Chapter III

Materials and Methods

3. MATERIALS AND METHODS

Plant Materials

In the Darjeeling Hills, aromatic rice (*Oryza sativa* L.) is cultivated organically by default in nature. *Oryza sativa* is a short-lived annual plant in the Gramineae family that dies after producing seeds during the onset of the winter season in November-December.All experiments in the current investigations have been conducted with stable and viable seeds of aromatic rice collected from nearby areas of Darjeeling district. During the initial collection of rice samples, more than 23 aromatic rice varieties were discovered during field visits. At the time of experimental set up, rice seeds from the local farmers (villagers) in the Darjeeling region were collected freshly, each weighing 3–4 kg of the 14 varieties **(Fig.1)**. Physiological and biochemical studies of these rice varieties were carried out in controlled conditions in the natural climate of the Darjeeling Hills. All the laboratory work has been carried out in the Post Graduate Department of Botany, Plant physiology and biochemistry Laboratory, Darjeeling Government College, Darjeeling, West Bengal, and the field work was carried out in the rice cultivation field at Sunsari Busty, Jamuney, Bijanbari, and Dist. Darjeeling.

During the experimental period, the environmental conditions in Darjeeling were as follows: Temperatures range from 2–9^{o}C to 20–35^{o}C, with a relative humidity of 84–93%.

Systematic Position

- ✓ Kingdom: Plantae [Plants]
  - ✓ Subkingdom: Tracheobionta [Vascular plants]
    - ✓ Superdivision: Spermatophyta [Seed plants]
      - ✓ Division: Magnoliophyta [Flowering plants]
        - ✓ Class: Liliopsida [Monocotyledons]
          - ✓ Subclass: Commelinidae
            - ✓ Order: Cyperales
              - ✓ Family: Poaceae [Grass family]
                - ✓ Genus: Oryza L.[rice]
                - ✓ Species: Oryza sativa L. [rice]

Details of seed morphology, external features of 14 varieties of aromatic rice

The harvested rice is known as "paddy". A paddy is a whole rice seed, and one cereal grain includes one kernel of rice. A grain of rice has four main parts: a seed containing an embryonic rice plant, stored food, and a protective coat. The inedible outer covering is the husk (hull), which is made up of two half-shells that are joined together. Each guards one half of the paddy. The husk is made mainly of silica and cellulose. Its weight accounts for roughly 20% of the total grain weight. Underneath the husk, the bran (germ) is concealed. The next layer is a very thin film of bran. The most nutritious part, fibre, vitamin B complexes, protein, minerals, and fat, make up the majority of the composition of bran. Each grain has an embryo at the base that will eventually develop into a new plant. Grain length, width, and thickness vary widely among varieties.

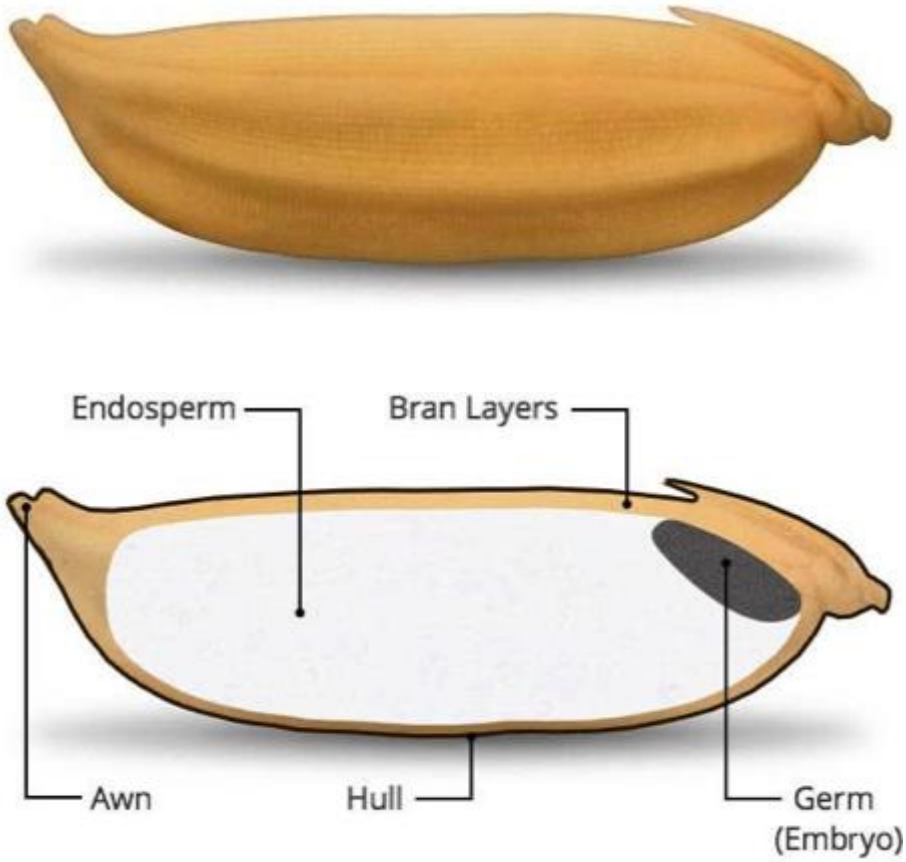


Fig. 4. The Anatomy of a Rice grain.

Three phonological stages of rice plants: (Table 3)

I. VEGETATIVE GROWTH

Stage 0 - GERMINATION - EMERGENCE

Germination and emergence are the first stages in the vegetative phase of growth. Germination begins with the appearance of the young shoot and roots through the seed coat at one end of the seed. The first leaf bursts through the coleoptiles on the second or third day following germination. Stage 0 concludes with the developing main leaf still coiled and an extended radical. **(Fig. 9)**

Stage 1 – SEEDLING

The planting phase begins immediately after emergence and continues until the first tiller appeared. Seminal roots and up to five leaves appear at this stage. Two additional leaves emerge and continue to develop as the seedling grows. Seedling stages occur during the first two to five weeks after planting and are ready for transplanting. **(Fig. 10)**

Stage 2 – TILLERING

This stage continues from the initial tiller appearance to achieve the maximum tiller number. Tillers sprout from the node's axiliary buds and, as they grow and mature, displace the leaves. Once the main tillers have emerged, subordinate tillers are generated. It happens around 30 days following the transplant. The plant is rapidly growing in length and tillering, which develops continually as the plant goes into the next phase, the elongation of stems. **(Fig. 13)**

Stage 3 - STEM ELONGATION

The quantity and height of tillers continue to rise without significant leaf senescence. The length of the stem is proportional to the length of the growth. Longer-growing variants have greater stem elongation. Varieties of rice are divided into 2 groups in this regard: one variety is short-duration (matures in 105 - 120days) and another variety is long-duration (matures in 150days).

II. REPRODUCTIVE GROWTH

Stage 4 - PANICLE INITIATION - BOOTING

Only around ten days after initiation, panicle primordium at the tip of the developing stalk will be apparent to the naked eye. Three leaves will still develop before the panicle appears at this point. The spikelets become distinctive while the panicle continues to grow. The juvenile panicle grows in size, causing the leaf sheath to bulge due to its upward expansion inside the flag leaf sheath, which refers to booting. Almost certainly, the main culm will be the first to fall. **(Fig. 14)**

Stage 5 - FLOWERING

Florets open, anthers emerge from the flower glumes due to stamen elongation, and pollen is discharged as the plant flowers. The florets then shut. It usually opens in the morning. In around 7 days, all of the spikelets in a panicle open. 3 - 5 leaves are still active throughout blooming. **(Fig. 15-18)**

III. GRAIN RIPENING

Stage 6 - MILK GRAIN STAGE

The ripening phase is made up of the last two stages of development, phases 6 and 7. The grain started to be filled with a milky substance at this point. A white, milky liquid fills the grain, which by pressing between the fingers squeezed out milky liquid. The panicle begins to bend and appears green. Senescence is developing at the tiller's base. The two lower leaves, as well as the flag leaf, are all green. **(Fig. 19-21)**

Stage 7 - DOUGH GRAIN STAGE

The milky part of the grain becomes soft dough initially, and firm dough afterwards. The panicle's grains turn from green to yellow. Leaves and tillers are remarkable in their senescence. When the panicle turns yellow, the field begins to look yellowish. **(Fig. 22 & 23)**

Methods of seed analysis

PHASE I

The rice grain quality of 14 aromatic rice varieties (collected for study) was evaluated using freshly harvested healthy rice seeds. Following collection, the seed batches were separated from the husk, and healthy and undamaged seeds were used for experiments. The assessment process was outlined by Cruz and Khush (1989).

EXPERIMENT NO. I

Rice Grain Quality

Rice is usually consumed as a whole grain, milled and cooked cereal. The idea of quality changes dependent on the preparations for which grains must be used, based on the dimension, form, and grain appearance, cooking patterns, softness, flavour, and taste. The desirable characteristics of rice may differ from one ethnic group or geographic location to the next (Juliano *et al*., 1964). The following information was used to assess the quality of rice grains:

Grain size and shape

The selection of large seeds has been a crucial aim during domestication, since grain size is one of the most important components of grain yield. Modern approaches to understanding the molecular and genetic processes of grain size control can increase rice yields. The final and full size of grains in the spikelet hull are maintained by cell proliferation and cell growth, which escalate storage capacity while restricting the filling of grains (Li *et al*., 2018).

In terms of rice quality, grain size, shape, length, breadth, and ratio of kernels (L/B) are all significant. There are short, medium, long, and long-slender types of aromatic rice grains available in all four categories, but the long-slender scented rice types fetch the largest market price worldwide. Based on physical characteristics, rice varieties are classed into two groups: long and short. The rice seed length is the most important factor to consider, and the length-to-width ratio determines the form. The grain can be visually graded based on its size and form. Grain length and form assessment standards vary by country and marketing

location. This approach was largely followed after Kaul (1970). The following is a classification for evaluating grain size and shape:

Grain size classification

SIZE CATEGORY	LENGTH (mm)	SCALE
VERY LONG	MORE THAN 7.50	1
LONG	6.61 - 7.50	3
MEDIUM OR INTERMEDIATE	5.51 - 6.60	5
SHORT LESS THAN OR EQUAL TO	5.50	7

Grain Shape Classification

SHAPE	LENGTH/BREADTH (RATIO)	SCALE
SLENDER	OVER 3.0	1
MEDIUM	2.1 - 3.0	5
BOLD	2.0 OR LESS THAN 2.0	9

Appearance of grains

The rice kernel size and form, as well as chalkiness, translucency, and the grain's "eye," all influence grain appearance. The rice samples are of low retail value and look bad with damaged eyes. In the same way, the higher the chalkiness, the lower the market acceptability. The customer can visually assess the existence or lack of a white belly, a white back, a white centre, a level of translucency and a fracture at the baseline-ventral end of the grain, known as the eye condition. The scales used to assess the endosperm of milled rice chalkiness are as follows:

% AREA WITH CHALKINESS	SCALE
NONE	0
LESS THAN 10%	1
10 TO 20%	5
MORE THAN 20%	9

Rice cooking and consuming characteristics

Many of the rice cooking and consuming qualities of milled rice are influenced by the starch properties, which comprise 90% of it. The starch qualities that influence cooking and consumption aspects are gelatinization temperature and amylose concentrations.

Gelatinization Temperature

10 entire milled kernels with no breaks are selected and placed in plastic containers along with a solution of 10 ml of potassium hydroxide (KOH) 1.7% (0.3035N). All samples are organised in such a way that there is enough room between kernels for spreading. The sealed containers are placed in an oven at 30 °C for 23 hours. On a scale of numerical 7, the starchy endosperm was evaluated visually. Any test includes standard rice type checks with low, moderate, and high gelatinization of endosperm. This approach was largely accepted by Little *et al*. (1958).

Scales for scoring gelatinization temperature are as follows:

SPREADING	ALKALI DIGESTION	GELATINIZATION TEMPERATURE	SCORE
Kernel not affected	LOW	HIGH	1
Swollen Kernel	LOW	HIGH	2
Kernel swollen; collar complete or narrow	Low or intermediate	High- intermediate	3
Kernel swollen; collar complete and wide	Intermediate	Inter-mediate	4
Kernel segregated or split; collar wide and complete	Intermediate	Inter-mediate	5
Kernel merging; dispersed with collar	HIGH	LOW	6
Kernel completely intermingled and dispersed	HIGH	LOW	7

Amylose content

The most significant intrinsic indicator of cooked and processed rice grain behaviour is the amount of amylose in the grain. It ultimately determines the firmness, rice-water ratio, and gloss of cooked rice. On the other hand, rice, which is not waxy or glutinous, is distinguished by moderate amylose, does not solidify, remains moist and tender while cooking, and is preferred. The majority of basmati and non-basmati rice have intermediate amylose (Sood *et al*., 1980). Rice with a high amylase concentration is dry and hard when cooked. These variations highlight the significance of amylose content as selection criteria.

To analyse the composition of amylase in 100mg of rice powder, 9ml of 1N sodium hydroxide and 1ml of 95% ethanol are put into a flask of 100ml. For the starch gelatinization, the ingredients are cooked in a water bath. After 1 hour of cooling, distilled water will be added and the ingredients will be properly mixed together. In a 100ml flask, 1ml of 1N acetic acid and 5ml of starch solution are added. In addition to the distillation water content, 2ml of iodine (2.0g Potassium iodide and 0.2g Iodine in 100ml of aqueous solution) is added and kept for twenty minutes. The absorbance of solutions is calculated at 620nm. The amylose content is calculated and reported using conversion factors in the dry weight result. The readings of optical density (O.D.) from the standard curve generated from potato amylose were compared to get a quantitative estimate. This method was adopted essentially after Juliano (1971). Rice varieties are grouped by Kumar & Khush (1986) on the basis of their amylose content into waxy (0–2%), very low (3–9%), low (10–19%), intermediate (20–25%), and high (> 25%). **(Table 2)**

Grain elongation

Rice grains absorb water while cooking, increasing their length, volume, and width. In high-quality rice, lengthwise elongation without a rise in girth during cooking is preferred. Both genetic and environmental variables, particularly temperature during ripening time, influence kernel elongation. During ripening, an ambient temperature during the day of around 25°C and at night of 21°C has been observed to assist in maximum grain elongation (Sood, 1978).

For elongation testing, 10 whole kernels were measured and immersed for 30 minutes in 20ml of dH_2O. All the samples have been put into a water bath and stored at 98° Celsius for 30 minutes. The rice was cooked and placed on filter paper-lined in Petridis. All cooked

grains are selected and measured. The average length of cooked rice grains compared to raw rice grains is proportionate to the average length of these grains. The method was largely embraced by Azeez and Shafi (1966). **(Fig. 25) (Table 2)**

Evaluation of aroma

1 gram of freshly milled rice is put in a centrifuge tube with 20 ml of dH_2O to test for the presence of aroma. All samples are then placed for 30 minutes in a water bath after the tubing has been wrapped in aluminium foil. The cooked samples must be allowed to cool before analysing the presence of fragrance in each sample. The samples were categorised as non-aromatic, somewhat aromatic, moderately aromatic, and strongly aromatic. For contrast, checking highly scented varieties are utilized. This fundamental laboratory technique was developed at IRRI in 1971. **(Table 2)**

PHASE II

Design of the experiment

Five aromatic rice types, namely Kalonunia, MohanBhog, Khemti, MasinoBasmati, and Musli, were chosen for this study based on their flavour, elongation of kernels, and gelatinization properties, out of fourteen kinds available. These qualities or properties are some of the most significant commercial parameters.

The experiments were designed to investigate the influence of long-term accelerated ageing (0-, 90-, 180-, 270-, and 360-days) on the five local aromatic rice grains chemically processed with Ascorbic acid (ASA), sodium dikegulac (NaDK), and Succinic acid 2, 2-Dimethyl Hydrazide (SADH). Chemicals such as ASA, NaDK, and SADH have been selected following the initial screening test.

Experimental condition and seed treatment

After being surface sterilised for 90 seconds with 0.1% mercuric chloride ($HgCl_2$), all 5 varieties of seeds (250gm) were individually pre-soaked for 6 hours in aqueous solutions of NaDK (1000 and 2000µg/ml), ascorbic acid (250 and 500µg/ml), SADH (150 and 300µg/ml) and dH_2O, then sun dried to their original seed weight with some moisture content. The soaking and drying procedures were performed three times in a 48-hour period, with a

cumulative pre-treatment time of 18 hours. This method of pretreatment allowed maximal chemical infiltration while preventing the start of germination. (Kanp, *et al*., 2021 & 2009; Pati, *et al*., 2020, 2019, 2014, & 1912; Das, *et al.*, 2003; Maity, *et al*., 2000; Bhattacharjee, *et al*., 1986 & 2006)**.**

This experiment was carried out in an artificially induced environment known as accelerated ageing in order to achieve a comparatively uniform and immediate outcome. The pretreated seed lots (250 gm) were placed into separate cloth bags after complete pre-treatment of the seed lots. In total, 35 muslin bags were placed in desiccators in which a relative humidity of 98.2% was pre-imposed by holding 250 ml of 5.96 percent Sulphuric acid (vol/vol) inside them. This experimental set-up allowed forced ageing and regularly (within 10–12 days) changed sulphuric acid periodically in order to restore the required relative moisture during the experimental time. After the imposition of an accelerated ageing condition on seeds in storage, tests were conducted at 90-day intervals (0-, 90-, 180-, 270-, 360-days) up to 360-days. The control group consisted of seeds that had been pre-treated with distilled water before being subjected to accelerated ageing. The techniques for sampling and the methods for analysing the parameters are outlined in considerable detail here.

In accelerated ageing treatments, vigour and viability of seeds are determined (Heydecker, 1972; Priestley, 1986; Chhetri, *et al*., 1993; Copeland and McDonald, 2001; Rai, 2000; Kapoor, *et al.,* 2011; Pati, *et al*., 2017; Patil, *et al.,* 2018; Henga, *et al*., 2019; Lama, *et al*., 2020; Alahakoon, *et al*., 2021). Under adverse conditions, experiments were designed to study such stable physiological and biochemical data in consecutive periods (0-, 90-, 180-, 270-, and 360 days, respectively) under the influence of three growth inhibitors, ASA, NaDK, and SADH.

EXPERIMENT NO. II

Physiological analysis of accelerated/stored seeds (0-, 90-, 180-, 270- and 360-days) of each pretreatment of ASA, SADH and NADK at an interval of 90 days

Analysis on response of seeds towards T_z salt (0.1%)

The viability of seeds is the degree to which the seed is metabolically alive and contains all the enzymes that can catalyse metabolic reactions for germination and seedling growth. To determine TTC-stainability, 5 varieties of aromatic rice seeds (100) were soaked

in 0.1% TTC solution (w/v) (2, 3, 5-Triphenyl Tetrazolium Chloride) in Petri dishes in darkness for 24 hours. The red-colored stained seed percentage is estimated using the total number of seeds for ASA, SADH, and NaDK treatments. This approach was largely introduced by Halder (1981). Data was collected at 90-day intervals starting from (0, 90, 180, 270- and 360 days, respectively). **(Table 1.4) (Fig. 24)**

Analysis of dehydrogenase activity

For dehydrogenase activity testing, 1g of standarised seed from each procedure was immersed in 10ml beakers containing TTC 5ml (0.1%) solution for 48 hours incubated in darkness. The generated atoms of hydrogen by full dehydrogenase activity engaged in the process of respiration convert tetrazolium in live tissues to a chemical known as formazen (red colour) (Moore, 1973). TTC is reduced in the seeds by a group of enzymes known as dehydrogenases. The formazen was extracted after incubation and the solution O.D. values were measured at 520nm. The Rudrapal & Basu (1979) tetrazolium chloride assay was used to evaluate the complete dehydrogenase activity in undamaged seeds. Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360-days, respectively). **(Table 1.5)**

Analysis of seed germination

To assess percentage germination, 100 rice seeds from all treatments were separately transferred to Petri plates containing soaked filter paper in 10 ml of distilled water. After 240 hours of seed soaking, germination findings in terms of percentage of seed germination were reported according to the ISTA (International Rules of Seed Testing, 1976). Analyses were performed at 90-day intervals (0-, 90-, 180-, 270-, and 360-days respectively) up to 360-days after seeds were subjected to an accelerated ageing condition in storage. **(Table 1.6)**

EXPERIMENT NO. III

Biochemical analysis of accelerated/stored seeds

Analysis of protein levels in seed kernels

Protein samples were collected from five varieties of aromatic rice seeds from each of the seven treatments and each ageing period (0-, 90-, 180-, 270-, and 360-days). Rice seeds (100 mg) were homogenised in a motar with 80% ethanol and centrifuged at 6000 g for 10

minutes.Following the process of Kar and Mishra (1976), pellets were washed twice with cold Trichloroacetic acid 10% (w/v), once with ethanol, with ethyl alcohol: chloroform once (3:1, v/v), with ethanol: ether once (3:1, v/v), and once with solvent ether finally. After that, the pellets were dried out by evaporation. After 1 hour of digestion at 80^0C with 0.5 (N) NaOH, the protein was isolated from the pellet and was rendered in a specific amount (4 ml). It was then calculated using the Lowry *et al*., (1951) process, which involved Folin Phenol reactions and calculating the O.D. values in a spectrophotometer at 650nm. For quantitative determination, the O.D. values were compared to a previously constructed standard curve based on BSA (Bovine Serum Albumin, Fractin-V-Powder, Sigma Chemical Company, USA). Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-and 360-days, respectively). **(Table 1.7)**

Analysis of soluble and insoluble carbohydrate in seed kernels.

Carbohydrate levels (soluble and insoluble fractions) were calculated using the McCready *et al*. (1950) approach with minor modifications. Every 10 minutes, 100mg of 5 different aged seed samples are homogenised at 6000g with boiling 80% ethanol. The supernatant was put in a watch glass. This was done three times, and the surface of the watch glass was placed in test tubes after being washed multiple times with 80% methanol and having the amount increased to 10 ml. This has been retained as a soluble carbohydrate source. **(Table 2.9)**

The residue following centrifugation of the sample was put in a water bath for 30 minutes with 5 ml of 25% H_2SO_4 at 80°C for the analysis of insoluble carbohydrates. The substance was removed as a source of insoluble carbohydrates after adequate dilution. **(Table 2.10)**

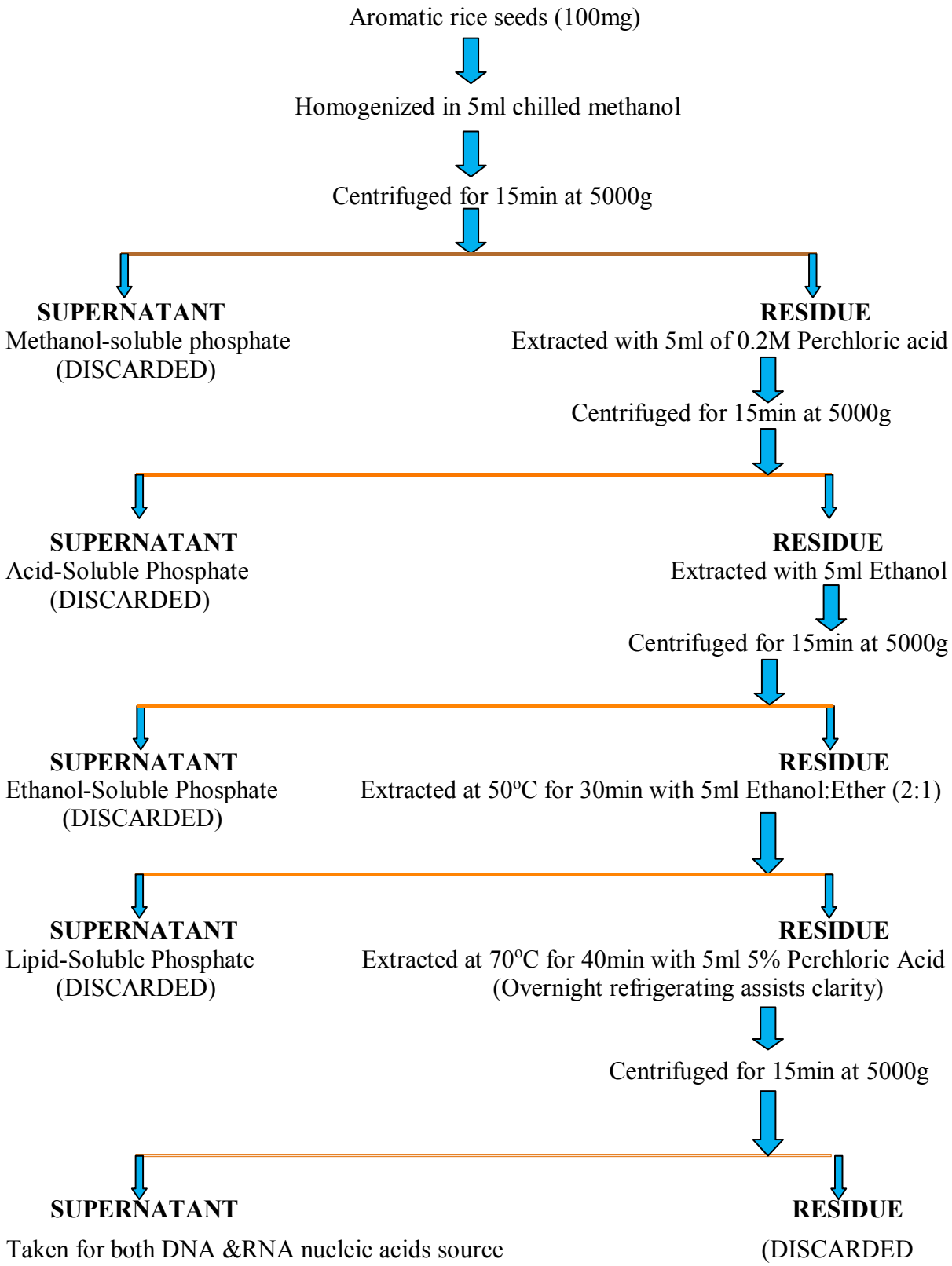
One millilitre of the source sample from each carbohydrate fraction was placed in a test tube, and four millilitres of freshly processed, pre-cooled, 0.2% anthrone reagent (200 mg of anthrone in 10ml of analar H_2SO_4) were applied to it for analysis and quantitative calculation. The spectrophotometer at 620 nm was used to calculate the strength of the green colour after 30 minutes. The actual contents were calculated using a regular curve made with glucose solution concentrations. Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.9 & 2.10)**

Analysis of free amino acids from seeds kernels

Using a clean, pre-chilled mortar and pestle, 100mg of rice seeds from various pre-treated cultivars were placed. The disorganised sample was centrifuged for 10 minutes at 5000rpm after homogenising the seeds of each (7) procedure and each (0-, 90-, 180-, 270-, and 360-day) ageing state with 5ml of 80 percent ethanol.The supernatant was then used as a source of free amino acid content by Dwivedi *et al*. (1979). Amino acid has been estimated using the Moore & Stein process from the stock solution (1948). For 15 minutes, a test tube containing 1 ml of 0.3% ninhydrin solution (80% ethanol) and marbles on the test tube tops were placed in a water bath. After the reaction had cooled to violet, the test tubes were removed and the volume was increased to 5 ml with 80 percent ethanol.A spectrophotometer was used to determine the solution's absorption at 570 nm. To get a quantitative estimate, the standard curve was constructed using glycine as the reference amino acid and compared. Data was collected at 90-day intervals beginning at (0-, 90-, 180-, 270-, and 360-days). **(Table 1.8)**

Analysis of nucleic acid levels from seeds kernels

Nucleic acid extraction (DNA and RNA) was performed on 100mg of kernel seed according to the Cherry method (1962). The DNA as well as RNA estimates were analysed by a standard stock, in which the sample was eventually collected using the Markham (1955) process, updated by Choudhuri and Chatterjee (1970) with perchloric acid (5%). The procedures for nucleic acid extraction are as follows (DNA and RNA):


Aromatic rice seeds (100mg)
Homogenized in 5ml chilled methanol
Centrifuged for 15min at 5000g
SUPERNATANT
Methanol-soluble phosphate
(DISCARDED)
RESIDUE
Extracted with 5ml of 0.2M Perchloric acid
Centrifuged for 15min at 5000g
SUPERNATANT
Acid-Soluble Phosphate
(DISCARDED)
RESIDUE
Extracted with 5ml Ethanol
Centrifuged for 15min at 5000g
SUPERNATANT
Ethanol-Soluble Phosphate
(DISCARDED)
RESIDUE
Extracted at 50ºC for 30min with 5ml Ethanol:Ether (2:1)
SUPERNATANT
Lipid-Soluble Phosphate
(DISCARDED)
RESIDUE
Extracted at 70ºC for 40min with 5ml 5% Perchloric Acid
(Overnight refrigerating assists clarity)
Centrifuged for 15min at 5000g
SUPERNATANT
Taken for both DNA &RNA nucleic acids source
RESIDUE
(DISCARDED

1. DNA

In an analysis, the extraction of 1 ml of nucleic acid was diluted with 5 ml in a test tube (100 ml of glacial acetic acid, BDH + 2.7 ml of conc. H_2SO_4 + 1 g of AR grade of diphenylamine) of freshly-prepared diphenylamine reagent. In a water bath, the mixture was boiled for 30 minutes with glass marbles on the test tube top. The blue colour intensity in the spectrophotometer was recorded after cooling in running tap water at 610nm. The O.D. values of the herring sperm DNA standard curve were quantified to the DNA content. Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.12)**

2. RNA

In order to estimate RNA, 3ml of freshly prepared Orcinol-reactive reactants (1gm AR dissolved Orcinol in con. HCl of 100ml containing 100mm 0.1 percent $FeCl_3$ and $6H_2O$) were treated with 3ml each of 5 different seed kernel treatments and each ageing period of diluted nucleic acid extract in separate test tubes for 20 minutes in a water bath with marbles on the test tubes' top. The cooled mixture was taken and the spectrophotometer was used to test the strength of the blue-green colour at 700nm. The blank was a combination of 3ml of distilled water and 3ml of orcinol, which were handled identically. The O.D. values from a regular curve made with yeast RNA were used to measure RNA level at 90-day intervals. Data was collected beginning at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.11)**

Determination of the activities of enzyme like catalase from seeds

For each treatment and ageing cycle, a homogenization of 500mg of seed kernels was obtained in 8ml of cold phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH 6.8). This homogeneous product has been centrifuged in the cold for 15 minutes at 3000g and then for 20 minutes at 10,000g. Using the same buffer, the supernatant was raised to 10 ml and analysed using the Snell and Snell (1971) method, which was revised by Choudhuri and Biswas in 1978. In the reaction of the catalase mixture, add 1 ml of H_2O_2 (0.05M) and incubate for 5 minutes at 37°C. The reaction was stopped by centrifuging the mixture for 15 minutes at 6000g with 0.1 percent titanium sulphate (2ml) in 25% H_2SO_4 (v/v).At 420nm, the golden yellow colour intensity was measured. The sample of blank was usually prepared with the addition of

titanium sulphate by inactivating (heat-killing) enzymes. Data has been collected at 90-day intervals beginning at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.13)**

Determination of the activities of enzyme like IAA-oxidase from seeds

This enzyme was extracted using 100mg of seed samples and 12ml of sodium phosphate (pH 6.1) buffer. At 10,000g, the homogenous product has been centrifuged for 15 minutes. The enzyme's rudimentary source was obtained from the supernatant. The method of Gordon and Weber (1951), modified later by Ramadas *et al*. (1968), was used to assess the behaviour of IAA-oxidase. The reaction mixture contains 1ml of 1mM 2, 4-dichlorophenol, 1ml of 1mM $MnCl_2$, 6ml of 0.03M sodium citrate (pH 4.5) buffer, and 1ml of enzyme extract.The process was halted after 1 hour of room temperature incubation by adding 1 mL of 20% $HClO_4$ to the reaction liquid. In a spectrophotometer, 1ml of the test mixture and 3 ml of Salkowski reagent (50ml of 35% $HClO_4$ + 1ml of 0.5 N $FeCl_3$) were combined, and the results were obtained at 525nm. Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360-days, respectively). **(Table 2.15)**

Analysis of enzyme alpha amylase in seeds

The seed kernels of each sample (100mg) were homogenised in 8ml of 0.1M phosphate (pH 6.5) buffer. The homogenate at 5000g was centrifuged for 15 minutes. For the analysis, the supernatant of the enzyme as a crude source was utilized. After mixing 2 ml of enzyme solution with a 0.2 percent starch solution, the mixture was incubated for 30 minutes at 37ºC.To stop the reaction, a 3ml of iodine-HCl solution was employed (60mg of KI and 6mg of iodine dissolved in 100ml of 0.1N HCl). In Biswas and Choudhuri's 1978 technique, the intensity of blue colour was calculated in a spectrophotometer at 610nm. Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360- days, respectively). **(Table 2.16)**

Analysis of catabolic enzyme protease in seeds

This enzyme had the same extraction process as the catalase one, with the exception that the used solution was buffered with 6.5pH. The protease activities were determined by incubating 1ml of enzyme extract, 0.1ml of 0.1 M $MgSO_4$, and 1ml of BSA (0.5mg/ml in dH_2O) at 37ºC for 1 hour, then adding 1ml of 50 percent trichloroacetic acid (TCA), and

analysing residual protein with the reagent Folin-phenol (Lowry, *et al*., 1951). This test was done in accordance with the updated Biswas and Choudhuri system (1978). The activities of all assayed enzymes were calculated as [(ATv)/(t v) g wt. of samples], where the O.D. value of A is the blank O.D. minus the O.D. of samples, Tv is the total volume of the filtrate, t (hour) is the incubation time with the substrate, and v is the filtrate volume taken for incubation after Fick & Qualset, 1975.Data was gathered at 90-day intervals beginning at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.14)**

Determination of the activities of enzyme like superoxide dismutase from seeds

To determine superoxide dismutase (SOD) activity, 200mg of seed kernals were crushed in 5ml of 0.1M phosphate buffer (pH7.8) containing 0.1 percent (W/V) insoluble PVPP (polyvinyl polypyrolidene) and at 4^{o}C centrifuged. As a source of an enzyme, the supernatant was employed. The enzyme capacity of nitro blue tetrazolium (NBT) was determined to block the reaction of photochemicals using a modified Giannopolitis and Ries (1977) technique (Roychowdhury and Choudhuri, 1985). The remaining components added to the 3ml reaction mixture were 0.05M Na_2CO_3, 0.1M EDTA, 63M NBT, 13M methionine, 20M enzyme extract, and 1.3M riboflavin. At a distance of 30 cm and at a temperature of 25^{o}C, the test tubes were mounted under two 4W florescent lamps. The light was turned off after 15 minutes, and the absorbance at 560nm was measured. Due to the maximum reduction in NBT, the non-irradiated sample would develop the most colour. The enzyme function was inversely proportional to the reduction of NBT. As a result, A560 of the particular set was subtracted from A560 of the blank set to obtain A (without enzyme). Data was collected at 90-day intervals starting at (0-, 90-, 180-, 270-, and 360-days). **(Table 2.17)**

EXPERIMENT NO. IV

After 360 days of pretreated seeds, seeds needed to be sown in order to find the field emergence capacities of various seed lots. All of the field experiments for this study were conducted in a rice field in Sunsari, lower Goke Busty, Jamuney, Darjeeling, at latitude 27.0951N, longitude 88.2301E, and altitude of 546.22m.

Soil Preparation and method of seeds sowing and transplanting

The field was ploughed 1-2 days before rice seeds were sown. The method is used in places with fertile soil, adequate rainfall, and a large supply of labour. Seeds from each treatment were manually scattered on the prepared flooded area **(Figs. 6, 7 & 8)**. Then water was drained out along with the floated non-viable seeds from the field. Normally, seeds germinate in 3–4 days, but since these seeds were chemically pretreated and stored in forced-accelerated conditions for 360 days, it took more than 10-15 days for them to germinate and seedlings to be prepared **(Fig. 9)**. Usually, seedlings that reached 3-4 inches in height were uprooted and planted on a damp puddled field that had been prepared for the purpose after 21–25 days **(Fig.12)**. Before transplanting, the area should be thoroughly puddled with bullock, which is a critical process. Puddling kills weeds and buries them in puddled soil. It also prevents weed germination during the crop's subsequent growing season. Puddling evens out the soil surface and creates favourable physical, biological, and chemical conditions for rice plant development. **(Fig.7)**

After 25 days of rapid growth and production, the seedlings were uprooted. The entire procedure was carried out by hand. As a result, it is a very complicated approach that necessitates a lot of input. However, it produces some of the highest yields. The winters in Darjeeling are too cold for rice cultivation, so the grain is only grown once a year. Data was obtained from the fields of physiological and biochemical studies.

Physiological field analysis of plant develop from pretreated accelerated/ stored seeds

Recording of field emergence capacity

Seeds were sown in well-prepared flooded soil mixed with a limited amount of cow dung to determine field emergence capability. The readings began the day after seed germination and lasted until the plants entered their senescence level.

Plant growth analysis

For the purpose of studying plant growth attributes, reading began shortly after seed germination and persisted until the plants entered their senescence period. Plant height,

internodal distance, and stem circumference were among the physiological parameters measured. For the purposes of documenting the growth data, the mean values of five plants were used. In case of plant height data were collected before shifting of rice plants in (0-5, 15- and 25- days) and after shifting of rice plants in (45-, 60-, 75-, 90-, 105-, 120- and 135-days) of plant age respectively. Each plant's first internode was used to measure the circumference of the stem. The distance between internodes was used to calculate internode elongation. **(See Tables 3.28 and 3.29)**

Biochemical field analysis of plant leaves develop from pretreated accelerated/ stored seeds

Biochemical analysis was performed for the five rice-plant stages, pre-flowering stage (P), Flowering stage (F), seed formation stage (S), seed mature stage (M), and pre-harvesting stage (H) corresponding to 67d, 86d, 105d, 120d and 132d respectively, taking samples from the leaves of each pretreated plant.

Analysis of macromolecules like DNA And RNA from leaves

Methods have already been described for extracting and estimating DNA and RNA. The samples and data were collected from the leaves of plants that had been grown from accelerated seeds at five stages of growth. The pre-flowering (P), flowering (F), seed formation (S), seed mature (M), and pre-harvesting (s) stages correspond to plant ages of 67d, 86d, 105d, 120d, and 132d, respectively.**(Tables 3.19 and 3.18.)**

Analysis of macromolecules like insoluble and soluble carbohydrates from leaves

Methods of extraction and estimation of insoluble and soluble carbohydrates have already been described. Here, the leaf samples were taken from plants raised from accelerated or aged seeds at four developmental stages. Data was recorded in five phases. Pre-flowering (P), flowering (F), seed formation (S), seed maturation (M), and pre-harvesting (H) stages **(Tables 3.21 and 3.20.)**

Analysis of some scavenging enzymes like catalase from leaves

200gm of leaf tissue from all treatments was homogenised in 8 ml of cold 0.1M phosphate buffer (Na_2HPO_4/NaH_2PO_4) (pH 6.5). The homogenate was, in cool circumstances, centrifuged at 3000g for 15 minutes and then 10000g for 20 minutes. The supernatant was diluted with the same buffer to a level of 10 ml and used as a source of crude enzyme. The activity of the enzyme was determined using the Snell and Snell (1971) technique, modified by Biswas and Choudhuri (1978). 1ml of the aforementioned extract and 2ml of 0.05M H_2O_2 were combined in a catalase reaction mixture and incubated at 37^0C for 2 minutes. The reaction was stopped by centrifuging the mixture for 15 minutes at 6000g with 2ml of 0.1% Titanium Sulphate in 25% H_2SO_4 (v/v).At 420 nm, a golden yellow colour intensity was observed. Prior to the addition of H_2O_2, the blank was prepared with the addition of titanium sulphate by inactivating (heat killing) the enzyme. Data was recorded in five phases, taking leaf samples from each pretreatment plant at the pre-flowering stage (P), flowering stage (F), seed formation stage (S), and seed maturity stage (M). **(Table 3.22)**

Analysis of some Scavenging Enzymes like Superoxide Dismutase from Leaves

Methods of extraction and estimation of enzymes of superoxide dismutase from leaves of plants raised from accelerated or aged seeds at four developmental stages have already been described. Data was recorded in five phases. Preflowering stage (P), flowering stage (F), seed formation stage (S), and seed mature stage (M) leaf samples were collected from each pretreated plant. **(Table 3.26)**

Determination of the level of some catabolic enzymes like IAA-oxidase from leaves

This enzyme was extracted using 100mg of leaf tissue and 12ml of cold 0.2M sodium phosphate buffer (pH6.1). The homogenous product was centrifuged for 15 minutes at 10,000 g. The enzyme was extracted from the supernatant as a crude source. The IAA-oxidase activity was measured using a technique developed by Gordon & Weber, 1951, which was modified by Ramadas *et al*., 1968. 1ml of 1mM 2, 4-dichlorophenol, 1 ml of 1mM $MnCl_2$, 6ml of sodium citrate 0.03M (pH 4.5) buffer, and an extract of 1ml of enzyme were added to the reaction mixture. The reaction mixtures were halted by adding 1 mL of 20% $HClO_4$ after they had been incubated at room temperature for 1 hour. In a spectrophotometer, 1 ml of reaction mixture was reacted with 3 ml of Salkowski reagent (50 ml of 35% $HClO_4$ + 1 ml of

N $FeCl_3$), and the reading was obtained at 525 nm. Data was recorded at five phases: pre- flowering stage (P), flowering stage (F), seed formation stage (S) and seed mature stage (M), taking leave samples from each pretreated plant. **(Table 3.24)**

Determination of the level of some catabolic enzymes like rnase from leaves

At 0^oC, 100mg of fresh leaves were homogenised in 5ml of 0.1M sodium phosphate buffer (pH6.4) and centrifuged for 20 minutes at 10,000g. The supernatant was used as a crude enzyme source and was diluted up to 10ml using the same buffer solution. The assay was carried out according to Biswas and Choudhuri's technique (1978).

The RNase reaction mixture contained 1ml of enzyme extract and 1ml of yeast RNA (1 mg/ml) diluted in 0.1M sodium phosphate buffer (pH5.7). After 30 minutes of incubation, the reaction was stopped by adding 0.2 mL of 70% perchloric acid at 37°C.The supernatant was combined with 5ml of BSA (0.5 micro/ml) diluted in a buffer of 0.1M sodium acetate (pH 4.0) after centrifugation at 6000g. After a few minutes, the developed turbidity was stabilised with 2ml of 0.1 percent gelatin and measured at 420nm.The activity of this enzyme was measured using the Flick and Qualset principle (1975). Data was recorded in five phases. Preflowering stage (P), flowering stage (F), seed formation stage (S), and seed mature stage (M) leaf samples were collected from each pretreated plant.**(Table 3.25)**

Determination of the level of some catabolic enzymes protease from leaves

This enzyme had the same extraction process as the catalase one, with the exception that the solution used was 6.5pH. The activity of proteases has been determined by incubating reaction mixtures comprising 1 ml of enzyme extract, 0.1 ml of $MgSO_4$, $7H_2O$ and 1 ml of BSA for 1 hour at 37^0C, followed by 1 ml of 50% trichloroacetic (TCA) and by using Folin-phenol protease residual can be determined (Lowry *et al*., 1951). This test was done in accordance with the updated Biswas and Choudhuri system (1978).

In all cases of enzyme assayed, the enzyme activities were expressed as [(ATv)/(t v) g wt. of tissue], where O.D. of A is the value of the blank O.D. minus the sample O.D., total volume of the filtrate Tv, incubation time t (hour) with the substrate, and volume v of filtrate after Fick & Qualset, 1975.Data was collected in five stages. Pre-flowering (P), Flowering

(F), Seed Forming (S), and Seed Mature (M) stages, with leaves collected from each pretreated plant. **(Table 3.23)**

Analysis of yield attributes

The following plant yield attributes were recorded from accelerated aged seeds with five varieties of rice aromatic seeds: total seed weight per panicle and 1000 seed weight per plant. The yield data that was presented was an average of three results. **(Table 3.27)**