1.00	"	72	80.85	64
0.50	12	74	84.20	70
0.75	"	75	81.80	68
1.00	"	70	75.84	66
0.50	15	69	73.48	63
0.75	"	66	71.70	67
1.00	"	67	72.75	59
Control	-	83	129.75	82

Table 3: Frequency of colchi-tetraploids produced after different seedling treatments.

(Total number of seedlings treated = 100)

Conc. (Per cent)	Duration (h)	No. of colchiploids*	No. of tetraploids
0.50	4 (1 day)	8	-
0.75	"	12	4
1.00	"	9	2
0.50	8 (4h. 2 days)	5	1
0.75	"	4	1
1.00	"	2	1
0.50	6	4	1
0.75	"	3	-
1.00	"	1	-

*Including mixoploids

Table 4. Mean and range of different characteristics in diploid and autotetraploid plants of *P. phaseoloides*

Character	Diploid	Tetraploid
Stomatal length (µm)	12.45 (17.50-22.50)	25.30** (23.00-27.50)
" width (µm)	14.70 (12.50-17.50)	16.90** (16.25-18.00)
" frequency/unit leaf area^{-1}	16.20 (14.00-21.00)	9.78** (9.00-12.00)
" index	21.50 (20.40-25.00)	23.00 (19.0-27.00)
Internodal length (cm)	22.50 (15.40-30.00)	20.89 (17.10-28.30)
Flower size (cm)	1.95 (1.70-2.10)	2.05 (1.70-2.41)
Pollen stainability (per cent)	90.50 (86.00-95.00)	46.21** (35.42-65.60)
Days for flowering	188 (180-198)	196 (182-214)
Inflorescence plant^{-1}	19.50 (15.00-22.00)	8.00** (3.00 - 11.00)
Flowers raceme^{-1}	12.00 (5.00-17.00)	15.00 (5.00-21.00)
Pods plant^{-1}	18.00 (12.00-22.00)	4 (0 - 5.00)
Pod length (cm)	8.34 (6.30-10.20)	5.18 (3.79-6.58)
Ovule fertility	97.00 (96.00-98.00)	-

*, ** Significant at 5 per cent and 1 per cent level respectively.

Table 5. Meiotic chromosome behaviour in the autotetraploid (2n = 4x = 44) of P. phaseoloides at metaphase-I.

No. of cells	Chromosome configuration
2	11IV
4	10IV + 2II
4	9IV + 4II
10	8IV + 6II
2	8IV +1III+4II + 1I
6	7IV + 8II
1	7IV +1III+ 6II + 1I
6	6IV + 10II
4	6IV +1III +8II + 1I
4	6IV + 9II + 2I
2	6IV +2III+ 6II + 2I
2	5IV + 12II
2	5IV +1III+ 9II + 3I
1	4IV + 14II
3	3IV +1III+13II + 3I
5	1IV + 20II
Total 58	
Mean ± S.E.	6.4 ± 0.33IV 0.27 ± 0.06III 8.36 ± 0.65II 0.58 ± 0.13I

Table 6. Chiasma frequency per cell in the diploids and autotetraploids of P. phaseoloides.

Ploidy	Cells examined	Chiasma per cell
2x	50	17.50 (16.00-19.00)
4x	35	31.75 (24.00-38.00)

Table 7. Frequency of cells showing anaphase-I chromosome disjunction.

Type of cells	Per cent
Without laggards	89.76
With laggards-1	6.34
-2	2.01
>2	1.28
Bridges	0.59

Fig.1. A young rubber plantation with a well established ground cover of *P. phaseoloides*

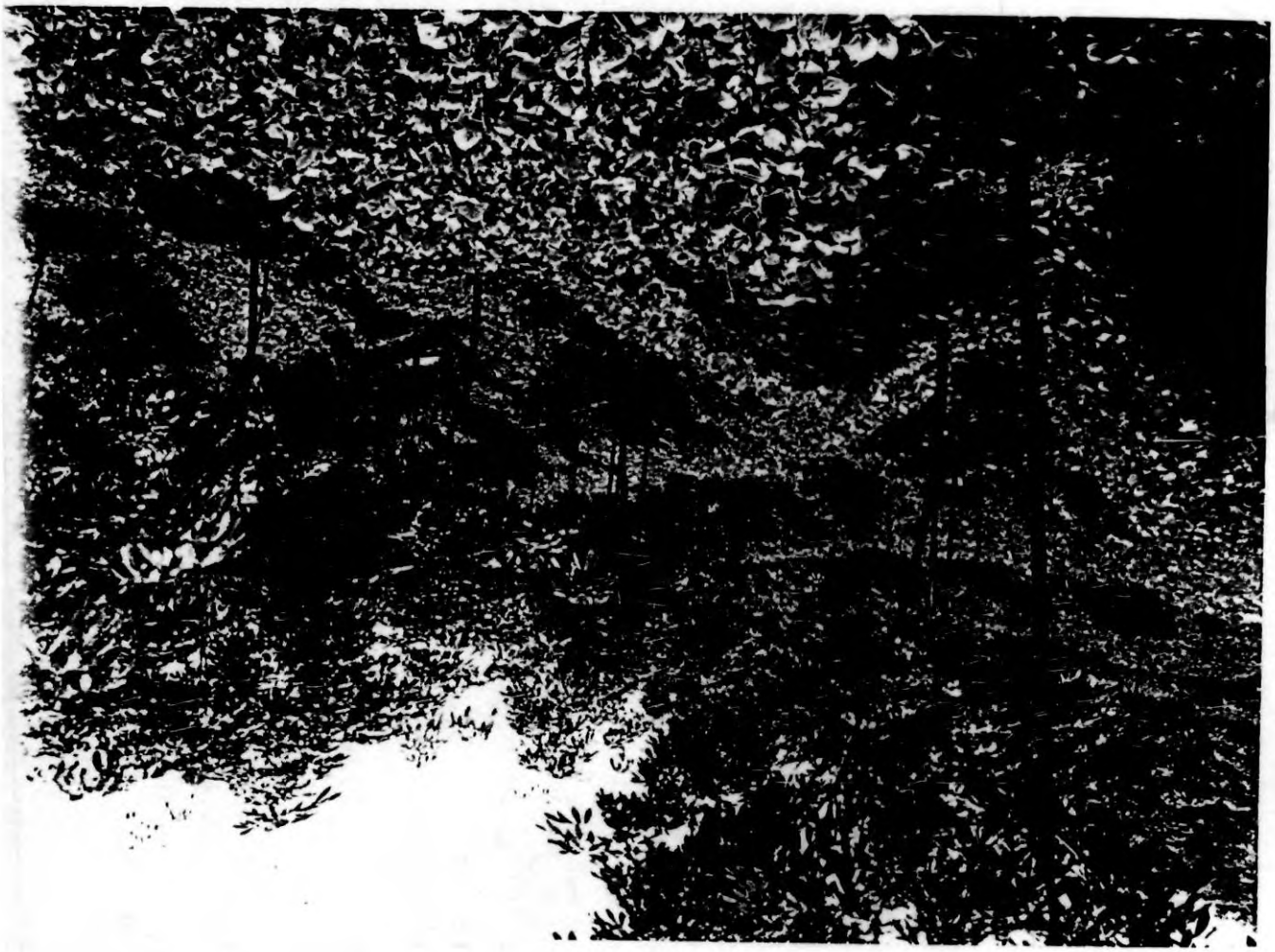


Fig. 2. Somatic metaphase of P. phaseoloides showing 2n = 22. X 3000

Fig. 3. Pollen mother cell (PMC) at diakinesis showing n = 11 bivalents X 1200

Fig. 4. PMC at metaphase-I showing 11 bivalents X 1200

Fig. 5. PMC at anaphase-I showing 11:11 chromosome disjunction X 1200

Figs. 6, 7. Flower, pod and seeds of P. phaseoloides

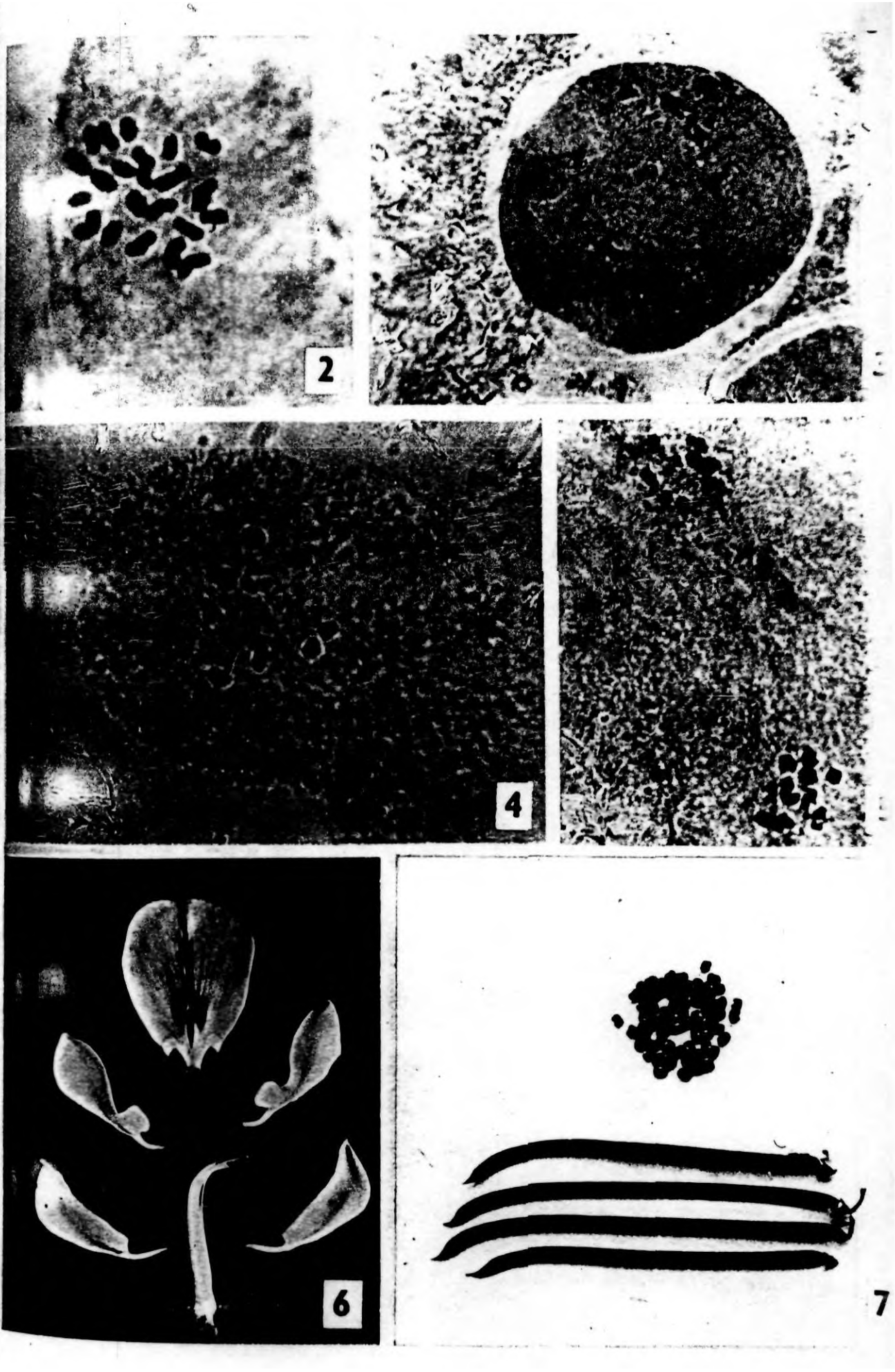

2
4
6
7

Fig. 8. (a) Retardation in growth rate in colchicine treated seedling and
(b) normal seedling

Fig. 9. (a) Six weeks old normal seedling and
(b) colchicine treated seedling

Fig. 10. A dwarf statured seedling

Fig. 11. (a) Trifoliate leaf of the diploid and
(b) polyphyllous leaf of the tetraploid

Fig. 12. (a) Branched inflorescence of tetraploid and
(b) inflorescence of diploid

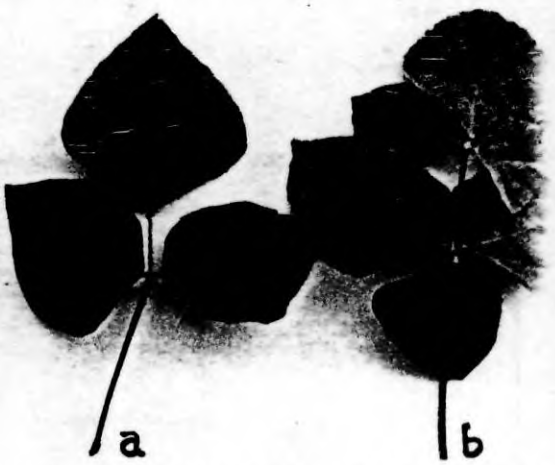
Fig. 13. (a) Stomata in the diploid and in
(b) the tetraploid (Arrow indicates base of the trichome) X 1200

Fig. 14. (a) Fertile pollen grains in the diploid
(b) Fertile and sterile pollen grains in the tetraploid X 1200


a
b
8


9a

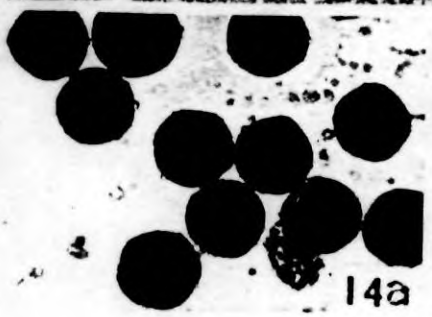

10


a
b


12


13a


1


14a

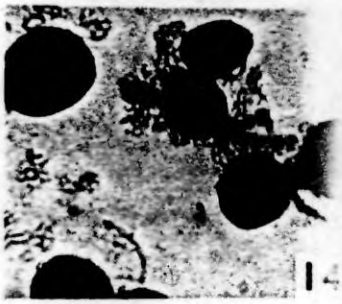

1

Fig. 15. Diakinesis in the induced tetraploid showing 7IV + 1III + 6II + 1I (arrowed)

Fig. 16. Diakinesis showing 6IV + 1III + 8II + 1I

Fig. 17. Diakinesis showing 2IIs attached to the nucleolus

Fig.18-20. Diakinesis showing varying degrees of chromosome configuration

[All figs. X 1200]

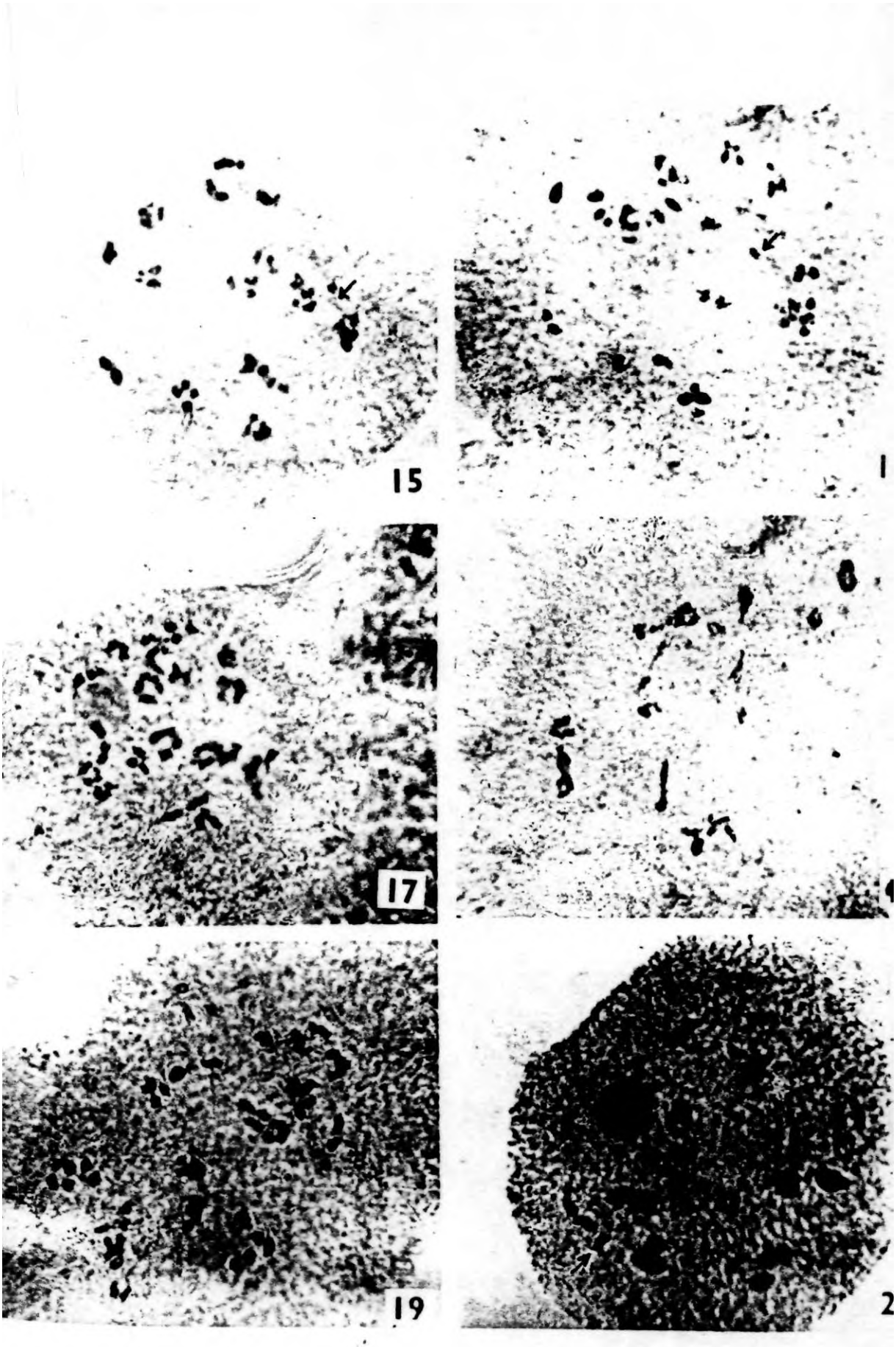
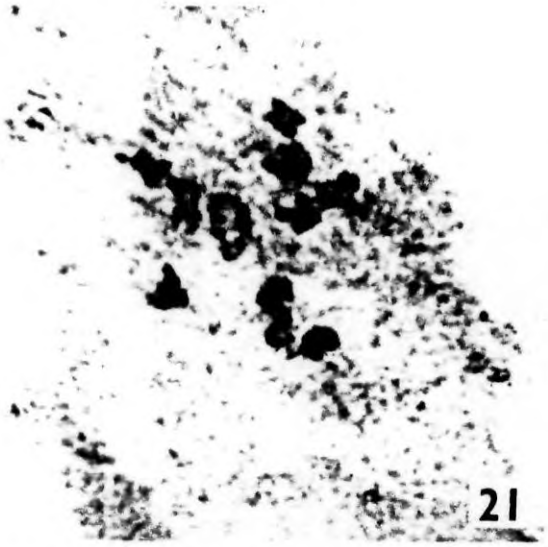

15
1
17
19
2

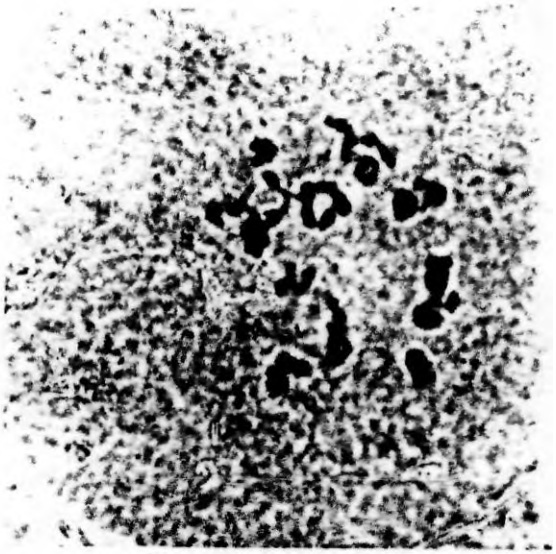
Fig. 21. Metaphase-I in the induced tetraploid showing 11IVs.

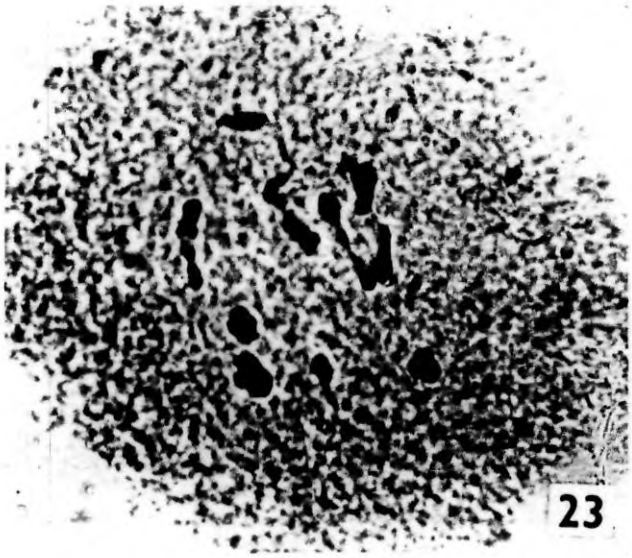
Fig.22-26. Metaphase-I showing varying frequencies of chromosome configuration

[All Figs. X 1200]

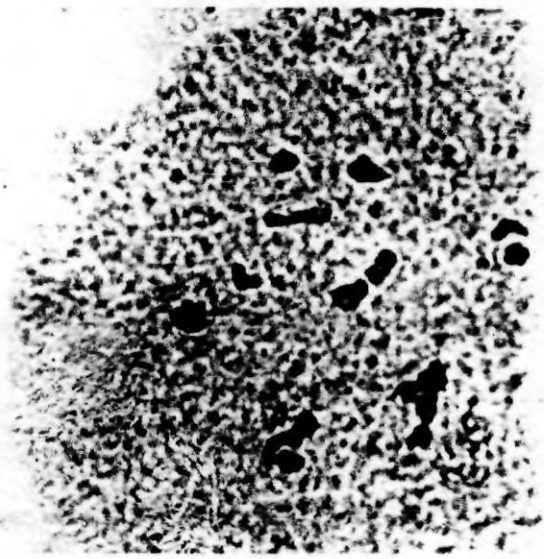


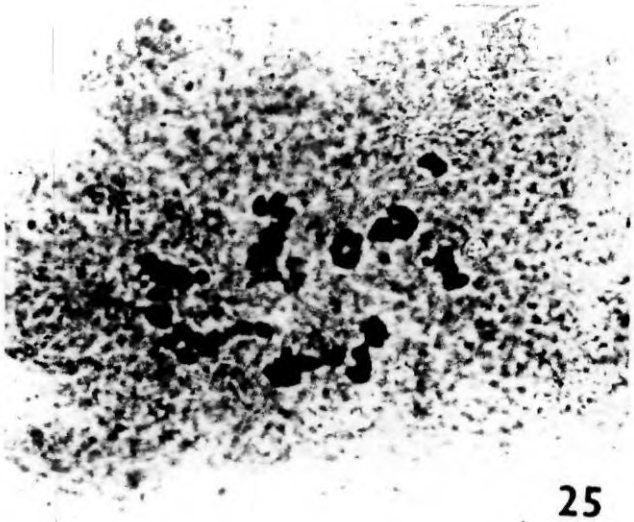
21





23





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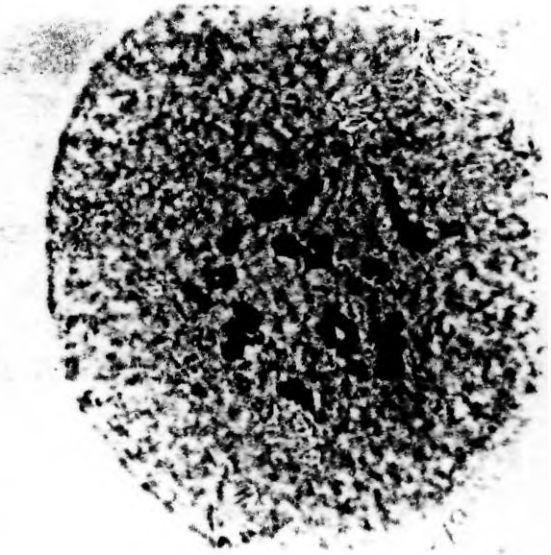


Figs.27-32. PMCs showing various chromosome configurations at metaphase-I in the induced autotetraploids of P. phaseoloides

Fig. 27. Cell showing secondary association between two bivalents (Dotted arrows indicate Is)

Fig. 28. Cell showing secondary association between four bivalents

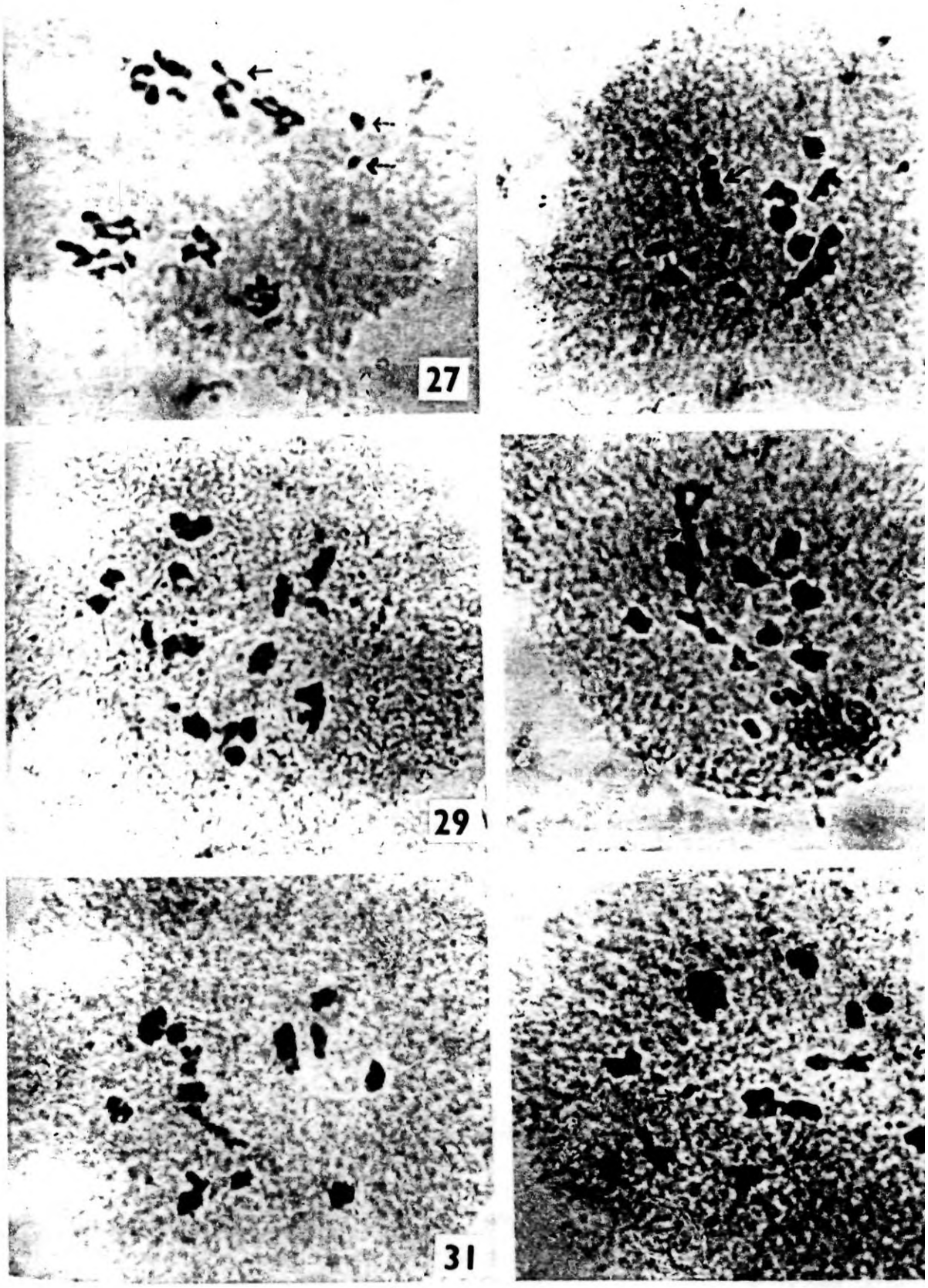
Fig. 29. 8IV + 6II

Fig. 30. 9IV + 4II

Fig. 31. 9IV + 4II (Arrow indicates secondary association)

Fig. 32. 8IV + 2II + 2I

[All Figs. X 1200]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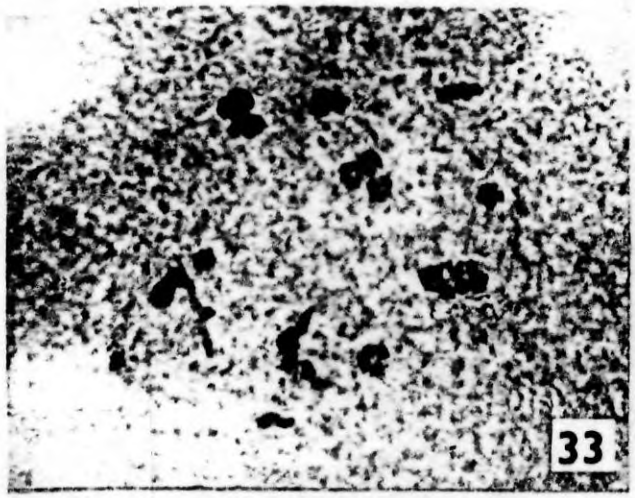
Figs. 33, 34. Metaphase-I showing 8IV + 6II

Figs. 35, 36. Amaphase-I showing varying degrees of stickiness

Fig. 37. Anaphase-I showing regular segregation of chromosomes

Fig. 38. Anaphase-I showing 22:1:21 chromosome separation [Arrow indicates laggard]

[All figs. X 1200]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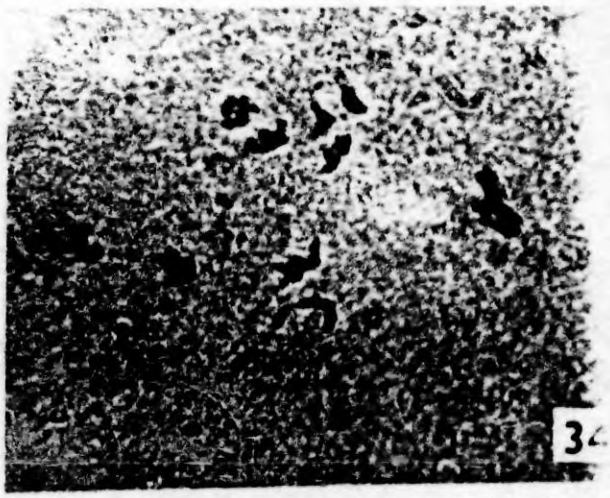
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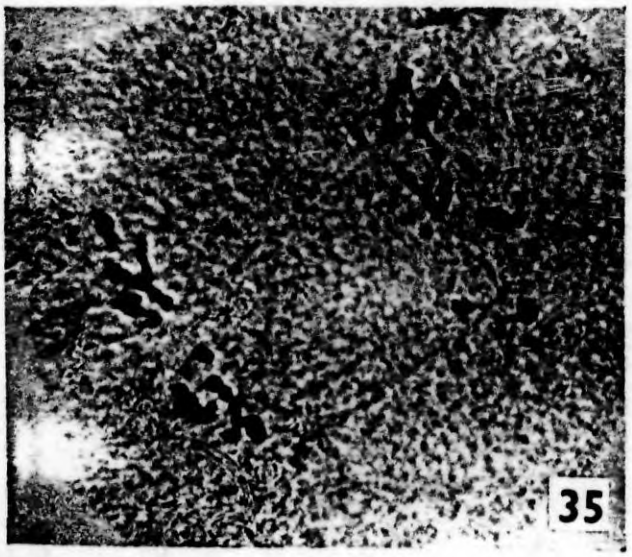
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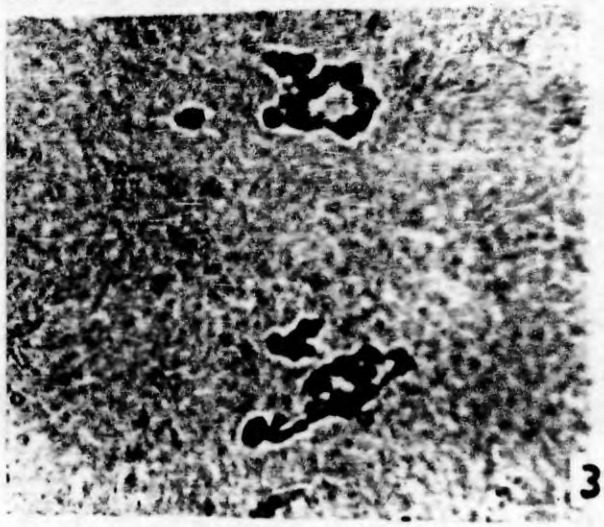
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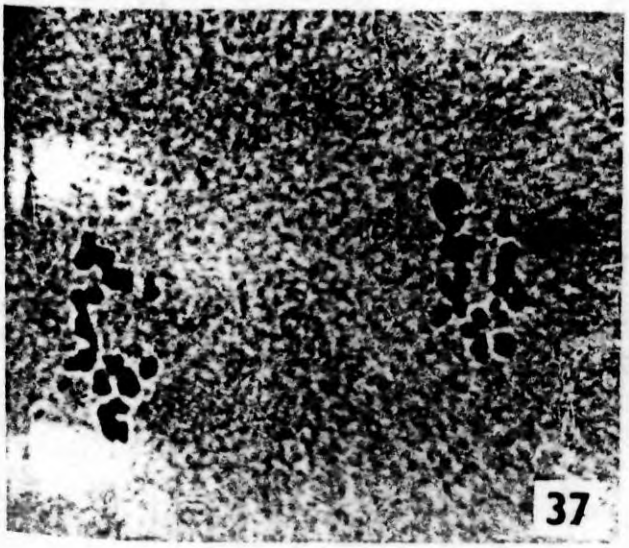
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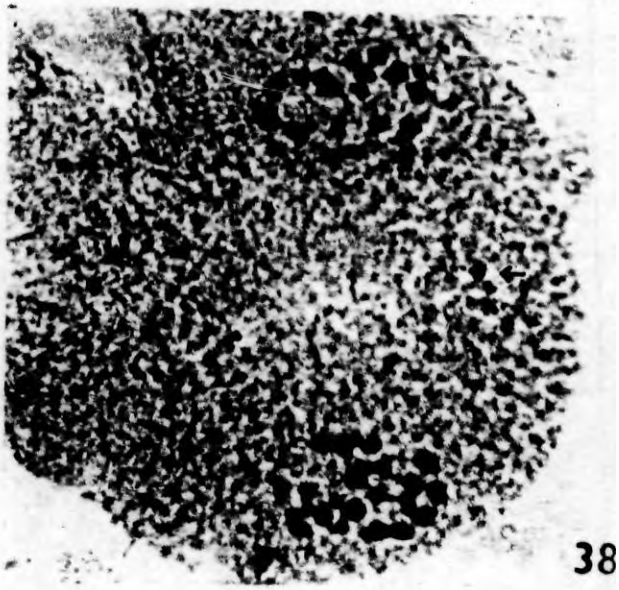

38

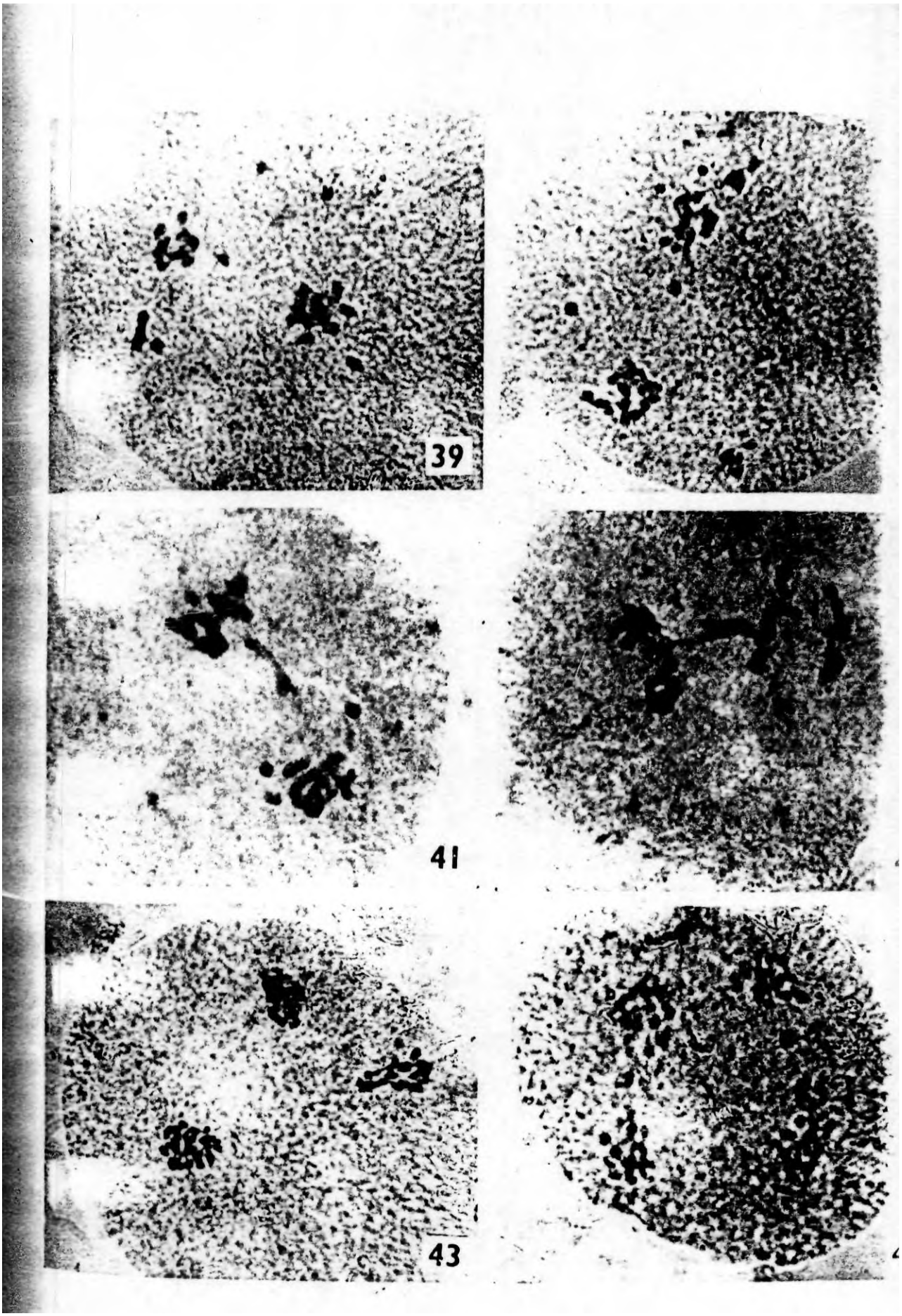
Figs. 39, 40. Irregular grouping of chromosomes at anaphase-I showing laggards

Figs. 41, 42. Sticky bridge at anaphase-I

Fig. 43. Tripolar orientation of chromosomes, at anaphase.

Fig. 44. Anaphase-II showing laggards

[All figs. X 1200]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43

2. PALYNOLOGY

RESULTS

Pollen grains of both the diploid and the autotetraploid plants were studied and the observations are summarised in tables 8-12. While the diploid plants were homogeneous with 3-zonocolporate grains, the tetraploids varied in different structural characteristics. The pollen descriptions and related information of each group are as follows:

2.1 Diploids

Grains were uniformly 3-zonocolporate, prolate spheroidal to subprolate with a predominance of the former (Table 8). The colporate aperture consisted of the outer ectocolpium (furrow) and the inner endocolpium (os; pl-ora). Ectocolpium narrow, 12 µm long; tapering with acute tips and tenuimarginate. Endocolpium lolongate/circular (Fig. 45 a, b). Amb (shape in polar view) triangular); exine surface reticulate; heterobrochate with variations in shape and size of brochi.

2.2. Induced tetraploids

The pollen mass generally consisted of three types of pollen grains with shapes varying from prolate spheroidal to prolate (Table 8). While diploid like grains were present in varying percentages in all the tetraploids, other pollen forms varying in apertural and ornamentation features also occur. The most significant characteristic was the occurrence of higher number of

grains with lolongate endocolpium measuring on an average 20.0 x 7.5 μm (length of the long and short axis respectively) (fig. 46a). Grains with circular ora showed an increase in size of the os than that of the diploids (fig. 46 b). The ectocolpium sometimes appeared to be short (brevicolporate) but in insignificant numbers (fig. 47). Yet other grains exhibited syncolporate condition (fig. 48). So also, 22.0 per cent of the grains were 4-zonocolporate (Fig. 49). In polar view, grains in the tetraploids were also triangular (Fig. 50). In addition to the above variations, micropollen having a size of 17.5 x 15.0 μm was observed in the tetraploid plants (Fig. 51). The micropollen apart from being small in size was characterised by a single spiral aperture occurring as a girdle around the grain and also the reticulum. SEM pictures showed reticulate exine ornamentation in both the cytotypes (Figs. 52-53) with bigger sized brochi in the tetraploids. A similar grain showing circular ora is shown in Fig. 54. A possible line of morphological evolution of different pollen shapes is proposed in Fig. 55.

A comparison of pollen morphological traits in the two cytotypes showed a significant increase in dimensions in the tetraploids than their diploid counterparts (Table 9). Pollen size index was 42.59 μm and 31.46 μm in tetraploids and diploids respectively. The location of measurements are as represented in Fig. 56 a. The palynogram of a typical pollen grain with its surface view as seen in the LM is given in Fig. 56 b. Apertures

are indicated by dotted lines.

The average pollen diameter of unacetolysed pollen grains i the diploids was 31.85 µm as against 37.82 µm in the tetraploid The chemical process of acetolysis ~~chemical process of acetolys~~ clearly revealed the morphology of pollen. Acetolysis resulted i an increase in grain size in all the tetraploids and diploids alik (Fig. 57). The mean increase in grain size in the diploids wa 3.45 per cent. The unacetolysed grains had a mean size of 37.9 µm whereas the mean size of acetolysed grains were 45.85 µm, thus resulting in a 20.95 per cent increase in pollen diameter in the latter (Table 10).

Frequency distribution of pollen grain diameters in colchicine treated plants, together with the corresponding ploidy (lower histograms) are given in Fig. 58. These histograms show the reliability of pollen grain measurements in determining ploidy. In order to determine the variation within the two groups of plants, the mean pollen diameter of ten tetraploids and diploids were analysed (Table 11). An ANOVA for this data (Table 12) showed appreciable variation between the two groups. However, the average pollen diameter of a few tetraploid plants was similar to that of the diploids. This was also indicated by the superposition of histograms for the two cytotypes (Fig. 58). Eventhough a precise demarcation is not possible, those plans with an average pollen diameter of 37.5 µm could be fairly selected for preliminary detection of tetraploidy.

DISCUSSION

Information on pollen morphology has been widely utilised in the studies on taxonomic and phylogenetic relationships of varieties, cultivars and cytotypes. Although the inheritance of pollen morphology in many species hybrids has been studied (Ravikumar and Nair, 1985) literature pertaining to the variation in size and shape of pollen with induced polyploidy are limited (Joshi and Raghuvanshi, 1967; Dnyansagar and Sudhakaran, 1972; SaraswathyAmma and Sethuraj, 1992). Ting (1961) from a survey of some members of Umbelliferae reported a high magnitude of pollen variability in the polyploids.

Among the various pollen shapes encountered in the present study, prolate spheroidal grains were more predominent, both in the diploids and induced polyploids. According to Nair (1970) and Nair and Ravikumar (1984) the shape of the pollen grains in many angiosperms being unfixed, pollen size, apertural variatioin and exine ornamentation are considered as more reliable parameters in morphological analysis. The wide range of variability in pollen size observed in the tetraploids might have resulted from chromosome disjunctional abnormalities expected during the course of meiosis. This was further established by the presence of micropollen, a resultant of lagging chromosomes. Joshi and Raghuvanshi (1967) studied the colchicine induced pollen variability in tetraploid Cuminum. They observed that in PMCs where a breakdown of spindle has taken place as well as a few in which normal four nuclei resulted after telophase II, there was no

clevaging into uninucleate microspores as is the normal case, bu all nuclei remained in a single cytoplasmic mass and the whol unit transformed into a giant grain. Also, the same PMC containe normal as well as variable grains.

Heslop-Harrison (1971) observed in Lilium, the sam phenomenon of spindle blockage by colchicine treatment, resulting in large sized individual spores with normal exine patterning; th colpus in such a spore is totally absent or it may develop randomly often in an irregular form. The occurrence of brevicolporate grains in the present study is indicative of such an abnormal development. Other additional pollen types included syncolporate, tetraporate or spiraperturate grains. Thus it is clear that most of the variations were the result of an increase in number or fusion of ectocolpium or else by a change in shape of the endocolpium. Clarke (1975 based on his study of forty species of Hypericum having irregular pollen with varying aperture number and configuration, also suggested that the production of such grains is associated with faulty meiosis. In P. phaseoloides some of the bigger pollen had a grain size of 55 x 50 µm.

Tetraporate grains observed in the polyploids could have been the result of meiotic disturbances leading to aberrant cytokinesis as suggested by Mujeeb et al. (1978). Tetramerous pollen has been commonly observed in many induced as well as natural polyploids (Nair and Sharma, 1966-67; Dnyansagar and Sudhakaran, 1972; Chadurvedi et al., 1990). According to Gupta

and Gupta (1978) the presence of 4-zonocolporate grains in the induced tetraploids of Crotalaria, is indicative that the material transgresses the limits of the family Leguminosae. Nair (1965 suggested that the members of the Leguminosae commonly exhibit 3-zonocolporate grains and the colporate or porate conditions might have been derived from the 3-colpate condition.

Considering both the diploids and induced tetraploids together, pollen grain variations with respect to different characters were evident, suggesting a certain evolutionary sequence. While applying pollen morphology for evolutionary considerations, characters with regard to the aperture are primary, the exine ornamentation secondary, and other tertiary in the order of their significance. In such a scheme, the basic morphotype in Pueraria is the 3-colporate form, as in most other Leguminosae, the ora being circular in shape. In a few grains in both the cytotypes, the change from circular to lolongate ora have been noticed, the quantum of which has been more in the tetraploids (ie., 21 per cent of the total grains scored, as against 16 per cent in the former). Further the change in the length of the furrow, from a long one (longicolpate) to a shorter one (brevicolpate) where the furrow remains within the circumference of the ora, in a few grains has been significant.

From the above observations, an evolutionary change from longicolpate to brevicolpate and from circular to lolongate ora

size as well as in the size of the reticulum was observed in the tetraploids. It may therefore be suggested that variations in the pollen grain are not only a projectile of genetic biodiversity, but also an indicator of pollen morphological evolution.

Acetolysis results in removal of protoplasm from the pollen grains, making them translucent and are hence more suited for morphological studies. The differential increase in grain size in the diploids and polyploids due to acetolysis, as presently observed, suggests the compactness or otherwise of the arrangements of sporopollenin materials in the elements comprising the exine wall (Ravikumar and Nair, 1985).

Scanning electron micrographs helps to understand the exact nature of exine ornamentation (Saraswathyamma et al., 1989) and to further substantiate the results obtained through light microscopy. Exine sculpturing was basically reticulate in both the diploids and tetraploids; nevertheless, there was an increase in the thickness of exine wall and the size of individual brochi in the latter. Many studies on the development of irregular pollen have shown that typical exine development takes place in reduced, unreduced and tetrakaryotic cells as well as in typical and abortive spores (Heslop-Harrison, 1971; Tara and Namboodiri, 1976; Nair and Ravikumar, 1984). Thus, it is clear that the basic programme for exine pattern are contained in the cytoplasm of the microspores, irrespective of the size of chromosome content (Ravikumar and Nair, 1985). At the same time, the reticular density varied widely,

within and between ploidy levels. Southworth and Pfahler (1992 while studying the spinule number and distribution in maize pollen in relation to ploidy, observed that the number of spinule initiation sites was more influenced by the genetic regulatory mechanisms of a given genotype rather than chromosome doubling per se.

i Pollen grain size has long been considered as a morphological indicator of polyploidy. It was in 1950 that Stebbins postulated the gigas effects of pollen of polyploids over their diploid counterparts. Later on, many authors have established a correlation between grain size and ploidy level (Kapadia and Gould, 1964; Speckmann et al., 1967; Tan and Dunn, 1973; Mathew and Philip, 1983). ANOVA for pollen grain size revealed that pollen diameter could be successfully used to identify the tetraploids to a considerable extent. Erdtman (1964) reported an increase of 15-29 per cent in equatorial diameter and 62-100 per cent in pollen grain volume consequent to the doubling of the chromosome number in species of Salvia and Eriogonum. The mean increase in grain size in the present study was 18.84 per cent. At the same time, the grain size of a few tetraploid plants (plant nos.10 and 38) were similar to that of the diploids. Hence it follows that pollen diameter alone should not be considered for determining tetraploidy in P. phaseoloides but chromosome counts are necessarily to be made.

Table 8. Occurrence of different pollen shapes in diploid and autotetraploid *P. phaseoloides*

Ploidy level	Per cent occurrence centre		
	Prolate Spheroidal	Subprolate	Prolate
2x	80.0	20.0	—
4x	64.5	32.0	3.5

Table 9. Morphological characteristics of pollen grains in diploid and tetraploid cytotypes.

Characteristics	2x	4x
Polar diameter (P) (µm)	32.75 ± 0.45	45.78 ± 0.92**
Equatorial diameter (E) (µm)	30.25 ± 0.41	40.65 ± 0.82 **
$\frac{P}{E}$ x 100	108.41 ± 1.20	112.65 ± 0.95
Pollen size index	31.46 ± 0.36	42.59 ± 1.02**
Exine thickness (µm)	2.50± 0.37	3.75 ± 0.43*
Ora diameter (µm)	9.49 ± 0.25	11.99 ± 0.38**

*, ** Significant at 5% and 1% level of probability respectively.

Table 10. Effect of acetolysis on grain size in P. phaseoloides

Ploidy	Mean diameter (µm)		Actual increase by acetolysis	Per cent increase
	Acetolysed	Unacetolysed		
2x	32.75 (30.00-42.50)	31.85 (20.00-37.50)*	+1.10	3.45
4x	45.78 (15.00-55.00)	37.85 (30.00-50.00)	+7.93	20.95

*Figures in parentheses relate to range

Table 11. Plantwise pollen diameter in ten tetraploids and their corresponding diploids

Diploids (2x)		Tetraploids (4x)	
Plant No.	Mean diameter (µm)	Plant No.	Mean diameter (µm)
1	33.50	10	31.24
2	35.00	12	41.33
3	33.50	15	36.45
4	33.25	16	38.75
5	31.75	27	39.30
6	29.50	32	40.45
7	30.50	33	42.00
8	31.75	38	35.80
9	32.75	45	41.10
10	33.50	50	42.41

Table 12. ANOVA for data presented in Table 11

Source of variation	DF	SS	MS	F	P
Plants	19	3408.49	179.39	58.81	**
2x X 4x	1	2143.16	2143.16	714.38	**
Error	180	549.73	3.05		
Total	199	3958.22			

** Significant at 1% level

Fig. 45. (a) Equatorial view of pollen grain in the diploid showing lolongate and

(b) circular ora

Fig. 46. (a) Equatorial view of pollen grain in the tetraploid showing lolongate and

(b) circular ora

Fig. 47. Brevicolporate grain - Tetraploid

Fig. 48. Syncolporate grain - Tetraploid

[All Figs. X 1200]

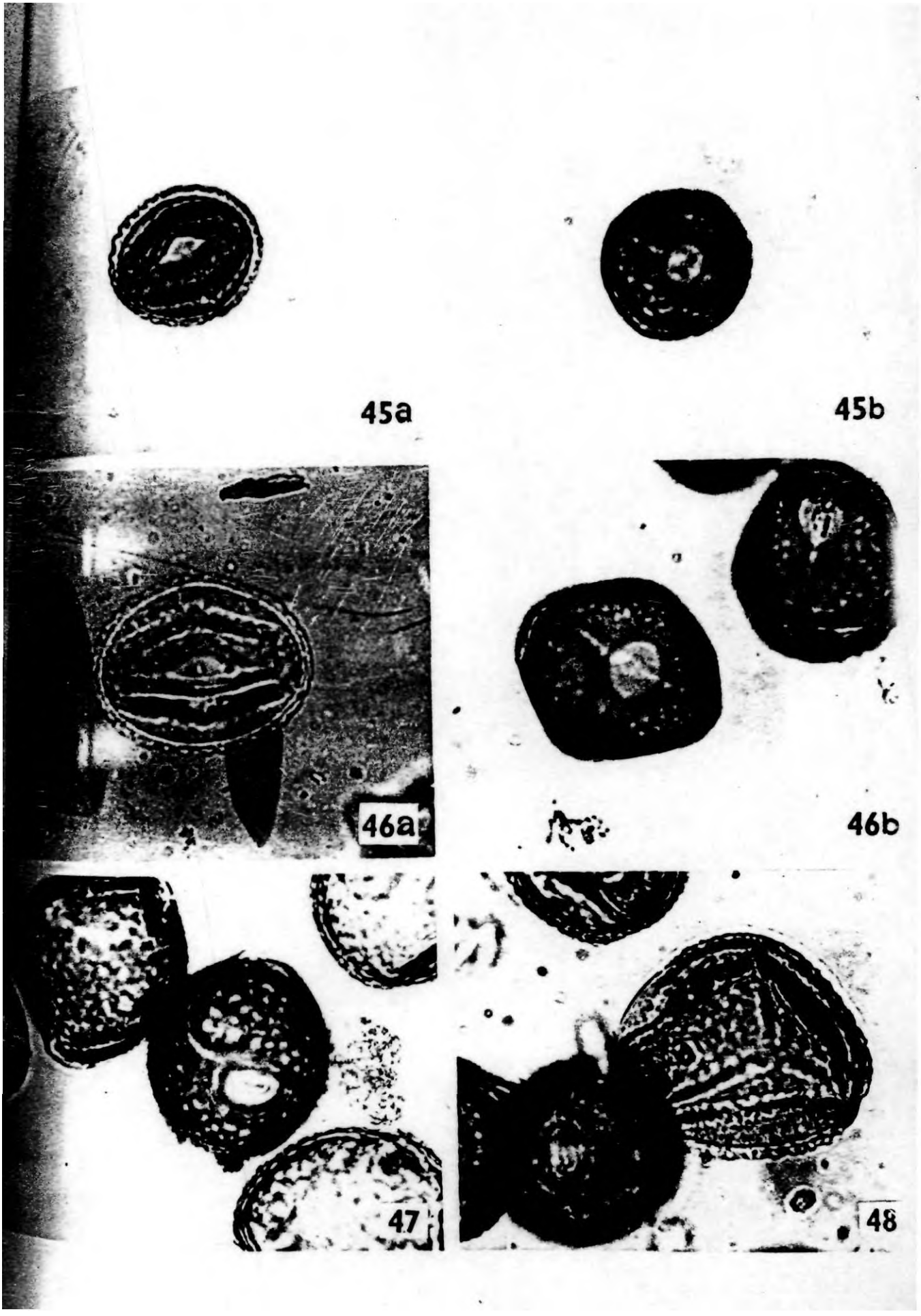

45a
45b
46a
46b
47
48

Fig. 49. 4-zonocolporate grain - Tetraploid (X 1200)

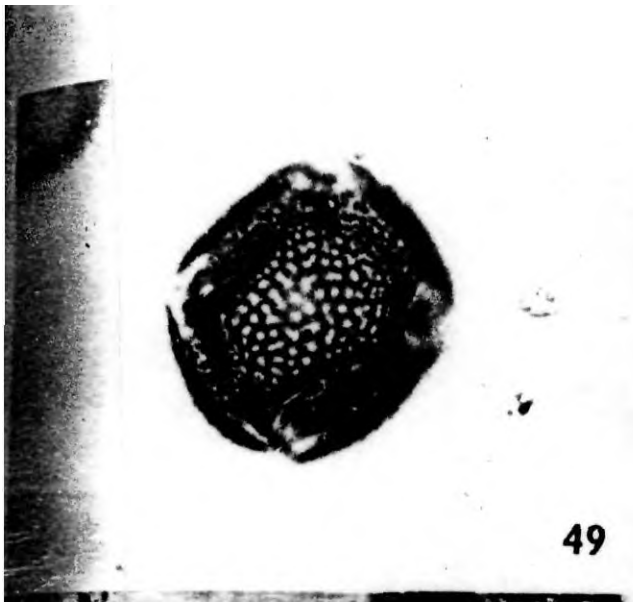
Fig. 50. 3-zonocolporate grain (Polar view) - Tetraploid (X 1200)

Fig. 51. 1-spiraperturate grain - Tetraploid (X 1200)

Fig. 52. Scanning electron micrograph (SEM) of a pollen grain - Diploid (X 1500)

Fig. 53. SEM of a 4-zonocolporate grain in the tetraploid (Note the shape of the pollen with bigger reticulations) (X 1500)

Fig. 54. SEM of a grain showing circular ora(X 7500)



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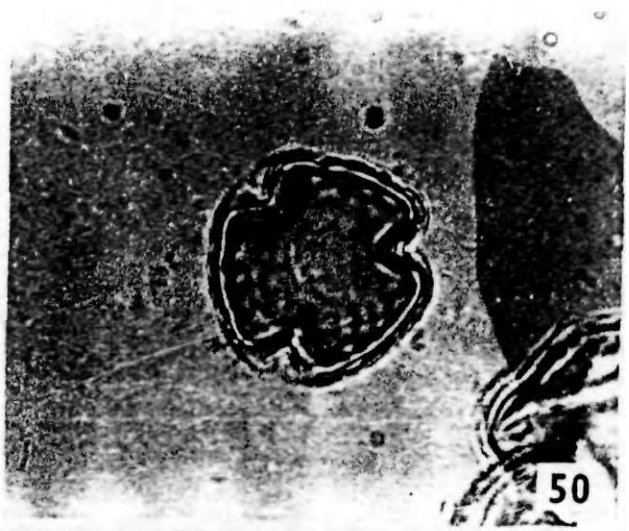

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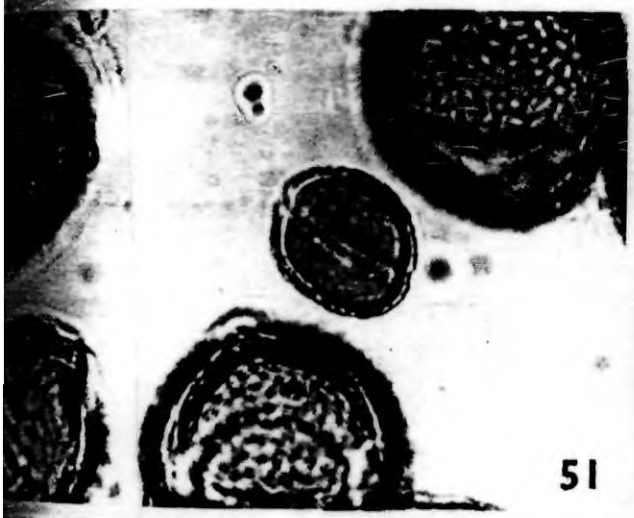

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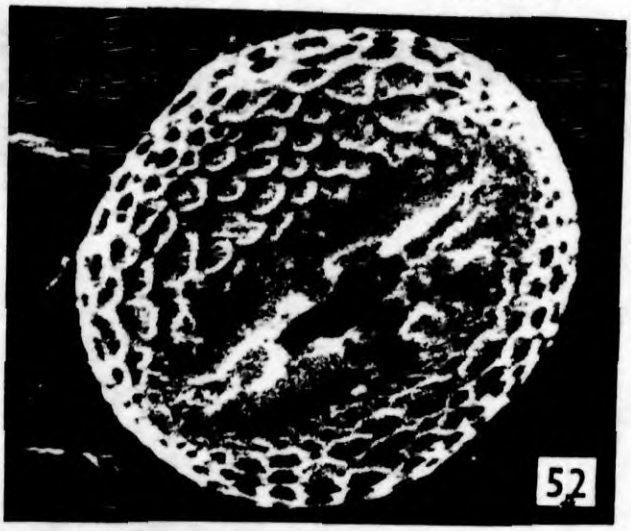

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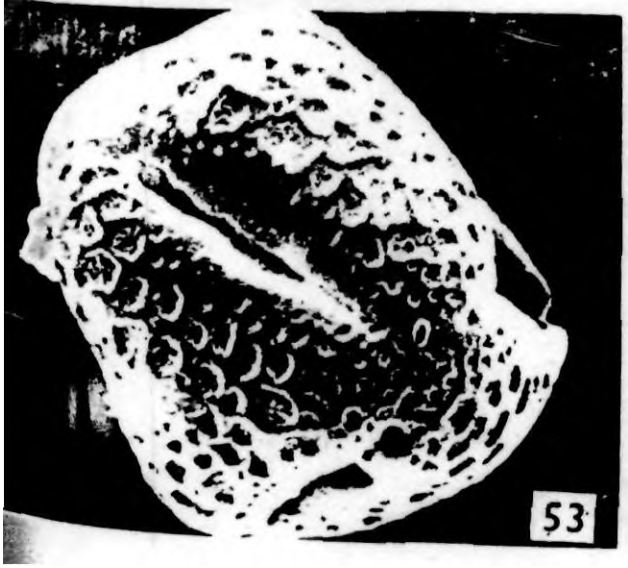

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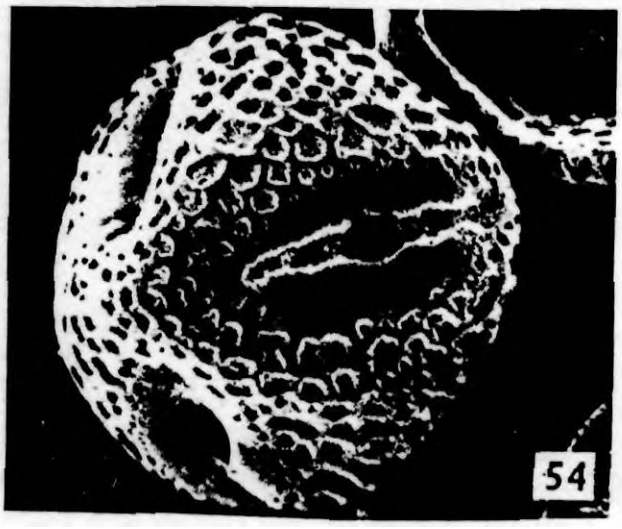


54

Fig. 55. Diagramatic representation of possible line of morphological evolution of pollen in the autotetraploids of P. phaseoloides. (CR - Circular ora; Po - Polar view; SY - Syncolporate; SR - Spiraperturate; LO - Lolongate; TR - Tricolporate, TT - Tetrazonocolporate; BR - Brevicolporate).

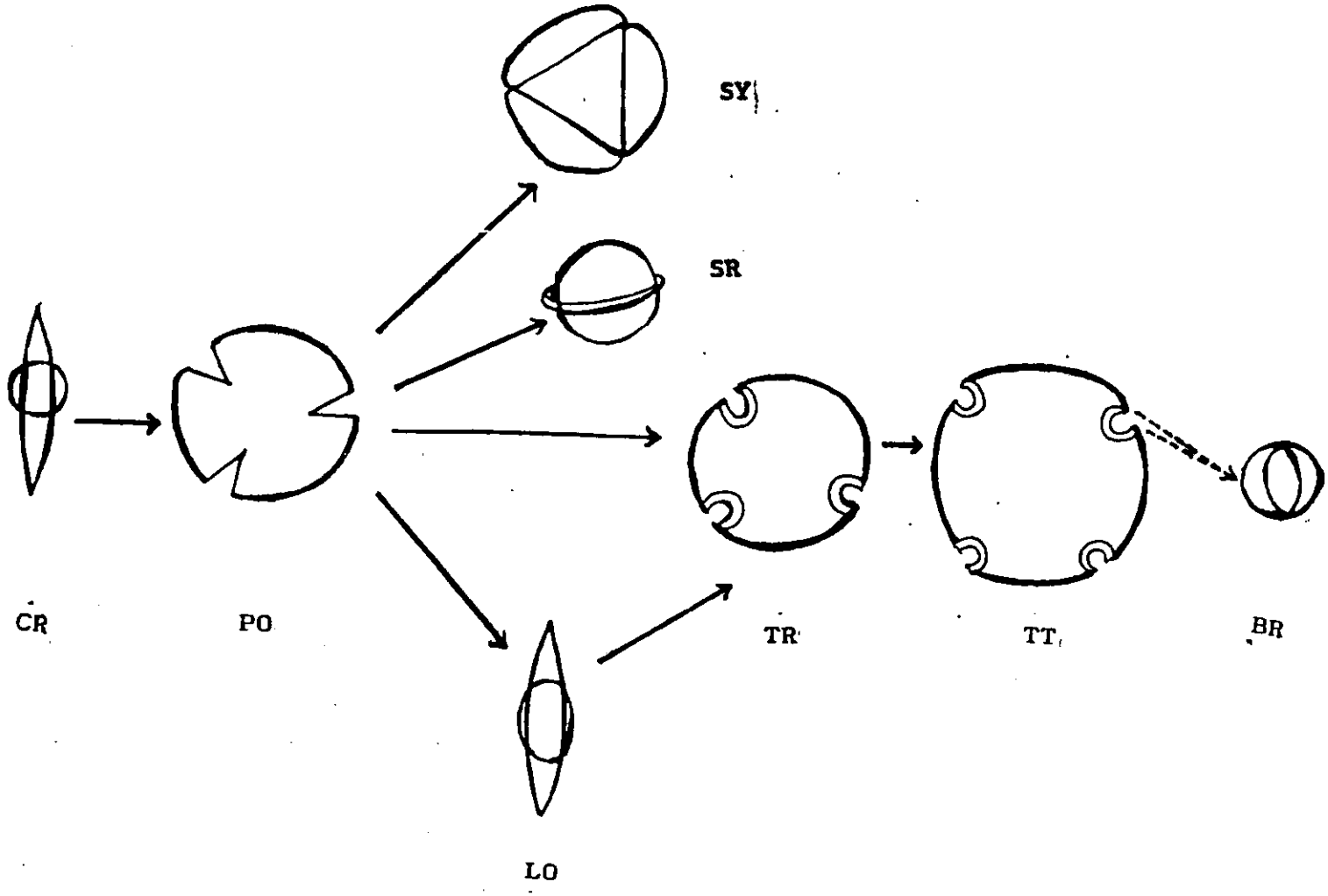
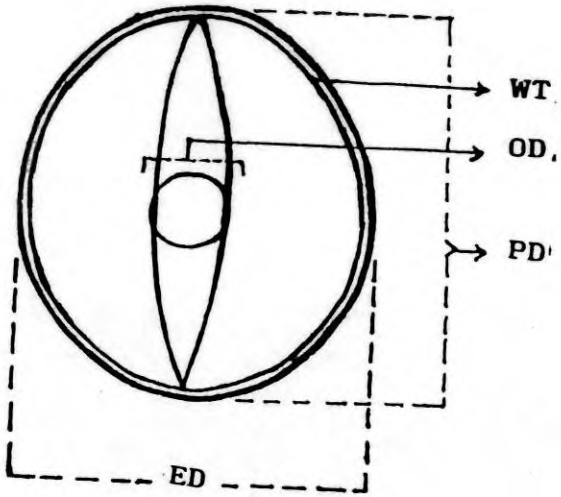
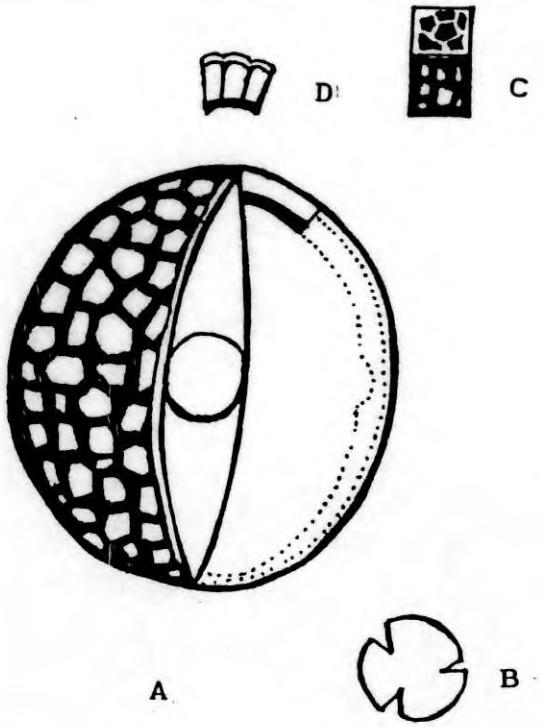

SY
SR
CR
PO
TR
TT
BR
LO

Fig. 56. (a) Diagrammatic representation of a pollen grain showing location of measurements. (WT - Wall thickness; OD - Ora diameter; PD - Polar diameter; ED - Equatorial diameter).

(b) Palynogram of P. phaseoloides. (A - Equatorial view; B - Polar view; C - Lo analysis; D - Exine strata).



(a)



(b)

Fig. 57. Effect of acetolysis on grain size in ten tetraploids of P. phaseoloides corresponding diploids.

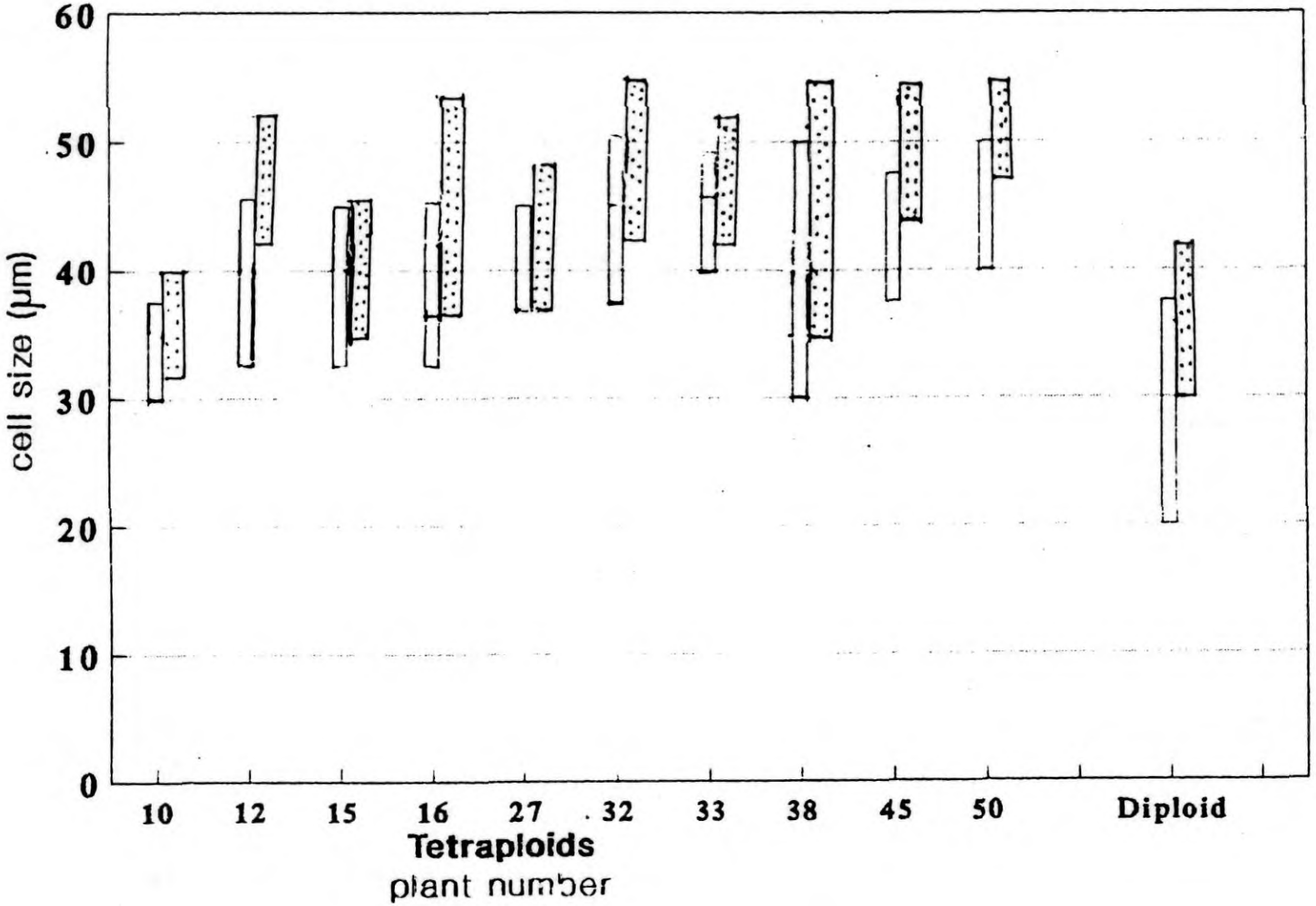
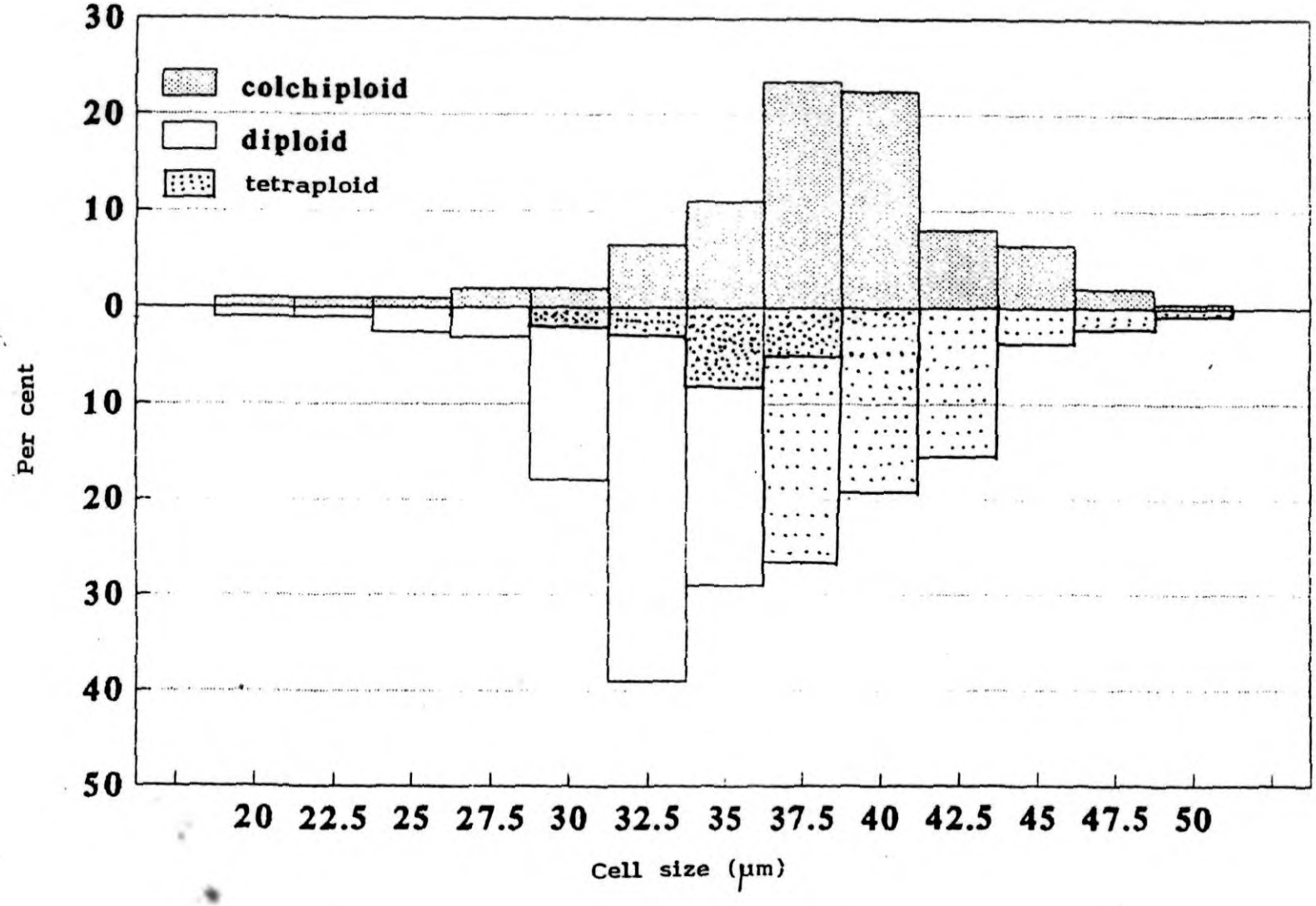

60
50
40
30
20
10
0
cell size (µm)
10
12
15
16
27
32
33
38
45
50
Diploid
Tetraploids
plant number

Fig. 58. Frequency distribution of pollen grain diameter in the colchiploids, diploids and induced tetraploids of P. phaseoloides.


colchiploid
diploid
tetraploid
30
20
10
0
10
20
30
40
50
Per cent
20 22.5 25 27.5 30 32.5 35 37.5 40 42.5 45 47.5 50
Cell size (µm)

3. FOLIAR ANATOMY

RESULTS

3.1. Diploids

Leaf of P. phaseoloides is dorsiventral. The internal structure of leaf lamina is organised into three different zones viz., epidermis, mesophyll and vascular tissue.

3.1.1. Epidermis

The leaf surface is slightly undulating with an upper and lower layer of epidermal cells (Fig. 59). Both the layers are covered externally with a cuticle. The epidermal cells are compactly arranged hexagonal cells which are almost uniform in size, except for a few cells against the veins, which appear to be larger in cross sectional view. In surface view the cell walls are wavy. This layer is interrupted by stomata and hairs.

(a) Stomata

Leaves are amphistomatic. However very few stomata appear on the adaxial surface, compared to their abundance on the abaxial side. Stomata are paracytic, occurring at the same level of epidermal cells.

(b) Trichomes

Trichomes appear as tubular extensions of epidermal cells and are of two types viz., glandular and nonglandular. Nonglandular hairs (Fig. 60) are simple, uniseriate and consisted of a basal cell, a middle cell and a long distal cell with pointed

tip. The basal cell was deeply stained and two to three times larger than the adjacent epidermal cells. Trichomes were more abundant in the lower epidermis. Often the foot of the trichome was covered by few radiating epidermal cells.

Glandular hair consisted of a stalk cell, a middle cell and a multicellular head covered by a cuticle (Fig. 61), stalk cell being larger than the epidermal cell. The middle cells have dense cytoplasm and a prominent spherical nucleus. In transection, the head consisted of three tiers of deeply stained cells.

3.1.2. Mesophyll

Mesophyll is differentiated into palisade parenchyma and spongy cells with a laterally oriented row of cells in between, designated as paraveinal mesophyll (PVM).

(a) Palisade parenchyma

This zone, comprised of two rows of compactly arranged elongated cells. The upper palisade layer is more uniform and regular, with the cells having an average length and width of 30 µm and 8.75 µm respectively. In the second palisade layer, cells were less regular with more of intercellular space and were in contact with the PVM cells. Some of these lower cells resembled the spongy cells in size and shape. The palisade layer was not continuous with the main vien.

(b) Spongy mesophyll

The cells were irregular in shape with most of the cells elongated along the vertical plane, producing protrusions on all

directions and thus resulting in large intercellular air spaces. This tissue forms an interconnecting network between the bundle sheath cells, lower epidermis, PVM cells and palisade parenchyma.

(c) Paraveinal mesophyll

Adjacent to the lower palisade layer there is a single row of laterally oriented elongated cells extending between the vascular bundles along the plane of the phloem. These cells differ distinctly from spongy cells in size and shape and appear weakly stained amidst the deeply stained mesophyll cells (Fig. 60).

3.1.3. Vascular system

The vascular bundles are collateral, surrounded by a parenchymatous bundle sheath with extensions between the epidermis. The PVM cells are continuous with the bundle sheath cells (Fig. 64).

3.2. Comparative anatomy of diploids and tetraploids

Leaf anatomical measurements revealed a significant increase in thickness of various tissues in the tetraploids, including epidermal, palisade and spongy cells compared to those of the diploids (Table 13). On an average the leaf lamina (excluding the major veins) in the tetraploids was 143.87 µm thick, as against 109.13 µm in the control plants. The columnar palisade cells possessed a total thickness of 69.00 µm and 56.03 µm in tetraploid and diploid plants respectively. Thus the palisade parenchyma alone occupied 47.98 per cent and 51.40 per cent respectively in the

two cytotypes, thereby accounting for about half of the total leaf thickness. Similarly the epidermal cells constituted 22 per cent and 19 per cent each in the tetraploid and diploid respectively. The spongy mesophyll cells (including PVM was comparable (29 per cent) in either ploidy. Palisade cell length and width and intervienal distance increased in the tetraploids. But the number of palisade cells per unit leaf area was considerably reduced in the tetraploids.

Correlation studies pointed out a highly significant positive correlation of palisade thickness and total leaf thickness. Palisade tissue thickness was again positively correlated with carbondioxide exchange rate ($r = 0.49$, $P < 0.05$). Among the polyploids, plant No.45 (Fig. 62) had closely spaced mesophyll cells with very little of intercellular space. On the other hand, plant No.33 (Fig. 63) possessed very long palisade cells and greater palisade thickness (86.95 μm) and also registered a higher rate of photosynthesis (21.55 $\mu m\ Co_2\ m^{-2}\ sec^{-1}$). Here the palisade cells were twice as long and were more loosely packed than in the leaves of the other tetraploids. Thus the polyploids varied among themselves with regard to various anatomical traits.

DISCUSSION

Diploids

Leaf anatomy of P. phaseoloides is typical of that described for many members of Papilionaceae (Metcalfe and Chalk, 1950) with only minor variations. Trichomes are almost universal in

Phaseoleae (Lackey, 1978). The probable function(s) of glandular hairs remain obscure, eventhough a secretory function has been generally associated with such type of hairs (Esau, 1965; Greulach, 1973). In the present species, however the glandular hairs appeared to be nonsecretory.

A notable feature of the mesophyll was the presence of a distinct layer of irregular cells between the palisade and spongy cells. This layer was first reported from a group of legumes in the 1890's (Soleredor, 1908). Later Fischer (1967) rediscovered it in soybean, as a one cell thick layer which extends between the veins in the plane of the phloem and hence named it 'paraveinal mesophyll'.

The lateral orientation of the PVM cells suggests their possible role in the transport of photoassimilates from mesophyll tissue to phloem (Fischer, 1967; Franceschi and Giaquinta, 1983 a, 1983 b). Further, these cells appeared to be poor in chloroplasts and hence considered to be non-photosynthetic (Franceschi and Giaquinta, 1983 c; Kevekordes et al., 1988). Probably, the feeble staining of these cells, thus resulted from the paucity of chloroplasts. Earlier, cells with almost similar function, referred to as the 'accessory transfusion tissue' were reported in some gymnosperms (Griffith, 1957; Esau, 1977; Fahn, 1982).

Comparative anatomy of diploids and induced tetraploids

A comparison of transverse sections of leaf blades of the diploids and induced polyploids revealed a significant influence of

ploidy with respect to cell size on different anatomical variables Apart from quantitative differences in various tissues, qualitativ variations with regard to mesophyll cell size, number of contact between cells, extent of air cavities etc. were also observed Differences in thickness were more strongly pronounced in th palisade and paraveinal mesophyll cells. Dornhoff and Shible (1976) worked out the partial correlations of leaf thickness (LT with thickness of various tissues in soybean leaves and found tha variations in thickness was strongly related to variations in thickness of palisade and paraveinal mesophyll cells. Studies on morphological and anatomical features of alfalfa leaves (Delaney and Dobrenz, 1974) indicated that leaf thickness was highly dependent upon palisade tissue thickness. On the contrary, increased LT due to increase in spongy mesophyll cells was observed in Phaseolus seedlings (Radoglou and Jarvis, 1992) due to enriched Co_2 supply, but the ratio of palisade to spongy cells remained unchanged. In the present study, however, the relative proportion of various tissues in the tetraploids were similar to that of diploids, as evidenced by the percentage of the total thickness, occupied by the various compartments.

The palisade cells in the tetraploids were longer than those in the diploids. Thus the ratio of length to width of palisade cells varied in the two cytotypes. The epidermal and mesophyll cell dimensions also increased, followed by a reduction in cell number per unit leaf area, thus indicating the apriori concept of ploidy on cell size. Similar results were also reported in the

induced tetraploids of chickpea (Fagerberg et al., 1990), where structural models based on leaflet size and tissue density showed greater productivity potential per unit leaf area in the tetraploids. Bryne et al. (1981) observed an increasing trend in the length and width of mesophyll cells with a natural polyploid series of tall fescue leaves, together with a linear increase in mesophyll cell volume. Similarly, in Triticum genotypes at several levels of ploidy, cell size appeared to be an overriding factor .(Jellings and Leech, 1984). The behaviour of these natural polyploids though not comparable with the induced ones in the strict sense also exhibits mean cell volume ratios in the order of 1.5-1.8, when compared to the diploids (Butterfass, 1987). In fact, in the case of induced polyploids, this ratio is even higher (Dijkand Delden, 1990).

The Co_2 exchange rate in polyploids of P. phaseoloides was significantly related to the thickness of palisade cells. Several authors have discussed in detail the relationship of internal leaf structure and Co_2 exchange rate (Mc Clendon, 1962; Esau, 1965, 1977; Delaney and Dobrenz, 1974; Dornhoff and Shibles, 1976; Bryne et al., 1981; Jellings and Leech, 1984; Ludlow, 1991; Soyza and Kincaid, 1991; Vogelmann, 1993). The palisade cells account for major part of the photosynthetic machinery. They contain at least twice or even 3 to 5 times as chlorophyll corpuscles, than the spongy cells - in which Co_2 exchange is only a subsidiary function (Haberlandt, 1914). Moreover, the elongated palisade cells exposes 1.6 to 3.5 times free surface area than the spongy parenchyma (Turrel, 1936), which means a higher proportion of palisade results

in a higher ratio of internal to external surface area thereby facilitating effecient gas exchange. Thus in the present study the individual palisade cell length combined with the total tissue thickness was indicative of the internal exposed surface area and thereby related to carbondioxide exchange rate. In alfalfa leaves, Delaney and Dobrenz (1974) obtained significant positive correlation between apparent photosynthesis and thickness of palisade tissue.

On a comparative basis, the polyploids with greater palisade thickness are expected to maintain a higher rate of gaseous exchange than the diploids. However carbondioxide exchange rate (CER) when expressed on a unit leaf area basis, did not differ significantly in either ploidy level. This could have been presumably due to density differences in other variables including total leaf thickness (Mc Clendon, 1962) chloroplast number and size (Jellings and Leech, 1984) and volume of air spaces (Dorhoff and Shibles, 1976). As a result, the more direct relationship of CER with the palisade cells, will not be reflected on a unit leaf area basis, even if the polyploid leaves are thicker. Wilson and Cooper (1967) observed in Lolium genotypes that apparent photosynthetic rate per unit leaf area is the result of an interaction between number of mesophyll cells per unit area and leaf thickness. Whereas on a per leaf basis, the increased palisade cell size coupled with greater leaf surface area, collectively contributed to the improved Co_2 exchange rate in the polyploids (See Section 4).

Regarding vascular tissues, the xylem vessels registered considerable cell enlargement than the mesophyll cells. Veins were thicker and more prominent. The intervascular distance in the

tetraploids were widened, probably due to bigger sized PVM ar mesophyll cells. Exceptionally, in one of the polyploids wit compactly packed mesophyll cells lacking intercellular spaces, th distance between veins were smaller. Thus the proportion o laterally oriented tissues (PVM and mesophyll cells) seemed t exercise some role in the spacing of vascular elements eventhough a clear correlation could not be established. From among a survey of 66 species of dicots, Wylie (1939, 1946) obtained a direc relationship of vein spacing with the amount of spongy cells and an inverse relationship with that of palisade cells. In polyploid tall fescue (Bryne et al., 1981) the observed decrease in the total number of veins from 4x to 8x was thought to be a function of leaf width, as the number of mesophyll cells between veins remained unchanged. However Jellings and Leech (1984), from a survey of nine Triticum genotypes, concluded that cell size rather than cell number is the major component of interveinal distance.

On the whole, polyploidisation could alter the cellular dimensions in P. phaseoloides. Neverthless, total leaf thickness seemed to be influenced by several interacting factors. Hence, apart from a positive association between palisade thickness and Co_2 exchange rate, the role of larger mesophyll cell compartment in internal regulation was not well understood. Although genetic parameters certainly influence the leaf anatomical frame work of crop species, our understanding of the effect of colchiploidy per se is meagre (Fagerberg, et al., 1990). Thus with the available information, it could only be assumed that not alone ploidy, but

also intrinsic genetic factors characteristic of individual lines are involved in determining the ultimate structure. The overall impact of genome doubling on anatomical traits indicate that variability could be induced. However detailed evaluation of each genotype is necessary to identify the best variant, as variability among the induced polyploids is noticed, though to a limited extent.

Table 13. Comparative foliar anatomy of diploid and autotetrapl of P. phaseoloides with the percentage occupied by respective compartments

Parameter	Diploid		Tetraploi	
	Thickness (μm)	Per cent of total	Thickness (μm)	Per ce of tota
Upper epidermis	12.5±0.17	11.45	18.73±0.42**	13.01
Palisade parenchyma	56.03±0.25	51.40	69.00±2.30**	47.98
Paraveinal mesophyll	10.00±0.50	29.18	15.50±0.93**	29.09
Spongy parenchyma	21.28±0.63		23.40±0.88	
Lower epidermis	8.75±0.20	8.01	13.25±0.37**	9.03
Total	109.13±0.50	99.98	143.87±3.10**	99.90
Palisade cell no. unit LA^{-1}	25.91±0.75	–	21.95±1.26*	–
Interveinal distance (μm)	23.33±0.42	–	28.68±0.69*	–

*, ** Significant at 5 per cent and 1 per cent respectively.

Figs. 59-65. T.S. of leaf

Fig. 59. Diploid

Fig. 60. Tetraploid; GH - Glandular hair; PVM - paraveinal mesophyll; PA - palisade; NGH - Non glandular hair (Arrow indicates single large epidermal cell against the vein ending)

Fig. 61. Glandular hair - enlarged

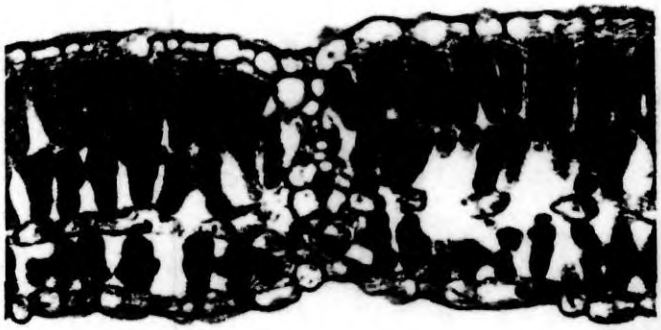
Fig. 62. A tetraploid showing compactly packed mesophyll cells

Fig. 63. A tetraploid showing loosely arranged mesophyll cells (Note the extremely long palisade cells)

Fig. 64. Midvein - Diploid

Fig. 65. Midvein - Tetraploid

[All Figs. X 1200]



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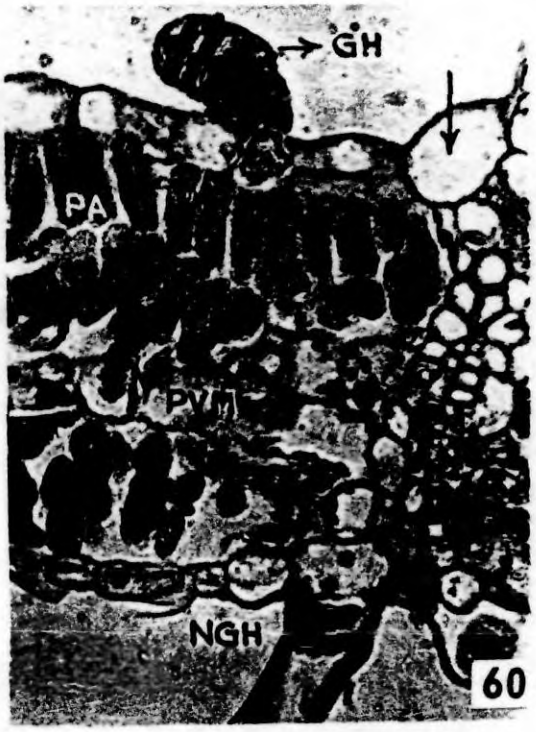

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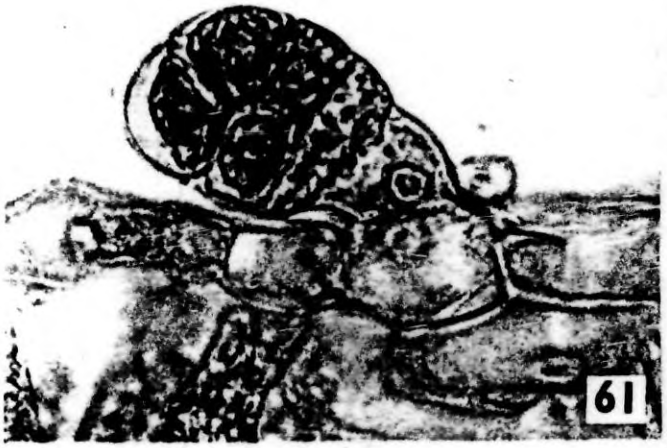

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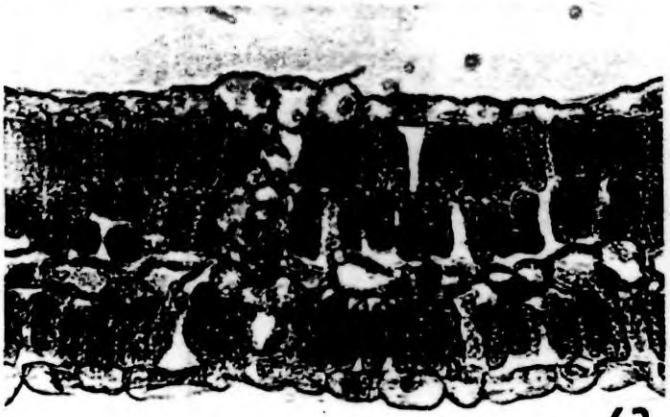

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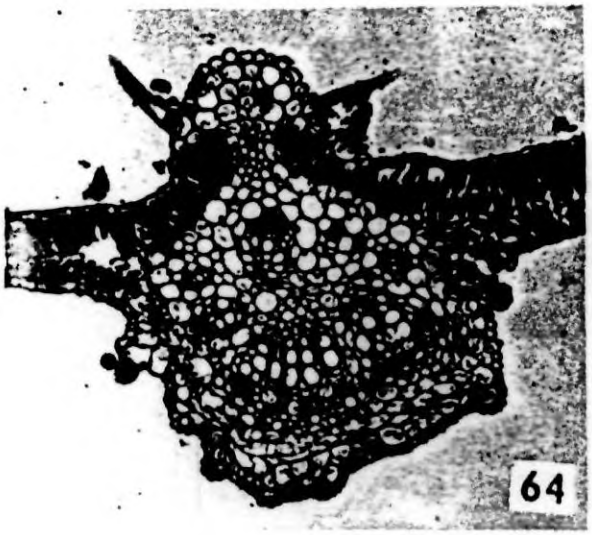

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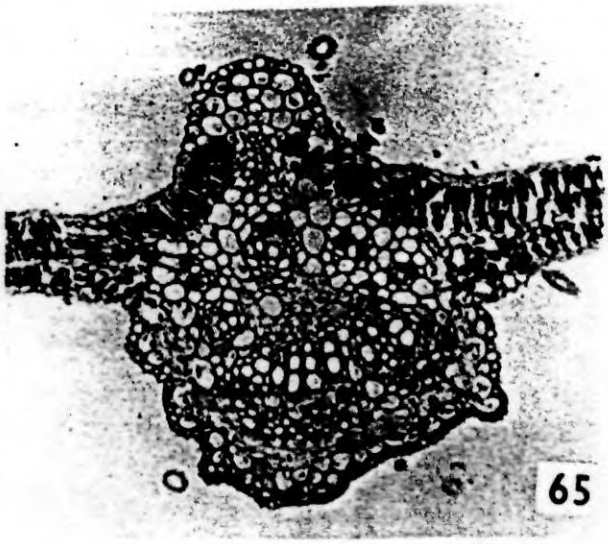

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4. LEAF PHYSIOLOGICAL TRAITS AND BIOMASS PRODUCTION

RESULTS

4.1. Leaf area, leaf weight and specific leaf weight

The tetraploids registered significant increase in foliage i terms of size, fresh weight and dry weight. The mean single lea area in the tetraploids were 150.83 sq. cm. as against 135.10 s cm. in the diploids. Mean fresh weight and dry weight pe trifoliate was 1.41 and 0.41 g respectively in the tetraploids a against 1.00 and 0.30 respectively in the diploid cytotypes Consequently colchiploidy also resulted in an improved total lea area per plant eventhough the total leaf number was not muc affected. Leaf thickness was also high in the autotetraploids tha the diploids, as may be recalled from Table 13. At the same time there was no significant difference in specific leaf weight betwee the two groups.

4.2. Transpiration and stomatal resistance

The stomatal guard cells in the tetraploids were bigger in size, but their density per unit leaf area was lesser than the control plants (See Table 4). The stomatal resistance was also higher in the former. The tetraploids also registered a higher rate of transpiration than the diploids on a unit leaf area as well as on a per leaf basis.

4.3. Carbondioxide exchange rate and canopy photosynthesis

Co_2 exchange rate (CER) was studied as a function of ploidy. There was no considerable difference between the two

groups when CER was expressed on a unit leaf area basi However, an increasing trend was evident for CER on a per le basis, with an increase in ploidy. Thus on a whole plant basi the canopy photosynthesis in the autopolyploids was 40.17 μmol plant^{-1} sec^{-1} as compared to 36.32 μmoles plant^{-1} sec^{-1} in t control.

4.4. Biomass production

Apart from an increase in foliage production, an increase i stem, root and nodule tissues in the tetraploids resulted in a improved biomass both in terms of green matter and dry matte production (Figs. 66, 67 a, b). On a per plant basis, the averag dry matter production in the tetraploids and diploids were 158.6 and 130.19 g respectively. The effect of colchi-tetraploidy on som of these morphophysiological traits are summarised in Table 14.

Simple correlations for leaf size and Co_2 exchange rate wit dry matter yield was worked out in the tetraploids (Table 15) an the regression analysis for this data are illustrated in Fig.68 Total leaf area was found to be highly correlated ($r = 0.710.71$ $P < 0.01$) with plant dry matter.

DISCUSSION

Leaf area, leaf weight and specific leaf weight

An increase of over eleven per cent in single leaf area wa observed in the induced polyploids of P. phaseoloides over thei diploid progenitors. Earlier studies have shown a clos relationship of leaf area with total crop yield. In alfalf

(Etzel et al., 1988) herbage yield was positively associated wit. leaf area per plant as leaves constitute between 30-60 per cent c. forage. An increase in leaf area suggests an increase in cel volume associated with ploidy, but the cell number per leaflet ma remain unchanged (Setter et al., 1978). In the present study a. increase in cell volume was indicated by an increase in stomata. and epidermal cell size. According to Delaney and Dobrenz (1974a the relationship between area per leaflet and leaflets : stem ratio: in alfalfa suggests that large leaved genotypes produced forage o: superior quality and were high in nutritional value.

Tetraploid P. phaseoloides showed a 41 per cent increase in leaf fresh weight over the diploids. Meyers (1982) reported an 80 per cent increase in leaf fresh weight in tetraploid alfalfa. Increase in leaf size and thickness have also resulted in an increased leaf dry weight in the present study. However, the proportion of dry weight to the total fresh weight was lower in the tetraploids. This could be attributed to the increased water content in the tetraploids as reported by Tal and Gardi (1976). Stebbins (1950) was of the opinion that an increase in ratio of volume to surface area can result in increased water content in polyploid cells.

Specific leaf weight is one of the many indicators of internal leaf anatomy (Pearce et al., 1969). The increase in specific leaf weight in polyploid P. phaseoloides was however not significant. Bhagsari and Brown (1986) reported lack of any correlation between

leaf area and specific leaf weight in many crop plants, as indicated by the negative relationship between thickness and area per leaflet. Turrel (1942) found larger leaves to be thicker, yet contained only fewer mesophyll cells per unit area. Perhaps, this discrepancy in cell size might have resulted in the nonsignificant difference in specific leaf weight in the present study. Physiological studies in alfalfa genotypes with large and small leaflets (Leavitt et al., 1979) also revealed a decrease in specific leaf weight in the former, despite the production of higher yield and increased leaf area per plant.

Stomatal traits and transpiration

P. phaseoloides showed a positive relationship of stomatal length and a negative association of stomatal density with ploidy (Table 4). Stomatal frequency and aperture are the prime factors controlling the porosity and conductance of leaves, thereby influencing two important plant functions photosynthesis and transpiration (Meidner and Mansfield, 1968). Transpiration and stomatal frequency in many species are positively related (Miskin et al., 1972; Tal and Gardi, 1976; Rutland and Chang, 1987). However, even with a reduced stomatal frequency, transpiration in the induced polyploids of P. phaseoloides was high. This could possibly be due to two reasons. The stomatal index (number of stomatal cells to number of normal epidermal cells) was almost the same in the diploids and polyploids, implying that the influence of lower stomatal frequency per unit leaf area in the polyploids will

be minimised on a per leaf basis, owing to their increased surfac area. Secondly, the aperture size and degree of stomatal openin are also equally important in determining the rate of water loss at a given time. Hence an increase in the total stomatal orific (size x frequency) in the polyploids might have resulted in the higher rate of transpiration. According to Waggoner and Zelitc (1965) stomatal pore area are of equal or greater importance tha stomatal density in regulating gas exchange. Higher stomata resistance in the polyploids under study could have resulted fron their lower stomatal density as observed by Bjurman (1959). Stomata and resistance associated with stomatal arrangement an activity, are important in determining the rate of Co_2 movement int the leaves.

Carbondioxide exchange rate and canopy photosynthesis

A few reports are available on the effect of polyploidisation on Co_2 exchange rates (Setter *et al.*, 1978). Joseph *et al.* (1981) reported a 54 per cent increase in carbondioxide exchange rate on a leaf area basis and a 47 per cent increase on a leaf weight basis when the ploidy level of tall fescue was increased from tetraploid to decaploid. Similar responses were also exhibited by diploid and tetraploid rye grass (Garrett, 1978). Though this is the case with most naturally occurring allopolyploids, in many derived polyploids photosynthetic rates are reported to decrease with polyploidisation, possibly due to inbreeding (Albuzio *et al.*, 1978; Bjurman, 1959). In *P. phaseoloides* photosynthetic rate per

unit leaf area was similar in both diploids and induce tetraploids. Nevertheless, on a per leaf basis carbondioxid exchange rate was higher in the latter. In many specie carbondioxide exchange rate and leaf area were negativel correlated (Hesketh et al., 1981; Bhagsari and Brown, 1986 However, Berdahl et al. (1972) observed similar rates of CER i small and large leaf lines of barley and thus the tota photosynthetic activity per leaf was twice as great in the larg flag leaves. In the present study increased rate of CER per le resulted from a concurrent increase in leaf area and leaf weight i the tetraploids. Similar observations were also reported by Sette et al. (1978) in polyploid alfalfa.

Canopy photosynthesis is a measure of the total leaf are per plant. Large leaflet alfalfa genotypes were found to expres higher rates of net photosynthesis (Leavitt et al., 1979).

Biomass production

Biomass production is the result of a number of interactin biological processes controlled by the genetic constitution of the plant species and influenced by the environment (Kuckuck et al., 1991). In P. phaseoloides dry matter production of tetraploids was 22 per cent more than that of the corresponding diploids. Dunbier et al (1975) obtained a tetraploid : diploid (T/D) ratio of 1.0 : 2.1 for dry matter yield in alfalfa.

A direct relationship between photosynthetic rate and biomass production has been reported (Sarkar, 1991). From the available

data on P. phaseoloides, CER was not related to dry matter (r = 0.23) whereas canopy photosynthesis exhibited significant positive correlation (r = 0.59 P < 0.01) with total dry matter However total leaf area was more highly correlated with total dry matter (r = 0.71 P< 0.01). Similar relationships have been reported in other crops also. Forage yield in alfalfa was positively correlated with total photosynthesis per plant (Delaney and Dobrenz, 1976b) yet the best selection criterion for yield was total leaf area per plant (Leavitt, et al., 1979). In Gossypium species (El-Sharkawy and Hesketh, 1965) with similar rates of photosynthesis dry matter varied linearly with variations in leaf area. Aase (1978) obtained a close relationship of leaf area with leaf dry matter and plant dry matter in winter wheat and suggested that dry matter could be substituted for leaf area.

The foregoing observations suggest that induction of polyploidy in P. phaseoloides is advantageous in the sense it increases dry matter production, which means more of organic matter and more of nitrogen, as a cover crop. Correlation figures point out a moderate association of canopy photosynthesis and a higher association of total leaf area with whole plant dry weight. Correlation and regression studies related to biological yield in many crop plants usually refer to the dry weight of aerial portion alone, whereas in the present study the root system was also considered. The root system is equally important, if not more, in a ground cover for plantation crop and is thus significant.

As many of these quantitative traits are influenced not o
by ploidy *per se*, but also on individual genotypes, it appears
that a combination of factors, rather than a single character h
produced this effect.

Table 14. Certain physiological traits and biomass production i autotetraploids and corresponding diploids of phaseoloides

Character	Diploid	Tetraploic
Single leaf area (cm^2)	135.10±2.29	150±7.50**
" leaf fresh weight (g)	1.0043±0.05	1.4125±0.08**
"leaf dry weight (g)	0.3060±0.02	0.4120±0.03**
SLW (mg cm^{-2})	2.39±0.83	2.74±0.93
Total leaf area $plant^{-1}$ (m^2)	1.72±3.41	2.30±4.11**
Stomatal resistance (Sec cm^{-1})	0.32±0.02	0.55±0.08**
CER (μmoles m^{-2} sec^{-1})	21.12±0.53	19.61±0.59
CER per leaf (umoles $leaf^{-1}$ sec^{-1})	0.28 ±3.30	0.28 ±6.41**
Canopy photosynthesis (μmoles $plant^{-1}sec^{-1}$)	36.32±2.21	40.17±4.11**
Transpiration rate (μmoles m^{-2} sec^{-1})	26.04±0.48	30.55±2.02**
Transpiration per leaf (μmoles $leaf^{-1}sec^{-1}$)	0.5290±2.66	0.6867±3.31**
Total dry matter (g $plant^{-1}$)	130.19±2.03	158.65±3.44**

**Significant at 1 per cent level of probability

Table 15. Correlation of total dry matter with growth parameters in the autotetraploids of P. phaseoloides

Character	Total dry matter 'r' value	Significance
CO_2 exchange rate (CER)	0.23	NS
Canopy photosynthesis	0.59	**
Total leaf area	0.71	**

NS - Not significant

** - Significant at 1 per cent level

Fig. 66. Diploid (a) and Tetraploid (b) plants of P. phaseoloides established from rooted cuttings.

Fig. 67. Detached roots of diploid (a) and tetraploid (b) plants of P. phaseoloides showing increased root proliferation in the latter.

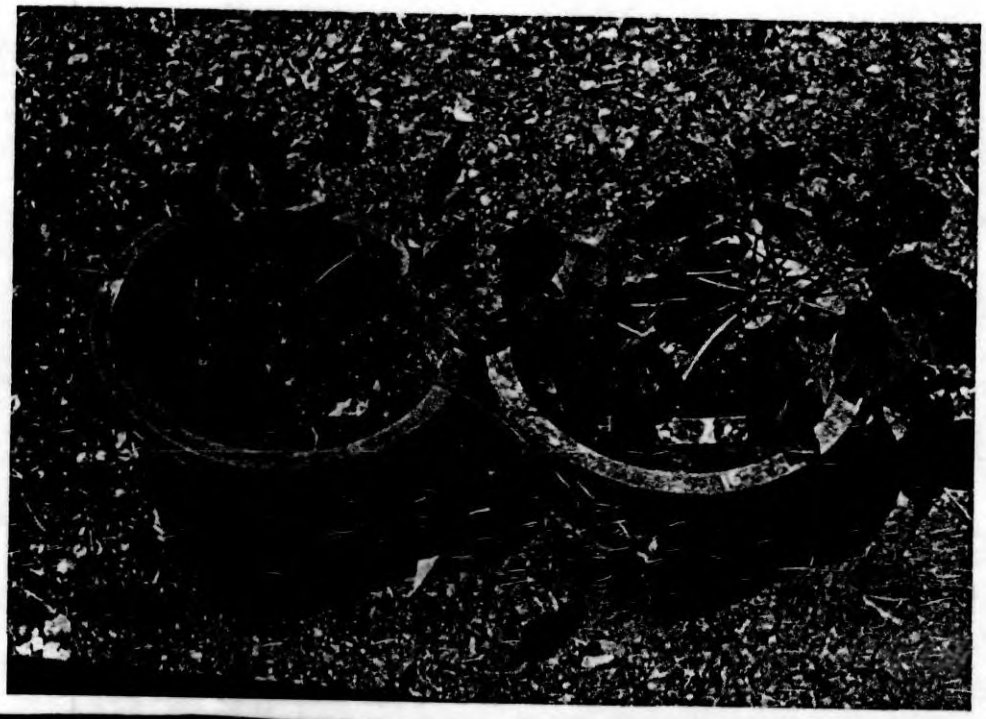


Fig 66

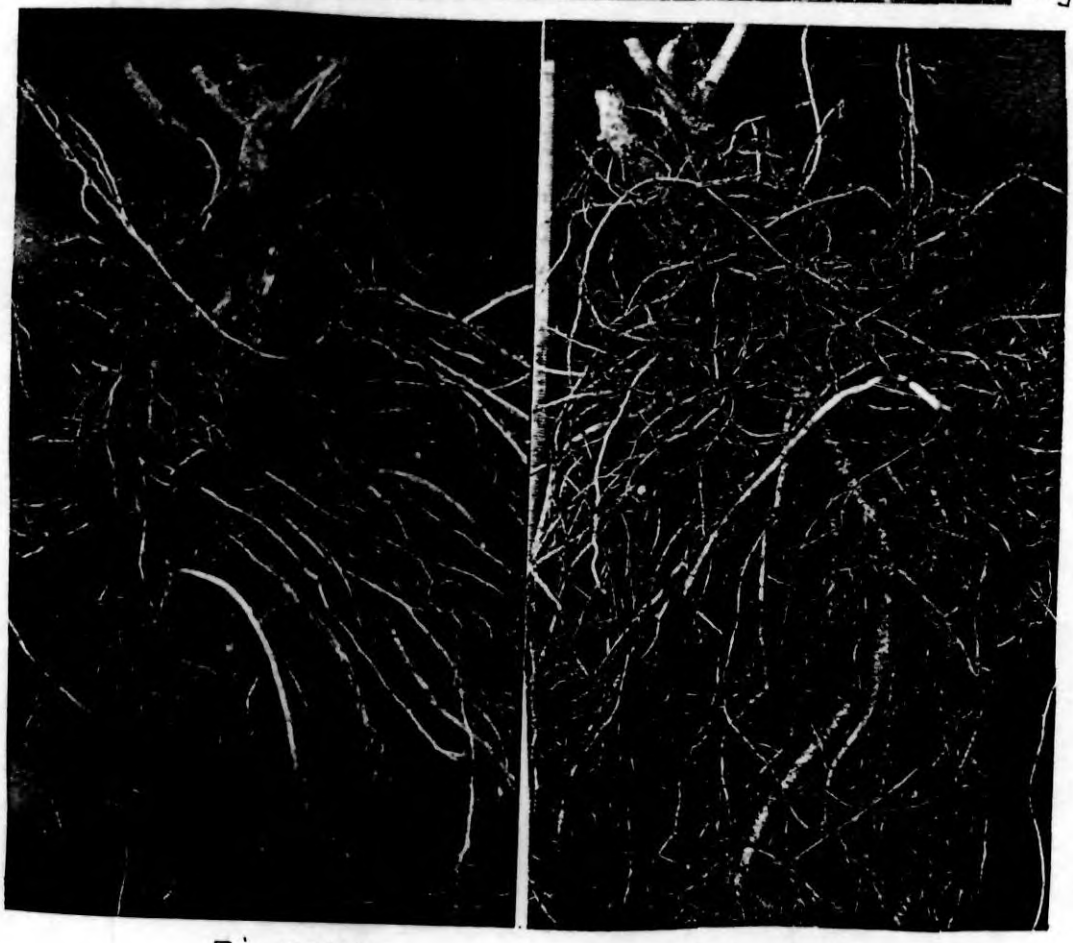
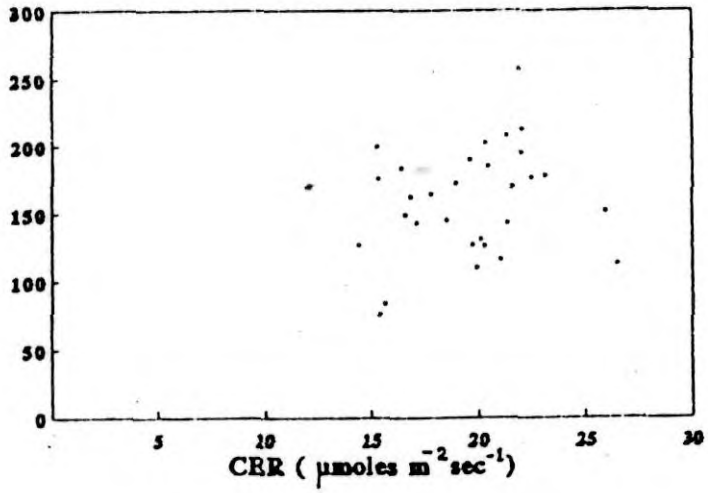
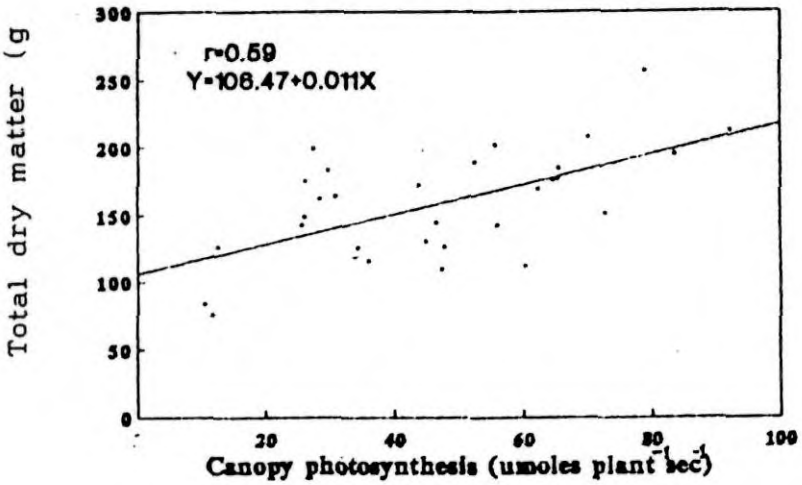


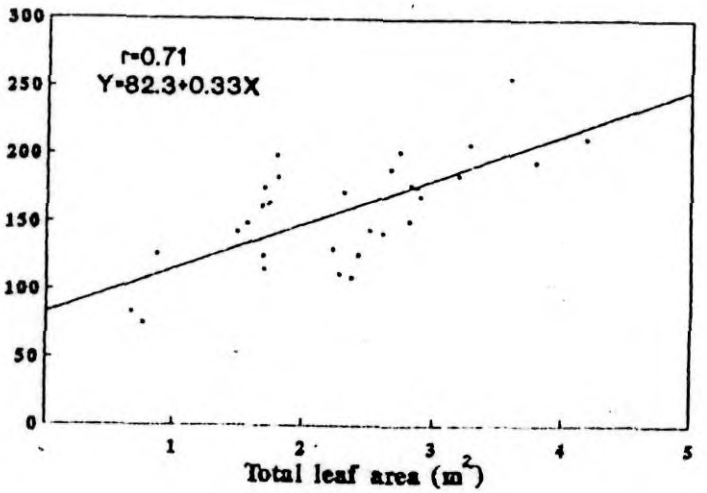
Fig 67(a)

Fig 67(b)

Fig. 68. Relationship of carbondioxide exchange rate (CER), canopy photosynthesis and total leaf area with total dry matter in the autotetraploids of P. phaseoloides


300
250
200
150
100
50
0
5
10
15
20
25
30
CRR (µmoles m^{-2}sec^{-1})


Total dry matter (g
r=0.59
Y=108.47+0.011X
300
250
200
150
100
50
0
20
40
60
80
100
Canopy photosynthesis (umoles plant^{-1} sec^{-1})


r=0.71
Y=82.3+0.33X
300
250
200
150
100
50
0
1
2
3
4
5
Total leaf area (m^2)

5. ESTIMATION OF NUTRIENTS

RESULTS

The level of various nutrient elements in different plant parts and those in the entire plant in the diploids and polyploids were compared. The concentration (percentage) of different nutrients in the two cytotypes were comparable (Table 16). But the uptake of nutrients (on a dry weight basis) and their distribution were found to vary in the two groups (Figs. 69, 70, Table 17).

5.1. Nitrogen

The total nitrogen uptake (on a dry weight basis) in the shoot, roots and nodules are represented in fig. 69A. Except for roots, all the other tissues in the tetraploids showed higher nitrogen uptake, than the corresponding tissues of normal plants. On an average the total N uptake in the former was 5.05 g per plant as against 3.95 g in the latter (Table 17) indicating an increase of 27.80 per cent. The distribution of total nitrogen in leaves, stem and roots were in the order of 38.98, 31.64 and 27.84 per cent respectively in the normal plants. On the other hand, the polyploid plants contained 42.61, 34.45 and 19.40 per cent of the total nitrogen in their respective tissues.

The N content in the root nodules of either ploidy does not differ much, the values being 5.40 and 5.33 for the tetraploids and diploids respectively (Table 18). However, the N uptake in the nodules of 2x and 4x plants were 0.06 and 0.16 g respectively.

Thus, with respect to leaves, stem and nodules, the polyploi plants contained comparatively more nitrogen.

5.2. Phosphorus

As is the case with nitrogen, the phosphorus content in th shoot and root tissues of either ploidy also did not vary muc (Table 16). However the P uptake was considerably higher in th polyploids (Fig. 70A, Table 17). Total P uptake per plant wa 0.56 and 0.42 g respectively. Among different plant parts irrespective of ploidy, stem tissues contained more P followed by leaf and root (Table 17).

5.3. Potassium

The potassium concentration in leaves of either ploidy was in the order of 0.70 to 0.80 per cent. The tetraploids registered increased uptake of K in all the tissues (Fig. 70B), but the increase was more pronounced in the leaf and stem tissues. Mean potassium uptake per plant in the 4x plants were 1.01 g as against 0.67 g in the control. But the distribution of potassium was more in the leaves and roots of normal plants and stem tissues of the polyploids (Table 17).

5.4. Calcium

The concentration of calcium in the plant tissues was more than that of phosphorus, potassium and magnesium (Table 16), but the distribution of calcium was similar to that of other elements (Fig. 70C). However, tetraploid roots contained comparatively less

calcium than the diploids. Mean calcium uptake in the two cytotypes were 1.26 and 1.12 g per plant respectively.

5.5. Magnesium

P. phaseoloides contained 0.30 to 0.80 per cent Mg in its tissues at either ploidy level (Table 16). The increase in the uptake of magnesium in the polyploids was lower than the case with other elements (Fig. 70D). Among the different plants parts, leaves of both normal and tetraploid plants contained more of magnesium followed by shoot and root. Mean magnesium uptake was about 0.4 per plant in both (Table 17).

The nutrient uptake was higher in general in the polyploids with respect to all nutrients studied (Fig. 69B). The results of the analysis of minerals in vegetative as well as the below ground parts of the two cytotypes, showed that in case of polyploids both stem and leaf tissues contained higher levels of nutrients than the roots. As a result, the shoot : root ratio, with respect to all the elements studied was higher in the polyploids (Table 19). However, the difference was less marked in the case of Mg.

DISCUSSION

A basic relationship between mineral nutrition in plants and biomass production has been reported (Smith, 1962) and the level of a particular nutrient in a crop species is indicative of its availability in soil. However, several other factors including climate, plant part, age and the supply of other elements also profoundly influence the nutritional profile. Regardless of all the

aforesaid reasons, varieties of a given species may differ widely i their mineral composition, even under indentical growth conditions This is primarily attributable to their genetic specificity, and thi aspect has been one of the least exploited areas in nutrition research (Saric and Loughman, 1983). The consequence o polyploidy on the major nutrient levels of P. phaseoloides wa examined in this context.

Nitrogen

Nitrogen is one of the major nutrients limiting crop production. Determination of total nitrogen in plants is considered as an important physiological parameter in estimating the nutritional status (Benton, 1987). As regards the diploid and tetraploid plants under study, the percentage N contained in the two groups was comaprable ie., P. phaseoloides contained 14-15 per cent N in their tissues. At the same time, the variation in actual N uptake observed between the two cytotypes could be attributed to their genotypic differences. So also, the distribution of nitrogen in different plant parts varied in either ploidy. Marked increase in N uptake through leaf and stem tissues of polyploids, clearly indicate the advantage conferred through their increased green and dry matter production.

Roots showed negligible variation in nitrogen level. In a pot culture experiment, the variability that could be expected in the root system is rather limited. Further, in the case of forage legumes, where the bulk of dry matter goes into the vegetative parts, the major nitrogen source comes through shedding of dead

plant materials (Sprent, 1979). In the case of P. phaseoloides it has already been observed that over 90 per cent of the total nitrogen fixed will be transferred to the aerial parts (Oke, 1967). Kothandaraman et al. (1984 while studying the dry matter production and nitrogen fixation of four month old ground covers observed 1.88 g of nitrogen gain per pot for P. phaseoloides plants with a dry matter production of 76.08 g. In another field experiment with P. phaseoloides the nutrient enrichment per ha through added biomass, at the end of three years was 108.02 kg (Kothandaraman et al., 1989). In the present study, the biomass production in diploid and tetraploid plants were 130.19 and 158.65 g respectively. A corresponding increase in nitrogen uptake ie., 3.95 and 5.05 g was also observed, strongly indicating the advantages of induced tetraploidy.

The level of nitrogen in the nodules of polyploid plants was higher than that of the diploids. According to Sprent (1979) only 1-4 per cent of legume biomass is in the nodules, which contain about 4-6 per cent N. But, the initiation, development and activity of nodules are highly genotype specific, and their distribution in the root system is often host determined (Caldwell and Vest, 1977; La Rue, 1980). This also explains the observed variation in nitrogen levels in the nodules of diploids and polyploids in the present study.

Phosphorus

The 2x and 4x plants contained 0.98 and 1.04 per cent phosphorus in their tissues respectively. The actual phosphorus

uptake per pot was greater in the polyploids. Several reports are available showing extensive genotypic variation for the concentration and content of P (Whitaker et al., 1976; Lindgren et al., 1977; Caradus, 1983). In various barley genotypes intervarietal differences in P absorption rates was found to be related to root length, shoot length and total biomass (Mego, 1983).

Nodulated legumes show an increased demand of P, which on defeciency may limit, nodule development, specific nodule activity and N_2 fixation (Graham and Rosas, 1979). De Mooy and Pesek (1966) suggested that high levels of phosphorus is required for maximum nodule activity than maximum nodulation. Hence, as is the case with other legumes, phosphatic fertilizers are recommended for leguminous ground covers of rubber (Pushparajah et al., 1977). Under conditions of uniform P fertilizer application, the variation in potassium levels in the diploids and polyploids is indicative of differential uptake. Variation in shoot : root ratio and differences in various physiological activities including the functioning of nodules, through altered nodule characters viz., nodule number and weight of nodules, could have contributed to this differential uptake of phosphorus.

Potassium

Potassium is of major importance in legumes in terms of yield, quality and stand longevity (Griffith, 1974) and affects nitrogen fixation, through its role in photosynthesis and translocation (Epstien, 1972). The observed physiological and anatomical variations between the diploid and tetraploid plants

point out the extent of variability possible in the production and supply of carbohydrates for efficient N_2 fixation.

The increase in potassium uptake in the polyploids, was higher than with the other elements. Apart from discernable morphological variations, the prolonged growth cycle in the polyploids could have contributed to their greater accumulation of potassium in the tissues, since the total length of the growing period has a bearing of K^+ uptake rate (Mengel and Kirkby, 1982). So also, the increased cell size, stomatal pore area and higher water content in the tissues of polyploids are all factors favouring the enhanced accumulation of potassium in plant tops. The role K^+ ions in regulating the turgour of guard cells during stomatal opening has been well emphasized (Fischer, 1968; Humble and Raschke, 1971).

Calcium

Calcium is important in maintaining cell rigidity and chromosome structure (Jones and Lunt, 1967) and Rasmusson (1967) suggested a relationship between calcium and strength of the leaves. Calcium is also an important nutrient for initiation and effecient functioning of nodules (Banath, 1966; Lowther and Loneragan, 1968; Munns, 1970 and Andrew, 1978). In P. phaseoloides the calcium content is about 2 per cent. This high Ca is all the more beneficial in the case of a ground cover, as in acid soils calcium exists as tricalcium phosphate - an unavailable form which is converted into an available form by the covers for uptake of rubber. The concentration of Ca in the root system of 2x

and 4x plants was relatively low when compared to the plant tops. The intensity of transpiration is thought to be a major factor controlling the upward translocation rate of Ca^{+} ions (Mengel and Krkby, 1982). In the autopolyploids of P. phaseoloides, the high rate of transpiration also appears to have contributed to their increased calcium uptake in the aerial parts.

Magnesium

Magnesium is considered to act as a bridge between the two protein complexes of the enzyme nitrogenase facilitating electron transfer during nitrogen fixation (Winter and Burris, 1979). Magnesium being a constitutent of chlorophyll molecule, the differences in Mg levels between the two cytotypes could be associated with altered leaf physiological traits including carbondioxide exchange rate.

The results indicate that the concentration (per cent) of various mineral elements in the diploids and polyploids showed little or no difference. In other words, the two cytotypes did not vary with respect to their nutrient use efficiency ie., mg dry weight per mg nutrient (Saric, 1983). Neverthless, obvious differences were observed in the nutrient uptake of the two groups, which could be attributed to their differential growth pattern. The polyploid plants after an initial period of retardation, shows vigorous growth subsequently. As a result, the dry weight of plant parts and consequently the total dry matter of the tetraploids was greater than that of diploids, and uptake efficiency is a function of both concentration and dry matter accumulation. Genetically controlled differences including ploidy variations, could

alter the uptake potential of roots, but the display of this potentiality may vary with crop and environment (Cacco, 1976). In P. phaseoloides the enrichment of soil with augmented level of organic matter by the polyploid plants also enhances the turn over of mineral nutrients in the soil.

Table 16. The nutrient content in different plant parts as affect by ploidy, in P. phaseoloides.

Component	Plody	Nutrient concentration (Per cent)				
		N	P	K	Ca	Mg
Leaf	2x	3.50	0.34	0.73	0.89	0.39
	4x	4.19	0.33	0.83	0.93	0.36
Stem	2x	2.79	0.38	0.50	0.95	0.31
	4x	2.69	0.42	0.67	0.76	0.29
Root	2x	2.78	0.26	0.28	0.76	0.24
	4x	2.52	0.29	0.40	0.74	0.27

Table 17. Uptake and distribution of total nutrients (on a dry weight basis) in different plant parts as affected by ploidy.

Nutrient	Ploidy	Uptake g plant^{-1}	Per cent in dry matter		
			L	S	R*
N	2x	3.95	38.98	31.64	27.84
	4x	5.05	42.61	34.45	19.40
P	2x	0.42	35.71	40.47	23.80
	4x	0.56	32.14	48.21	19.64
K	2x	0.67	47.76	32.83	19.40
	4x	1.01	42.57	42.57	14.85
Ca	2x	1.12	34.82	38.39	26.78
	4x	1.26	38.09	43.75	23.01
Mg	2x	0.40	42.50	35.00	22.50
	4x	0.47	40.42	38.29	21.27

L - leaf ; S - Stem ; R - Root

Table 18. Nitrogen content and uptake of nitrogen by the root nodules of diploids and autotetraploids of P. phaseoloides

Ploidy	Nitrogen content (Per cent)	Nitrogen uptake g plant^{-1}
2x	5.33	0.06
4x	5.40	0.16

Table 19. Shoot : root ratio for different nutrients in the diploids and tetraploids of P. phaseoloides

Ploidy	Nutrients				
	N	P	K	Ca	Mg
2x	2.53	3.20	4.15	2.73	3.34
4x	3.96	4.09	5.73	3.34	3.37

Fig. 69. (a) Uptake of Nitrogen in different plant parts and (b) total uptake of various nutrients in whole plants of diploid and tetraploid *P. phaseoloides*.

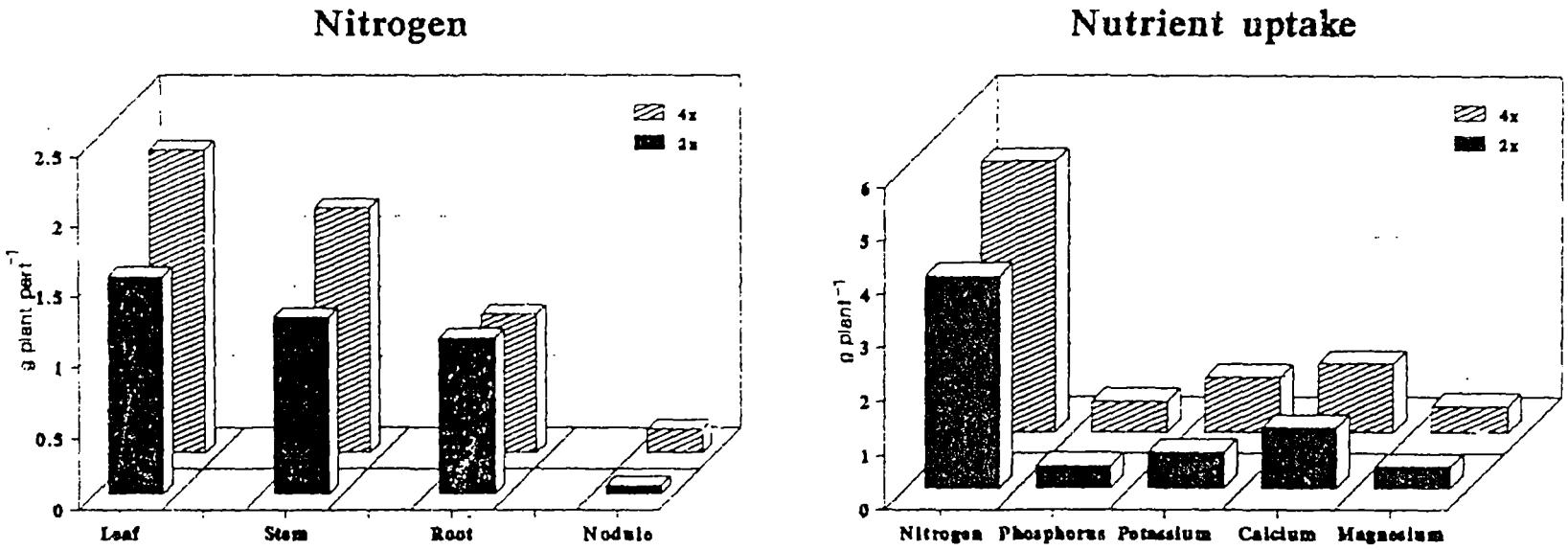
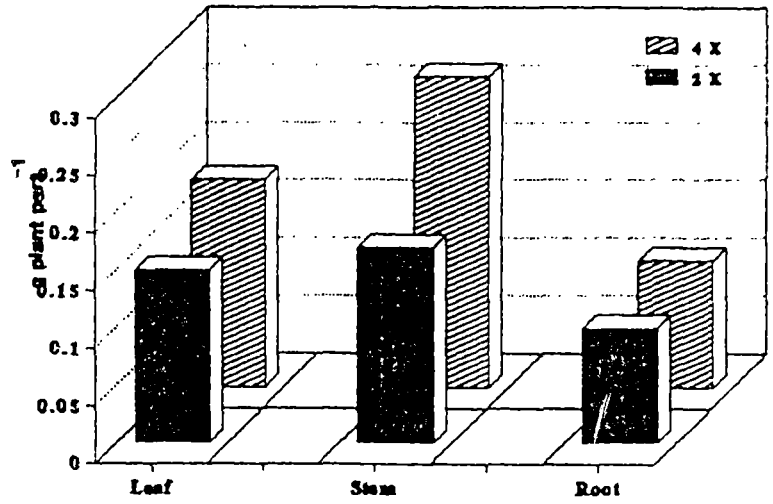

Nitrogen
4x
2x
2.5
2
1.5
1
0.5
0
g plant part⁻¹
Leaf
Stem
Root
Nodule
Nutrient uptake
4x
2x
6
5
4
3
2
1
0
g plant⁻¹
Nitrogen
Phosphorus
Potassium
Calcium
Magnesium

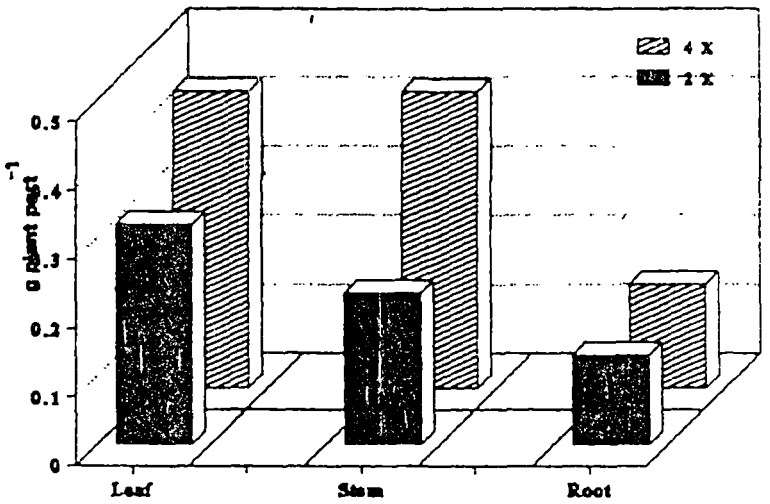
Fig.69

Fig. 70. Uptake of (a) Phosphorus; (b) Potassium; (c) calcium and (d) magnesium in various plant parts of diploid and tetraploid P. phaseoloides.

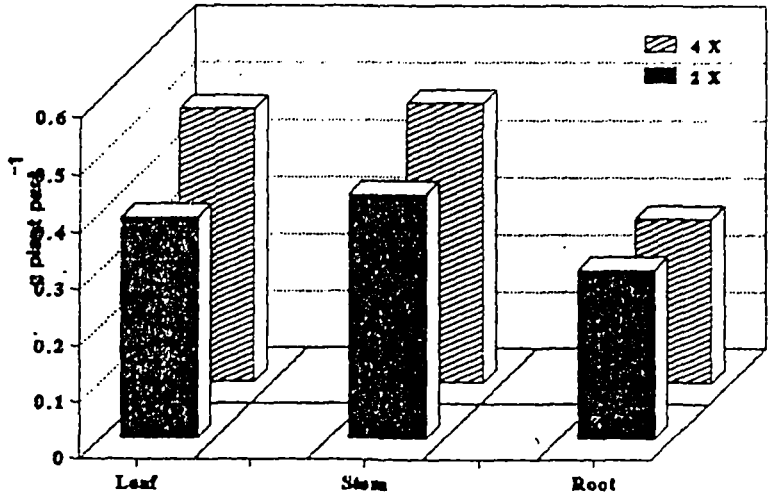
Phosphorus



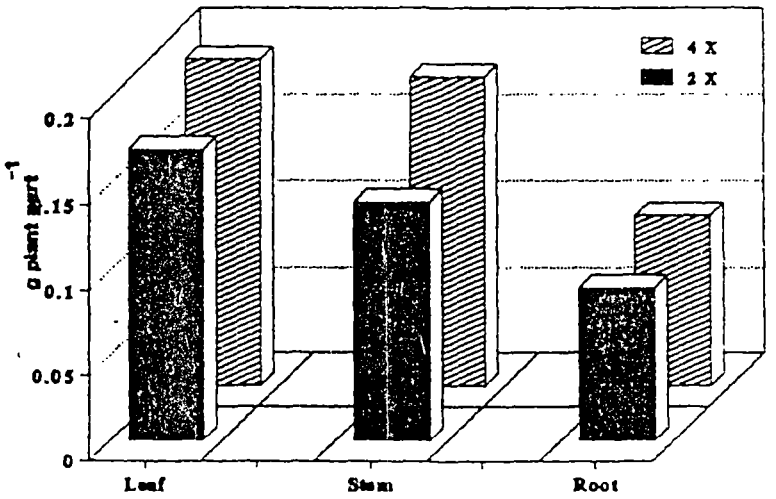
Potassium



Calcium



Magnesium



6. NODULATION AND NITROGENASE ACTIVITY

RESULTS

The polyploid plants varied among themselves and with the diploids in nodule characters.

6.1. Nodule numbers and nodule score

The first formed nodules in the tetraploids were larger and fewer in number than the diploids (Fig. 71). However on maturity, the former showed an increased root proliferation than the latter and the total number of nodules in the tetraploids showed a significant increase over that of the diploids. While in the diploids the nodule number ranged from 134 to 161, the variation in that of the polyploids was 112 to 194 (Table 20). Categorisation of the nodules based on their individual size, revealed that smaller nodules (measuring less than 2 mm diameter) accounted for the bulk of the variation in the total number of nodules in the polyploids. The number of medium sized nodules were comparable in the two cytotypes. The number of larger nodules decreased with increase in ploidy. At the same time, a few very large nodules, exceeding 6 mm diameter, was a characteristic feature of all the tetraploid plants. Such very large nodules were totally absent in the diploid plants (Figs. 72 a, b). Due to the high discrepancies in nodule size in either ploidy, arbitrary nodule rating (score) was worked out for the two groups. The average nodule score in the tetraploid was 504 as against 461 in the diploids.

6.2. Nodule weight

The tetraploids registered significant increase in both the fresh weight and in the dry weight of nodules than those of the control plants. The nodule fresh weight of individual polyploid plants ranged from 6.6 g to 16.91 g. The same pattern was also observed with respect to dry weight. The mean root dry weight in the polyploids was however similar to that of diploids.

6.3. Nitrogenase activity

The nitrogenase activity was assayed at two different time intervals for both the ploidy types and the results are presented in Table 21. When the plants were assayed at 6 weeks growth the rate of acetylene reduction activity (ARA) in the polyploids were 203.78 µmols C_2H_4 $plant^{-1}$ h^{-1} as compared to 163.80 µmols C_2H_4 $plant^{-1}$ h^{-1} in the diploids. When the same plants were further assayed after 9 weeks growth, the polyploids did not show a linear increase in nitrogenase activity and the two cytotypes reduced acetylene almost at the same rate. Similar to other nodulation traits, the acetylene reduction activity varied considerably within the polyploids, as evidenced by the high standard error observed for this trait.

The pattern of variation in acetylene reduction when compared to that of nodulation, showed no direct relationship with either nodule number or dry weight of nodules (Fig. 73). On the other hand, the nodule scores for different plants closely followed the varying trend in ARA, indicating the positive influence of nodule size on nitrogenase activity.

DISCUSSION

The nodulating behaviour of many polyploid legumes, including a few induced ones, have been studied by several workers (Bhaskaran and Swaminathan, 1958; Wier, 1961; Van der Starre-Van der Molen et al., 1967; Kabi and Bhaduri, 1978). In P. phaseoloides such observations assume greater significance, in that, an efficient symbiotic N_2 fixing system is an essential prerequisite for a ground cover.

The number of nodules in the tetraploids exceeded that of their diploid counterparts. According to von Bulow (1978) nodule number is a polygenic trait, whose response to ploidy levels may vary from species to species. In tetraploid Phaseolus, (Kabi and Bhaduri, 1978) number of nodules increased during preflowering stage, but decreased during flowering. In P. phaseoloides the observations were recorded, just prior to the onset of flowring, as nodulation and nodule activity will be at the peak during this stage.

In accordance with the general concept in polyploids, an increase in cell size resulted in an increased nodule size in the tetraploids, accompanied by a reduction in nodule number during the early period of establishment. Later on, further root proliferation in the tetraploids produced variable number of root hair infections, resulting in wide variation in nodule number. The more number of small sized nodules in the polyploids contributed substantially to an increase in their total number. The competing

ability of rhizobium strains to nodule sites is also reported to be affected by the ploidy status of the host (Wier, 1961; Kabi and Bhaduri, 1978).

The concept of 'Polyploid-cell Hypothesis' in nodule formation (Libbenga, 1974) is worth considering in this context. According to earlier theory, pre-existing disomatic cells in the root cortex are the sites of bacterial infection and nodule initiation (Wipf and Cooper, 1938, 1940). Van der Starre-Van der Molen et al. (1967) observed an increase in the percentage of deformed root hairs and number of infections, when colchicine solution was incorporated into the rooting medium. However, Bhaskaran and Swaminathan (1958) reported an incease in nodule number in the tetraploids of berseem and senji to be a consequence of increased root surface than that of the diploids. They further suggested that infection may occur in both the diploid and tetraploid cells alike, but the latter if present in more number, may get preferentially infected, particularly if the auxin content in the cells is important for nodule formation. The possible cause/effect relationship of polyploidy on nodule initiation however, still remains an area of uncertainty (Vincent, 1984).

In P. phaseoloides which nodulates profusely under tropical conditions (Bogdan, 1977) it is difficult to assume that a good percentage of its root cortical cells are disomatic. Also, mitotic studies in the root meristem cells of the diploids, did not reveal any kind of aberrations. But in the polyploids propagated by

rooted cuttings, as is the present case, the influence of ploidy variations in root cortical cells on the number of nodule initiation sites cannot be totally ruled out, especially since the first formed effective nodules are thought to inhibit further nodule formation, by a self regulatory mechanism of the host (Pierce and Bauer, 1983; Nutman, 1984; Giller and Wilson, 1991).

Irrespective of nodule number, the aggregate nodule scores in all the tetraploids was higher than the diploids. Mytton and Jones (1971) observed from selections on white clover, that the size of the nodule unit was more relevant to its symbiotic efficiency and also indicative of plant vigour. So also, Cralle et al. (1987) while studying high and low nodule mass alfalfa genotypes observed that the former facilitated greater N_2 fixation supporting greater vegetative growth and higher photosynthate supply to the nodules.

Several direct and indirect methods are currently in use for measuring nitrogen fixation by legume nodules (Bergersen, 1980). The acetylene reduction activity is one such method, based on the principle that the nitrogenase enzyme involved in dinitrogen fixation, has also the property of reducing acetylene to ethylene, which can be quantified by gas chromatography (Hardy et al., 1968). Eventhough the ARA measurements pose several constraints in linking with the actual amount of N_2 fixed (Witty and Minchin, 1988) it is a rapid non-destructive technique, best suited for laboratory and greenhouse grown plants (Denison et al., 1983; Wani et al., 1984) and is adequate for comparative assessments

(Hassan et al., 1987). In P. phaseoloides the enhanced rate of of acetylene reduction by the tetraploids in the initial weeks of establishment might be accomplished by the formation of larger effective nodules than the diploids. The vigorous growth rate of the polyploids also could have contributed to this effect (Hoffmann and Melton, 1981; Wynne et al., 1982). However, this increase was not linear throughout with the growing period and the reduction in acetylene reduction activity during the second sampling in the polyploids apparently reflects their differential nodulating behaviour. In the diploids, nodule formation was more or less regular resulting in uniform distribution of nodules in the root system during vegetative growth. But in the case of tetraploids, after an initial development of few larger effective nodules, there was an abrupt decline in active nodulation. Thus nodule initiation and development and the pattern of nodulation, have their implications on N_2 fixation (Graham, 1982; Pueppke, 1986). A few reports are available relating ploidy variations with ARA. In alfalfa, Laps et al. (1980) observed early and higher ARA in the tetraploids during the first ten days and in the diploids during the next five days. In their study, the tetraploids had fewer number of nodules than the diploids.

The plant to plant variation associated with ARA might well reflect the assessment of N_2 fixation too (Hardy et al., 1973). In P. phaseoloides ARA was not related to nodule number or weight of nodules in the polyploids. But the trend in variation in nodule rating and ARA followed a similar pattern, indicating that size of

the individual nodules gives a better prediction for rating the AR. whereas the fresh weight and dry weight of nodules was more of measure of their total number, regardless of their effectivity Variability in ARA with several related traits including number weight and volume of weight and volume of nodules, top dr weight, total nitrogen in top growth, Co_2 exchange rate and roo morphology were observed in different forage and grain legume (Duhigg et al., 1978; Sheehy et al., 1980; Barnes et al., 1984;)84; Hoffmann and Melton, 1981; Nutman, 1984; Arunachalam et al., 1984; Rosas and Bliss, 1986; Arrendell et al., 1990; Bliss, 1992). Nodulation score has been a common associative factor in all these cases.

In P. phaseoloides improved N_2 fixation leading to enhanced biomass production is advantageous to the rubber growers, by providing better coverage of interspace between the plants (Kothandaraman et al., 1993). In the present study, early development of effective nodules in the polyploids, not only facilitates considerable fixation in the upper soil profile, but also contributes substantially to early vegetative growth.

Table 20. Nodulation traits in diploid and tetraploid P. phaseoloides

Characteristics	2x		4x	
	Mean	Range	Mean	Range
No. of nodules				
Small (<2.0 mm)	44.66	(38-49)	73.9	(55-104)
Medium (2.0-4.0 mm)	53.67	(46-61)	51.2	(31-69)
Large (4.0-6.0 mm)	43.33	(36-53)	30.4	(16-41)
Very large (<6.0 mm)	-	-	9.5	(6 - 14)
Total No. of nodules	141.00	(134-161)	165.00	(112-194)
Nodule score*	461.00	(431-529)	504.00	(308-634)
" Fresh weight(g)	7.04	(5.98-8.01)	11.26	(6.60-16.9
" Dry weight (g)	1.11	(0.96-2.30)	2.98	(1.25-4.92
Root dry weight (g)	38.76	(33.71-40.90)	39.74	(34.2-45.2

* Arbitrary units

Table 21. Acetylene reduction activity at two different time intervals for the diploid and tetraploid plants.

Ploidy	Time of assay	ARA (μmoles C_2H_2 plant^{-1} h^{-1})
2x	6 weeks	163.80 $\pm$ 7.51
	9 weeks	408.01 $\pm$ 8.22
4x	6 weeks	203.78 $\pm$ 18.18
	9 weeks	426.00 $\pm$ 21.88

Fig. 71. Rooted cuttings of (a) diploid and (b) tetraploid P. phaseoloides showing initial development of a few, but larger nodules in the latter.

Fig. 72. (a) Detached nodules in the diploids graded as small, medium and large and (b) very large nodules in the tetraploids.

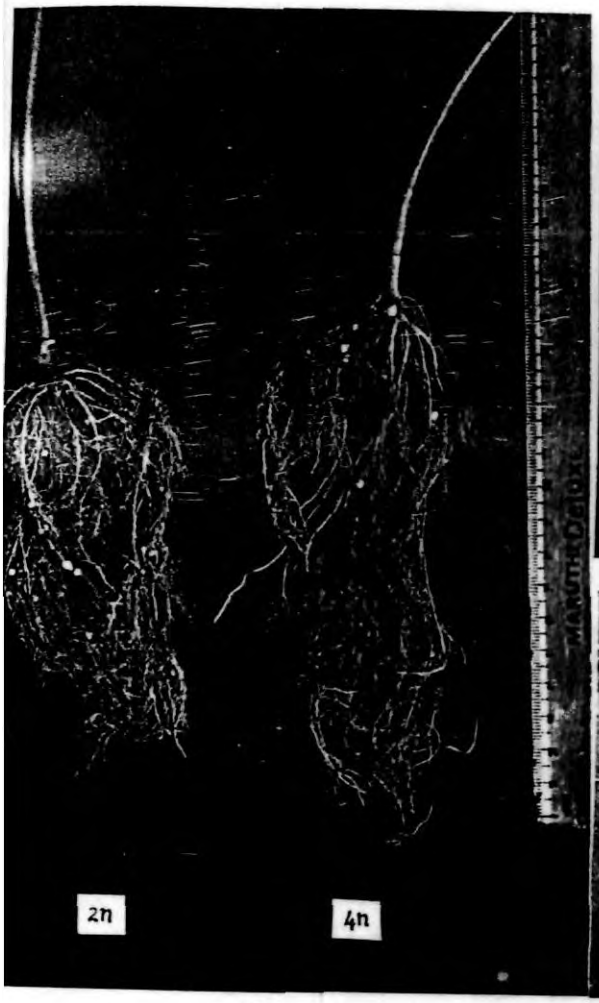


Fig 71

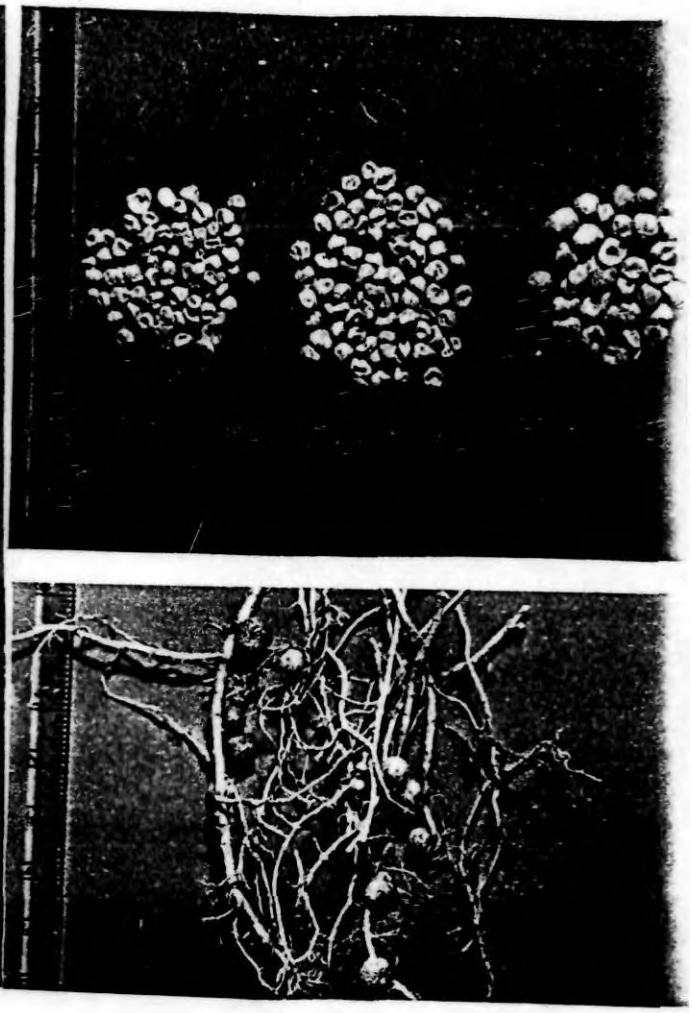
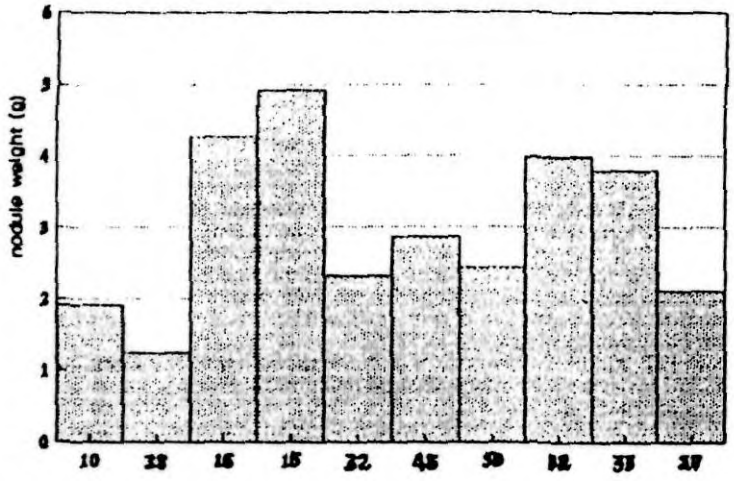
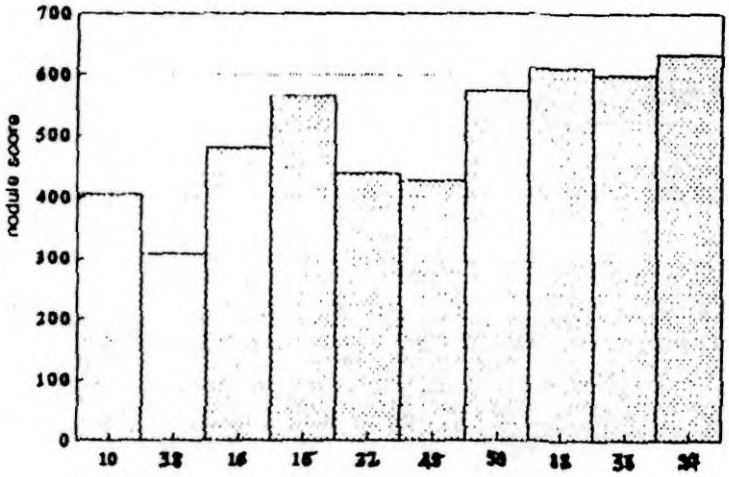


Fig 72

Fig. 73. Variation in nodule weight, nodule score and acetylene reduction activity within the autotetraploids of P. phaseoloides.


nodule weight (g)
0
1
2
3
4
5
6
10
38
16
15
32
45
50
12
35
37


nodule score
0
100
200
300
400
500
600
700
10
38
16
15
32
45
50
12
35
39

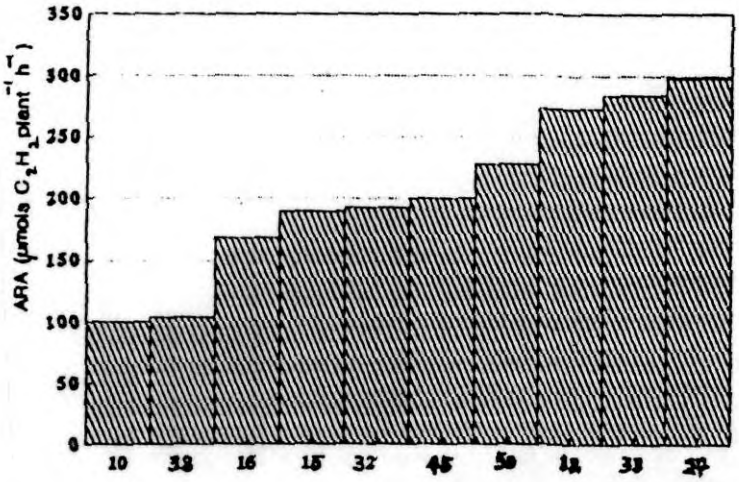

ARA (μmols C_2H_4 plant^{-1} h^{-1})
0
50
100
150
200
250
300
350
10
38
16
15
32
45
50
12
35
39

Fig.73

SUMMARY AND CONCLUSION

Induction of polyploidy was successfully attempted in Pueraria phaseoloides (Roxb.) Benth (2n = 2x = 22) and the resultant int autotetraploids (2n = 4x = 44) were evaluated for different nt cytomorphological and physiological traits. Diploid (untreated) P. phaseoloides served as the control, for comparison.

Seeds and seedlings were treated with various concentrations of aqueous colchicine for different durations and application of 0.75 per cent colchicine for 4 h yielded the highest number of tetraploids. Seed treatment failed to induce polyploidy.

Manifestation of genome doubling with respect to various traits studied was bidirectional. The autotetraploids registered a slow but vigorous growth rate with bigger leaf size, thicker leaves with intensified colour and an increase in number of leaflets per leaf. They also showed an increase in size of the guard cells, trichome length and pollen diameter and a reduction in internodal length, number of inflorescence and pollen stainability as compared to the diploids. These parameters could be utilised for the preliminary screening of tetraploids to a considerable degree. Pod set was drastically reduced in the autotetraploids and seeds obtained were nonviable.

Cytologically the 'raw' tetraploids were characterised by a high frequency of quadrivalents and bivalents and a low frequency of trivalents and univalents. Data on chromosome configurations at diakenesis, metaphase-I and abnormalities in anaphase I and II were analysed and their possible role in lowering pollen stainability and reduced pod set are discussed.

Pollen morphological studies revealed that 3-zono colporate grains were common in the diploids. Additional pollen types including 4-zono colporate syncolporate, brevicolporate and spiraperturate grains appeared in the tetraploids indicating pollen structural and sculptural variations through induced polyploidy. A possible line of evolution of these different types from the normal ones has been proposed.

Foliar anatomical studies showed profound increase in cell dimensions in various zones including epidermis, palisade, paraveinal mesophyll and spongy cells. Moderate correlation was established between palisade thickness and carbondioxide exchange rate but not with the total leaf thickness. Increased leaf thickness with prominent vascular bundles serves to boost up the functional efficiency of leaves in the tetraploids.

With respect to gas exchange properties, the tetraploids and diploids showed similar rates of photosynthesis on a unit leaf area basis. However carbondioxide exchange rate on a per leaf basis, and canopy photosynthesis (on a per plant basis) was higher in the tetraploids. The tetraploids also showed a higher rate of transpiration, on a per leaf basis, which might be due to increased stomatal pore size. An increase in leaf, stem and root tissues resulted in an improvement in total biomass in the tetraploids. Correlation figures pointed out a moderate association of canopy photosynthesis ($r = 0.59$ $P < 0.01$) and a higher association of total leaf area ($r = 0.70$ $P < 0.01$) with whole plant dry weight.

The effect of ploidy on the level of major nutrients has also been worked out. The nutrient content in the leaf, stem, root and nodule tissues of both the cytotypes with respect to N, P, K, Ca and Mg were

comparable. However the better growth of tetraploids facilitated greater uptake of nutrients.

The tetraploids varied with the diploids in the pattern of nodulation and nitrogenase activity. Nodules were graded visually intc three categories as small, medium and large and arbitrary nodule scores were worked out for the two cytotypes. The tetraploid plants produced a few, very large nodules than the diploids and the total nodule scores were also higher in the former. Nitrogenase activity in the tetraploids were higher during the first sampling date, six weeks after their establishment, but showed comparable rates with that of the diploids during the second sampling which was done nine weeks after establishment.

An optimum level of ploidy exists for every crop species, above which an increase in chromosome number results in a decline in productivity. The vigorous growth exhibited by the induced tetraploids in P. phaseoloides indicates that the plant is promising upto the tetraploid level. The production of large sized nodules in the second vegetative generation was indicative of the persistence of polyploid characters. The enrichment of soil with augmented level of organic matter in the tetraploids might facilitate better aeration and water retension capacity of soil. The prolonged growth cycle in the polyploids might also be beneficial in extending the period of ground coverage and also in leaf litter addition. The possibility of vegetative multiplication in P. phaseoloides is an added advantage in multiplying the tetraploids. More over, the induced autotetraploids serves to enrich the genetic stock of P. phaseoloides, which in turn could be incorporated in future crop improvement programme.

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