Material and methods

MATERIAL AND METHODS

The experimental material used in the present investigation is ipecac (Cephaelis ipecacuanha, the wonder drug plant known in tropical countries as 'the plant producing green gold'. Against depleting wild sources in the Central American forests, organised cultivation of ipecac ensures production under controlled conditions and maintenance of better-yielding plants for the production of raw-materials of the emetine-industry.

Commercial cultivation of ipecac is still less in vogue. In India too, ipecac cultivation is a recent industry, rather concentrated at the foot-hills of Darjeeling; plantations being maintained at different localities of Rongo, Mungpoo, Munsong and Latpanchor hills where the climatic and physiographic factors are favourable for the growth of this plant species. The elevations of these plantations range from 500 to 1000m and the plant favours valley regions between hills with warm and humid climate and sufficient precipitation. The optimum temperature ranges from 15° to 35°C. Though direct rainfall is injurious to the growing plants, the optimum annual precipitation should be in the range of 150-300 cm a year. Soil for ipecac cultivation should be well-drained, loamy to sandy; pH remaining neutral to slightly acidic and slopes should preferably be south-east facing.

Under normal harvest pattern, the economic life cycle of the plant passes through seven well-defined phases viz., vegetative (V), reproductive I (R I), post-reproductive I (PR I). reproductive II (R II), post-reproductive II (PR II), reproductive III (R III) and post-reproductive III (PR III) mainly distinguishable on the basis of the extent of development of flowers and fruits as has been shown in the following table :

Dev. phase	Age (days)	Temp. range (°C)	Flower/ Fruit	Remarks
V	Upto 240	Min : 10-15 Max : 15-19	Nil	Plants with active growth
R I	270 - 420	Min : 13-22	30 : 1	Plants start flowering apically; flowering sparse.
PR I	450 - 600	Min : 10-23 Max : 15-29	16 :1	Fruiting sparse, mostly sterile with nonviable seeds or fruits do not mature.
R II	630 - 780	Min : 13-22 Max : 20-28	2.8 : 1	Plants flower for the second time; flowering profuse.
PR II	810 - 960	Min : 10-23 Max : 15-29	1 : 6	Fruiting occurring second time and higher in number; produce viable seeds.
R III	990 - 1140	Min : 13-22 Max : 20-28	2 : 1	Plants produce flowers for the third time; flowers comparatively lesser than in R II.
PR III	1170 - 1260	Min : 10-23 Max : 15-29	1 : 9	Profuse fruiting; fruits with viable seeds.

The major categories of the study area and salient details about the experimental materials and parameters of observation are given in the following table :

Category of study		Experimental material	Observation made on
I.	General growth and developmental physiology	5-month old seedlings grown at 900m altitude, studied till the plants became 42 -month old.	(a) Extension growth, laminar formation, flowering, dry matter accumulation. (b) Changes in chlorophyll, RNA, SN and TN contents in leaf and TA content in root.
II.	Pharmacognosy	12, 24 and 36-month old plants grown at two altitudes viz., 500 and 900m.	Root : (a) Morphology and annulation patterns. (b) Histology Stem : (a) Morphology. (b) Histology Leaf : (a) Morphology. (b) Stomata, vein, trichomes. (c) Histology
III.	Growth regulator effects	20-month old plants from 900m altitude.	(a) Extension growth, laminar formation, dry matter accumulation. (b) Changes in chlorophyll, RNA, SN and TN contents in leaf and TA content in root.
IV.	High temperature stress effects	20-month old plants from 900m altitude : normal and growth regulator treated plants.	(a) Extension growth, laminar formation, dry matter accumulation. (b) Changes in chlorophyll, RNA, SN and TN contents in leaf and TA content in root.

Contd...

Category of study	Experimental material	Observation made on
V. Low temperature stress effects	20-month old plants from 900m altitude : Normal and growth regulator treated plants.	(a) Extension growth, laminar formation, dry matter accumulation. (b) Changes in chlorophyll, RNA, SN and TN contents in leaf and TA content in root.
VI. P^{32} - uptake studies (a) High temperature (50°C)	10-month old seedlings from 900m altitude : Normal and growth regulator treated plants.	Uptake and incorporation of P^{32} in different parts of the seedlings.
(b) Low temperature (5°C)	10-month old seedlings from 900m altitude : Normal and growth regulator treated plants.	Uptake and incorporation of P^{32} in different parts of the seedlings.

I. Studies on general growth and development

Viable seeds were collected, soaked in water for 72 hours and sown on raised beds during middle of February. The germination started from middle of May and different growth and developmental data were collected at pre-determined intervals commencing with 180 days after germination. Number of plants included in the study was sufficiently high to undertake a thorough statistical analysis.

The growth data included the following parameters :

a) Extension growth : The growth characteristics were noted as per the procedure described by Gupta(1970). The linear growth of the plants was measured from the soil surface to the tip of the stem taking care to avoid parallax error. The data were collected at intervals of 30 days and mean length calculated.

b) Leaf number : The leaves were counted at intervals of 30 days separately for each plant and means calculated.

c) Leaf dry matter accumulation : The fresh weight of representative samples were taken and oven-dried at 80°C until the dry weight became constant. Mean dry weight (DW) was determined and recorded at 30 days interval.

d) Root biomass production : Representative plants were harvested and roots were carefully separated, cleaned and fresh weight determined. The roots were oven-dried at 80°C till constant weights were obtained. Dry weight were noted separately for each plant and mean calculated. The data were recorded at intervals of 30 days as in previous cases.

e) Flowering behaviour : The initiation of flowering was examined in each plant under study in different experimental plots and the flowering phenomenon was separately recorded at intervals of 30 days. The flowering behaviour was expressed on percentage basis, calculated as under :

$$\text{Flowering percentage} = \frac{\text{No. of plants with flower}}{\text{Total no. of plants}} \times 100$$

Flower number was also recorded at an indentical interval .

f) Fruiting behaviour : The study of fruiting behaviour of ipecac was undertaken in the pattern similar to that of the flowering at an interval of 30 days.

The biochemical data included total chlorophyll(Chl) content, ribonucleic acid (RNA) content, soluble and total nitrogen(SN & TN) contents of leaf and total alkaloid (TA) content of root. These data were recorded at intervals of 30 days as per the methods described below :

a) Determination of chlorophyll content : Two fresh leaf samples of 100 mg each were taken. One sample was oven-dried at 80°C for 72 hours and dry weight was determined. The second sample was used for chlorophyll estimation. It was extracted with 80% acetone and the supernatant after centrifugation was decanted. The residue was again extracted with solvent and centrifuged. The decants were mixed and the total volume of the extract was made upto 10 ml with acetone. The optical density of the acetone extract was measured at 660 nm in a photoelectric colorimeter (Erma AE 11 N). The amount of chlorophyll was determined as mg per 10 ml following Arnon's formula (Arnon, 1949) :

$$\text{Chlolophyll 'a' \& 'b'} = \frac{\text{Optical density at 660 nm}}{34.5} \times 10 ;$$

and calculated as microgram chlorophyll per mg dry tissue (µg/mg).

b) Determination of RNA content : Extraction of nucleic acids from fresh plant tissue was undertaken following Cherry(1962) and quantitative estimation of RNA was done following the method described by Markham(1955) with some modifications.

100 mg of fresh leaf tissue was homogenised in cold methanol and the extract was centrifuged for 10 minutes. The supernatant was discarded and the residue was extracted with 8 ml of cold 0.2 M perchloric acid, centrifuged for 10 minutes. The residue was extracted with 5 ml ethanol and again centrifuged. The residue was extracted in 10 ml ethanol : ether (2 : 1) at 50°C for 30 minutes and centrifuged. The residue was finally extracted with 10 ml of 5% perchloric acid at 70°C for 30 minutes and kept overnight in cold condition and centrifuged.

From the supernatant 0.3 ml was diluted to 3 ml with distilled water and to it 3 ml of orcinol reagent was added. Orcinol reagent was prepared by dissolving 1 g of orcinol (3,5-dihydroxytoluene) in 100 ml of concentrated hydrochloric acid containing 0.1% ferric chloride and heating the mixture in boiling water-bath for 30 minutes. Marble spheres were put on the mouth of each tube containing the reaction mixture to prevent escape of the mixture while boiling. Tubes were later cooled at room temperature and the intensity of green colour developed due to ribose sugar was measured colorimetrically. The readings were compared with the calibration curve of known concentration of yeast RNA and expressed as µg per mg dry tissue.

c) Determination of SN content : Nitrogen content was estimated colorimetrically using Nessler's reagent according to the method described by Vogel (1961) with necessary modifications.

100 mg fresh leaf tissue was extracted in distilled water. The volume of the extract was made upto 9 ml; centrifuged, and 1 ml of trichloroacetic acid was added to the supernatant to precipitate colloids; kept overnight in cool condition.

The extract was centrifuged and the supernatant decanted. The total decant was digested with 1 ml concentrated sulphuric acid for 2 hours. When a homogeneous dark cockroach colour was attained, it was cooled and hydrogen peroxide was added drop-wise. The aliquot was again reheated till it became colourless. The clear solution was diluted with distilled water to a specific volume depending on the colour reaction with Nessler's reagent. To 1 ml of the digested solution, 1 ml mixture (1 : 1) of 10% sodium hydroxide and 10% sodium silicate was added. 5 ml of freshly prepared alkaline Nessler's reagent was added to the mixture and allowed to react for 10 minutes. Yellow to orange colour was developed, the intensity of which was measured colorimetrically at 420 nm wavelength against a blank prepared identically with 1 ml of distilled water instead of digested sample solution. Quantitative estimation was done from the standard curve prepared from known concentration of potassium sulphate and ammonium sulphate solutions and expressed as µg per mg dry tissue.

d) Determination of TN content : 10 mg of oven-dried leaf tissue was digested with 1ml of concentrated sulphuric acid till a cockroach coloured homogeneous solution was formed. The solution was cooled; hydrogen peroxide was added dropwise and again heated till a clear solution was obtained. Rest of the procedure followed was as described earlier in the case of SN estimation.

Preparation of Nessler's reagent : 150 g of potassium iodide and 110 g of iodine were put in a litre flask. 140 g of metallic mercury was added and shaken vigorously until the colour of iodine nearly disappeared and a greenish colour developed. This was filtered and the volume of the filtrate was made upto 2 l with distilled water. 75 ml of the above solution was mixed with 350 ml of 10% sodium hydroxide solution and the alkaline Nessler's mixture was used in nitrogen estimation.

e) Determination of TA content : Extraction of alkaloids was done following Seth and Ray (1967). 500 mg of powdered root moistened with dilute ammonia, packed loosely in a thimble, was extracted in a soxhlet apparatus with a mixture (1 : 1) of petroleum ether (b.p. 40-60°C) and benzene for 8 hours. The extract was concentrated to a volume of 10-20 ml. The concentrate was shaken first

in a separating funnel with 20 ml of sulphuric acid (N) and then with a mixture (3 : 1) of sulphuric acid (0.1N) and alcohol (95%) till complete extraction of alkaloids as confirmed with Mayer's reagent.

The quantitative estimation of alkaloids from ipecac roots was carried out according to *British Pharmacopoeia (1980) with suitable modification. The acid extract* was shaken with 10 ml of chloroform and it was run off to a second separating funnel containing 0.1 N sulphuric acid (20 ml); shaken well; allowed to separate and the chloroform layer was discarded. The extracted solvent was repeatedly shaken with chloroform taking 5 ml each time and separating the chloroform layer to the second separating funnel. The acid layer of the second funnel was transferred to the first separating funnel and made alkaline with 10% sodium hydroxide solution. To it 20 ml of chloroform was added; shaken well; *allowed to separate and the chloroform layer was transferred. The process* of addition of chloroform, shaking the mixture and transfer of chloroform layer to the beaker was repeated several times till complete extraction was ensured. Chloroform was evaporated and the residue dissolved in 5 ml of alcohol which was again evaporated to dryness. The residue, containing the alkaloids, was later dissolved in 5 ml of 0.1 N sulphuric acid and titrated against 0.1N sodium hydroxide using methyl red as indicator. Each ml of sulphuric acid was taken as equivalent to 0.02403 g of total alkaloids.

II. Studies on pharmacognosy

Pharmacognosy of ipecac (C. ipecacuanha) in relation to morphology, histology and alkaloid content was studied with plants of different age groups viz., 1,2 and 3 years from two different altitudes viz., 500 and 900 m. The methods followed were as follows :

A. General morphology

Plants of 1,2 and 3 years were collected from 500 and 900 m altitudes and general morphology of the plant along with nature of stem, root, leaf, flowers and fruits were studied.

B. **Macroscopic studies :**

Stem : The length and breadth of stem of 1,2 and 3 - year old plants, collected from two different altitudes, were measured with the help of a centimetre scale. Measurements were taken from at least 50 plants and mean values calculated.

Leaf : Number of leaves of 1,2 and 3 - year old plants at two different altitudes were noted. The total area of leaves of each plant of different age-groups was determined by graphical method (Acharya Choudhury and Gupta, 1976). The number of leaves and total laminar area per plant were determined on randomised basis to satisfy statistical requirements.

Root : The dimensions of root were measured with the help of a centimetre scale, separately from plants of different age-groups growing at two different altitudes. Volume of roots was measured by water displacement method. For each study, sufficient number of plants were taken and mean volume was calculated. The roots were then separated into different components like main root, branches, root tip and root hairs. The number of annulations per cm of root length was counted. Roots were dried at 80°C for 72 hours and dry weight taken. Total alkaloid content in each part of the root was determined following standard method of B.P. (1980). TA contents were determined separately and mean values were calculated and expressed on percentage dry weight basis.

C. **Histology :**

To study tissue systems of stem, root and leaf 2 - year old plants were collected and uniform microscopic sections were made from stem, root and leaf. The sections were dehydrated in alcohol grades and double-stained with safranin and light green according to the schedule described by Johansen (1940); mounted in Canada balsam and observed under microscope. Dimensions of cells of different tissue systems were measured with the help of ocular micrometer and expressed in μm.

D. **Quantitative microscopy**

Studies relating to stomatal index, stomatal frequency and area, palisade ratio, vein-islet and vein-termination number were conducted in plants of different age-groups growing at two different altitudes (500 and 900 m).

a) Stomatal index : The percentage proportion of the ultimate divisions of the epidermis of a leaf which have been converted into stomata is termed the stomatal index (Trease and Evans, 1983).

For determination of stomatal index, fragments of leaves (other than the extreme margin and midrib portion) of different age-groups were cleaned by boiling in chloral hydrate solution, washed with distilled water and later mounted in 10% glycerine. The lower surface of the leaf was examined and the number of epidermal cells and stomata was counted to determine the stomatal index according to the formula,

$$I = \frac{S}{E + S} \times 100;$$

where I is the stomatal index;
S is the no. of stomata per unit area; and
E is the no. of epidermal cells per unit area.

b) Stomatal frequency : The average number of stomata per mm^2 of the epidermal surface is called stomatal frequency. Two methods were followed for its determination :

(1) Method no. 1 : Leaf peels were collected from the middle of lamina, mounted in 10% glycerine and observed microscopically. The number of stomata present per mm^2 was determined separately.

(2) Method no. 2 : Fragments of leaf from the middle of lamina were cleared with chloral hydrate solution, washed in distilled water and mounted in 10% glycerine. The number of stomata present in a mm^2 area was counted separately in each case by tracing with the help of a camera lucida.

Sufficient replications were made for each observation to satisfy statistical requirements. Stomatal frequency of 1,2 and 3 - year old plants growing at two different altitudes (500 and 900m) were determined separately.

c) Stomatal area : Fragments of leaf from the middle of lamina were cleaned with chloral hydrate solution, washed and glycerine mounts were prepared separately for plants of three different ages at two different altitudes. Area of stomata was traced with the help of camera lucida and length and width of stomata were measured and expressed as μm^2. Replications were sufficiently made to satisfy statistical requirements.

d) Palisade ratio : The average number of palisade cells beneath each upper epidermal cell is termed as palisade ratio (Trease and Evans, 1983).

About 2 mm^2 pieces of leaf were cut from the middle of lamina, boiled in chloral hydrate solution and mounted in 10% glycerine. Camera lucida drawing of a group of four or five epidermal cells were made and the palisade cells lying beneath each group were traced. The palisade cells in each group were counted and cell covering more than half of its area was included in the counts. Palisade ratio was calculated by dividing the total number of palisade cells by the total number of epidermal cells. Observations were sufficiently replicated and statistical analysis done.

e) Vein-islet number : The vein islet is the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein-islet per mm^2, calculated from contiguous square millimeters in the central part of the lamina, midway between midrib and the margin, is termed vein-islet number (Trease and Evans, 1983).

Fragments of leaf from the middle of lamina were cleared by boiling in chloral hydrate solution in a test tube placed in a boiling water bath. After clearing, a temporary mount was prepared and with the help of a stage micrometer, areas were demarcated into mm^2 units in the drawing sheet of paper. The stage micrometer was then replaced by the mounted preparation and the veins were traced in each square. The total number of vein islets

per mm^2 was determined by dividing the total number of vein-islets by the total number of square millimetre area.

f) Vein-termination number : A vein-termination is the ultimate free termination of a veinlet or branch of a veinlet (Trease and Evans, 1983). The vein termination number is the number of veinlet terminations per mm^2. Temporary glycerine mount was prepared and veins were traced in each mm^2 area and the number of vein terminations was counted. The number of vein terminations per mm^2 was later determined by dividing the total number of vein-terminations by the total number of squares.

g) Leaf trichomes : Detailed observation was made on size and pattern of trichomes, their distribution and frequency of occurrence at two different altitudes (viz., 500 and 900 m). For trichome study, epidermal tissue from the leaves of 1, 2 and 3-year old plants, collected from two different altitudes were carefully peeled off, mounted in 10% glycerine and observed under microscope. Measurements of trichomes were taken, number of trichomes were counted per field of vision and calculated as number of trichomes per mm^2. For each observation, sufficient replications were made for statistical analysis.

h) Pollen grains : Pollen grains from mature stamens were dusted on clean slide, mounted in 10% glycerine and observed microscopically. Characteristics like shape, size, surface features and other characteristics were examined carefully.

E. Distribution of alkaloid in different tissues

Uniform thin sections of stem, root and leaf were made and stained with alkaloid specific stains (Trease and Evans, 1983). The intensity of colouration was observed and noted separately for different types of tissues. Parallel tests were also made on fresh sections which were washed off of their alkaloid contents by treating in 5% tartaric acid and 95% ethyl alcohol for several days.

F. **Examination of powder drugs**

a) Preliminary tests : The root samples collected from two different altitudes were powdered and the colour, odour, taste and texture of the powder drug were examined thoroughly.

b) Microscopical examination : Powder drug was moistened with alcoholic solution of phloroglucinol in a slide and kept until nearly dry. Few drops of concentrated hydrochloric acid was added to the moistened samples, covered with coverglass and observed microscopically.

III. **Studies on effects of growth regulators**

To study the effects of different growth regulators on growth and biochemical parameters of ipecac, plots with 20-month old healthy plants with luxuriant growth were selected and sprayed with 100 mg solutions of GA_3, KN, NAA, MH, ABA and KSCN at the rate of 5 ml per plant at 24-hour interval for 7 days. After each spray schedule, the plants were covered with transparent polyethylene for 6 hours to maintain high humidity for ensuring proper foliar absorption. The plants were then allowed to grow under normal conditions following conventional cultural practices.

IV. **Studies on high-temperature stress**

Plots with 20-month old healthy and luxuriantly growing plants were selected and their shoots subjected to high temperature shock treatments according to the following schedules.

a) **High temperature stress without chemical treatments**

The apical portions (8-10 cm) of the experimental plants were immersed in hot water, the temperature of which was kept steady at 50°C (±1°C) for 10 minutes in one set of experiment and 30 minutes in another set. Approximately 2 cm^2 of stem area and 120 to 150 cm^2 of leaf area were exposed to high temperature treatment. After temperature treatment, the plants were allowed to grow under normal conditions following conventional cultural practices.

b) **High temperature stress after pre-treatment with different growth regulators**

To study the protective effects of different growth substances and inhibitors against high temperature-induced damages on growth and endogenous biochemical parameters of ipecac, the plants were sprayed with 100 mg/l of GA_3, KN, NAA, MH, ABA and KSCN at the rate of 5 ml per plant at 24 hours interval for 7 days. After each spray-schedule the plants were bagged with transparent polyethylene for 6 hours to maintain high humidity for ensuring proper foliar absorption of the sprayed chemicals. After 24 hours of the last spray, high temperature shock was given to the apical 9 - 10 cm portion of the plants by immersing in hot water maintained at 50°C (± 1°C) for two different durations viz., 10 and 30 minutes.

c) **High temperature stress followed by treatments with different growth regulators**

Apical shoot portions (9 - 10 cm) of experimental plants were immersed in hot water (50°C ± 1°C) for 10 and 30 minutes separately as described earlier. Later, the high temperature treated plants were sprayed with 100 mg/l of GA_3 NAA, KN, MH, ABA and KSCN at the rate of 5 ml per plant at 24-hour interval for 7 days. After each spray, the plants were bagged as described earlier. The plants after treatment were allowed to grow under normal conditions and conventional cultural practices.

Following growth and biochemical data were collected from different experimental sets mentioned above :

i) growth data including plant height, laminar formation and leaf dry matter accumulation, and

ii) biochemical data including chlorophyll, SN, TN and RNA contents in leaf and TA content in roots.

V. <u>Studies on low-temperature stress</u>

Plots with 20-month old healthy and luxuriantly growing plants were selected and their shoots exposed to low temperature shock treatments according to the following schedules.

a) **Low temperature stress without chemical treatments**

The apical portions (9 - 10 cm) of the plants were immersed in cold water maintained at 5°C (± 1°C) for 10 minutes in one set of experiments and 30 minutes in another set of experiments. Approximately 2 cm^2 of stem area and 120 to 150 cm^2 of leaf area were subjected to low temperature treatment as in the case of high temperature treatment. After the low temperature treatment, plants were allowed to grow under normal conditions and conventional cultural practices.

b) **Low temperature stress after pre-treatment with growth regulators**

To study the effects of different growth substances and inhibitors against low temperature-induced damages on growth and endogenous biochemical parameters of ipecac, the plants were sprayed with 100 mg/l of GA_3, KN, NAA, MH, ABA and KSCN at the rate of 5 ml per plant at 24-hour interval for 7 days. After each spray-schedule the plants were bagged with transparent polyethylene to ensure complete foliar absorption of sprayed chemicals. After 24 hours of the last spray, low temperature shock was given to the apical 9-10 cm portion of the plants by immersing in cooled water maintained at 5°C (± 1°C) for two different durations viz., 10 and 30 minutes.

c) **Low temperature stress followed by treatments with different growth substances**

In this set of experiments, apical shoot portions (9-10 cm) of experimental plants were immersed in cooled water (5°C ± 1°C) for 10 and 30 minutes separately. The plants were then sprayed with 100 mg/l GA_3, NAA, KN, MH, ABA and KSCN at the rate of 5 ml per plant at 24-hour interval for 7 days. After each spray, the plants were bagged as in earlier cases for 6 hours and then allowed to grow under normal conditions following conventional cultural practices.

From different experimental sets described above, the following growth and biochemical data were collected :

i) growth data including plant height, laminar formation and leaf dry matter accumulation; and

ii) biochemical data including chlorophyll, SN, TN and RNA contents in leaf and TA content in roots.

VI. Studies on radioactive phosphorus (P^{32}) uptake

To study the uptake behaviour of P^{32} by ipecac seedlings and its subsequent incorporation into different parts of root and shoot, 10-month old seedlings were selected, the morphological attributes of which have been included in the following table :

Age of seedling (months)	Height (cm)	Leaf (no.)	Dry weight (mg)
10	8	4	15.20

Seedlings with apical pair (A-leaves) and pair of second node leaves (II-leaves) aged between 30 - 45 days were dipped in 25 ml of 2.5 μci radio-active phosphorus solution in the form of orthophosphoric acid in phosphate buffer (prepared from 0.2 M disodium hydrogen phosphate and 0.1 M citric acid in the proportion of 13:87) for 24 hours. The experiments were undertaken at room temperature.

After 24 hours, the seedlings were removed and root portions were washed thoroughly with buffer solution followed by distilled water several times. The seedlings were then separated into its component parts viz., A-leaves, II-leaves, stem and roots, cut into small pieces and dried separately under infra-red source. The process of drying was continued till the plant materials attained constant weight. The radio-activity of the dry materials were measured with the help of Geiger - Muller Counter (Model GCS 41) . Counts were taken per 10 seconds and mean value of five readings for each sample was calculated. Each count was corrected against background count and radio-activity of the lead chamber. Data of P^{32} was calibrated with respect to the radio-activity count of the blank consisting of a fixed quantity of P^{32} solution and each value was expressed as counts per minute per mg dry tissue (cpm/mg). Radioactivity - data were collected according to the following schedules :

a) **Uptake of P^{32} by different parts of seedlings**

Uptake of P^{32} by different parts of ipecac seedlings was determined separately in both control and treated plants and increase (+) or decrease (-) over control was calculated as follows :

$$\% \text{ increase (+)/decrease (-) over control} = \frac{a - b}{a} \times 100;$$

where, a = uptake by a component of control seedling, and
b = uptake by the same component of treated seedling.

b) **Incorporation of P^{32} in different parts of seedlings**

Percentage incorporation of P^{32} in different parts of seedlings was determined by dividing P^{32} uptake of an individual part by the total uptake of P^{32} of the whole plant and later expressed as percentage.

$$\% \text{ incorporation} = \frac{b}{a} \times 100 ;$$

where a = total uptake by individual seedlings, and
b = uptake by a particular plant part.

The radioactive P^{32} uptake studies in this investigation have been conducted separately under the following three categories of experiments.

A. **Uptake and incorporation of P^{32} in seedlings treated with different growth regulators**

10-month old ipecac seedlings (8 cm long) having two pairs of leaves (average age-group of 30 - 45 days) were thoroughly washed and the root portions were dipped in 50 ml of 100 mg/l of GA_3, KN, NAA, MH, ABA and KSCN for 24 hours. The roots were later washed thoroughly with distilled water and were dipped in 25 ml of 2.5 µci radio-active phosphorus solution for 24 hours. After feeding, root portions were washed thoroughly with buffer solution followed by distilled water several times. The plants were separated into different components, dried and uptake by component parts was separately measured as described earlier. Uptake and percentage incorporation by individual part were calculated as described under (a) and (b).

B. **Uptake and incorporation of P^{32} in high temperature (50°C) treated seedlings**

i) Without chemical treatment : 10-month old seedlings (8 cm long) with 2 pairs of leaves were washed and later exposed to high temperature stress by immersing the root system in hot water maintained at 50°C (±1°C) for 10 and 30 minutes separately. After temperature treatment the plants were fed with radio-active phosphorus solution for 24 hours. The roots were later washed thoroughly with buffer solution followed by distilled water several times. Treated seedlings were then separated into different components, dried and uptake by component parts was separately measured as described earlier.

Uptake and percentage incorporation in different plant parts were determined as described in (a) and (b).

ii) Chemical treatments followed by high temperature : To examine the combined effects of pre-treatments with some growth regulators and high temperature on radio-active phosphorus uptake and incorporation, the root portions of the seedlings were immersed in 50 ml of 100 mg/l solution of GA_3, KN, NAA, MH, ABA and KSCN for 24 hours, washed, exposed to high temperature by dipping the root portion in hot water maintained at 50°C (± 1°C) for 10 and 30 minutes and thereafter the plants were fed with P^{32}.

Uptake and percentage incorporation in different parts were determined as described in (a) and (b).

C. **Uptake and incorporation of P^{32} in low temperature (5°C) treated seedlings**

i) Without chemical treatment : 10-month old healthy seedlings (8 cm long) having two pairs of apical leaves were washed and exposed to low temperature by dipping the root system in chilled water maintained at 5°C (± 1°C) for 10 and 30 minutes separately. After temperature treatment, the plants were fed with 25 ml of 2.5 µci radio-active phosphorus solution

for 24 hours. The root portions were later washed thoroughly with buffer solution and distilled water several times. The treated seedlings were then cut into different components and dried. Radio-activity of the component parts was measured as described earlier.

Uptake and percentage incorporation by individual components were determined as described in (a) and (b).

ii) With chemical treatments followed by low temperature : To study the combined effects of pre-treatments with some growth regulators and low temperature on P^{32} uptake and incorporation, the root portions of seedlings were first immersed in 50 ml of 100 mg/l solutions of GA_3, KN, MH, ABA and KSCN for 24 hours. The roots of these pre-treated plants were washed thoroughly and exposed to low temperature environment by immersing them in cold water maintained at 5°C (± 1°C) for 10 and 30 minutes separately followed by P^{32} feeding. Subsequent measurement of P^{32} uptake was determined as described earlier. Uptake and percentage incorporation in different parts was calculated as described in (a) and (b).

Statistical studies

Before entering into the final interpretation of any of the above experimental observations, sufficient statistical measures were adopted wherever necessary. To attain this objective sufficient care was taken while maintaining homogeneity within plant populations and general growth conditions except the subjective treatments concerned in each case, before and during experiments. In general, the variability of different plant characters was analysed in every group of data. Amongst different statistical criteria taken into consideration in the present analysis, main emphasis was given in :

- determining the correlation between different growth and developmental characteristics on one hand and alkaloid content on the other;
- testing the significance of different treatments undertaken.

The pattern of the present study and its experimental designs demanded

these two biometric analyses mainly. The methods adopted in different analyses were those after Snedecor (1946) and Panse and Sukhatme (1978).

Correlation coefficient

It was determined according to the following formula :

$$r = \frac{\Sigma [(x - \bar{x}) \times (y - \bar{y})]}{\sqrt{[\Sigma (x - \bar{x})^2 \times \Sigma (y - \bar{y})^2]}}$$

where, x and y are the two variable plant character;

$\bar{x}$ and $\bar{y}$ represent the means of the variable, x and y, respectively.

From the above formula :

$$\Sigma (x - \bar{x}) \times (y - \bar{y}) = \Sigma xy - \frac{\Sigma x \times \Sigma y}{x};$$

$$\Sigma (x - \bar{x})^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}; \text{ and}$$

$$\Sigma (y - \bar{y}) = \Sigma y^2 - \frac{(\Sigma y)^2}{n};$$

where, Σx and Σy are the sums of variables, x and y respectively;

Σxy is the sum of the products of the variables; and

n is the number of replications.

Test of significance

It was undertaken to distinguish whether the observed difference between

- treated plants and untreated controls; or
- plants of different growth conditions (viz., attitude, age etc.)

connote any real difference or the observed difference can be ascribed to mere sampling fluctuations and/or natural variations in the plant population. The test was carried out by determining 't-values' and comparing that in the standard 't-table' (Fisher and Yates, 1963), as per the formula :

$$t = \frac{M_A - M_B}{\sqrt{(SE_A^2 + SE_B^2)}}$$

where, M_A and M_B are the means of samples A and B, respectively; and SE_A and SE_B represent standard errors of samples A and B, respectively.