II MATERIALS AND METHODS

1. Method of cultivation :

The plants required for the experimentation were raised in the garden. The seeds from various localities such as Kolhapur, Solapur, Satara and Pandharpur were sown in the beds as well as in the pots in Botanical garden. The seedlings raised were uniformly fertilized with farm yard manure. But in the case of Tribulus terrestris seeds, in order to find out the relative suitability of soil various types of soil substrata were correlated. Plants grown in pots were kept in diffused sunlight. It was found that none of the seed germinated in the clay soil. The pots containing sandy loam, loam and gravel soils showed good growth of Tribulus terrestris L.

In order to study mode of propagation in nature, the seeds were sown at the depth of 1.0, 2.0, 3.0 and 5.0 cm, in pots with sandy loam soil. The seeds sown at the depth 3.0 cm or more than that depth do not germinate. However, the seeds sown superficially or at depths of 1.0 and 2.0 cm showed the sprouting of seedlings.

2. Meiotic studies :

A) Fixation of bud : The method followed is adapted from Hegde and Lugade (1985). Young flowerbuds of Tribulus terrestris L. were fixed in freshly prepared Carnoy's fluid (mixture of Absolute alcohol and Glacial acetic acid in the proportion of 3 to 1), in the morning hours between 6 to 7.00 a.m. Twenty four hours after fixation they were transferred to 70% ethanol and stored in the refrigerator or squashed.

B) Preparation of Stain : For meiotic study so called anther smear technique has been employed. The stain used is Belling's 1% iron aceto carmine. It is prepared by taking 1 g certified carmine into 100 ml of freshly prepared 45% acetic acid, and then refluxing for several hours on a waterbath. After cooling, the stain was filtered. One drop of saturated solution of iron acetate was then added and stored in refrigerator.

C) Preparation of slide : Prior to the preparation of anther smear, the anthers of appropriate bud size was carefully selected and were dissected out and transferred to 45% acetic acid. To ensure complete removal of ethanol several changes of acetic acid were given and then taken on a clear microslide, with a drop of freshly prepared iron aceto carmine. It was horizontaly cut with a sharp razor blade and squeezed by pressing with stainless steel needle. This step not only should have brought out sac of pollen mother cells from anther

but brightened direct contact of them with the stain. After ensuring that clean coverglass was placed on it, it was warmed on a spirit lamp to effect proper spreading. Excess of the stain was squeezed out by pressing a clean blotting paper with the thumb. Inverted technique was employed to squeeze out excess of stain. The slide was sealed with bee wax for temporary observation. Keeping of the slide overnight, intensified the stain and chromosome and exibited brilliant staining. Different stages of meiosis were studied from this preparation. Photomicrograph was carried out at this stage.

For this study five well spread meiotic plates were microphotographed. The photographic film used was ORWO 22 DIN, 100 ASA 35 negative. The sharp negative were enlarged and chromosome and different stages of meiosis were studied. Slides are made permanent by the method of Lavania (1979). Here the paraffin seal of the cover glass was carefully removed with blade and the slides are inverted in Petri-dish containing tert-butyl alcohol for dehydration. After the coverglass fell off it was transferred further to give two more changes of pure tert-butyl alcohol for complete dehydration. Slides and coverglasses were mounted separately with DPX mounting medium.

3. Pollen studies :

A) Pollen fertility : Studies were carried out in two ways. The methods followed are as described by Hegde and

Lugade (1986). First method is by testing the stainability of pollens by Muntzing's aceto-carmine. In this method fully matured freshly collected anthers were broke open in a drop of 1% aceto-carmine on a clean microslide. The pollen grains were allowed to spread uniformly. They were covered with coverslip. The slide was warmed and excess of stain blotted out. The preparation was observed under microscope. The sterile pollen did not take stain. The fertility percentage was scored by counting the fertile and sterile pollens per unit area.

$$\% \text{ Pollen fertility} = \frac{\text{No.of stained pollen grain per unit area}}{\text{Total No.of pollen grain counted in that area}} \times 100$$

B) Pollen germination : Second method adapted to study pollen fertility is that of Bishop (1979). In this method conditions of a compatible stigma was simulated in artificial condition and pollen grains were allowed to germinate. To accomplish this hanging drop method was followed. A drop of 2-6% sucrose solution was taken on a clear microslide and freshly harvested matured pollen grains were smeared on it. The microslide was held inverted on the surface of Distilled water in petri-dish covered with lid to saturate with humidity. The ambiant temperature ranged from 23°C to 25°C. After 24 hour the germinating pollens were scored under the microslide and percentage of fertile pollen was determined from it.

$$\% \text{ Pollen germination} = \frac{\text{Total No.of germinated pollen per unit area}}{\text{Total No.of pollen counted in that area.}} \times 100.$$

C) Method of pollen morphological studies :

Acetolysis : Pollen grains of Tribulus terrestris L. were studied for their exine ornamentation and structure under scanning electron microscope. The method followed is same as described by Hegde and Lugade (1986 a).

The fully matured freshly harvested anthers were taken as source of pollens. The pollen grains were acetolysed by the method of Erdtman (1964), to accomplish it fresh pollens were collected and dispersed in 70% alcohol in a vial. The vial was throughly shaken and centrifused at 5000 rpm. The supernatant was decanted. The step was repeated once again to ensure complete removal of oily substances. The pelleted pollen grains were washed with water and centrifuged. To ensure complete removal of colouring substances it was repeated. In the third step the pelleted pollens were washed with glacial acetic acid and centrifuged again. The step was repeated. The pellets were then dispersed in 5 ml. freshly prepared mixture of acetic anhydried and sulphuric acid as given below.

Nine parts of acetic anhydride was mixed with one part of conc. sulphuric acid in a corning testtube and poured on the

polleted pollens and kept in a waterbath at 70°C for 3-4 min. till the medium turns brown. This step enables dissolution of protoplast of the pollens. The pollens were then centrifuged to pellet out in the same way as before and acetolysing mixture was decanted. The pollen pellets were again resuspended in glacial acetic acid and centrifuged. The supernatant was decanted. The pelleted pollens were then washed in distilled water for several times to remove traces of acid and resuspended in water, so suspended pollens are now ready for microscopic study.

D) Scanning Electron microscopic (SEM) study : SEM studies of acetolysed pollens were carried out ~~in~~ by the course of a Director NIO (National Institute of Ocenography, India) Goa. The preparation of pollen for SEM studies was carried out as under :

The acetolysed pollen mass is mounted and dried on the stubs. The mass was covered with colloidal silver solution. They were then coated with thin carbon film in vacuum with the help of vacuum coating unit. It was then coated with gold. The mounted stubs were then rotated while being coated. The pollens were then studied with the help of cambridge stereoscan-150 Scanning Electron Microscope at various magnifications ranging from 500 to 3000 x from various angles for their morphology and exine ornamentation and photographed.

4. Flavonoid glycosides of T.terrestris :

A) Chromatographic Separation of glucoside of Tribulus terrestris L. leaves and seed :

Method adapted is of Borwski and Latomski (1960). The methyl alcohol extracts of both seed and leaves were evaporated to dryness in vacuo. The residue was treated with 5% acetic acid followed by alkali treatment with ammonia solution. Subsequently, it was reextracted with chloroform and finally condensed to 1 to 2 ml. Fifty µl. fractions from each of fruit and leaves extracted were spotted on Whatman Filter Paper No.1. The chromatogram was run unidirectionally. The solvent system used consist of mixture of carbon tetrachloride, Benzene/ methyl alcohol mixed in a proportion of 4:1:1. Alternatively BAW (4:1:5) was also used. The chromatogram was then dried and was sprayed with the freshly prepared Dragendorffs reagent. It was again dried. The yellow brown spot developed matched with the Rf. values of standards.

a) Preparation of Dragendorffs reagent : The reagent was prepared by the method of Harborne (1973). Two stock solutions 'a' and 'b' are prepared as follows :

b) 0.6 g of Bismuth sub-nitrate dissolved in 1 ml of conc. HCl and 20 ml of water.

c) 6 g of potassium iodide dissolved in 90 ml. of water. The solutions (a) and (b) were mixed and to it 7 ml of conc.

HCl and 15 ml. distilled water were added and the reagent thus prepared was diluted to 400 ml. with distilled water.

B) Assay of glycosides of Tribulus terrestris seed and leaves :

To determine the efficiency of separation method was also examined. The method of extraction is same as that employed for chromatographic technique.

a) Separation : The TLC plate was prepared as per the method of Stahls (1958). The Kisselgur (silicagel G) of Emerk previously mixed with binder was used for the purpose. The powder was mixed with distilled water and slurry was prepared. This slurry was uniformly spread over the TLC glass plate with the help of applicator. After setting of the film on the plate was activated by heating at 120°C for 3 hours in an oven. The known quantity of standard as well as the sample was loaded side by side and was run in a glass chamber using the solvent system. MeOH : NH_4OH . mixed in proportion of (200 : 3) solvent system.

After allowing the solvent and the solute to run completely the plate was removed and dried to detect the spots of flavanol by spraying Stahl's Reagent No.11.

b) Preparation of Stahl's Reagent No.11 : The spray reagent was prepared as per the method of Inamdar et al. (1989). It was prepared by mixing 50 ml of glacial acetic acid in 0.5 ml of anisaldehyde and 1 ml conc. sulphuric acid in a conical flask. The mixture was thoroughly shaken to facilitate better mixing.

5) Foliar application of micronutrients :

In order to study the effect of Mn^{++} and Zn^{++} on drug yield, foliar spray method was adapted. This trial was under taken in plants grown in garden prior to plantation soil beds were prepared in small plot by mixing farm yard manure in each of the bed.

The treatments were applied to each plot separately. For foliar spray $ZnCl_2$, $MnCl_2$ 4 H_2O are used as source of Zn and Mn respectively.

The solutions of desired concentrations are made in distilled water. They are sprayed in the following concentrations and combination :

	$ZnCl_2$		$MnCl_2$
1)	15	+	15
2)	20	+	20
3)	25	+	25

The control was given spray of distilled water. The foliar spray was started when seedlings were 15 days old and continued periodically at the interval of week. In all 3-sprays were given during their growth period. The leaves and seeds were harvested and drug yield was determined by paper chromatogram. The method adapted was as per Boywski and Lutomski (1960).

6) Estimation of Nitrogen :

To determine the Nitrogen content of leaf and seed oven dried samples were taken. Half gram leaf and the seed samples were separately Kjeldahl digested in 10 ml. of 50% sulphuric acid with a pinch of microsalt. The digested sample was made to volume. The nitrogen content was estimated titrometrically by distilling against alkali in the following way :

Reagents :

1. Boric acid, 4 percent. Dissolve 40 g H_3BO_3 in 1 litre of distilled water. The concentration of this reagent need not be precise as long as the amount of boric acid is more than chemically equivalent to the amount of ammonia to be absorbed.

2. Mixed indicator : Dissolve 0.3 g of bromcresol green and 0.2 g methyl red in 400 ml of 90 percent ethanol. The indicator color will change from red in acid solution to blue in alkaline solution.

3. Sodium hydroxide, 40 percent. Under a fume hood, dissolve 400 g of technical grade NaOH in a beaker containing 600 ml of distilled water. Place the beaker in a cold water bath to dissipate the heat produced. When cool, store the solution in a screw-top bottle.

4. Sodium carbonate. Transfer 10 to 20 g of AR grade Na_2CO_3 to a pyrex beaker and heat at 270 C for 3 hours. Cool the beaker in a dessicator.

5. Methyl orange indicator. Dissolve 0.1 g of methyl orange in 100 ml of distilled water.

6. Standard hydrochloric acid, 0.1 N. Dilute 9 ml of concentrated HCl to 1 liter with distilled water. Standardize this approximate 0.1 N HCl solution as follows : Dissolve exactly 0.530 g of the sodium carbonate reagent in 20 ml of distilled water. Dilute to 100 ml. Transfer 10 ml of this 0.1 N sodium carbonate solution to a 125-ml Erlenmeyer flask. Add two drops of methyl orange indicator. Titrate the approximate 0.1 N HCl solution into the 0.1 N sodium carbonate until the methyl orange indicator turns reddish-orange. Boil the solution gently for 1 minute and then cool to room temperature by running tap water over the outside of the flask. If the color changes back to orange, titrate more HCl until the first faint but permanent reddish-orange color appears in the solution.

Calculation :

$$\text{Normality of HCl} = \frac{0.1 \times 10}{\text{ml of HCl titrated}}$$

7. Standard hydrochloric acid, 0.05 N. Transfer 500 ml of the standardized 0.1 N HCl to a 1-liter volumetric flask and make up to volume with distilled water.

Procedure

Distillation. Empty the Kjeldahl flask containing the digested sample into the micro-Kjeldahl distillation apparatus. Rinse

the flask three times with distilled water, each time emptying the rinse water into the distillation apparatus. Use a minimum amount of water. Then with a quick delivery pipette, add 10 ml of the 40 percent NaOH to the distillation apparatus.

Prepare a 125 ml Erlenmeyer flask containing 10 ml of 4 percent boric acid reagent and three drops of mixed indicator. Place the flask under the condenser of the distillation apparatus, and make sure that the tip of the condenser outlet is beneath the surface of the solution in the flask.

Allow steam from the boiler to pass through the sample, distilling off the ammonia into the flask containing boric acid and mixed indicator solution.

Distill the sample for 7 minutes. Then lower the flask and allow the solution to drop from the condenser into the flask for about 1 minute. Wash the tip of the condenser outlet with distilled water.

Titration. Titrate the solution of boric acid and mixed indicator containing the "distilled off" ammonia with the standardized HCl.

Note :

a) Use the standardized 0.1 N HCl for samples containing 1.5 to 4 percent nitrogen.

b) Use the standardized 0.05 N HCl for samples containing less than 1.5 percent nitrogen.

c) Try to have a titration value of more than 2 ml so that the titration error will be negligible.

d) Determine the titration value of a blank solution of boric acid and mixed indicator.

Calculation :

$$\% \text{ nitrogen in sample} = \frac{(\text{sample titer} - \text{blank titer}) \times \text{normality of HCl} \times 14 \times 100}{\text{Sample weight (g)} \times 1000}$$