MATERIAL AND METHODS
Isolation of triacontanol from rice bran wax

Triacontanol was isolated from rice bran wax. The raw material, rice bran wax was collected from V Kumar and Sons factory, Aurangabad. Triacontanol was isolated by using 2 step saponification and extraction method. Rice bran wax (200g) was heated until melted. The melted sample was saponified by adding NaOH (20%) and ethyl alcohol (15ml). These solutions were added and heated with stirring. The saponified material was dried. After complete drying, the material was extracted with acetone in a soxhlet extractor using extra ordinary thimble. As the solvent was cooled to room temperature the crystallization of the products was carried out. The crystallized compound was then filtered under vacuum and dried. The melting point was found out at 84°C. The sample was further characterized by GC and purity was confirmed with standard sample where 23% purity was obtained.

Formulation of triacontanol

Triacontanol was formulated by Welebir (Welebir et al., 1982) method. The triacontanol was mixed with the acetone and heated at boiling temperature with care taken not to form vapours. It was then dissolved afterwards in large amount of water to develop colloidal solution. It was then diluted at different concentrations such as 2 ppm, 5 ppm, 6 ppm, 8 ppm and 9 ppm. The different concentrations of triacontanol were sprayed on the foliage of green gram with sprayer as per the spraying schedule given below.

1. After the development of two to three leaves 1st spray
2. At flowering stage 2nd spray and
3. During panicle initiation 3rd spray
1) Effect of triacontanol on the growth of green gram in presence light.

Green gram [*Vigna radiata* (L.) Wilczek var. *sublobata* (Roxb.) Verdc.] variety, BPMR-145 seeds were collected from Agricultural Research Station, Badnapur. Healthy seeds were sown in pots filled with black soil, gypsum and cow dung (20 seeds per pot) and irrigated. The germinated plants were kept in light and shade. The foliar spraying of triacontanol at various concentrations was employed at the age of 14 and 30 days and after flowering. The growth of the plants was monitored by recording height of the plants and number of leaves after 37 days and at maturity. At the time of harvesting, root length, leaf area, number of seeds per pod and average seed weight was recorded.

2) Effect of different concentrations of triacontanol on the growth of green gram

Healthy seeds were selected and sown in the pot (15 seeds per pot) and irrigated. The germinated plants were kept in light. After developing trifoliate leaves the triacontanol was sprayed on it. Triacontanol was formulated as above. The foliar spraying of triacontanol with various concentrations was done after 5 days (1\textsuperscript{st} spray). During flowering stage the 2\textsuperscript{nd} spray and at the pod formation 3\textsuperscript{rd} spray was applied. The growth of the plants was monitored by recording height of the plant and number of leaves. At the time of harvesting, root length, leaf area, number of seeds per pod and average seed weight was recorded.

3) Effect of different spectra of light on the growth of triacontanol treated green gram.

Six chambers were made by applying colored gelatin papers. Each section was covered with different colored gelatin paper, red, green, blue, yellow and 5\textsuperscript{th} one was white light and 6\textsuperscript{th} section was under the shade. 30 pots were taken.
Material and methods

Each pot was filled with soil and soil conditioners. In each pot 30 seeds were sown. After germination (when two leaves were developed) in each section 5 pots were kept under different colored gelatin papers at vegetative stage. The spray of triacontanol was applied to the whole plant body by using different concentrations i.e. 2 ppm, 5 ppm, 6 ppm and 9 ppm along with control. After 30 days the number of leaves was counted and the height of the plants was also measured. The plants were watered daily early in the morning.

4) Effect of different plant growth regulators on green gram combined with triacontanol

Healthy seeds were selected and sown in the pots (3 seeds in each pot). The pots were irrigated. After 5 days trifoliate leaves were developed on the plant. After 21 days different combinations of plant growth regulators with triacontanol were sprayed on the plants as follows.

1. NAA 40 ppm + Triacontanol 5ppm
2. GA 50 ppm + Triacontanol 5ppm
3. IBA 40 PPM + Triacontanol 5ppm
4. Cytokinine 6 ppm + Triacontanol 5ppm
5. Triacontanol 5 ppm
6. Control (water)

The foliar spraying was done at vegetative and flowering stage and at pod initiation stage after 32 days and 48 days. The plants were irrigated early in the morning.
The number of leaves, height of the plant was measured at the vegetative stage of growth. Number of pods, leaf area index, dry matter content and yield components were recorded at the time of harvesting. The data was statistically treated for analysis of variance (ANOVA) and the values for critical difference (C.D) were calculated at P=0.05.

**Quantitative characters**

The different quantitative traits were analyzed as given below

**Plant height**- the height of 3 selected plants was measured by using scale before harvesting and at the time of harvesting in centimeters from base to apex of plants.

**Number of leaves**- total number of leaves in each plant out of selected 3 was counted individually and average was recorded.

**Leaf area index**- leaf area index per plant was determined by gravimetric method (Shahane and Mungikar, 1984; Mungikar, 1986).

**Number of pods** – total number of pods per plant was counted individually and average was recorded.

**Pod length** – the length of 10 pods per plant was measured. Average was calculated for each time and for each treatment.

**100-seed weight**- The weight of 100 seed was recorded in gram from each treatment.

**Biomass**- fresh and dry weight of seed was measured in grams.

**Yield**- the total seeds yield per plant was recorded and expressed in tone per hectares. By using following formula yield per hectare was calculated.

**Seed yield**= no of harvested plant x no of pods/ plant x number of seed per plant x weight of 1000 seed (g) x10⁻⁵
Material and methods

The data was statistically treated for analysis of variance (ANOVA) and the values for critical difference (C.D) were calculated at p=0.05 (Mungikar et al., 1997)

Biochemical analysis

At the end of three months the moisture, ash, fats, proteins, carbohydrate and calories were estimated by adopting standard analytical methods as follows.

Moisture content:-

The total moisture was calculated as follows. 10 gram of the sample was taken in Petri dish and heated in hot air oven at 100\(^0\) C for about 2 hours. It was then cooled in desiccators and weighed. It was again heated for half an hour, cooled and reweighed. The process of heating cooling and weighing was repeated till constant weight was obtained. The moisture percentage was calculated as follows.

\[
\text{Moisture \% by weight} = \frac{W_2 - W_1 \times 100}{W}
\]

Where \(W_2\) = weight (g) of Petri dish + sample before heating.

\(W_1\) = weight of Petri dish+ sample after heating

\(W\) = weight (g) of the sample taken for test.

Total Ash:-

The residue, after incineration of sample at 550-600 \(^\circ\)C is known as ash. For this purpose the sample was subjected to high temperature up to 600\(^\circ\)C and then the ash content was determined as follows. 2 g of oven dried sample was taken in a previously weighed vitrosil silica crucible and 2 drops of the mixture of \(\text{H}_2\text{SO}_4 : \text{HNO}_3\) (2:1) was added to it and heated on hot plate for about 30 minutes, till the sample sufficiently charred and turns black. The lid was replaced and kept in muffle furnace and allowed the
Material and methods

temperature raise up to 600°C. It was kept constantly for 2 hours. The crucibles were removed and were transferred directly to desiccators, cooled and weighed immediately. Weight of the ash was measured as percent dry matter (DM)

**Estimation of crude fat**

The crude fat in the seed sample was estimated by the standard soxhlet method given in Official methods of analytical chemistry (Latimer, 2010). The fat present in the seed material was extracted in the solvent consisting of petroleum ether. This was done in soxhlet extraction assembly and after complete evaporation of the solvent the amount of extracted fat was measured as follows.

Two gram dry seed powder was placed into thimble prepared with Whatman filter paper no.1. The mouth of thimble was plugged with fat free absorbent cotton. A 250 ml dry clean receiver was taken from the soxhlet assembly and the solvent was added to it, just to reach the level of the neck. The thimble with sample was placed into the soxhlet assembly. It was kept on heating mantle with temperature controlling device. The water condenser was fixed at the top of the soxhlet.

The fat was extracted for 8 hours at 60°C. When the extraction is over, the thimble was removed from soxhlet and assembled the apparatus again and heated to recover most of the solvent from the receiver flask. When the receiver flask contains about 25 ml solvent along with the extracted fat the receiver flask was disconnected. The solvent was transferred in a clean previously weighed beaker 3 times dried in a hot air oven at 95°C, cooled in a desiccators and the final weight was measured. The amount of fat was extracted per 2 grams of the sample and the amount of crude fat was calculated as percent dry matter.
Material and methods

**Estimation of nitrogen:**

The dry sample was digested with concentrated sulphuric acid (H$_2$SO$_4$) in the presence of catalyst. During the digestion, nitrogenous compounds were converted to ammonium sulphate [(NH$_4$)$_2$SO$_4$]. It was then made strongly alkaline with the addition of sodium hydroxide (NaOH). The released ammonia (NH$_3$) was distilled into boric acid (H$_3$BO$_3$) solution. The ammonium tetraborate formed was then titrated with 0.035 N hydrochloric acid for the determination of nitrogen.

The reagents were prepared as follows:

1. Concentrated H$_2$SO$_4$, AR grade or specific gravity 1.85.
2. Catalyst: A mixture of copper sulphate (CuSO$_4$), potassium sulphate (K$_2$SO$_4$) and selenium dioxide (SeO$_2$) in ratio 1:9:0.02.
3. NaOH solution: 400g NaOH was dissolved in 1000ml distilled water.
4. Mixed indicator: 300mg bromocresol green and 200 mg methyl red were dissolving in 95% ethyl alcohol. The volume was made to 500 ml with 95% ethyl alcohol.
5. Boric acid solution: 2% H$_3$BO$_3$ solution was made by dissolving 10g H$_3$BO$_3$ in 480ml of glass distilled water. To this about 5 ml mixed indicator was added and the volume was made to 500 ml.
6. 0.035 N HCl: 25ml of concentrated HCl was taken in volumetric flask and diluted up to 500 ml with distilled water. This served as a stock solution of HCl. To determine the normality of this solution, 1g sodium tetra borate [(Na$_4$)$_2$BO$_3$] was taken in 50 ml conical flask. To it 10 ml glass distilled water and 2-3 drops of mixed indicator were added. This was titrated with the stock solution of HCl to calculate the normality using following equation.
Material and methods

Normality of the stock solution of HCl = \frac{1000}{\text{Titration valu(ml)} \times 190.72}

After determining the normality of stock solution 0.035 N HCl was prepared by appropriate dilution. The 300 mg of dry plant material was transferred in a K-jeldhal flask. Pinch of catalyst was added with the help of spatula, accurately measured 7.5 ml of concentrated sulphuric acid (H₂SO₄) was added slowly. The flasks were heated gently on a digestion stand until the fumes of H₂SO₄ were freely evolved. The heat was increased until acid boiled vigorously and digested for a time till the mixture has been cleared i.e. apple green colored. During digestion, care was taken to avoid particles of undigested carbon sticking on the sides of the tube. The contents of the flask were cooled and used for the distillation. For this purpose the digested material was made up to volume of 50 ml in volumetric flask with distilled water. The distillation was carried out with the Markham’s steam distillation apparatus. The steam boiler was heated to produce steam. 50 ml conical flask was kept containing 10 ml boric acid solution at the delivery end of the condenser. The condenser was timed off which was just beneath the surface of H₃BO₃ solution. 5 ml of previously diluted, digested sample was taken into the distillation flask through funnel. The funnel was closed. 10 ml NaOH solution was added in the funnel and introduced it slowly into distillation flask. The ammonia was formed due to the treatment of NaOH. This was passed with the steam and was absorbed by H₃BO₃ at the condenser outlet to form ammonium tetraborate [(NH)₄BO₃]. This resulted into the change in colour of H₃BO₃ solution from pink to green. The distillation was continued till the volume become 20 ml. The ammonium borate was titrated with 0.035 N HCl till pink colour reappeared and titration values were recorded. The strength of NH₃ with 0.035 N
Material and methods

HCl was tested till pink colour reappears and titration values were recorded. The strength of NH$_3$ in the distillation was calculated using equation: 1ml 0.035 N HCl = 0.5 mg of nitrogen. The amount of nitrogen was calculated for 50 ml of the sample 300 mg of dry plant material was the nitrogen computed in percent of dry matter.

**Crude protein**

On an average, most proteins have 16% nitrogen in their composition. In other words, 1 mg nitrogen equals 6.25 mg protein. Thus the amount of nitrogen content, when multiplied by 6.25, the crude protein content of the sample was determined.

**Estimation of carbohydrate**

The Carbohydrates were measured by adding percentage of moisture, total ash, fat and protein and subtracted this from 100.

**The total calories were estimated as follows.**

\[
\text{Total no. of calories/ 100gram.} = \% \text{ of carbohydrates} \times 4 + \% \text{ fat} \times 9 + \% \text{ protein} \times 4
\]

The addition of calories of carbohydrates fat and proteins was made, from this the calories value was calculated on dry weight basis as follows.

\[
\text{Cal/ 100gram, on dry weight basis} = \frac{\text{Calories/100gX100}}{100-\text{Moist}}
\]

**Mitotic index**

The green gram seeds were treated using different concentration of triacontanol These were 2 ppm, 5 ppm, 6 ppm, 8 ppm and 9 ppm having control with tap water. First formulation of triacontanol was made by dissolving in 20 ml acetone then dissolved in water and stirred well. With each concentration in beaker 10 seeds were soaked in 20 ml solution. This was kept for 2 hours. After soaking seeds these were placed in Petri plates.
Material and methods

for germination in dark room. After 2 days percentage of germination was recorded. At the end of germination the roots were cut off early in the morning and then fixed in an ethanol: acetic acid (3:1) solution for 24 hours at room temperature. The fixed roots were soften with 1N HCl at $60^\circ$C for 4 min, acid was removed by rinsing the tips with water and tips were stained with 2 % acetocarmine stain. The slides were prepared by putting a drop of 45% acetic acid on the root tip by and squashed the root tip, by placing the cover slip over the material and tapped with a pencil to disperse the cells uniformly. The slide was warmed and observed the under light microscope at 100 x and 60 x magnification. The mitotic index (MI) was calculated with the formula.

$$MI = \frac{\text{The cell under division}}{\text{Total no of cells observed}} \times 100$$

**Growth of green gram in vitro.**

Healthy seeds were potted in the glasshouse of the Botanic Garden. After 37 days primary leaves were collected for *in vitro* studies. In the studies different concentrations of HgCl$_2$ were tried for sterilizing the explants. 0.5% was found better for sterilization. The leaves were surface sterilized and cut which were chosen as explants.

The explants were first washed thoroughly in running tap water for 15 minutes and then with distilled water for 30 min for surface sterilization. Chemical such as HgCl$_2$ (0.5%) was used. The explants were surface sterilized using 0.5% HgCl$_2$ for 10 minutes followed by three rinses with sterilized double distilled water in a laminar flow hood.
Leaf was cut into small pieces of equal size and then treated so that maximum part was exposed to medium.

The explants were inoculated into Murashinge and Skoog (MS) culture medium supplemented with 16 gram sucrose per liter with vitamins. The plant growth regulator, triacontanol combined with 2-4D and 2-4D alone were used for the treatment. The medium was solidified with 3 grams/L agar. The pH of the medium was maintained at 5.8 throughout the experiment. For adjustment of pH 0.1N KOH and 1N HCl was added prior to boiling and adding the agar. Culture tubes and conical flasks containing medium were plugged with non absorbant cotton and autoclaved at 120 °C for 15 min. Each treatment was with 5 replicates and experiment was repeated two times till the constant result were obtained. The response was recorded by measuring the weight of the callus and microscopic observations of the cells and the best responses observed were photographed.

**Callus induction**

For callus induction initial explants were consisted of leaves halves (20 mm)² with abaxial side in contact with the medium. The explants for culture on MS basal medium was supplemented with 3% (w/v) sucrose, 2-4D (5 mg/L) and 2-4D + triacontanol (5 ppm) were found to give profuse callusing and when callusing was observed in entire explants, the callus was cut into small pieces and transferred to MS medium supplemented with 2-4D and 2-4D + triacontanol in same concentration for callus induction. Sub culturing was done after 2 weeks. Data was recorded based on callus characteristic and microscopic observation of the callus cells. Microscopic observation was done by using simple method in which callus was squashed and stained.
with acetocarmin stain. The slides were warmed and observed under light microscope at
40 X and 60 X magnifications. By using occular micrometer, length and breadth of the
cells was measured and average was recorded. Motic microscope plus image processing
calibration software was used for the measurement of length and breadth of the cells.