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

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. $\text{H}_2\text{SO}_4$ for 10 min. washed thoroughly in water and kept for germination.

Cytological studies in the diploids

(1) 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 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, 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 ± 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.

(1) 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 Å. Observations were made with the help of an HU5-5B, 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 earthen 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 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 carbon dioxide 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 carbon dioxide 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 sieve, 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:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Nodule size</th>
<th>Points awarded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Small (&lt; 2.0 mm diameter)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Medium (2.0-4.0 mm diameter)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Large (4.0-6.0 mm diameter)</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Very large (&gt;6.0 mm diameter)</td>
<td>10</td>
</tr>
</tbody>
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Nitrogenase activity:- Nitrogenase enzyme action was estimated by measuring the acetylene (C₂H₂) 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
Siliene 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 ± 2°C) for 1 h. A 0.5 ml gas sample was withdrawn after flushing twice and fed into a Shimadze 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:

\[
\text{\textmu moles } C_2 H_4 \text{ plant}^{-1} \text{ hour}^{-1} = \frac{[\text{sample ethylene after 1 h.} \times \text{acetylene at 0 time} - \text{ethylene after 1 h.} \times \text{acetylene after 1 h.}]}{[\text{sample ethylene at 0 time} - \text{ethylene at 0 time}] \times \frac{GV (ml) - VCF \times VPM}{22.4 (T_1 - T_0) h.}}
\]

where

- GV = gas volume at container
- VCF = vacutainer correction factor
- VPM = ethylene concentration (standard sample)
- \(T_1 - T_0\) = 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 (1957).

**Photography**

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