CHAPTER-5

Part-1

REGENERATION STUDIES BY SEEDS AND VEGETATIVE CUTTINGS

Introduction and Review of Literature :

"Seed is the start,
Seed is the end,
And what takes place between the two
is biochemical mystery" (Osborn, 1981)

Seed dipicts the most important stage of plant. It is a device for reproduction and dissemination of plant. Efficiency and success of raising plant depend upon the quality of seeds. There are so many methods for plant propagation but seed is the easiest tool and represents the living unit of plant. It is said that seed is the greatest miracle ever created by nature, only a few milligram in weight for creation of a huge tree, weighing several tonnes. Germination test is the best way to test the seed viability. The process of germination comprises the capacity to develop into a normal plant under optimum conditions. It is not easy task to define seed germination. Various workers defined it variously in their own way :

Toumey and Stevens (1928) : reported that germination occurs when the radicle begins to protrude beyound the seed coat.

Fowell (1953) and Evenari (1956) : reported germination as three over lapping processes :

(a) Imbibition or absorption of water through seed coat and micropylar region causes the seed coat to swell and eventually break.

(b) Enzymatic activities and increased respiration, assimilation, indicate the use of stored food and translocation to the growing region.

(c) Cell enlargement and cell division result in the emergence of radicle and plumule.

Krawar and Kozlowski (1960) : stated that seed germination is the basic aspect of ecophysiological studies because functionally seed is a device for reproduction, perpetuation and multiplication of a species.

Justice (1979) : reported that "Germination is the embryonic emergence and development of those essential structures that enable a normal plant to develop under favourable conditions.

The transformation of seed into seedling is accomplished by the process known as germination. Germination may be defined as the sequential series of morphogenetic events that result in the transformation of an embryo into seedling. The emergence is the first indication of germination, when plumule is developed and other primordia are expressed, the germination is completed.

Better aeration, moisture and temperature are three important requisites associated with germination process. Requirement for aeration, moisture and temperature can vary from species to species. With a little variation in these conditions, seed germination is affected to a great extent.

In fact, seed germination represents the growth phase and physiological and biochemical changes occur during the process. The most important external

conditions necessary for the germination of seed are water and higher concentration of oxygen. Seed germination in a laboratory test is the emergence and development of those essential structures from seed embryo, which indicate the ability to develop into a normal seedling under favourable conditions in soil (ISTA, 1976). However, germination process consist of different steps : Imbibition, Hydration of tissues, absorption of oxygen, enzymatic activity, cell division and enlargement and emergence of radicle and plumule.

A lot of work has been done in India on various aspects of seed germination. But studies related with seed germination of some important plant species which are important from medicinal point of view is still lacking. With this end in mind the present study is aimed to study the effect of Imbibition, temperature, storage and other factors in seed germination of two species of genus *Calotropis* (Linn). R Br.

The process of germination is largely affected by the various internal conditions of the seed and various external factors under which plant is growing. It is a process which causes sudden transformation of dry seed into the young seedling.

Imbibition is the first step of seed germination. It is a most critical and preliminary process in seed germination. Water availability in liquid or gaseous form control imbibition. The process of imbibition makes seed coat soft due to absorption of water, hence enhance the germination. The phenomenon of water

absorption in seed has been the subject of wide spread investigations. While ample supply of water is essential for germination. Kidd and West (1918) reported that excessive soaking is frequently injurious to seeds. Various workers have put forward different views to excessive soaking.

Toumbey and Durland (1923) studied the effect of soaking on coniferous seeds of upland species and reported that soaking more than 3 to 5 days were generally injurious. Each species has a critical soaking period for the germination as indicated by promptness and completeness of germination. Resuher (1941) reported that injurious effect of presoaking seed of soyabean due to high water holding capacity. Orphanos and Heydecker (1968) soaking of seed before sowing has been reported to enhance germination and suggested that soaking injury is caused at a critical stage of germination by deficient oxygen supply to the enterior of soaked seed, because during soaking the cavity between the cotyledon is flooded with excessive of water. Sherwin and Siman (1969) reported aerobic conditions of soaked seeds. They reported the accumulation of lactic acid and enhanced lactate dehydrogenase activity in beans, germinated under wet condition.

Ghosh *et. al.* (1974) reported that soaking of seeds of *Pinus petula* for 18 hours at room temperature resulted in high germination. Gupta and Kumar (1977) reported that in seed of *Dendrocalamus strictus*, germination of over 95% was achieved at 30^{0}C with 50-75% moisture level. Yadav and Mishra (1982) described the best imbibition time for germination of *Poplus ciliata*. Verma and Tandon

(1984) discussed that water potential stimulated seed germination and seedling growth in *Pinus kesiya* seeds but higher water potential inhibits the germination.

Yadav (1989) also studied the effect of imbibition of seed germination in 8 tropical forest species. Souza and Filho (1993) studied the effect of imbibition on seed germination of *Calopogonium mucunoids* and found that as the imbibition period progressed within the range of 1-7 hrs. differences among the sample lot expressed in term of percentage of imbibed seeds. Imbibition rates were associated with the physiological quality of the seeds. Low quality seeds were first to imbibe.

Temperature is also one of the most important factor, it plays the most vital role in the germination of seed. Excessively high and low temperature adversely affects the germination process by hindering metabolism and eventually causes death of the organism. With the increase in temperature upto an optimum value the germination percentage increase in most of the species which may be due to rapid water absorption at high temperature. Brown and Worley (1921) reported that seed of barley absorbed water 3-4 times faster at 21°C as compared to water at 38°C and 2-4 time faster at 34°C as compared to water absorption at 21°C. Edward and Collin (1834); Teitz (1976); Kienitz (1879) were perhaps amongst the first workers to study the effect of temperature on tree seeds.

Harting (1878) reported that germination did not begin below 7.5°C. Siegal (1950) studied the effect of different temperatures on seeds of herbaceous plants

and suppression in germination was recorded in some cases, while in other temperature and light promoted germination. Datta (1961) reported the seeds of *Nerium oleander* germinated successfully within temperature range of 17°C-28°C. Kamra and Simak (1968) tested different constant and alternating temperature for germination of *Pinus silvestris* and found that constant temperature (20°C) was better for obtaining higher germination. Vyas and Agarwal (1972) reported that the optimum temperature for germination in seeds of *Indigofera cordifolia* was 30°C. Modiwala and Dubey (1976) reported that with increase in temperature of pretreatment from 30°C to 40°C, there was a subsequent increase in the germination of seeds in *Corchorus olitorius, Alysicarpus regosus* var. *Heyneanus* and *Desmodium diffusum* respectively.

Gupta and Pattanath (1976) investigated optimum temperature requirements in several tree species and found 30°C, as the most suitable temperature for seed germination. Bhoojh and Ramakrishnan (1981) reported the germination response of seeds of two closely related species of *genus sehima. S. khesiana* which occur at higher altitude germinate better at lower temperature and *S. wallichii* from lower altitude responded to higher temperature.

Yadav (1989) studied the seed germination behaviour of nine species of forest tree species and found that eight species *A. catechu A. lebbek, A. procera, B. variegeta, D. sissoo, D. strictus* and *S. robusta* give best germination percentage at 30°C-35°C whereas in *L. parviflora* the optimum germination was found at 20°C-

30°C alternating temperature. Dubey (1991) also tested seeds of ten plant species and found that temperature affected seed germination in all species.

Most of the previous reports on seed germination (Wright, 1931; Niizuma, 1936; Igusa, 1943; Spring field, 1972; McDonough and Harniss, 1974 and Bare *et. al.*, 1978) describe merely the effect of low, high or optimum temperature on seed germination of some species.

Seed differ enormously in temperature range when they germinate. However, better germination occurred between 30°C to 35°C temperature in most of the species. The temperature above and below this temperature range was found to be negatively correlated with germination. At a very low and high temperature germination is mostly prevented.

Storability of seed may be defined as the preservation of seeds from the time of collection until they are required. Holmes and Buszewicz, (1958) reported that during storage of seed, suitable atmosphere is required to maintain their viability and vigour for a long duration.

There are three main objects for storage of plant seeds :

(1) To preserve seed under condition that retain germination energy during the interval between collection and time of sowing.

(2) To protect the seeds from damage by rodents, birds and insects.

(3) To preserve quality of seeds collected during year of heavy seed crops to furnish supply during lean seed year (Mishra, 1992).

The period for which seed can remain viable is greatly affected by their treatment during storage period and conditions in which they are stored. Germination capacity depends on the storage viability . Roberts (1972) reported that viability can be controlled by storage. Seed can be stored for longer duration, if optimum suitable conditions of moisture, temperature, light, aeration etc. are being provided. The seed storage physiology has been demonstrated by Roberts (1973a), who separated seed into two categories i.e. Orthodox and Recalcitrant seeds. Recalcitrant seeds are those which cannot be dried below a relatively high moisture content without immediate damage and these seed cannot be stored for a long time (King and Roberts, 1979). Suitable combination of temperature and moisture content are required to maintain acceptable viability level during storage. High temperature during storage hastens the germination energy.

Oxygen and carbondioxide concentration of atmosphere, surrounding the seeds and light also influence seed longevity to a lesser extent during storage period (Harrington, 1973).

Moisture content is one of the most important factor maintaining geminability of seed in storage (Crooker and Barton 1957; Holmes & Buszewicz 1958; Barton, 1961; Heit 1967a; Harrington 1972). Seeds stored for long periods should be kept at temperature above freezing (Huse 1954; Bernett 1969).

Notable work on seed storage and germination behaviour of tropical tree species have been done by Dent (1948); Anon (1966); Gopal (1968); and

Maithani *et. al.* (1987). Rehackova (1954) studied the variations in germination capacity and germination energy of pine seed, on monthly basis storage, Gvozdikov and Umarov (1968) tested seed viability of *Haloxylon aphyllum* affected by duration of storage. They reported that initial viability was 50% to 60% but after 12 months of storage, viability fell to 17-24%. Pintaric (1970) reported that in *Picea omorika* seed germination percentage declined to 87% after 6 years 6 months, who tested from 1 year 6 months upto 6 years 6 months.

Popovaski (1972) studied germination of seeds of *Ulmus campestris* and *Ulmus minor* under different storage conditions.

Muttiah (1975) reported considerable fluctuation in seed germination of *Tectona grandis*, highest germination was reported 2-4 months stored seed after collection, after which it decreased . Kumar and Bhatnagar (1976) studied germination behaviour of *D. sissoo* seeds under laboratory condition for fresh and one year old seeds. Shukla and Ramakrishnan (1981) studied seed germination of *L. parviflora* and found that viability and germination both of the seeds declined with storage period.

Pathak and Gupta (1984) reported that there was no decline in seed germination, in *Hardwickia binnata*, upto 14 months after that seed germination decreased by 9%. Williams (1984) reported that in *Quercus alba* seeds stored in polythene bags, when tested after 13, 26, 41, 54 days and found that in all cases germination decreased significantly with increased storage.

Bhagat (1986) found that in Silver fir, after 18 months of storage period viability increased from 22 to 65% in comparison to freshly collected seeds, which gave only 25% germination. Schaefer (1990) reported storage and germination of *Podocarpus milanjanus* seeds. Kandya (1990) studied in *Cassia glauca* that two years storage is beneficial to increase germination capacity.

Influence of storage conditions and containers on seed germination has also been studied by many workers. Khare *et. al.* (1990); Jindal *et. al.* (1990); Rao *et. al.* (1991) Jagdish *et. al.* (1994) and Martinez *et. al.* (1994) etc.

The effect of storage temperature on germinability of tree seeds has been studied by many workers (Roe 1940, Heit 1967a, 1967b); Allen 1957; Tang and Tamari 1973; Gupta and Sood 1978; Yadav *et. al.* 1987a).

Bahuguna *et. al.* (1968) studied seed germination behaviour of *Berberis lycium* in the nursery pots, filled with soil, sand and GYM in 2:1:1 ratio. They found 50% germination in the nursery and 33% under laboratory conditions. Speed of germination was rapid (germination completed in 15 days) in nursery as compared to germination completed in 18-21 days to that in laboratory. Recently, Mishra and Kandya (1991) reported seed germination of *Dalbergia sissoo* under laboratory and field conditions and found that field conditions were better than those obtained under laboratory conditions.

Variation in seed coat colour are common in seeds of many crops. The relationship of seed coat colour on seed germination has been discussed in

different agricultural crops (Steward and Carlson 1932; Nel and Burger, 1967; Gugnani, *et. al.* 1975; Bonner, 1976; Edwards, 1980).

Williams (1958) found that black seeds of *Halogeton glomeratus* may had strong viability and excellent germination in comparison to brown coloured seed. Bonner (1976) found that in *Liriodendron tulipifera* colour change of fruit was best indicator to assess the seed maturity. Penafiel *et. al.* (1982) found in *Pinus kesiya* that seeds with black discoloration had higher germination percentage (80%) than brown (15.6) or white (21.1%).

Seeds usually germinate when conditions become suitable else they are capable to withstand adverse environment conditions for long period. The length of the time that embryo retain their life span, referred to as their viability, varies enormously. The longest period of viability ranging from a few days to several thousand year have been reported. For example in *Nelumbo nucifera* (Lotus) seeds were found to be viable after being buried for about 1000 years in peat moss under dry lake bed.

The term 'viability' has been defined by several scientists of different times Baldwin. (1942) suggested seed viability as the possibility of growth and referred it to as the potential capacity of seed to germinate. Barton, (1961) pointed out that viability is a condition of seeds in the sense of being capable of growth and survival. Schupmeyer, (1974) stated that viability is potentiality of seed to germinate. Agrawal, (1980) described viability as the ability of seed to

live, grow and developed. Bonner (1984) defined seed viability as the state by being capable of germination and subsequent growth and development of normal seedling.

Hence, in all probability viable seed is one which is capable of germination under proper external and internal conditions.

Viability of seed can be evaluated by physical and biochemical tests. There are many ways to test the viability of seed and have been in practice since very long time. Floating test to separate dead and viable seed has been utilized for a long time with a view that the former float and later sink in water. This method is quite old and is not applicable for light weighted and winged seeds. It is successful for large seeded species. But in some cases viable as well as dead seeds float on water due to some morphological characters.

The oldest method to test viability is by cutting test. Seeds from a lot are taken randomly, cut into two halves and observed directly. This method is suitable to eliminate empty seeds. The main drawback of this test is that where seeds, otherwise appear sound in every way, may still be incapable of germination due to immaturity or invisible injury. Turesson, (1921) and Barton, (1961) reported that methylene blue was used to test the viability of seeds. Neljubov, (1925) used Indigocarmine to stain endosperm and embryo to determine the seed vigour and viability. This method was applied to the seeds of a number of tree species by Safer-Safonova (1934, 1937). This method depends upon the ability of certain

organic dyes to penetrate and stain dead tissues much more rapidly then live ones. The technique involves extraction of the embryo from the seeds before treatment with the dye. Indigocarmine has been widely used in the USSR for the determination of viability of tree and shrub seeds. Gost SSSR (1968) applied Indigocamine test for forest trees & shrubs. Gadd, (1944) used malachite green to test viability in pea seeds.

Heit, (1955) has described the exised embryo test as 'efficient' accurate and timely for the seed analyst. Robocker *et. al.* (1969) determine the viability of *Heteropogon* seed by excised embryo test method and compared with germination test.

Simak (1957) developed the use of x-rays contrast method for testing the viability and since then, this method has been used with various modifications.

Babeley and Kandya (1986) studied excised embryo to determine the viability of six tropical tree species. Mathews and Powell (1981) and Yadav *et. al.* (1987) used electrical conductivity as a measure of seed viability of six tropical species. Use of electrical conductivity measurements of seed leachates to determine seed viability was based upon the fundamental work of Osterhout, (1922) who established the relationship between cell death and release of electrolyte, using this technique .Frick and Hibbard, (1952) studied the relationship between seed viability and electrical conductivity.

The importance of tetrazolium salt test seed viability was first reported by Kuhn and Jerchel (1941) Lakon (1942) demonstrated that the location and degree of staining could be correlated with seed vigour the "Topographic tetrazolium method for determining the germination capacity of seed" was published by Lakon (1949). After that many improvements have been made in staining techniques.

After the successful application of tetrazolium chloride for the determination of seed viability, almost all the seed viability tests were performed with the help of tetrazolium chloride. Various workers worked on various plant species and tested viability of the seeds with the help of tetrazolium salt.

Porter *et. al.* (1947) successfully assessed the seed viability of several leguminous seeds with tetrazolium stain method. Venkatanathan (1951) tested seed viability of paddy seeds. Mukherji (1956) also applied tetrazolium chloride test in many agricultural and vegetable crop seeds. More (1964, 1966, 1968, 1970, 1973, 1975, 1976) contributed a lot for the tetrazolium testing of seed viability. Unalcin (1979) found a simple linear correlation between sunflower seed viability and tetrazolium staining test. Yadav (1989) has described tetrazolium colouring in numerous tropical forest tree species. Schatral and Fox (1994) tested viability of Hibbertia seeds using tetrazolium chloride. Recently, Balaiya (1996) also tested the TTC on seeds of leguminous tree species.

Material and Methods :

Seed germination studies on two species of Calotripis viz., *C. gigantea* and *C. procera* have been done by taking the healthy seeds. Germination trials of fresh as well as stored seeds were conducted simultaneously in the laboratory , to find out the effect of imbibition, temperature, moisture, storage, colour differentiation and hormones. etc. on seed germination

Germination test : In different studies of germination 400 seeds of each species in four replicates were taken for germination. Different seeds lots were mixed thoroughly. After imbibition seeds were placed on moistened filter paper in petridishes in seed germinator incubator. Petriplates were sterilized and lined with double layer of filter paper. The petriplates were moistened with distilled water and seeds were kept on filter paper. All the petriplates were incubated at 35°C. The filter papers of petriplates were moistened with 2 ml of distilled water on alternate days. For germination studies sound seeds were sterilized by keeping them in 0.1% $HgCl_2$ for five minutes for the removal of external microflora.

(1) Seed storage **:** Seeds were stored in sealed polythene bags soon after their collection and stored at room temperature, 10(±1)°C, 15(±1)°C, 25(±2)°C, 45(±2)°C, at 90(±1) % relative humidity (with constant temperature of 30°C observation were taken at an interval of 4; 6; 8; 12;..24, months and germination per cent and plant per cent was calculated.

(2) Imbibition : Different sample of 2x100 seeds were soaked in distilled water upto 4, 6, 8, 12, 16, 18, 24 and 36 hours, after completion of different imbibition period, seeds were placed on moistened sterilized filter paper in petriplates ,then petriplates were placed in seed germinator incubator. Observations were recorded daily upto 30 days. Germination per cent and plant per cent were calculated after 30 days.

(3) Towel paper method : Seed lots of 50x4 were placed on moistened sterilized towel paper in seed germination incubator. Observation was made daily upto 15days. Radicle emergence was taken as a criterion for germination (ISTA, 1985).

(4) Silica sand : Seed lots of 50x4 were placed on moistened sterilized silica sand in seed germination incubator. It was placed on trays. Observation was made as above.

(5) Artificial growth media : The media used during investigation along with their ingredients are given below : (Jensen's Seedling Agar, Jensen, 1942).

$CaHPO_4$	1.0g
K_2HPO_4	0.2g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$FeCl_3$	0.1g
Distilled water	1000ml
pH	7
Agar	8-15 g/l according to use of deep as slope.

In 1000ml distilled water all chemicals were mixed and shaken by glass rod. P^H of this solution, if less than 7, alkli was added and if high, above 7, dil HCl was added. This solution was heated for 5 minutes and weighted quantity of agar was added and heated till agar formed a gel. It was sterilized in an autoclave at 15 psi, to a temperature 121°C for half an hour.

(6) Temperature : Studies on the effect of temperature on seed germination was done by fixing the temperature of seed germinator (Sew, India) at following constant temperatures 10°C, 20°C, 25°C, 30°C, 35°C and 40°C.

(7) Moisture content : Soon after seed collection and after definite storage period, seed moisture content was determined. Determination was carried out in triplicate on three independently drawn samples (10 gram seeds) for each species. The seed weight was determined by analytical balance. Seeds were kept inside paper packets in an oven at constant temperature 60°C for 24 hours, cooled in desicator and reweighed.

Moisture content % was calculated and expressed as follows :

$$\text{Moisture content \%} = \frac{\text{Fresh weight. - Oven dry weight.}}{\text{Fresh weight}} \times 100$$

(8) Colour differentiation test : Seed population of each species was minutely observed under dissecting microscope and different coloured seeds of each species were separated and germination per cent was noted by testing seeds in seed germinator incubator.

(9) Viability test : All the collected seeds were thoroughly washed with or distilled water in separate beakers and rinsed with sterlized water for 2-3 times and ultimately kept in distilled water in separate beakers for 12 hrs for imbibition.

(i) Cutting test : Imbibed seeds were cut into longitudinally, into two halves with the help of blade. Cut halves were placed on glazed glass plate for further observation. The evaluation was done by naked eye to find out healthy, sound, empty and immature seeds in the sample. Seeds with normal colour of endosperm and well developed embryo have been considered viable and germinable. Whereas seeds without embryo or abortive embryo or with milky, mouldy, decayed

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shriveled were considered as non-viable (Bonner, 1974).

(ii) Biochemical test : The biochemical test of seeds was done by 2,3,5....triphenyl tetrazolium chloride (TTC) following the method first given by Lakon (1949) and ISTA (1985).

The various steps for testing viability of seeds by TTC solution is described in brief below:-

(a) Pre-moistening of seed samples : The seeds were imbibed in distilled water for a period of 8 to 12 hours. (ISTA, 1976, 1985, Rule 6.5 2A). Imbibed seeds were placed on filter paper to drain excess water and were kept exposed to air for some time to allow easier penetration of TTC.

(b) Decoating of seeds prior to staining : The seed coat was removed with the help of forceps or thumb nail or needles in case of swollen seeds. The seeds were kept

moist until the whole replicate was completed and seeds were bisected while on filter paper with the help of a blade to expose the main structure of the embryo. One half of each seed was immediately transferred from the filter paper to the solution with forceps to avoid drying while other half was discarded.

(c) Preparation of staining solution : 2, 3, 5-triphenyl tetrazolium chloride (TTC) is a light yellow water soluble powder. To get the pH range of 6.5 to 7.5, TTC. salt was dissolved in a phosphate buffer solution prepared in the following way. Solution 1-9.078gm of KH_2PO_4 was dissolved in 1000ml of water. Solution 2-11.876gm of $Na_2HPO_4.2H_2O$ was dissolved in 1000ml of water. Then 400 ml of solution 1 was mixed with 600 ml of solution 2 to give buffer of pH ranging from 6.5-7.5. 01 and 1% solutions of TTC were prepared and stored in two different umber coloured glass bottles in dark at room temperature up to 6 months.

(d) Incubation : For TTC test the cut halves of the seeds as prepared above were immediately immersed in TTC solution of desired dilutions taken in different beakers. Coloured beakers were incubated at 30°C in an oven in dark. After incubation, seeds in the test were immediately washed with water 2-3 times to drain the excess staining solution.

(e) Evaluation of staining pattern : Stained seeds were placed in glazed glass plate under a dissecting microscope for examination. Evaluation was done as per Rule 6.5, 2A.4 (ISTA, 1985). All essential structures such as meristem and embryo were examined carefully for staining. Seeds with weakly stained embryo non-stained essential structures was considered non-viable (Leadem, 1984).

(10) Hormone treatment : 4 months old seeds of both species were soaked in different concentrations (1ppm, 2ppm, 5ppm, 10ppm, 20ppm 50ppm and 100ppm) of different hormones viz. Indole-3 acetic acid, (IAA), Indole-3-butyric acid, (IBA) and Gibberellic acid, (GA_3) for 24 hours then they were kept for germination on moistened filter papers in seed germinator incubator after 30 days, seed germination per cent and plant per cent and seedling length were calculated.