3. MATERIALS AND METHODS

The present research work was carried out in the research laboratory of the Department of Botany, Meerut College, Meerut (U.P.) during the academic sessions 2003-2004 and 2004-2005. The materials used and the methods adopted are being described hereunder the following heads.

3.1 MATERIALS

3.1.1 Seed

Seeds of the following medicinal plants were obtained from National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi 110012 and other reliable sources for this study.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Common Name</th>
<th>Botanical Name</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Isabgol</td>
<td>Plantago ovata (Horsk.)</td>
<td>Plantaginaceae</td>
</tr>
<tr>
<td>2</td>
<td>Senna</td>
<td>Cassia angustifolia (Vahl.)</td>
<td>Caesalpinaceae</td>
</tr>
<tr>
<td>3</td>
<td>Ashwagandha</td>
<td>Withania somnifera (Linn.)</td>
<td>Solanaceae</td>
</tr>
<tr>
<td>4</td>
<td>Muleth</td>
<td>Glycyrrhiza glabra (Linn.)</td>
<td>Fabaceae</td>
</tr>
<tr>
<td>5</td>
<td>Aonla</td>
<td>Emblica officinalis (Gaertn.)</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>6</td>
<td>Bael</td>
<td>Aegle marmelos (L. Corr.)</td>
<td>Rutaceae</td>
</tr>
<tr>
<td>7</td>
<td>Kanghi</td>
<td>Abutilon indicum (Linn.)</td>
<td>Malvaceae</td>
</tr>
<tr>
<td>8</td>
<td>Reetha</td>
<td>Sapindus trifoliatus (Linn.)</td>
<td>Sapindaceae</td>
</tr>
</tbody>
</table>
3.1.2 Chemicals

All the chemicals used in this study were of analytical grade.

3.2 METHODS

The seeds of the medicinal plants were subjected to the following treatments in order to achieve the desired objectives of the study pertaining to seed viability, germinability and causes of dormancy:

1. Seed coat structure/seed morphological characteristics
2. Imbibition rate of seeds
3. Seed viability test
4. Electrical conductivity of seed leachates
5. Seed germination and seedling vigour

3.2.1 Seed coat structure/seed morphological characters

The testa is a very effective water proofing tissue which, because of this property, can delay germination of hard-coat seeds for considerable period of time. Many species have seeds with extremely hard coats which by preventing the entry of water delay germination for many years. Seeds of many species of the leguminous such as species of Albizzia, Acacia and Cassia, have impermeable seed coats. Impermeability is generally due to various impervious substances deposited in the testa such as suberin, lignin or cutin.

The mechanical resistance of the seed coat prevents the endosperm from absorbing water and expanding. These never get close to one of the basic requirements for germination i.e. water, notwithstanding hard-coated seeds. In which water entry is the factor limiting germination are generally
considered under the heading of dormancy. The fossil is generally responsible for impeding water uptake.

The coats of many seeds are hard, tough tissues which may be expected to offer considerable resistance to the emergence of the embryo, for hard shells of different kinds of seeds units are outstanding examples, and obviously if the embryos can not generate enough force to penetrate these tissues, they can not germinate.

This mechanical resistance can be overcome by physiological process occurring in the seed during stratification even if the seed coat is not treated physically. Impermeable seed coats have to be softened before planting to permit a more uniform water uptake and germination.

Thus seeds of several species, which are apparently permeable to both water and gases are prevented from germinating through mechanical resistance of the seed coat to the expansion of the growing embryo. The seed coat resistance can be decreased by warm, moist storage. Mechanically resistant seed coats can also be softened by mechanical abrasion alternate freezing and thawing or by acid scarification.

Hence in view of the above the seeds of different medicinal plants under study were assessed for their size, shape, colour, test weight and the nature of seed coat i.e. hard and permeability to water through physical observation and the imbibition rate, seed viability and germinability for determining the appropriate treatments to be applied accordingly in order to overcome seed dormancy.
3.2.2 Imbibition rate of seeds

The uptake of water by seeds is called imbibition, which is an essential initial step towards germination. The imbibition of water by seeds depends on the seed composition and seed coat structure. Hence the assessment of the seed coat permeability was worked out by comparing the rate of imbibition by the seeds with intact seed coat and the chipped-seeded seeds by taking their equal initial weight. The seeds were soaked in water on moist filter paper and weighed daily for water uptake during the initial germination period and the adequate amount of water was added to the growing medium.

3.2.3 Quick viability test (TTC test)

Since viability test by seed germination takes long time the experimental seeds under various treatments were also assessed by quick viability method, i.e., 2, 3, 5 triphenyl tetrazolium chloride test (Lakin, 1942). The seeds were soaked for 8-10 hr. in water to soften the seed. The cotyledons were opened to expose the embryo and were dipped in 0.5 percent and 1 percent tetrazolium chloride solution of pH 7.0. These samples were kept in dark at about 20°C for 6-8 hr. depending on the size of the seed. The seeds were then rinsed 2-4 times with distilled water. The red colour staining pattern of the embryo was assessed for viability determination.

Kitchin and Law (1936) contributed to the acceptance of the tetrazolium method for vigour by reporting 99.9% correlation between tetrazolium readings of individual wheat seeds and field emergence. There was a good correlation between staining intensity and field emergence of seeds.

Tetrazolium salt is an oxidation-reduction indicator and the development of a non-diffusible red colour in tissue is a result of reduction of
the chemical by enzymatic action. One or more of the dehydrogenase systems appear to be involved in the reaction. The reaction is as follows:

![Diagram of the reaction](image)

The dehydrogenase enzymes are involved in the respiration activity of biological systems. During the respiratory process intermediates are produced which serve as substrate for the hydrogen ions are transferred to tetrazolium which acts as hydrogen acceptor. Tetrazolium is then reduced to an insoluble red coloured formazan. Since the reaction occurs within the cells and the pigment is non-diffusible, there is a rather sharp demarcation between respiring (viable) tissue and non-respiring (non-viable) tissue. The former takes on a characteristic red colour while that of the latter its natural colour.

### 3.2.4 Electrical conductivity of seed leachates

As seeds age and natural deterioration proceeds, degradation and disorganization of cellular membranes may occur, allowing nutrients to be leached from them in the presence of water. It is also known that the loss of seed vigour can be detected by increase in seed leachates in the presence of distilled water. The concentration of ions in the leachates was measured by electrical conductivity meter.
The seeds (e.g., were soaked in 20 ml of distilled water and incubated at 25°C for 4 h. Seed techmics was collected and electrical conductance (EC) was measured using a conductivity bridge (Elco CN-82T). Measurements were recorded in 5 replicates: each EC of distilled water was taken as control.

3.2.5 Seed germination and vigour

Germination begins with water uptake by the seed and ends with the start of elongation by the embryonic axis usually the radicle. The degree to which germination has been completed in a population is usually expressed as a percentage, normally determined at time intervals over the course of the germination period.

Germination test was conducted with four replicates of 25 seeds each, following the ISTA method at 27°C (Anonymous 1989). The germinated seeds were evaluated into normal and abnormal seedlings and dead and hard seeds were counted every day. Germination percentage was recorded on the basis of normal seedlings only.

Seeds were germinated by different techniques depending on size and type of the seeds

3.2.5.1 Rolled towel test

The large seeds were tested for germination by this procedure. Towel papers were spread on top of each other and then soaked in water for 2-3 hr. Waxy paper kept below the towel paper. Normally 20-25 seeds depending on size were placed on top of the uppermost sheet in a fairly regular pattern so that they were approximately equidistant. The towels were then gently and loosely rolled to form a tube of about 50-30 mm and tied with rubber band and
kept in germinator at 27±3°C for 7-15 days. The rooted towers were held with rubber band and kept up right in cage within the germinator. In incubators where relative humidity is not controlled, water was sprayed over the towers every 2-3 days.

3.2.5.2 Filter paper

Normally 9 cm diameter filter paper were used. The small seeds were placed over filter paper. The filter papers were soaked in water and placed in the petri dishes. Normally two filter papers were used for each dish. Seeds were placed on top of the filter paper. Each petri dish was marked with the name of the accession, treatment and the date. All these petri-dishes were maintained in germinator at 27±3°C for recording the germination percentage of accessions.

3.2.5.3 Germination test

The speed of germination is an important test and provides a reasonably good index of vigor. Each individual species has its own rhythm of germination and some species take a lesser time than others. On the whole, however, the pattern of germination is the same. Depending on the species germination starts after two to ten days after the date of sowing. The number of seedlings that emerge increases with each day till the peak period when the maximum number of seedlings emerge. After that number that emerges slows down with each passing day till none more seedlings emerge. The last day after which no more seedlings emerge is known as the final count. The peak period is known as the first count and the number of seeds germinating at the first count is a fairly good indication of the vigour of the seed lot, provided they are germinated under optimum conditions.
3.2.5.4 Vigour index

Vigour index was calculated as the product of seedling vigour on the shoot length and germination percentage.

3.2.5.5 Speed of germination

For computing the speed of germination, germination counts were taken every 24 hours and the seeds were considered germinated when the first radical emerged. An index was calculated for each treatment or by dividing the number of seedlings removed each day by the day after planting on which they were removed.

\[ \text{Speed of germination} = \frac{\text{No. of seedlings removed daily}}{\text{Days after planting}} \]

3.3 TREATMENTS FOR OVERCOMING DORMANCY

3.3.1 Scarification

It is a technique for overcoming the effect of an impermeable or hard seed coat. The following scarification techniques were adopted to overcome the germination constraints.

3.3.1.1 Mechanical

Mechanical scarification was done by rubbing seeds between two pieces of sandpaper (Schmidt, 1980) using a file, a pin, or a knife to rupture the seed coat. Care was taken not to injure the embryo. For this purpose, it was considered necessary to open a couple of seeds to see where the embryo is located in relation to the micropyle, the former point of attachment to the fruit. The large seeds were easily scarified with a knife, but in hot water treatment was adopted for small seeds as suggested by Emery (1987).
3.3.1.2 Hot water treatment

For small to medium-sized seeds or large quantity of seeds, the hot water treatment was considered more practical than mechanical scarification. For the treatment seeds were dropped into about six times their volume of 70°C pre-heated water. They were left to cool and soak in the water for 12 to 24 hours after which they were used for sowing. The container used for this treatment was not made up of aluminium for avoiding toxic effect to the seeds. After the hot water treatment and soaking the seeds were sown promptly and not stored again.

3.3.1.3 Acid treatment

Acid treatments are often used to break down especially thick impermeable seed coats. Seed seeds placed in concentrated sulfuric acid (H₂SO₄) will become charcoal in time; the temperature of the acid and the length of time of the seed soaking are very important. The acid was used at room temperature for a period of few minutes (5-15) depending upon species. The seeds were immersed in acid in a glass or porcelainware container and were stirred occasionally with a glass rod. The seeds were removed from the acid just before acid penetrates the seed coats. After a allotted time the seeds were removed promptly and washed thoroughly with several changes of water to remove the remaining acid. Since sulfuric acid is caustic and dangerous to skin hence the precautions were taken during handling.

Seeds of medicinal plants were soaked in concentrated H₂SO₄ (100%), 50%, 25%, 10N, 5N and 1N: concentration for varying duration (5 to 15 minutes) to different seeds according to the nature of their seed coat.
3.3.1.4 Other chemical treatments

About 50 years ago researches and private seed industry began conducting experiments with chemicals to overcome dormancy conditions present in seeds and concluded that the presence of inhibiting factors in one or more parts of the seed can be neutralized by the application of chemicals.

Three chemicals that have proven very helpful in breaking certain types of dormancy are gibberellic acid (GA$_3$), potassium nitrate and thiourea. The aqueous solutions of these chemicals were used at room temperature. The concentration and length of treatment were provided as per species treated as follows.

i. **KNO$_3$**: The seeds were soaked in 0.1, 0.5 and 1.0% solution of KNO$_3$ for 5, 12, 24 hr prior to plate them on the moist filter tissue paper for germination.

ii. **Gibberellic acid (GA$_3$)**: The seeds were soaked in 100, 200 and 300 ppm solution of GA$_3$ for 6, 12 and 24 hr prior to plate them on the moist filter tissue paper for germination.

iii. **Thiourac treatment**: Seeds were soaked in 0.1, 0.5 and 1.0% thiourac for 5, 12 and 24 hr prior to plate them on the moist filter tissue paper for germination.

The seeds soaked in GA$_3$ or thiourac were stirred occasionally and rinsed after wards unless specified and sown immediately. The main advantages of these chemicals are speed, ease of use and unaltered physical condition of the seeds following treatment (Emery 1987).

3.3.1.5 Cold stratification or prechilling

Cold stratification or prechilling for seeds with internal dormancy
simulates cold winter conditions. The embryo of many seeds fails to germinate because oxygen does not diffuse through the seed coat.

At cold temperatures, more oxygen is dissolved in water so the oxygen requirement of the embryo is better satisfied. Coping with stratification involves overwintering in a cool, sealed environment (Young and Young, 1964).

The seeds were mixed in a ratio of 1:3 straw:peat:most soil. Moss or most vermiculite was placed in a tightly sealed polyethylene bag or glass petri dish and stored in the refrigerator at an temperature of 35-41°F for an winter period of seven days and the impact of chilling was assessed on germination percent.

3.4 STATISTICAL ANALYSIS

The data recorded for different parameters were analyzed statistically by fitting of Analysis of Variance as described by Cochran and Cox (1967) method, randomized design. The results are presented at 5 per cent level of significance. *P* < 0.05. The standard error of mean was given in all the cases. Where in two F-test were found significant at a per cent level of significance, the corresponding critical difference (CD) values were calculated to compare the significant results.
4. EXPERIMENTAL FINDINGS

The observations recorded on the seed weight, size, shape, seed coat nature, seed viability and germinability were assessed in order to formulate the strategy to be adopted for overcoming the seed dormancy. Accordingly, the seeds were provided mechanical (acid scarification), hot water treatments, moist seed pre-chilling and chemical treatments like GA (100, 200 and 300 ppm), KNO₃ (0.1, 0.5 and 1.0%), and indoleacetic acid (1, 5 and 10%). The chemical treatments were given for different durations of soaking i.e., 6, 12, and 24 hr to workout their impact on germination pattern and seedling vigour in different medicinal plant seeds. In some plant seeds, the aforesaid treatments were also applied in combinations like mechanical scarification plus chemicals to Cassia angustifolia, Emblica officinalis, Sauromatum tinctorium, Glycyrrhiza glabra, and pre-chilling plus chemicals to the seeds of Plantago ovata in order to observe their synergistic effects on seed germination and other parameters. The data recorded under different treatments pertaining to the various aspects under study were statistically analysed and are being described here under the following heads.

4.1 SEED CHARACTERISTICS

4.1.1 Plantago ovata (Isabgol)

The seeds of Plantago ovata are low weight categorized into small seeds. The 1000 seed weight is 0.18 g. The size of the seed is 0.3 x 0.2 cm.