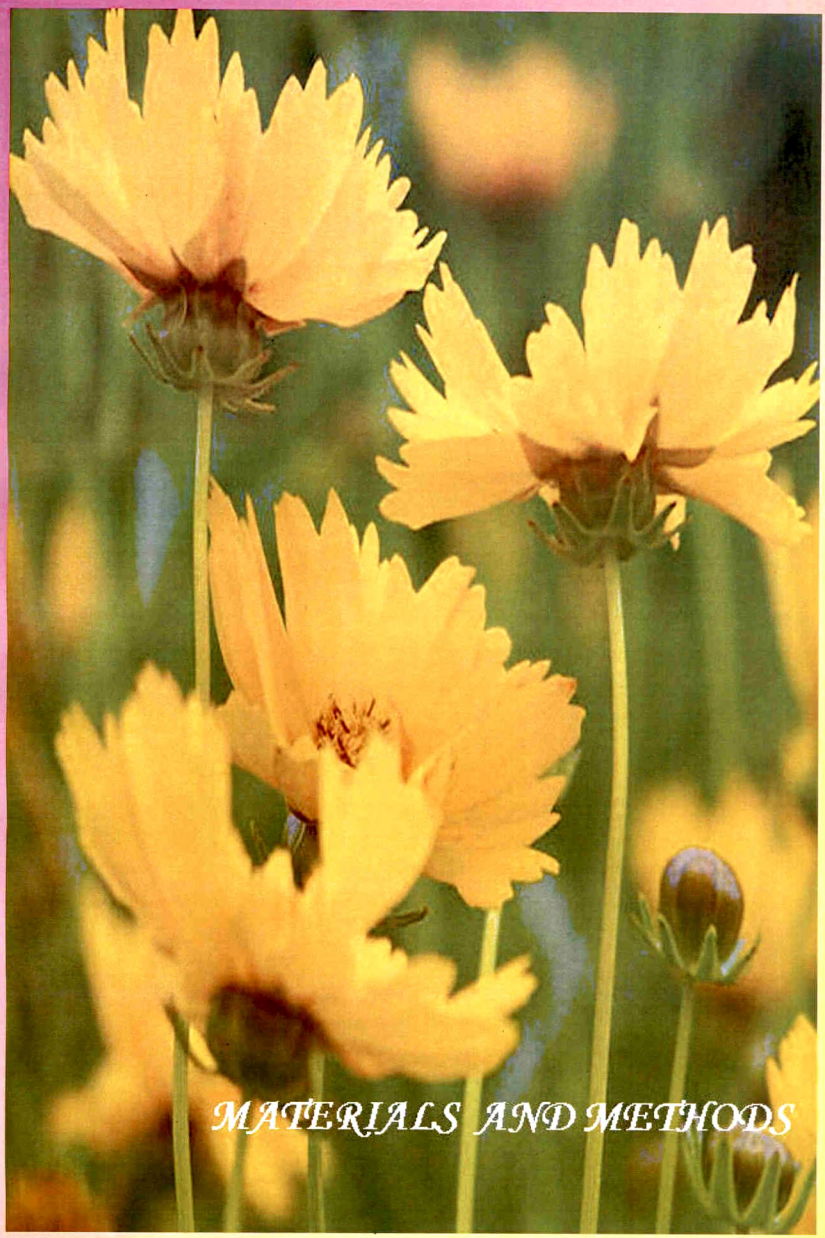
MATERIALS AND METHODS

Material

The slips of local cultivar of Vetiver grass were obtained from Agriculture College, Kolhapur. The slips of popular cultivar KS1 of Vetiver grass were obtained with special permission from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, UP for research purpose. Plants of these variety were raised in both large sized pots (45cm in diameter) and field plots of 1 sq m one. The plants in different pots and plots were supplied with equal amount of water once in a week. Every care was taken for proper growth of plants in the field. The experiment was conducted in months of March.

Foliar spray of plant growth regulators

After establishment of slips and satisfactory growth of plants of both varieties, the foliar treatments of three plant growth regulators namely chlorocholine chloride (CCC), salicylic acid (SA) and Vipul (triacontanol) ~~was~~ were undertaken. The concentrations of the PGRs chosen were 100ppm and 200ppm and spraying was carried out twice in a week. The plants sprayed with distilled water served as control.

The biochemical alteration caused (pigment status and enzyme activities) by the foliar sprays of the three PGRs were investigated after 15 sprays. At the end of 11 months the plants were carefully uprooted and the roots were washed thoroughly and the growth analysis was performed with respect to fresh weight, dry weight, number of tillers etc.

Growth parameters

The growth parameters of local and KS 1 variety like dry weight of root and shoot and number of tillers per plant ~~is~~ was carried out.

Pigment status

Chlorophyll

Chlorophylls were estimated by following the well known method of Arnon (1949).The leaves were brought to the laboratory washed thoroughly and blotted to dry. The midrib was removed. The leaves were cut into small segments with razor and these segments were randomly mixed. Five hundred milligrams of leaf segments of each treatment and control sample were accurately weighed. These pieces were then homogenized in chilled mortar with pestle in chilled 80% acetone. During crushing addition of a pinch of magnesium carbonate was made to neutralize acids released from leaf material during crushing. After fine crushing, the homogenate was filtered through Whatman filter paper no.1 using Buchners funnel. Filtration was continued till the residue becomes whitish brown. Final volume of filtrate was made 50 ml with 80% acetone. The filtrate was poured into a conical flask wrapped with a black paper. The absorbance of the filtrate was taken at 663nm and 645nm on spectrophotometer (UV 190) using 80% acetone as blank. By using following formula chlorophyll a, chlorophyll b and total chlorophyll in the leaf material was estimated.

Formula

Chlorophyll a= 12.7 X A663 – 2.69 X A645 = x

Chlorophyll b= 22.9 X A645 – 4.68 X A663 = y

Total chlorophyll (a+b) = (8.02 X A663) + (20.2 X A645) = z

$$\text{Chla/ chl b/ total chl (mg } 100^{-1} \text{ fresh weight)} = \frac{\text{x/y/z X vol. of extract X 100}}{\text{1000 X weight of plant material (g)}}$$

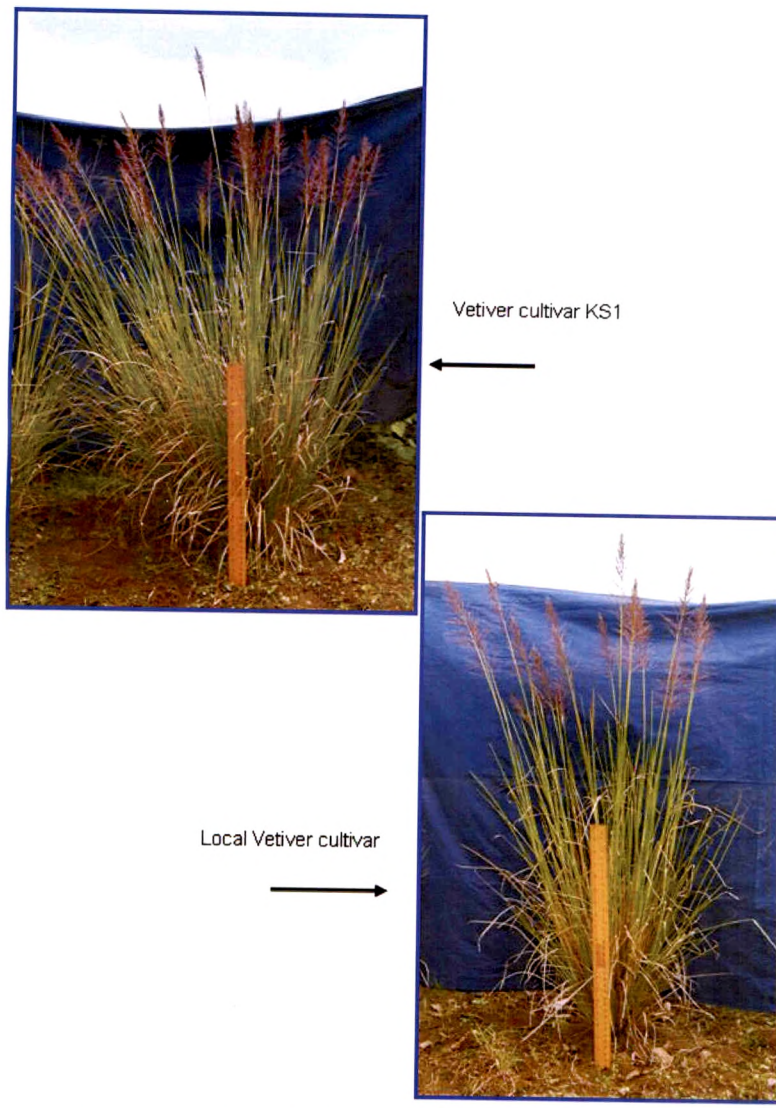

Plate No. 1. Habit of *Chrysopogon zizanioides* (L.) Roberty

Carotenoids

The method for extraction of carotenoids and filtrate preparation for accessory pigments carotenoids was same as explained earlier in case of chlorophylls. The absorbance of filtrate was taken at 480nm at spectrophometer against 80% acetone as a blank and then using formula, the value of carotenoids was accessed. According to the method described by Kirk and Allen (1965), the formula is as follows-

Formula

$$\text{Total carotenoids} = \frac{A480 \times \text{vol of extract} \times 10 \times 100}{2500 \times \text{weight of material (g)}}$$

Where, 2500= average extinction

Anthocyanins

Estimation of anthocyanin content was carried out following the method of Murray and Hacket (1991). The leaf material excluding midrib was cut into pieces. Five hundred mg of leaf pieces of each treatment and control plant material were accurately weighed and was incubated in prechilled (-20^{o}C) 0.1% (v/v) methanol-HCl solution for 24 h in dark at low temperature (2^{o}C for 24h). After 24h the plant material was crushed in the chilled mortar with pestle. Then homogenate was filtered through four layers of muslin cloth., the absorbance was taken at 532nm and 653nm after 20 times dilutions of filtrate on spectrophotometer (UV double beam- Shimadzu). The content of anthocyanin was calculated by using following formula

Anthocyanins

(Abs. unit) = (A532nm) - (0.24 X A653nm)

Polyphenols

To determine the total polyphenol, the method of Folin and Denis (1915) was employed. The leaves were brought to the laboratory washed thoroughly and blotted to dry. The midrib was removed. The leaves were cut into small segments with razor and these segments were randomly mixed. Five hundred mgs of leaf material was homogenized in 80 % acetone and filtered through Buchners funnel. The residue was washed repeatedly with 80% acetone and final volume of extract was made 50ml with 80% acetone. Two ml of plant extract along with a series of standard tannic acid (0.1mg/ml) were taken in separate Nessler's tube and then in each tube; 10ml of 10% Na_2CO_3 was added. Then two ml Folin Denis reagent (100g of sodium tungstate mixed with 20g phosphomolybdic acid in about 800ml of distilled water to this 200ml 25% phosphoric acid was added and this mixture was refluxed for 2-3h to room temperature and final volume was made 1000ml with distilled water) in each test tube were added. The final volume of reaction mixture was made 50ml with distilled water. Absorbance was read at 660nm after 20 minutes using regent blank. Total polyphenol were calculated with the help of standard curve of tannic acid and the results were expressed as mg $100g^{-1}$fresh weight.

Enzymatic study

Nitrate reductase (EC 1.6.6.1)

The *in vivo* method of Jaworski (1971) was followed for determination of nitrate reductase activity in the leaves and roots of Vetiver plant.

The weighed amount of plant material like root pieces and leaf segments of different treatments and control sample were separately incubated in paraffin sealed test tubes containing assay medium (1ml KNO_3, 2ml 5% n propanol, 5 ml 0.2 M phosphate buffer(pH 7.5) and 2 ml, 0.5% Triton X 100) were kept in dark for one hour. After the desired incubation period 1 ml reaction mixture was taken out and mixed with 1ml 1% sulphanilamide(1% in 2N HCl) along with 0.02%

NEEDA (N-1-(napthyl)- Ethylenediamine dihydrochloride) from blank (without plant material) reaction mixture, 1 ml solution was taken out and mixed with 1 ml of 1% sulfanilamide and 0.02%NEEDA, which served as a blank. The absorbance was read at 540nm on UV-VIS double beam spectrophotometer (Shimadzu 190). By taking different concentrations of KNO_2, standard curve was prepared and enzyme activity was expressed in terms of µg of NO_2 liberated $h^{-1}g^{-1}$ of fresh tissue.

It was noticed during the *in vivo* assay of NR activity in the roots that the reaction mixture becomes foggy and somewhat nontransparent and this created problems in recording accurate absorbance values. This problem was tackled by keeping the root pieces in a small portion of dialysis tubing and putting a knot at each end of the tubing with the help thread. Such tied dialysis tubes containing root pieces were placed in the incubation medium and the assay of enzyme activity was satisfactorily carried out.

Peroxidase (1.11.1.7)

The method described by Horiguchi (1988) was followed as study of peroxidase activity in the roots. Roots of Vetiver grass (local variety and KS1variety) subjected to different treatments and control root pieces were brought to laboratory, washed and blotted dry. Five hundred mg of root pieces were weighed and homogenized in 10ml ice cold (1/15 M) phosphate buffer (pH 6.8) and filtered through four layers of muslin cloth. This homogenate was then centrifuged at 10,000 rpm for 20 minutes and supernatant was used as the source of enzyme. Afterwards assay reaction mixture was made by adding 5ml of 1/15 M acetate buffer(pH - 5), 0.5ml of 0.1% guaiacol, 0.5ml of enzyme extract, 2ml distilled water, 0.5ml 0.08% H_2O_2 and then it was incubated at 30^0C. Zero minute reaction was immediately terminated by addition of 1ml, 1N H_2SO_4 with burette. In other test tubes, reaction allowed to proceed for 15 minutes and then it was terminated by addition of H_2SO_4. The absorbance was taken at 470nm. By

following the method of Lowry *et al* (1951), the soluble proteins in enzyme extract were determined and the enzyme activity was expressed as unit h^{-1} mg^{-1} protein.

Acid phosphatase (EC 3.1.3.2)

Acid phosphatase activity in Vetiver roots was assayed by adopting the method of McLachlan (1987). In this assay, weighed amount (500mg) of Vetiver root material of each treatment of local and KS1 variety with control was homogenized in 10ml of ice cold 0.1M acetate buffer pH 5. This homogenate was filtered through four layered muslin cloth which is followed by centrifugation at 10,000rpm for 20 minutes. The supernatant was served as source of enzyme. Enzyme assay mixture was prepared by adding 3ml p-nitrophenyl phosphate per ml of acetate buffer (pH 5) and 0.5 ml enzyme. Reaction was immediately terminated in zero minute test tubes by adding 1.5ml, 1.68 N NaOH with burette. The another set of test tubes were kept for 30 minutes to allow reaction and then it was terminated by adding 1.5ml , 1.68 N NaOH. Optical density of pale yellow coloured p- nitrophenyl was read at 420nm. Soluble proteins in the enzyme extract were determined by the method of Lowry *et al.,* (1951). The enzyme activity was expressed as μ moles p-nitrophenyl liberated h^{-1} mg^{-1} protein.

Carbohydrate analysis

Soluble sugars, reducing sugars and starch

Estimation of sugars was performed according to the method described by Nelson (1944). One gram of fresh Vetiver roots and leaf material from each treatment and control was extracted in 80% neutral alcohol and then it was filtered through Buchners funnel by using Whatman no. 1 filter paper. The filtrate was condensed upto 5ml on boiling water bath, cooled and 2g of lead acetate and potassium oxalate (1:1) were added for decolourization purpose. After adding 40 ml of distilled water, resulting aliquot was filtered through Bucheners funnel. The volume of filtrate (a) was measured and it served as an extract for determination of

reducing sugars. For the starch estimation, insoluble residue on filter paper which was obtained at the first step after filteration was transferred to 100ml conical flask. This was followed by addition of 50 ml distilled water and 5ml concentrated HCl and these contents were hydrolyzed at 15 lbs pressure for half an hour. Then the conical flasks were cooled to room temperature and the contents were neutralized by addition of unhydrous sodium carbonate bit by bit. Then it was followed by filtration through Bucheners funnel. After measuring the volume of filtrate (b), which contains glucose (reducing sugar) formed as a result of hydrolysis of starch. This amount of glucose is equivalent to the starch content in the residue.

For estimation of both starch and reducing sugar, 0.1 ml (b) and 0.4 ml (a) were taken in a set of respective test tubes. Different concentration of glucose were taken in another set of test tubes (glucose 0.1 mg/ ml).Then in each test tube, appropriate amount of distilled water was added to make final volume 1 ml. In blank, instead of filtrate or standard solution, 1 ml of distilled water was used. Afterwards addition of one ml Somogyi's alkaline copper tartarate reagent (4g $CuSO_4$, $5H_2O$, 24g anhydrous Na_2CO_3, 16g Na-K tartarate and 180g anhydrous Na_2SO_4 dissolved in 1 liter distilled water) was carried out then this set was kept in boiling water bath for 10 minutes. One ml Nelson Arsenomolybdate reagent (25g Ammonium molybdate dissolved in 450ml distilled water, 3g sodium arsenate dissolved in 25ml distilled water, 21ml concentrated HCl. These ingredients were mixed well and digested for 48h at 37^0C) was added with burette in each test tube after cooling the set of tubes to room temperature. The final volume of this reaction mixture was made to 10ml by adding the distilled water. The absorbance was read at 560nm on a double beam spectrophotometer (Shimadzu UV 190). The amount of reducing sugar was estimated with the help of calibration curve of standard glucose (0.1mg/ ml) and the values were expressed as g $100g^{-1}$ fresh tissue.

The extract containing both reducing sugars and non-reducing sugars were used for the further estimation of soluble sugars. For this purpose the method of Dey (1990) (phenol sulphuric acid) were used with slight modification. For the estimation, 0.2ml plant extract was taken in a test tube in which 1ml 0.5% phenol was added carefully and mixed thoroughly. It is followed by careful addition of analytical grade conc. sulphuric acid. This was mixed thoroughly by agitating it with glass rod. These contents were cooled in air and its absorbance was read at 485nm. The amount of soluble sugar was estimated with the help of standard glucose (0.1mg/ml). The values were expressed in g $100g^{-1}$ fresh weight basis.

Estimation of Inorganic constituents

Preparation of acid digest

For the extraction of inorganic constituents, method of Toth *et al.* (1948) was followed. Plant material like roots and leaves were washed thoroughly and blotted to dry. Then it was subjected to drying at 60°C for 10 days till the plants dried up to a constant weight. This oven dried plant material was fine powdered and then 500mg powder of each treatment and control was weighed. This plant material was transferred to 150ml capacity beaker in which 20ml of concentrated HNO_3 were added. These beakers were covered with watch glass and heated slowly to dissolve solid particles. After cooling to the room temperature, 10ml 60% perchloric acid was added and mixed thoroughly. On hotplate the colorless 2-3ml solution was obtained. Then it was allowed to cool and diluted with 20ml distilled water and transferred into 100ml capacity volumetric flask made final volume (100ml) with distilled water and kept overnight. In next day this extract was filtered through Whatman No. 44 (ash less) filter paper. Filtrates so obtained were used for estimation of different inorganic constituents.

The level of magnesium, iron and calcium were estimated by using Atomic Absorption Spectrophotometer, by doing dilutions if required. Potassium and Sodium levels were estimated by using flame photometer.

Study of Vetiver oil composition

The Vetiver roots (air dried) of each treatment and control were analyzed to investigate influence of foliar application of PGR on quality of essential oil with the help of thin layer chromatographic technique(TLC) according to the method described by Somchai *et al.,* (2008). Accurately weighed one g of root material was mixed in 20ml methanol and refluxed for 20min. Then the solution was transeferred to evaporating dish to evaporation at RT. Afterwards the concentrate remained in the dish was dissolved in 0.5ml methanol and used as source of extract for TLC. The synthetic TLC aluminium sheet used and 20µl extract was loaded with micropipette at equivalent points. This TLC plate was developed into toluene: ethyl acetate (90:10) solvent system for sufficient period in glass jar. When the chromatogram was sufficiently developed, taken out, air dried and then sprayed with vanillin-sulphuric acid spraying reagent, in segmental manner i.e. first solution A (1% ethanolic vanillin) was sprayed, this was followed by spraying of solution B (10% ethanolic sulphuric acid). Then this TLC was dried in oven for few minutes at105^{0}c to develop spots. The Rf values of detected spots were then compared with standard values of the authentic standard (Wagner and Bladt, 2003).