MATERIALS AND METHODS
PLANT MATERIAL

The present investigation was carried out on two varieties (cultivars) of niger (*Guizotia abyssinica* (L.F) Cass). Good quality niger seeds of the cultivar IGP-76 variety grown in Southern Gujarat (Dharampur) and the Ootacamund-5 (Ooty-5) seeds grown in Orissa were procured from Department of Agronomy, Orissa University of Agriculture and Technology, Bhubaneswar, India and used for the investigations.

CHEMICALS

The growth regulators were procured from the following sources:

- Gibberellic Acid (GA₃) SIGMA USA
- Ascorbic Acid (AA) SARABHAI, INDIA
- Indole-acetic acid (IAA) C.H.Boehringer Sohn, West Germany
- Kinetin (KIN) SIGMA, USA

All other chemicals used in the study were of Analytical grade and procured from BDH India.
SEEDS TREATMENT AND SOWING

The 1000 ppm stock solutions of Colchicine, Gibberellic acid (GA3) and Ascorbic acid (AA) were prepared in distilled water, Indole-acetic acid (IAA), in 0.1N NaOH and Kinetin (KIN) in 0.1N HCL.

The stock solutions of GA3, AA, IAA and KIN were further diluted in double glass distilled water to prepare 25, 50, and 100 ppm concentrations. In case of colchicine 5, 10 and 25 ppm concentrations were used.

Two grams of niger seeds were weighed for each treatment and soaked in different concentrations of all the four growth regulators (GA3, AA, IAA and KIN) and growth retardant (colchicine) in petri-plates, kept for 8 hours and then dried on Whatman filter paper in another set of petri-plates (Inamder J.A. & Gangadhara, M. 1975). The soaking and drying treatment was repeated for three days continuously in the same manner. One set of seeds was soaked in distilled water and dried for three days in a similar way to serve as control. On the fourth day, the partly germinated seeds treated with growth regulators were sown in 10x10 sq feet plots prepared in the Sardar Patel University Botanical Garden and the seeds treated with growth retardants were allowed to grow in petri plates for further studies. The plants
were raised using normal agronomic practices. IGP-76 var. was grown in winter as well as in monsoon seasons whereas Ooty-5 was grown in monsoon season alone.

IGP-76 winter crop
- Date of sowing: Jan 6, 1989
- Date of harvesting: March 29, 1989

IGP-76 winter crop
- Date of sowing: Jan 11, 1990
- Date of harvesting: April 2, 1990

IGP-76 monsoon crop
- Date of sowing: June 26, 1989
- Date of harvesting: Oct 12, 1989

Ooty-5 monsoon crop
- Date of sowing: June 26, 1989
- Date of harvesting: Oct 12, 1989

After harvesting, seeds were collected, winnowed and used for various biochemical analysis.

MORPHOLOGICAL STUDIES

Growth measurements

Ten plants from each treatment (plot) were tagged at random and the total height of the plant, total number of leaves
per plant, number of branches (primary, secondary and tertiary) per plant and no. of capitula per plant was recorded at an interval of 7 or 15 days. The dates of flowering and fruit formation were also recorded.

**Leaf area**

Five fresh leaves were picked up at random from the different plots and the outline was drawn on the centimeter scale graph paper in which each centimeter unit was further divided into 10 unit of mm each. The number of centimeter squares covered by each leaf were counted. The part squares were added to give equivalent full cm squares and the sum of fully covered squares and equivalent of part squares was taken as the total area of the leaf in centimeter squares.

**PHYSIOLOGICAL STUDIES**

**Chlorophyll Estimation**

Fresh leaves from the field were picked up fortnightly at random from each plot and after determining their areas, chlorophyll was estimated by the method of MacLachlan and Zalik, (1963). Leaves were weighed (approximate 1 g) and 10 % homogenate was prepared in 80 % acetone using pestle and mortar.
The Optical density was measured at two different wavelengths of 645 and 663 nm using UV spectrophotometer (Shimadzu double beam spectrophotometer UV-150-02). The experiments were conducted in duplicates and mean value was used for chlorophyll estimation using the following formulae.

Chlorophyll a in mg / gm of leaf

\[ \text{Chlorophyll a in mg / gm of leaf} = [12.7 \times (\text{Q.D at 663}) - (2.69/(\text{Q.D at 645}))] \times \frac{V}{1000 \times W} \]

Chlorophyll b in mg / gm of leaf

\[ \text{Chlorophyll b in mg / gm of leaf} = [(22.9 \times \text{Q.D at 645}) - (8.02 \times \text{Q.D at 663})] \times \frac{V}{1000 \times W} \]

Total Chlorophyll in mg/gm of leaf

\[ \text{Total Chlorophyll in mg/gm of leaf} = [(20.2 \times \text{Q.D at 645}) + (8.02 \times \text{Q.D at 663})] \times \frac{V}{1000 \times W} \]

where

- \( W \) = weight of leaf taken in grams
- \( V \) = volume of acetone used in ml.
- \( \text{Q.D} \) = optical density at 645 or 663 nm.

BIOCHEMICAL STUDIES

Oil Extraction by Soxhlet Extraction

Two gram of uniformly ground seeds was taken into a thimble and refluxed with petroleum ether (60°C) for 8 hours on a water bath in a soxhlet extractor. The solvent was removed from
the miscella, dried under vacuum in an oven at 105°C for 3 hours and the residue was weighed (AOCS official method).

Nuclear Magnetic Resonance Method for determining the Oil Content

The oil content of the niger seeds was determined by the NMR using New Port Analyser Model 4000 by a multi point calibration using a linear regression. The equipment was calibrated using authentic niger seeds as well as niger seed oil. The oil content of seed samples was estimated using seeds directly in the sample tube in between the magnetic field of 90 radio frequency. The percentage oil in the sample is arrived automatically comparing the echo signal of pure oil with that of the sample to be measured. The average of the two determinations by the equipment was reported for the oil content in the niger seeds (International standard ISO/DIS 10565.2 oil seeds 1992).

Extraction of oil by press

Clean niger seeds (15 kg) containing 5 to 7% moisture and about 40% oil were crushed in a laboratory expeller using an imported Komet Single Spindle Press C-A 59 with a drive electromotor 220/380, 50 Hz and 0.37 KW at 45 rpm. The clean seeds were fed to the above expeller at a constant rate of 0.5 kg per 10 minutes at cold conditions. The recovery of the oil was as follows:
Weight of the seed fed to the expeller = 15 Kg
Oil content in the seeds = 40 %
Oil obtained = 5 Kg
Weight of the oil cake = 10 Kg
Oil content in the cake = 7 %
Recovery of oil from the seeds = 83.3 %

Total Oil Content

The total oil content was determined by Soxhlet Extraction Method and confirmed by using NMR method.

Moisture Content

The moisture content was estimated using I.S.I. procedure by heating a known amount of oil on an electric hot plate till there was no condensation of water droplets seen on the covering watch glass and calculated as follows:

Per cent moisture content in oil = 100 w / W

where w = loss in weight in g of material upon drying
W = weight of oil in g taken for the test.
Fatty acid profile by G.L.C.

Fatty acid composition of the samples was determined as methyl esters (Christopherson and Glass 1969) with a Howlette Packard (4ozs) G.L.C. fitted with flame ionization detector. A column (2mmx3mmI.D.) packed with 15% diethylene glycol succinate at 80/100 diameter to post 'S' was used. Column temperature was maintained at 200°C. Nitrogen was used as a carrier gas (30ml/min) and Hydrogen served as fuel gas. Relative percentage of fatty acids was calculated.

Colour of the oil

The colour of the oil was determined by using Lovibond Tintometer (A.O.C.S procedure) in which colour is determined by comparison with Lovibond glasses of known colour characteristics. The colour is expressed as sum total of yellow and red units (slides) used to match the colour of the oil in the cell of specified size in tintometer. The colour is reported in terms of Lovibond units.

Colour reading = Y + 5R
Refractive Index

The refractive Index of oil was determined at 40 \( \pm 0.1 \) C by Abbe's Refractometer. The temperature of the refractometer was controlled within \( \pm 0.1\)°C. The instrument was standarized using distilled water as a standard (1.3330 at 20°C). Oil was filtered to remove impurities and last traces of moisture and few drops of oil were put on lower prism. After adjusting the light and the instrument, the most distinct reading was taken and refractive Index was found from the standard tables.

Saponification value

Standard AOCS method was used, about 1.5 to 2.0 g of oil was accurately weighed in a 250 ml conical flask and 25 ml of alcoholic KOH (3.5 to 4.0 %) solution added to it. The flask was connected to the reflex air condenser and heated on a water bath for one hour till the sample was completely saponified as indicated by absence of any oily matter. The contents were titrated with 0.5 N HCL. Distilled water was used as blank and saponification value calculated as follows:

\[
\text{Saponification value} = \frac{56.1 \times (TB - TS)}{W}
\]
where \( W \) = weight of the oil in gram
\[ TB = \text{ml of 0.5N HCL used for blank} \]
\[ TS = \text{ml of 0.5N HCL used for titration of the sample} \]
\[ N = \text{normality of standard Hydrochloride solution} \]

Unsaponifiable Matter

About 5g of the oil was boiled with 50 ml. of alcoholic potassium hydroxide under a reflux condensor for about an hour. The contents were transferred to a separating funnel. The flask was washed 2-3 times with ethyl alcohol and mixed. The lower soap solution layer was separated and extraction was repeated 4-5 times using 50 ml. petroleum ether every time.

After washing the ether layer was transferred to a flask and evaporated to dryness on a water bath and removed the last traces of ether by keeping the flask in an air oven at 80 C for one hour. A few ml. of acetone was then added to the residue. The residue was put in 50 ml. of warm neutral ethyl alcohol containing a few drops of phenolphthalein and titrated with standard sodium hydroxide solution. Calculated the unsaponifiable matter according to following formula.

\[
\text{Weight in g of fatty acids} = B = 0.282 \times V \times N
\]
in the extract as oleic acid
where \( V \) = Volume in ml of standard NaOH  
\( N \) = Normality of standard NaOH

Unsaponifiable matter Per cent by Weight = \( 100 \left( \frac{A - B}{W} \right) \)

where \( A \) = weight in g of residue  
\( B \) = weight in g of fatty acids in the extract  
\( W \) = weight in g of material taken for test.

(AOCS procedure)

Free fatty acids

AOCS procedure was employed, about 4-5 gm of oil were weighed in a 200 ml conical flask. Thirty ml of freshly neutralised hot ethyl alcohol was added to the oil in the conical flask. The mixture was boiled (70°C) for about five minutes and titrated while as hot as possible with standard aqueous alkali solution (0.1N NaOH) using phenolphthalein as indicator.

Percentage of FFA as oleic acid = \( \frac{V \times N \times 28.2}{W} \)

where \( V \) = volume of NaOH used in ml  
\( N \) = normality of NaOH used  
\( W \) = weight of oil in gm
Iodine Value (WIJS)

AOCS procedure was employed, about 0.2 g of oil was weighed in a 500 ml Iodine flask and mixed with 25 ml of carbon tetrachloride. 25 ml of the WIJS solution was added and glass stopper was replaced on the flask after wetting with potassium iodide solution. The mixture was thoroughly mixed by swirling the contents. The contents were kept in dark for 30 min. Blank was also run simultaneously. After 30 minutes, 15 ml of Potassium iodide solution and 100 ml of water were added, rinsing the stopper. The liberated Iodine in the contents was titrated with standard 0.1N sodium thiosulphate solution shaking the flask thoroughly until the colour of the solution was straw yellow. Then 1 ml of starch solution was added and titration continued until the blue colour formed disappeared after thorough shaking with stopper on and calculated iodine value as follows:

\[
\text{Iodine Value} = 12.69 \times \frac{(B-S) \times N}{W}
\]

where,

- \( B \) = Volume of standard Sodium thiosulphate required for blank in ml
- \( S \) = Volume of standard Sodium thiosulphate required for sample in ml
- \( N \) = Normality of standard sodium thiosulphate solution
- \( W \) = Weight of the material in gm

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**Total Protein**

The estimation of total protein content was done according to the method of Lowry et al (1951). 10% homogenate was made with 0.25 M sucrose. Protein was precipitated using equal volume of 10% TCA and centrifuged at 3000 rpm for 10 minutes. The precipitate was washed twice with 10% TCA and dissolved in 0.1 N NaOH. 1 ml aliquotes of this solution were mixed with 5 ml of freshly prepared copper reagent prepared by mixing 98 ml of the reagent A (2% Sodium carbonate in 0.1 N NaOH) and 2 ml of reagent "B" (0.5% CuSO4 in 1% Sodium potassium tartarate). 0.5 ml of 1 N alkaline Folin ciocalteau reagent (CDH Analytical grade) was added rapidly with simultaneous mixing. After 30 minutes incubation at room temperature, the optical density was read at 660 nm in a Shimadzu double beam Spectrophotometer UV-150-02. A reference standard graph was similarly prepared using bovine serum albumin (Sigma grade).

**HISTOLOGICAL STUDIES**

The seeds of *Guizotia abyssinica* IGP-76 variety were cultured following the method described by Inamdar and Gangadhara (1975) using 5, 10 and 25 ppm colchicine as substrates. The epidermal peels were taken from young and mature cotyledons. The
peels were stained in Delafield's haematoxylin and mounted in glycerine. The observations and photomicrographs were taken on Carl Zeiss photomicroscope-I with plane apochromatic objective using blue filter and ORWA-NP-55 film.

NUTRITIONAL ASPECTS

Healthy male albino rats weighing between 110-140 g were selected from the animal house maintained by Biochemistry department of the University and used for nutrition experiments. Two rats were put in each metabolic cage. The rats were divided into three groups and were fed with standard synthetic diet (Pollack et al., 1965) as follows: Group Diet - Type of protein and oil

A Casein and Groundnut oil
B Niger Protein and Groundnut oil
C Casein and niger oil

The standard synthetic diet consists of:

Sucrose = 22%
Starch = 50%
Protein/ niger protein = 20%
Salt mix = 4%
Groundnut/ niger oil = 3%
Vitamin mix = 1%

The salt mixture consists of: The Vitamin mixture consists of the following per 100 gm of diet:

CaCO3 = 55.35%  Vit. A = 200 I. U.
K3PO4 = 31.75%  Vit. K methodine = 0.01 mg
KCL = 11.400%  Vit. E a tocopherol = 6.00 mg
NaCL = 7.05%  Thiamine = 0.13 mg
MgCO3 = 2.550%  Riboflavin = 0.25 mg
MgSO4.7H2O = 1.650%  Pyridoxine = 0.12 mg
NaF = 0.100%  Niacine = 1.50 mg
CuSO4 = 0.090%  Choline Chloride = 75.0 mg
MnSO4 = 0.035%  Cobalamin = 0.50 ug
AlKSO4 = 0.017%
KI = 0.006%

The albino rats were fed with the above diet and tap water ad libitum for 75 days. The weighed amount of diet was given to the rats every day and the left overs were weighed on next day. The body weight of the animals was taken on alternate days.
The faecal matter and urine was collected weekly and used for nitrogen estimation by Kjeldahl method.

At an interval of every fifteen days, the blood from the eyes of the treated and control rats was collected. Serum was separated by centrifuging the blood at 2000 rpm using REMI RBC centrifuge for 10 minutes and stored at -80°C till it was used for further study.

At the end of 75 days period, the animals were sacrificed, livers were taken out, washed thoroughly in distilled water, dried on a whatman filter paper and stored in an aluminium foil at -80°C to be used later for the study.

**Nitrogen estimation**

Nitrogen estimation was performed using I.S.I. procedure. 25 ml of concentrated sulphuric acid was added to .5 g of material in a the flask alongwith 0.2g of CuSO4. The flask was heated below the boiling point of the acid until frothing ceased. About 10.0 g of potassium sulphate or anhydrous sodium sulphate was added and heating was increased till the acid started boiling vigorously and the contents were digested for 30 minutes. Even after the mixture became clear (pale green or colourless) the digestion was continued for another 60-90 minutes. The contents
were cooled and diluted to 250 ml with distilled water. About 50 ml. of 1N NaOH solution was added along the sides of the flask, Contents were mixed thoroughly and distilled till all the ammonia had passed over into standard H2SO4. The contents were titrated with standard NaOH solution using methyl red indicator. A blank was also carried out using all reagents and 0.5 g of sucrose in place of experimental material.

Per cent Nitrogen = \( \frac{8.93 \times (B - A) \times N}{W} \)

where,

\( B \) = Volume in ml. of standard NaOH used to neutralise the acid in blank

\( A \) = Volume in ml. of standard NaOH used to neutralise the acid in test with material

\( N \) = Normality of standard NaOH

\( W \) = Weight in grams of material taken

Protein efficiency ratio (PER)

The protein efficiency ratio of the niger cake protein was estimated by the method of Osborne and Mendal (1917). The experimental albino rats were fed with the known amount of synthetic diet every day in which 20% casein was replaced with niger cakes and the left overs were weighed next day. The control rats were fed with synthetic diet in the same way. The body
weights of the rats were also taken and protein efficiency ratio of the niger protein was calculated as such.

\[
\text{PER} = \frac{\text{Weight gain (gm)}}{\text{Protein intake (gm)}}
\]

**Biological value (apparent)**

The term is defined as that proportion of the digested (and absorbed) protein that is not excreted in the urine i.e. percent of the absorbed nitrogen retained by the body for maintenance and/or growth. The faecal and urinary nitrogen was calculated separately by Kjeldahl method. The total nitrogen intake was also calculated from the food intake per day and B.V. was calculated by method of Mitchell (1925) as follows:

\[
\text{BV} = \frac{[\text{N intake} - (\text{Faecal N} + \text{Urinary N})] \times 100}{[\text{N intake} - \text{Faecal N}]}
\]

**Cholestrol estimation**

Total cholestrol in the serum was estimated using enzymatic method, 0.05 ml. of serum was added to 1.5 ml. of working enzyme reagent (Span Diagnostics) and after mixing it well incubated for 15 minutes at 37°C. The reaction was stopped by adding 1.5 ml. of distilled water, mixed well and optical density was read at 510 nm against distilled water with the help of
Shimadzu double beam Spectro-photometer immediately. A blank was run simultaneously by taking 1.5 ml of working enzyme reagent and 1.5 ml of distilled water. The total cholesterol in the serum was calculated as follows:

\[
\text{Total Cholesterol as mg / 100 ml.} = \frac{[\text{O.D.}(\text{Experimental})-\text{O.D.}(\text{Blank})] \times 200}{\text{O.D.}(\text{Standard})-\text{O.D.}(\text{Blank})}
\]

Preparation of liver homogenate

2% (w/v) homogenate of liver tissue was prepared with 0.25 M sucrose solution and used for the enzymatic study.

Serum Glutamate Oxaloacetate Transaminase

(L-aspartate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.1)

The enzyme catalyses the reversible transamination of aspartate and α-ketoglutarate to oxaloacetate and glutamate respectively generating intermediates of Kreb's cycle. The reaction is as follows:

\[
\begin{align*}
\text{L-Aspartate} + \text{α-Keto-glutarate} &\rightarrow \text{Oxaloacetate} + \text{L-Glutamate} \\
\text{COO}^- &\quad \text{COO}^- \quad \text{Glutamate Oxaloacetate} \quad \text{Transaminase} \quad \text{COO}^- &\quad \text{COO}^- \\
\text{NH}_2\text{-CH} &\quad \text{C}=\text{O} \quad \text{Transaminase} \quad \text{C}=\text{O} &\quad \text{NH}_2\text{-CH} \\
\text{CH}_2 &\quad + \quad \text{CH}_2 &\quad \text{CH}_2 &\quad + \quad \text{CH}_2 \\
\text{COO}^- &\quad \text{COO}^- &\quad \text{COO}^- &\quad \text{COO}^-
\end{align*}
\]
The enzyme assay was done according to the method of Wootton (1964). The substrate used was 0.2M L-aspartic acid and 0.002M α-Ketoglutarate prepared together in 0.05 M phosphate buffer, pH 7.4. In the experimental and control tubes 1 ml. substrate was added and the reaction was initiated by the addition of 0.1 ml. enzyme preparation in the experimental tubes only. All the tubes were incubated at 37°C for 60 minutes. The reaction was terminated by the addition of 0.05 ml. of aniline citrate prepared by dissolving 5 g of citric acid in 5 ml. of distilled water and adding equal amount of redistilled aniline. The enzyme was added in the control tubes after the addition of aniline citrate. The tubes were allowed to stand for 20 minutes at room temperature after thoroughly shaking the contents. After 20 minutes, 1 ml. of 0.001 M 2,4-dinitrophenyl hydrazine (DNPH) was added in all the tubes and after keeping the tubes for 20 minutes at room temperature, 10 ml. of 0.4 N NaOH was added to each tube and mixed. The reddish brown colour developed after 10 minutes was read at 520 nm on Shimadzu double beam spectro photometer UV-150-02 and pyruvate formed was calculated by comparing with standard Sodium pyruvate processed simultaneously.
Serum Glutamate Pyruvate Transaminase

(L - Alanine: 2 - Oxoglutarate aminotransferase, E.C. 2.6.1.2)

This enzyme causes reversible transamination of alanine and a-Ketoglutarate to pyruvate and glutamate generating intermediates of Kreb's cycle.

\[
\begin{align*}
\text{COO}^- & \quad \text{COO}^- & \quad \text{COO}^- & \quad \text{COO}^- \\
\text{CH}_2 & \quad C = 0 & \quad C = 0 & \quad \text{NH}_2-\text{CH} \\
\text{NH}_2-\text{CH}_2 + & \quad \text{Glutamate pyruvate} & \quad \text{Transaminase} & \quad \text{CH}_3 + \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 & \quad \text{CH}_2 \\
\text{COO}^- & \quad \text{COO}^- \\
\end{align*}
\]

L - analine  a-Ketoglutarate  Pyruvate  L - Glutamate

The procedure adopted for glutamate pyruvate transaminase was essentially similar to that described for glutamate oxaloacetate transaminase except that the substrate used was 0.2 M dL-analine instead of aspartic acid and the enzyme assay was conducted for 30 minutes instead of 60 minutes.

Acid Phosphatase

Friske and Subbarow's (1925) method was adopted for the assay of these two enzymes in the serum and liver homogenate. The
substrate used was freshly prepared p-Nitrophenyl phosphate (8 u/ml. / 1 D/W). 1.2 ml. acetate buffer (pH 4.5) was taken in all the experimental and control tubes and the reaction in the experimental tubes was initiated by the addition of 0.1 ml. of the enzyme. 0.5 ml. of p-nitrophenyl phosphate (PNP) was then added in all the experimental and control tubes and the contents were mixed thoroughly. All the tubes were incubated for 10 minutes at 37°C. The reaction was terminated after 10 minutes by the addition of 2.0 ml. of alkaline Tris-HCL buffer (pH 9.0). The enzyme was now added to the control tubes and the colour developed was read at 405 nm with Shimadzu double beam spectrophotometer UV-150-02. The enzyme activity was calculated with reference to a standard curve of p-nitrophenol (50 u/ml. / 1 in alkaline tris - buffer).

Alkaline phosphatase

The enzyme activity was measured in serum and liver by the method of Bowers et al. (1967). 0.8 ml. of carbonate-bicarbonate buffer was added to 2.0 ml of the p-nitrophenyl phosphate substrate (5 u/ml / 1 in alkaline carbonate-bicarbonate buffer) and mixed thoroughly. At zero time, added 0.2 ml. of serum/ homogenate and followed the change in the extinction at 405 nm at an interval of 30 seconds for 3 minutes. In the blank tubes, the enzyme was replaced by 0.2 ml. of buffer. The curve was
prepared along with by preparing a range of \( p - \) nitro phenyl concentrations and plotting extinction at 405 nm against concentrations.

The amount of \( p \)-nitrophenol released was calculated by subtracting the extinction of the blank from the experimental and calculating it from the standard curve. The activity of alkaline phosphatase was expressed in terms of units per millilitre of serum or / mg protein in liver homogenate.