Effect of Stratification and Gibberellic acid on seed germination of Capsicum(*Capsicum annum)*

A Thesis

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Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University

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Submitted by

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Examination Roll No: MS-160718

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Biotechnology and Genetic Engineering Discipline, Life Science School,
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Khulna-9208, Bangladesh

May, 2018

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Declaration

This thesis reports the original research work of the author, except as otherwise stated. The materials presented have not been submitted either in whole or in part for a degree from this or any other university. The findings of the research have not been/will not be published anywhere without the concurrence of the concerned supervisor.

Tahera Khatun

Tahera Khatun
Student ID: MS-160718
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Dedicated to

My beloved parents

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At the very beginning, I offer my deepest gratitude to the almighty Allah for all his merciful blessings bestowed upon me Allah, to whom all praises, goes for enabling me to complete my review work.

It is my pleasure to express my greatest and profound gratitude and heartfelt with best regards to my honorable supervisor, Dr. S. M. Abdul-Awal, Professor of Biotechnology and Genetic Engineering Discipline for his active guidance, keen interest, continuous inspiration and constructive criticism for which the work comes to light.

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The Author

May, 2018

Abstract

Capsicum or Sweet pepper (*Capsicum annum*) is a perennial herbaceous plant in the nightshade family (Solanaceae). It is one of the world's most widely used spices as well as vegetable, colorant and pharmaceutical. The climate, environmental condition and other important agronomic attributes of Bangladesh has promising aspects for successful cultivation of Capsicum. But at the very beginning, it is necessary to identify the challenges and take proper measures to accelerate the production of Capsicum in our country. The first challenge for cultivating this plant is long germination time which takes 2-3 weeks in compared with model plant Arabidopsis that requires only 3-4 days for germination. In this regards, the application of stratification or cold treatment and gibberellic acid (GA3) can be a productive solution to combat this challenge. Therefore, the sterilized seeds were sown on petri dishes containing filter paper and MS media, separately. Then the seeds were incubated for 2 days and 4 days at three different temperatures viz. 4°C, 20°C and 27°C and identified appropriate GA3 concentration to reduce the number of days required for Capsicum seed germination. A high seed germination rate (90%) was found within 4- 5 days when seeds were incubated for 4 days at 27°C temperature compared to 4°C and 20°C temperature. GA3 at 40 mg/L concentrations showed a very high response for germination (100 %) of Capsicum seed compared to other concentrations (0 mg/L, 5 mg/L, 10 mg/L, 20 mg/L). A Combination of high temperature (27°C) and GA3 at 40mg/L concentration showed 90% germination within 5 days compared to 0 mg/L of GA3 at 27°C where only 65% seeds were germinated within 5 day.

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List of Abbreviations

ABA	Abscisic acid
et al	et alia and other people
gm	Gram(s)
ml	Milliliter
mg	Milligram
mg/L	Milligram(s) per liter
mM	Mille molar
MS	Murashige and Skoog(1962) medium
Viz	Videlicet=namely
µM	Micro molar
°C	Degree celcious
pH	Acid base Measurement of Solution
M	Molar
v/v	volume per volume
w/v	weight per volume

Chapter one

Introduction

Capsicum annuum also known as bell or sweet pepper is a perennial herbaceous plant of Solanaceae family, which is native to Central and South America and was domesticated over thousands of years ago. It is now cultivated around the world and one of the world's most widely used spices, vegetable, ornamental and medicinal plant (Barchenger & Bosland).Capsaicin (8-methyl-N-vanilly-6-noneamide), a pungent compound found in Capsicum has significant therapeutic properties. In addition, the fruit contains coloring pigments, resin, protein, cellulose etc. The fruit also possesses a unique flavor, aroma, taste, and contain a high level of beta carotenoids, vitamins C, B-complex, vitamins E and minerals (Ochoa-Alejo & Ireta-Moreno 1990).

Capsicum is a warm climate crop and requires 25-30°C for optimum seed germination (Carter & Vavrina, 2001). It is sensitive to frost, therefore the crop can only be grown successfully in the regions with more than three frost-free months in a year. Flowering begins one to two months after seed germination and one month later many fruits are ready for harvest at the green-mature stage. Fruit yields are higher when temperature ranges from 20 to 30°C while extremely high temperatures result in poor fruiting (Bodhipadma, 2002). In the Mediterranean region, Capsicum is established using seedling transplants for open field production in early spring or into protected cultivation in late summer (Demir *et al.*, 2008). Bangladesh has huge prospects of producing Capsicum and some advanced farmers started to grow this crop periodically to fulfill the local demand of city areas (Rahman *et al.*, 2014). The climate, environmental condition and other important attributes are available in some regions of Bangladesh for successful production of Capsicum.

An experiment conducted in the plant biotechnology laboratory at Biotechnology and Genetic Engineering Discipline, Khulna University indicates that Capsicum seeds take 2-3 weeks to germinate which is very high in comparison with the model plant Arabidopsis. Arabidopsis takes only 3-4 days for germination. Seed coats and surrounding structures may influence the ability of a seed to germinate through interference with water uptake, gas exchange, diffusion of endogenous inhibitors, or by mechanical restriction of embryo growth (Ikuma and Tmmann, 1963, Mayer and Shain, 1974). Late seed germination is an obstacle for the evaluation of the seed vigor

of crops as well as the crop requires a long time from sowing to harvest. To combat this challenge, stratification or cold treatment and gibberellic acid (GA3) treatment of the seeds of Capsicum can be a productive solution for seed germination within short period of time. Stratification is the process of treating stored or collected seed before sowing to provide natural winter conditions that a seed must endure before germination. After reaching physiological maturity, seeds of many trees enter a state of deep dormancy, and for germination they require moist and cold treatment (stratification) (Pawłowski *et al.*, 2004). In the process of stratification, seeds are subjected to both cold and moist conditions. This cold moist period triggers the seed's embryo, its growth and subsequent expansion which eventually break through the softened seed coat in its search for sun and nutrients. The seeds of *C. annuum* cv. *sivri* germinated well at constant temperatures of 15°C (83%), 20°C (100%), and 30°C (88%). The germination was 100% in the seeds placed in distilled water and left at 20°C (Cavieres and Arroyo, 2000). Another germination inducing factor of plants is the Gibberellic Acid (GA3). GA3 is a very potent hormone and treatment with high concentrations of GA3 is effective in overcoming dormancy and causing rapid germination of seed. Two different mechanisms of action have been proposed to explain the role of this endogenous hormone in the control of germination. The first one is the induction of the expression of genes encoding enzymes for hydrolyzing and weakening the endosperm cells. This tissue confers part of the mechanical resistance to radicle protrusion, as demonstrated in tomato, tobacco, and barley (Debeaujon and Koornneef, 2000). The second mechanism consists of a direct stimulating effect on the growth potential of the embryo, as suggested for Arabidopsis. GA3 is involved both in overcoming dormancy and in controlling the hydrolysis of starch and protein reserves (Harvey and Oaks, 1974). The presence of adequate levels of this acid in seeds stimulates the synthesis, activation and secretion of hydrolytic enzymes, mainly α-amylase, releasing reducing sugars and amino acids which are essential for embryo growth (Debeaujon and Koornneef, 2000, Vieira *et al.*, 2002).

The overall goal of the present study is to evaluate the effect of stratification and GA3 on germination of Capsicum in order to explore the optimum conditions required for large scale production of this crop in Bangladesh.

The main objective of this project is

- To reduce the number of days required for Capsicum seed germination under different temperature conditions at different incubation periods.
- To identify an ideal concentration of GA3 which will accelerate the germination process.

Chapter Two

Review of Literature

2.1 *Capsicum annum*

Capsicum (*Capsicum annuum*) is also called as bell pepper or sweet pepper and is one of the most popular and highly remunerative annual herbaceous vegetable crop. Its species are native to the Americas, where they have been cultivated for thousands of years. Capsicum is cultivated in most parts of the world, especially in temperate regions of Central and South America and European countries, tropical and subtropical regions of Asian continent mainly in India and China. (Sreedhar *et al.*, 2013). It is the world's second most important vegetables after tomato (Ara *et al.*, 2007) .

Fig.1: *Capsicum annum*

Bell pepper is grown nearly all over Bangladesh especially in Bogra and Norsingdi, (Careaga *et al.* 2003)Sylhet in rabi season and area under 259383 acres. Most of the varieties cultivated in Rabi season and production is about 176134 metric ton (Hasanuzzaman *et al.*, 2013). Ideal growing conditions for peppers include a sunny position with warm, loamy soil, ideally 21 to 29 °C, that is moist but not waterlogged. Extremely moist soils can cause seedlings to "damp-off" and reduce germination. The plants will tolerate temperatures down to 12 °C and are sensitive to frost. For flowering, *Capsicum* is a non-photoperiod-sensitive crop. The flowers can self-pollinate. However, at extremely high temperature, pollen loses viability, and flowers are much less likely to pollinate successfully. *Capsicum* consists of

20–27 species (Walash and Hoot, 2001) five of which are domesticated: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. (Heiser *et al*, 1969).

The crop is very sensitive to environmental factors (Bhatt et al., 1992). Owing to its sensitivity, its yield is affected significantly. Capsicum is the most important summer crop of temperate regions but now a days effort are being made to grow sweet pepper in Bangladesh (Paul, 2009). Some advanced farmers grow capsicum sporadically to meet the demand of the periphery of Dhaka city (Saha and Salam, 2004). Sweet pepper is a minor vegetable in Bangladesh and its production statistics are not available (Hasanuzzaman, 1999). Small scale cultivation is found in peri-urban areas primarily for the supply to some city markets in Bangladesh. It has got a good demand to some big hotels in the capital city to feed the foreigners residing in Bangladesh (Rashid, 1999).

2.2 Health benefits of capsicum:

In addition to use as spices and food vegetables, *Capsicum* species have also been used as medicines and lachrymatory agents. Peppers are highly nutritious. They have more Vitamin C than an orange, and a typical bell pepper contains more than 100% of the daily recommended value for Vitamin C. They also have relatively high amounts of Vitamin B6. Fresh fruit is 94% water (Rakshit *et al.*, 2008)

Capsicum is one of the most nutrient-dense foods available. By itself, capsicum has so many healing properties, but when taken together with other herbs, fruits and vegetables, the nutrients absorption are multiplied hundredfold.

- **Anti-bacterial and anti-fungal:** The anti-septic properties in capsicum makes it effective in fighting food poisoning. Coupled with a good supply of probiotics, yeast and fungal infection problems, like ring-worm, shingles, athlete's foot, etc. can be easily eliminated (Careaga *et al.* 2003).

- **Anti-aging:** Capsicum has antioxidant property which is highly effective to protect the skin from free radical damage known to cause signs of aging (Khan et al.)

- **Blood clots, prevent:** The very high content of vitamin C in capsicum makes it very effective in preventing blood clot, thus preventing strokes ,(Al-Snafi)

- **Cancer, prevent:** All the colored capsicums contain very high antioxidant and phyto-nutrients that are especially helpful in preventing cancers of the bladder, cervix, pancreas and prostate (Prasad *et al.* 2006).
- **Cholesterol:** These colorful juices can significantly help to reduce cholesterol. The concentrated antioxidant fights oxidative stress that is the main culprit in oxidizing the LDLs in our blood. In the process, it also retards the development of atherosclerosis (hardening of the arteries) and lower blood pressure (Robinson, 2013).

- **Digestive system:** Capsicum is a stimulant herb. It helps relieve gastrointestinal problems like indigestion, stomach ulcers, colic, dyspepsia, diarrhea and even help reduce excessive flatulence (Rolland , 2006).

- **Immune system:** The strong content of vitamin C stimulates white cells to fight infection, naturally building a good immune system(Rasul,2004)
- **Metabolism, enhanced:** Increases our body metabolism by lowering triglycerides which are stored in our body fats. This helps to burn calories more effectively (Martinez *et al.*, 2014)
- **Nose bleeding:** The rich vitamin C helps to heal, repair strengthen the lining of the mucous membranes to prevent nose bleeds. Mixing capsicum juice with lotus root juice gives better effect (Klemic *et al*, 2012).
- **Optical system:** The high vitamin C and beta-carotene makes capsicums especially beneficial in preventing eye problems like astigmatism, cataracts and macular degeneration(Pal *et al.*,2007)
- **Pain relief:** Capsaicin in capsicum blocks transmission of pain, so it can help relieve pain to a certain degree. It is also effective for eliminating headaches and migraines(Venugopal,2010)
- **Respiratory problems:** The high level of vitamin C coupled with flavonoids make capsicum a very good food that helps prevent respiratory problems like asthma, emphysema, wheezing, lung infections, etc(Ito *et al*,.2005)

2.3 Demand of foreign vegetables in Bangladesh:

Farmers in Bogra are cultivating various types of foreign vegetables these days as their demand is high. Every year, about 400 metric tons of foreign vegetables are produced in three upazilas of the district. Foreign vegetables like lettuce leaf, Chinese leaf, capsicum, beet root, broccoli, red cabbage, squash, French bean, sweet corn, baby corn, Thai ginger, Thai Basil, lemon grass, celery leaf, Chinese cabbage etc are highly demandable these day. The farmers sell them at hotels like Pan Pacific Sonargaon Dhaka, Dhaka Westin, and many Chinese restaurants, super shops of the capital city. The farmers can harvest lettuce leaf, lemon leaf and Chinese leaf in 40 days and broccoli in 70 days, French beans in 60 days. Broccoli, red cabbage cost Tk 20,000 per bigha and the profit is Tk 40,000 in an average (The daily star, 2015) .

2.4 Germination of seeds:

Germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (Bewley and Black, 1994). The visible sign that germination is complete is usually the penetration of the structures surrounding the embryo by the radical, the result is often called visible germination. Subsequent events, including the mobilization of the major storage reserves, are associated with growth of the seedling. Virtually all of the cellular and metabolic events that are known to occur before the completion of germination of non-dormant seeds also occur in imbibed dormant seeds (Bewley, 1996).

2.5 Germination of *Capsicum annum* L.:

Germination and emergence of pepper (Capsicum annum L.) seeds is often slow and non-uniform under normal as well as stress temperatures (Demir and okcu, 2004). Seed invigoration treatments have been developed and used extensively to improve germination and seedling emergence in a wide range of species. Generally, it takes 2-3 weeks for germination. This erratic and non-uniform emergence often leads to non-uniform transplants, poor crop stands, and may necessitate crop replanting. Often, reduced yields and delayed harvest (losing early high market prices) can be caused by slow stand establishment. Seed dormancy could be considered simply as a block to the completion of germination of an intact viable seed under favorable conditions (Hilhorst, 1995).

A completely non-dormant seed has the capacity to germinate over the widest range of normal physical environmental factors possible for the genotype (Baskin & Baskin, 1998). Besides the basic requirement for water, oxygen and an appropriate temperature, the seed may also be sensitive to other factors such as light and/or nitrate. Germination commences with the uptake of water by imbibition by the dry seed, followed by embryo expansion. The uptake of water is triphasic with a rapid initial uptake (phase I, i.e. imbibition) followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs as the embryo axis elongates and breaks through the covering layers to complete germination (Manz *et al* .,2005). In typical angiosperm seeds the embryo is surrounded by two covering layers: the endosperm and testa (seed coat). Cell elongation is necessary and is generally accepted to be sufficient for the completion of radicle protrusion (visible germination) (Kucera *et al*, 2005).

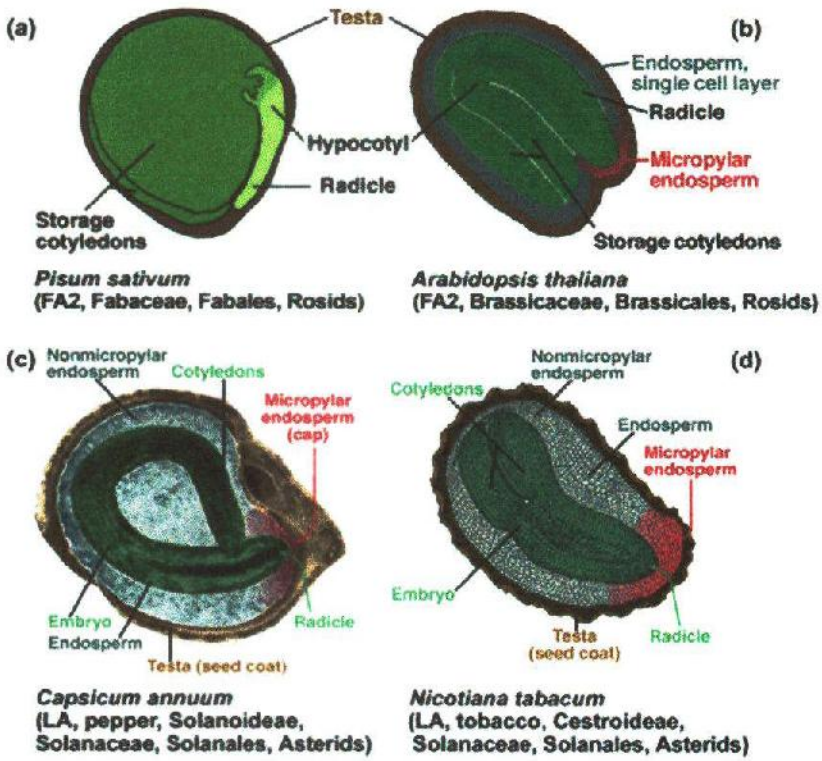


Fig.2: Biodiversity of the structure of mature seeds of angiosperms and the importance of the seed covering layers. Mature seeds of (a) pea (*Pisum sativum*) (without endosperm) and (b) *Arabidopsis thaliana* (single cell layer of endosperm) are characterized by embryos with storage cotyledons. The micropylar endosperm (several cell layers) is known to be a germination constraint of Solanaceae seeds (c, d). The diploid embryo is surrounded by two covering layers: the triploid (in most species) endosperm

(nutritive tissue; mostly living cells) and the diploid testa (seed coat; maternal tissue; mostly dead cells). In several species the endosperm is completely obliterated during seed development and the nutrients are translocated to storage cotyledons ,FA2 and LA are seed types(Finch-Savage, W. E., & Leubner-Metzger,2006)

2.6 Stratification or cold treatment:

Stratification is the process of treating stored or collected seed before sowing to provide natural winter conditions that a seed must endure before germination. To overcome late germination problem, stratification at low temperature for a sufficiently long period is required. The stratification period and temperature required for breaking seed dormancy varies from cultivar to cultivar due to genetic and climatic factors (Carlson and Tukey, 1945). Seed coat also plays an important role in seed dormancy and it has been possible to induce germination of un-stratified seeds either by removing the seed coat or by subjecting them to running water before sowing (Flemion, 1934). Stratification might act simply to lower the rate of enzymatic reactions taking place in the seed, and might cause differential changes in enzyme concentrations or in enzyme production (Bewley and Black, 1994).

The treatments involve a period of controlled hydration of the seed to a point close to but before the emergence of the radicle, after which the seeds are dried back to their initial moisture content before sowing (Matthews & Powell, 1988) After reaching physiological maturity, seeds enter a state of deep dormancy, and for germination to occur they require moist, cold treatment (stratification) (Pawlowski et al ,2004). The mechanisms of dormancy breaking at low temperature are virtually unknown. The process of seed dormancy breaking involves many parallel and concomitant pathways (Ross, 1996). The highest increase in the number of proteins was observed after hydration. The proteins associated with dormancy breaking in the last weeks of cold stratification appeared just before seed germination (Ross and Bradbeer, 1971). This cold moist period triggers the seed's embryo, its growth and subsequent expansion which eventually break through the softened seed coat in its search for sun and nutrients. The seeds of *C. annuum* cv. *sivri* germinated well at constant temperatures of 15°C (83%), 20°C (100%), and 30°C (88%). The germination was 100% in the seeds placed in distilled water and left at 20°C (Cavieres and Arroyo, 2000).

The response of seeds to germination at low temperatures has been grouped into three general categories: 1) Seeds which will germinate at temperatures near freezing 2) seeds which fail to germinate at low temperatures but if moved to a more favorable temperature will germinate normally, and 3) seeds which

when imbibed at low temperatures showed injury symptoms when subsequently grown at warmer temperatures. This injury is known as chilling injury (Simon, 1979).

Goodall and Bolas (1942) reported that chilled imbibed tomato seeds at temperatures of to 11°C for periods of 10 to 20 days, get germinated than untreated seeds at more favorable temperatures. The plants grown from the chilled seeds produced more fruit and had a concentrated yield at the beginning of the cropping season.

Pepper seeds germinate slowly at less than optimal temperatures. Cochran (1935) reported that no peppers germinated after 45 days in a greenhouse with a temperature range between 4 and 10°C. However, with each 6° increase in temperature range, the speed of germination increased until germination was obtained in five to six days in a greenhouse with a temperature range between 32 and 38°. Heydecker (1974) reported that when imbibed pepper seeds were held for three weeks at 3, 7 and 10°, they failed to germinate. But, when they were transferred to 30° they germinated faster than untreated seeds placed at 30° from the outset, with no apparent injury.

Stratification might act simply to lower the rate of enzymatic reactions taking place in the seed, and might cause differential changes in enzyme concentrations or in enzyme production (Bewley and Black, 1994)

2.7 Gibberellic acid and seed germination:

Gibberellic acid (actually a group of related substances called gibberellins) was discovered as a metabolic byproduct of the fungus *Gibberella fujikuroi*, which causes the stems of growing rice to elongate so rapidly the plant collapsed. Synthetic forms of gibberellic acid are available commercially. Gibberellic acid (GA) is a very potent hormone whose natural occurrence in plants controls their development. Since GA regulates growth, applications of very low concentrations can have a profound effect.

GA3 is a very potent hormone and treatment with high concentrations of GA3 is effective in overcoming dormancy and causing rapid germination of seed. Two different mechanisms of action have been proposed to explain the role of this endogenous hormone in the control of germination (Karlsson *et al*, 2006). The first one is the induction of the expression of genes encoding enzymes for hydrolyzing and weakening the endosperm cells. This tissue confers part of the mechanical resistance to radicle protrusion, as demonstrated in tomato, tobacco, and barley (Debeaujon and Koornneef, 2000). The second mechanism consists of a direct stimulating effect on the growth potential of the embryo, as suggested for Arabidopsis. GA3 is involved both in overcoming dormancy and in controlling the

hydrolysis of starch and protein reserves (Harvey and Oaks, 1974). The presence of adequate levels of this acid in seeds stimulates the synthesis, activation and secretion of hydrolytic enzymes, mainly α-amylase, releasing reducing sugars and amino acids which are essential for embryo growth (Debeaujon and Koornneef, 2000; Vieira *et al* ., 2002).

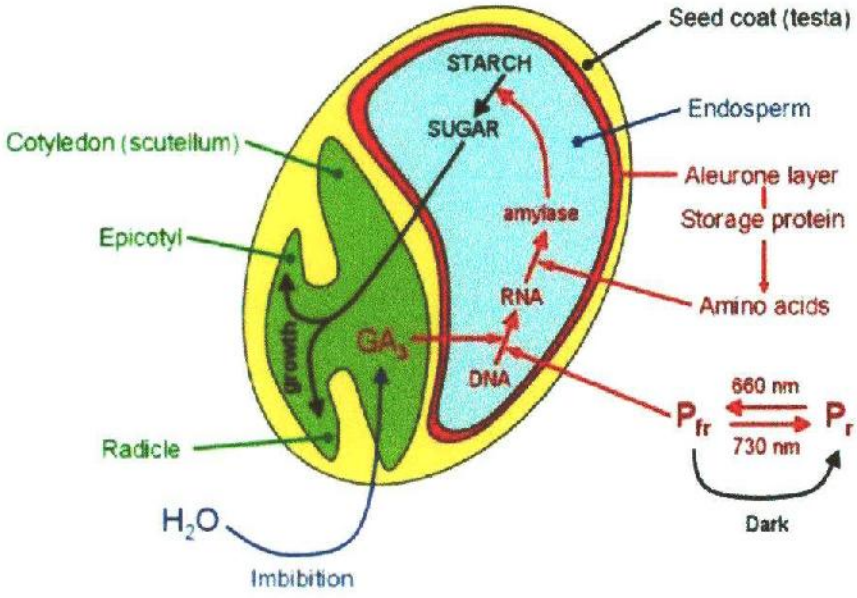


Fig.3: Breaking seed dormancy by GA3. (In barly with the imbibitions of water, the hormone signal in barly, gibberellic acid (GA3) is carried from the embryo to the aleurone of the endosperm. The GA activates the DNA for the gene encoding alpha-amylase in the aleurone cells. Transcription and translation of that gene results in the production of alpha- amylase inside the aleurone cells. This enzyme is shipped by ER into the golgi, sorted and packaged into vesicles, and exported into the vesicles and exported through the cell membrane by exocytosis. The amylase is dumped into the endosperm area. There the amylase break down starch into the maltose which is transported to the embryo. The sugar fuels respiration the embryo so it can grow. The radical protrudes from the seed coat and germination accomplished) (Baskin and Baskin,1998).

Gibberellic acid (GA3, GA4, and GA7) has been shown to break dormancy and increase germination in seeds of several genera (Bewley and Black, 1985) including certain penstemon (Meyer *et al*., 1995). Kitchen and Meyer (1991) reported that five concentrations of GA3 (0 to 500 mg/ml) in combination with a cold treatment (2°C) seeds that were treated with GA3 and stratified had higher germination

percentages. Raeber and Lee (1991) found that soaked seeds of Penstemon parryi in six concentrations of GA3 (0 to 500 mg/ml) for 24 h showed 87% to 97% seed germination.

Koyuncu F, (2005) reported that Pre-treatment with GA3 significantly enhanced germination of non-stratified black mulberry seeds. GA3 treatments at 1000 and 2000 mg/l concentrations yielded the highest germination percentage (60–67%). Increasing the concentration of GA3 resulted in an increase in germination percentage.

Chapter Three

Materials and Methods

3. Working Place

The study was conducted in the Plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna- 9208, Bangladesh.

3.1 Materials

3.1.1 Plant Materials

In this experiment mature seeds of bell pepper *(Capsicum annum)* were purchased from local market of Khulna used in germination test.

3.1.2 Chemicals

i. MS media (Murashige and Skoog ,1962) (Appendix A)

ii. Plant growth regulator: Gibberellic acid (GA3)

iv. Absolute Ethanol, Spirit, Hydrochloric acid (HCl), Sodium Hydroxide (NaOH), Agar powder, Sucrose, Sodium hypochlorite, Triton x-100 etc.

3.1.3 Instruments

Petri dishes ,Test tubes ,Beakers, Conical flask, Measuring cylinder, Magnetic stirrer, Electrical balance ,pH meter, Autoclave, Spirit lamp, Autoclave, Refrigerator, Laminar airflow cabinet, Incubator , Plant growth chamber etc.

3.2 Methods

3.2.2 Preparation of Stock Solution for MS media

At first, stock solutions were prepared for the final preparation of standard MS media. Freshly prepared stock solutions containing necessary ingredients were needed for very frequent preparation of culture media. Therefore, six types of stock solution (I, II, III, IV, V and VI) were prepared at the very beginning of the experiment and stored in a refrigerator at 4°C.

3.2.2.1 Stock Solution I (Nitrogenous Compounds)

200 ml (v/v) of stock solution was made up to 50 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask and 16.5 gm NH_4NO_3 and 19 gm KNO3 were added and dissolved with the help of magnetic stirrer. Finally, distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in refrigerator at 4°C.

3.2.2.2 Stock Solution II (Sulphur Compounds)

200 ml (v/v) of stock solutions was made up to 100 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask. Then $MgSO_4.7H_2O$ (7.4 gm), $MnSO_4.4H_2O$ (0.446 gm), $ZnSO_4.7H_2O$ (0.172gm) and $CuSO_4.5H_2O$ (0.0005 gm) were weighted accurately and dissolved with the help of magnetic stirrer. Finally, distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in refrigerator at 4°C.

3.2.2.3 Stock Solution III (Halide Compounds)

200 ml (v/v) of stock solutions was made up to 100 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask and then appropriate amount of $CaCl_2.2H_2O$ (8.8 gm), $CoCl_2.6H_2O$ (0.0005 gm) and KI (0.0166 gm) were weighted accurately and dissolved with the help of magnetic stirrer. Finally, distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in refrigerator at 4°C.

3.2.2.4 Stock Solution IV (PO_4, BO_3, MoO_4 Compounds)

200 ml (v/v) of stock solutions was made up to 100 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask and then appropriate amount of KH_2PO_4 (3.4 gm), HBO_3 (0.124gm), $Na_2MoO_4.2H_2O$ (0.005gm) were accurately and dissolved with the help of magnetic stirrer. Finally, necessary amount of distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in a refrigerator at 4°C.

3.2.2.5 Stock Solution V (Fe-EDTA)

200 ml (v/v) of stock solutions was made up to 100 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask and then appropriate amount of $Na_2EDTA.2H_2O$ (0.746 gm), $FeSO_4.7H_2O$ (0.556 gm) were weighted accurately and dissolved with the help of magnetic stirrer. Finally, necessary amount of distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in a refrigerator at 4°C.

3.2.2.6 Stock Solution VI (Organic Compounds and Vitamins)

200 ml (v/v) of stock solutions was made up to 100 times of the final concentration of the medium. At first, 100 ml of distilled water was taken in a 250 ml conical flask and then appropriate amount of

pyridoxine HCl (Vit-B6) (0.001 gm), nicotinic acid (Vit-B3) (0.001gm), thiamin HCl (Vit-B1) (0.002 gm) and Inositol (0.02 gm) and glycine (0.04 gm) were weighted accurately and dissolved with the help of magnetic stirrer. Finally, necessary amount of distilled water was added to adjust the final volume to 200 ml and stored in reagent bottle in a refrigerator at 4°C.

3.2.3 Preparation of GA3 Stock solution

GA3 is not dissolved in H_2O.Therefore, 0.025 gm of GA3 powder was first dissolved in 300µl of 100% Ethanol. Then sterilized distilled water was added to make the final volume of 50ml which gives the stock Concentration of 0.5mg/ml .The required amount is calculated using the following formula:

V1S1 = V2S2

Where, V1= Final volume of media to be prepared

S1= Target concentration of growth regulator in media

S2= Stock concentration of growth regulator solution

V2= Required growth regulator volume

3.2.4 Preparation of MS Media

Preparation of 200 ml standard MS media without any special modifications was done using the following flow:

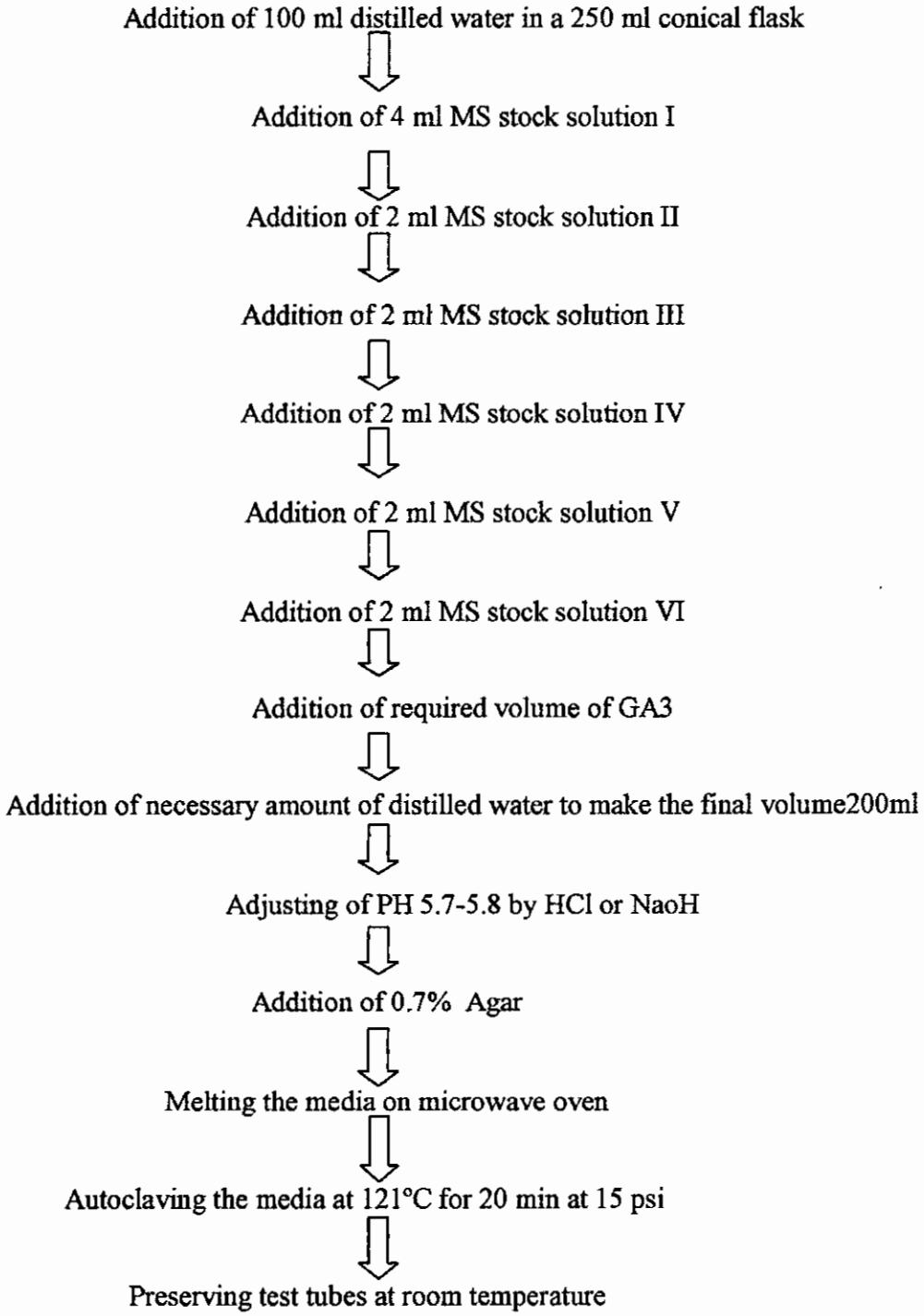


Fig.4: Flowchart for MS medium preparation (200 ml)

3.5 Seed Collection and Sterilization:

Seeds of Capsicum were sterilized by using 70% (v/v) ethanol followed by 10% sodium hypochlorite solution containing 0.05% (v/v) Triton X-100. Seeds were washed four to five times by sterilized distilled water.

3.6 Germination tests under cold treatment or Stratification:

Sterilized seeds (20 seeds/petri dish) were transferred into petri dishes containing water soaked double layer of filter paper and MS media separately.In both cases the petri dishes were transferred into 27°C (room temperature; control), 4°C, and 15°C in dark for two to five days. After stratification seeds were sown into Murashige Skoog (MS) medium from filter paper containing 0.8% bactoagar in absence of Sucrose. Seeds were grown in growth cabinet (27°C) for germination scoring. Alternatively, seeds were transferred into soil containing organic compost. In both cases, seeds were grown in growth cabinet (27°C) for germination scoring.

3.7 Germination tests under different concentration of GA3

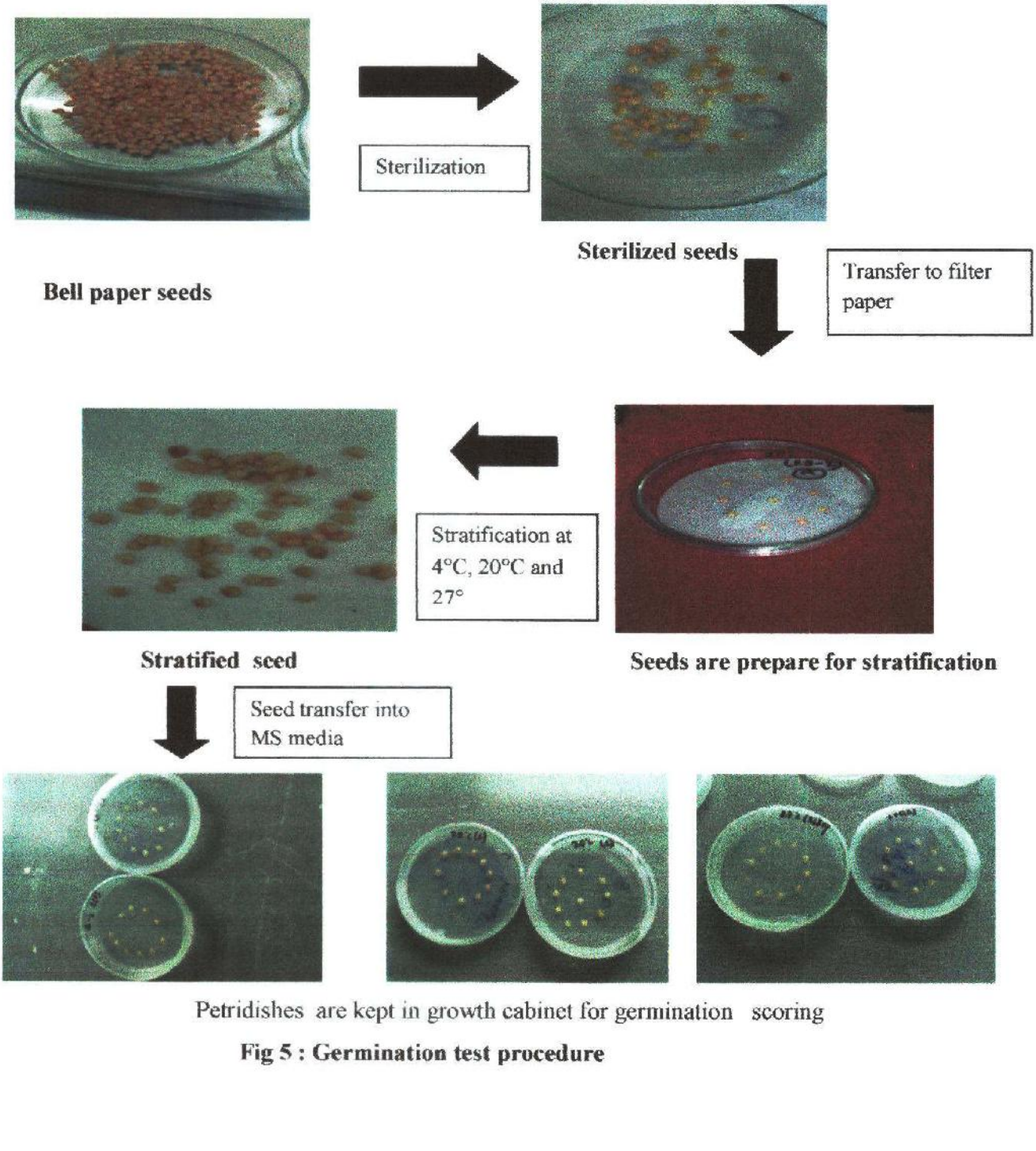
Sterilized seeds (20 seeds/petri dish) were directly sown into Petri dishes containing MS medium in absence of GA3 (control) and in presence of different(0,5,10,20,40mg/l) concentrations of GA3. Seeds were stratified at 27°C (room temperature; control) and 4°C for 3 days and then petri dishes were transferred into growth cabinet (27°C) for germination scoring.

3.8 Germination tests under the combined effect of Stratification and GA3

Sterilized seeds (30 seeds/petri dish) were directly sown into Petri dishes containing double layer filter paper and stratified under suitable temperature (27°C) identified in section 3.6. Afterwards, seeds were transferred into MS medium in absence of GA3 (control) and in presence of suitable concentration of GA3 (40mg/l) according to section 3.7. The petri dishes were transferred into growth cabinet (27°C) for germination scoring.

3.9 The overall procedure of germination test:

The steps of germination have been shown in the following figure:



Petridishes are kept in growth cabinet for germination scoring

Fig 5 : Germination test procedure

3.10 Germination in soil:

Sterilized seeds (20 seeds/petri dish) were transferred into petri dishes containing water soaked double layer of filter paper. The petri dishes were transferred into 27°C (room temperature; control), 4°C, and 20°C in dark for two to five days. After stratification seeds were sown into soil which containing soil ,vermicompost and sand as ratio of 2:1:1 t0 check the germination efficiency of Capsicum seeds.

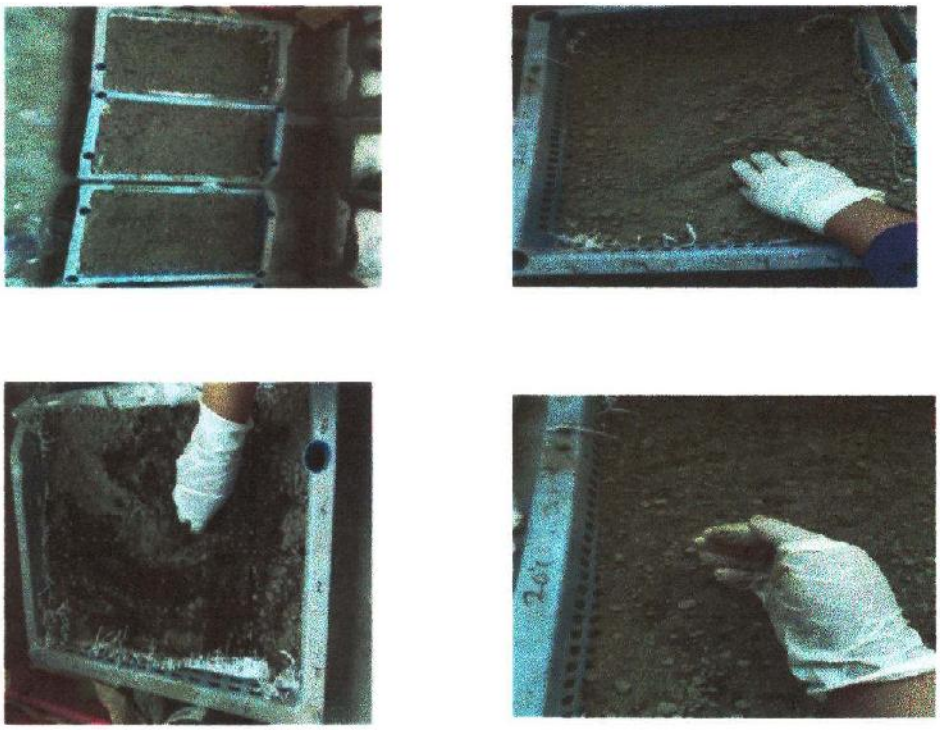


Fig.6: Preparation of soil for seed germination

3.11 Germination Scoring

Germination was scored visually in each day at the specific time point till 15 days. Graphs were prepared by percentage of germination versus no of days required for the germination. Data were analysed by Excel XP.

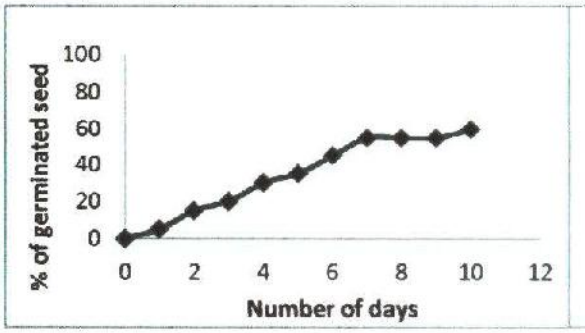
Chapter Four

Results

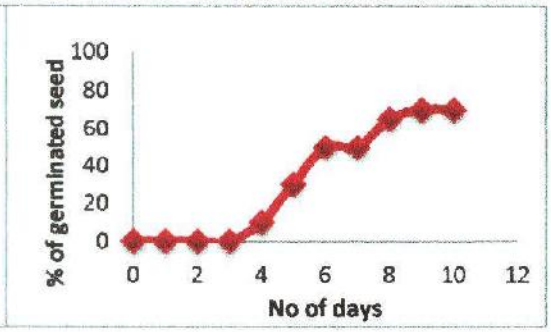
4.1 Effect of Stratification on seed germination at 4°C

Seeds directly sown in agar media and incubated at 4°C for 2 days and 4 days took a bit longer time to get germinated 70% seed showed germination within 10 days when stratified at 4°C for 2 days (Fig 1A), whereas 80% seed showed germination within10 days when stratified at 4°C for 4 days (Fig 1B). Alternatively, seeds directly transferred in water soaked double layer filter paper and incubated at 4°C for 2 days and 4 days. Then seeds were transferred into agar media and scored for germination. Seeds showed 90% germination in 10 days (Fig. 1C) and get germinated 100% in 11 days.(Fig:1D)

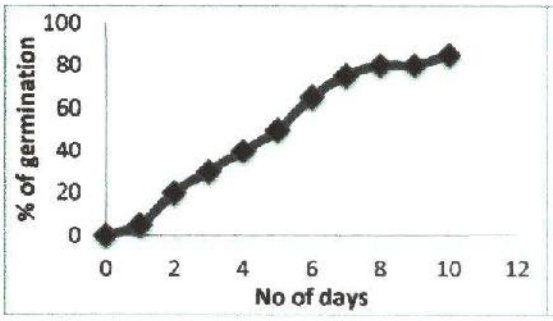
A.



B.



C



D

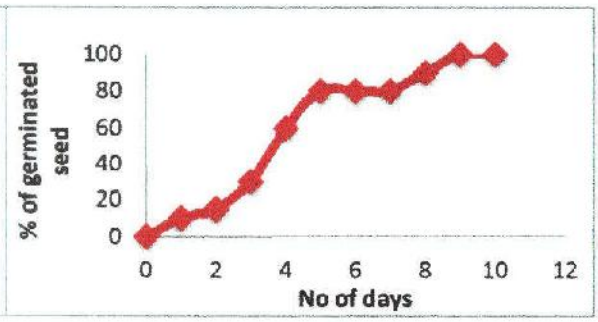


Fig.7: Germination response of seed at 4°C. A. Seed stratified for 2 days at 4°C in agar medium. B. Seed stratified for 4 days at 4°C in agar medium C. Seed stratified for 2 days at 4°C in water soaked double layer filter paper. D. Seed stratified for 4 days at 4°C in water soaked double layer filter paper.

4.2 Effect of Stratification on seed germination at 20 ^{0}C

Seed directly sown on agar media showed germination of 75 % within 7 days when stratified at 20°C for 2 days (Fig. 2A), whereas 80 % seed showed germination within 5 days when stratified at 20°C for 4 days (Fig. 2B). Seeds stratified on water soaked double layer filter paper for 2 to 4 days and then transferred into agar media. 70 % seed showed germination within 8 days (Fig. 2C) when incubated for 2 days, however 90 % seed showed germination within 5 days when incubated for 4 days (Fig. 2D).

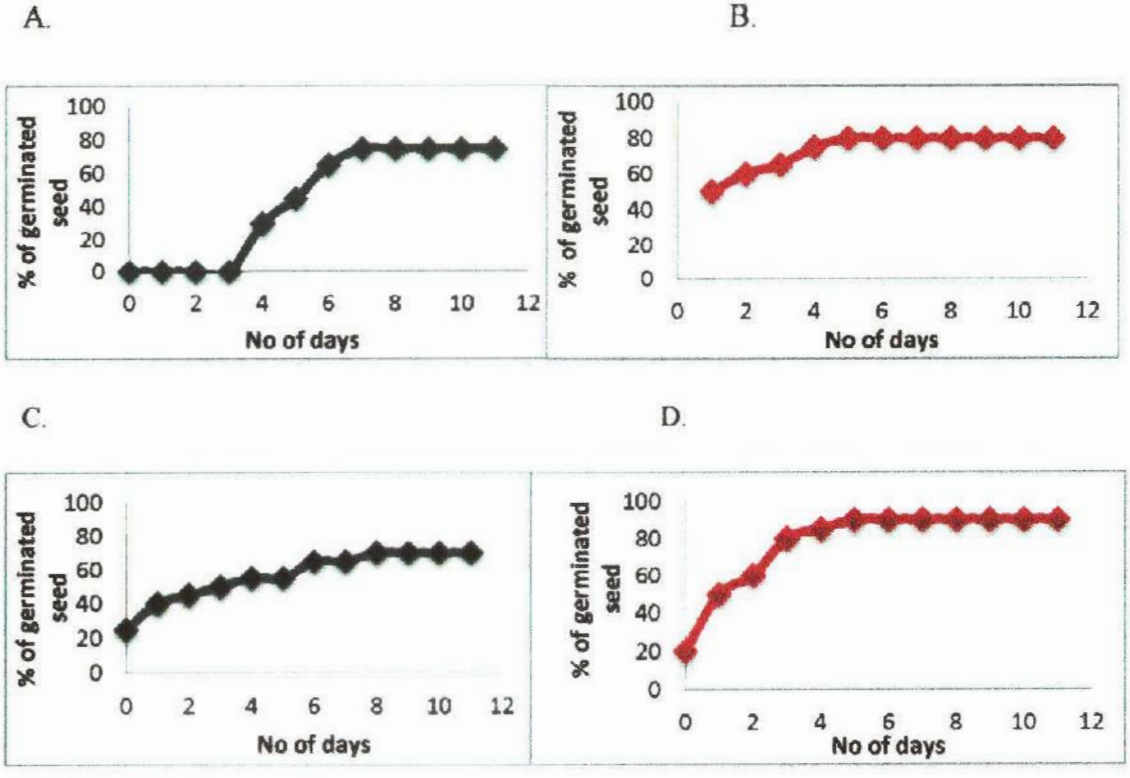


Fig .7: Germination response of seed at 20°C A. Seed stratified for 2 days at 20 ^{0}C in agar medium. B. Seed stratified for 4 days at 20°C in agar medium C. Seed stratified for 2 days at 20°C in water soaked double layer filter paper. D. Seed stratified for 4 days at 20°C in water soaked double layer filter paper.

4.3 Effect of Stratification on seed germination at 27 ^{0}C

Seed directly sown on agar media showed germination of 90 % within 9 days when stratified at 27°C for 2 days (Fig. 3A), whereas similar percentage of seeds showed germination within 4 days when incubated at 27°C for 4 days (Fig. 3B). Seeds stratified on water soaked double layer filter paper for 2 to 4 days and then transferred into agar media. 90 % seed showed germination within 6 days (Fig. 3C) when incubated for 2 days, however similar percentage of seed showed germination within 4 days when incubated for 4 days at 27°C. (Fig. 3D).

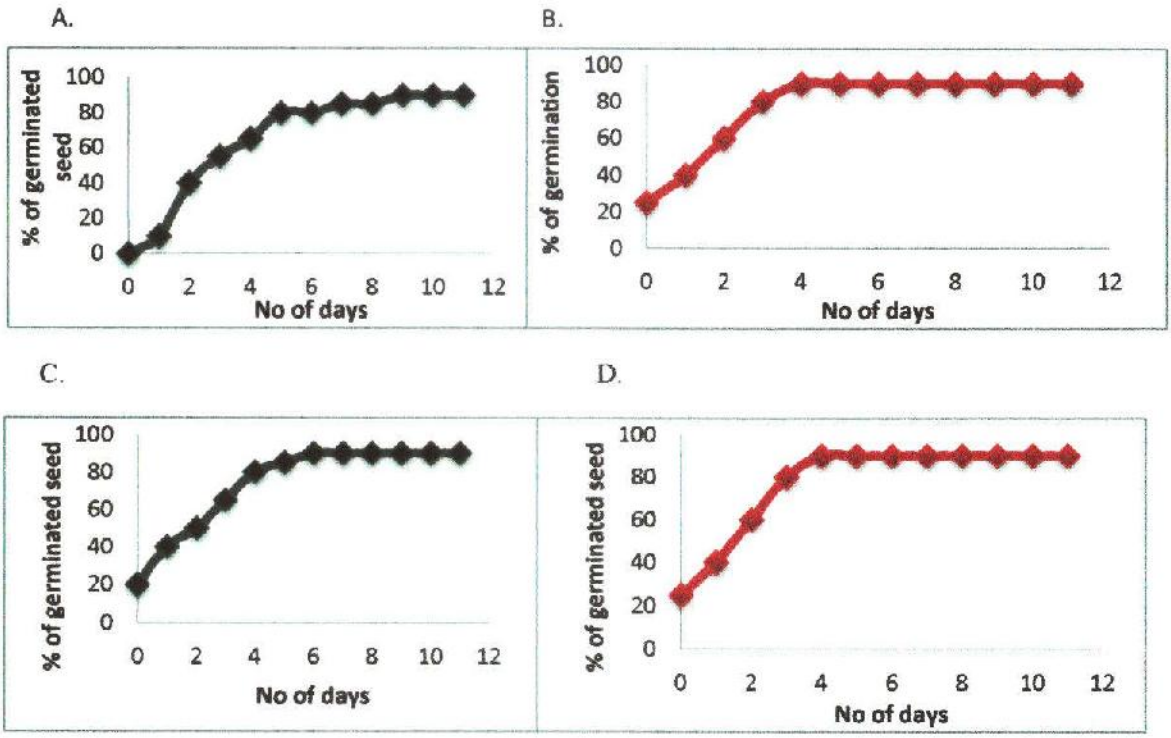


Fig 8. Germination response of seed at 27 ^{0}C. A. Seed stratified for 2 days at 27°C in agar medium. B. Seed stratified for 4 days at 27°C in agar medium C. Seed stratified for 2 days at 27 °C in water soaked double layer filter paper. D. Seed stratified for 4 days at 27°C in water soaked double layer filter paper.

4.4 Effect of GA3 on seed germination at 4 ^{0}C

Seeds sown in agar media containing 0, 5, 10, 20, 40 mg/L GA3 showed germination of 60-80% within 7 to 10 days. However, seeds started germination earlier in agar media containing 10 and 20 mg/L GA3 (Fig. 4C and 4D) compared to other concentrations of GA3 (Fig. 4A, 4B and 4E). 0 and 20mg/L of GA3 was effective for early germination and it take 9-10 days to reach maximum germination (70-80%). However, 70% of seeds were germinated within 7 days at 40mg/L of GA3

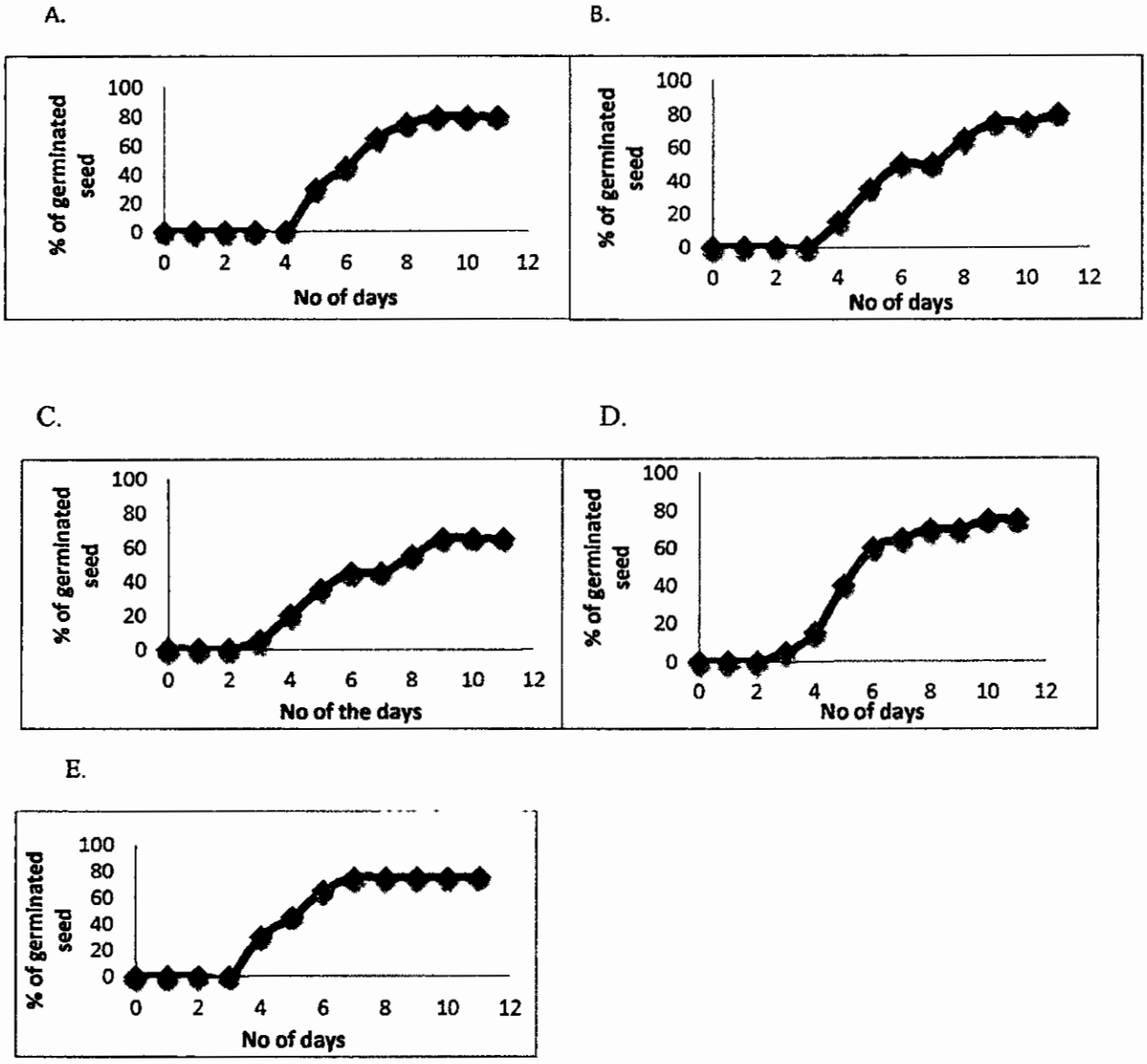
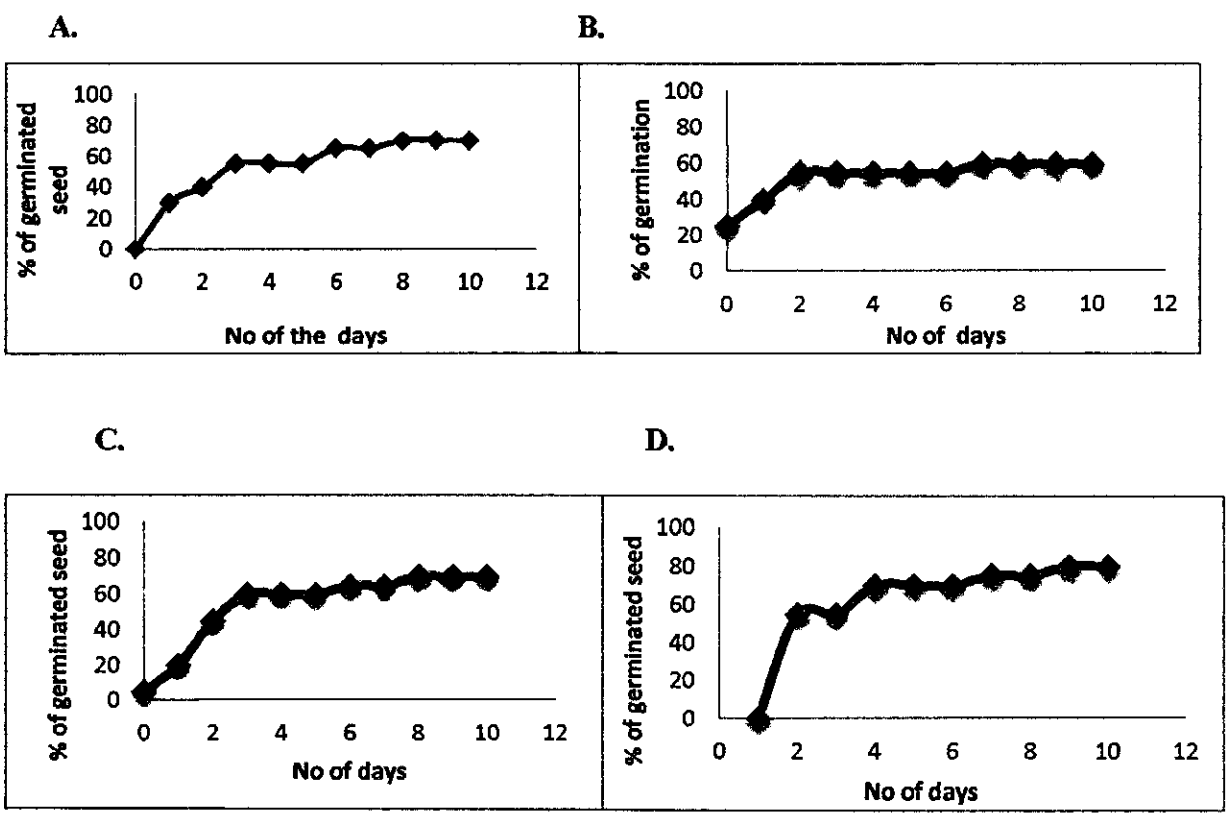


Fig.9: Germination response of seed in presence of GA3 at 4 ^{0}C.

A. Seed stratified for 3 days at 4°C in agar medium containing 0 mg/ml GA3. B. Seed stratified for 3 days at 4°C in agar medium containing 5 mg/ml GA3. C. Seed stratified for 3 days at 4°C in agar medium containing 10 mg/ml GA3.D. Seed stratified for 3days at 4°C in agar medium containing 20 mg/ml GA3. E. Seed stratified for 3 days at 4°C in agar medium containing 40 mg/ml GA3.

4.6 Effect of GA3 on seed germination at 27 ^{0}C

High concentration of GA3 showed good response for germination. Seeds showed 70-80 % germination within 4-6 days when sown in agar media containing 20mg/ml GA3 (fig:1D). Maximum percentage of germination (90%) was found within 6 days in agar media containing 40mg/ml of GA3 (Fig.5E).



E.

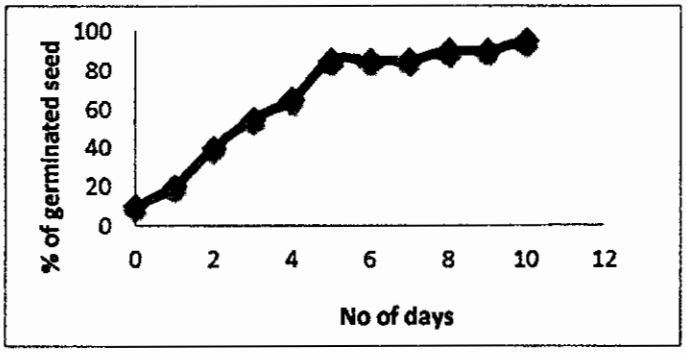


Fig.10: Germination response of seed in presence of GA3 at 27°C

A. Seed stratified for 3 days at 27°C in agar medium containing 0 mg/ml GA3. B. Seed stratified for 3 days at 27°C in agar medium containing 5 mg/ml GA3. C. Seed stratified for 3 days at 27°C in agar medium containing 10 mg/ml GA3. D. Seed stratified for 3 days at 27 °C in agar medium containing 20 mg/ml GA3. E. Seed stratified for 3 days at 27°C in agar medium containing 40 mg/ml GA3.

4.7 Effect of Stratification of seed (4°C) in soil:

Seeds stratified in water soaked double layer filter paper for 2 days and 4 days at 4°C and then directly sown into soil which containing 2:1:1 vermicompost and sand. Seeds showed 50% germination (fig: A) in 7 days whereas seed stratified for 4 days showed 50% (fig:B) germination in 6 days.

A. B.

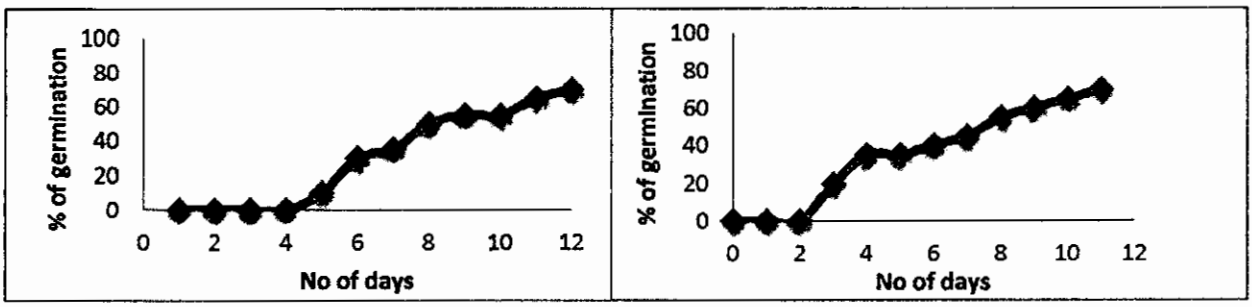


Fig.11: Germination response of seed stratified in 20°C

A. Seed stratified for 2 days at 4 in water soaked filter paper B. Seed stratified for 4 days at 4°C water soaked filter paper and then sown into soil.

4.8 Effect of Stratification of seed (20°C) in soil:

Seeds were stratified in water soaked double layer filter paper in 20°C for 2 days(figure A) and 4 days in 20°C (figure B).Germination response was good. Seeds stratified for 2 days at 20°C showed 80% germination (fig:A) in 7 days in contrast to seeds which are stratified for 4 days in same temperature . It took a bit longer time to get germinated. 50% germination showed in germination in between 15 days.

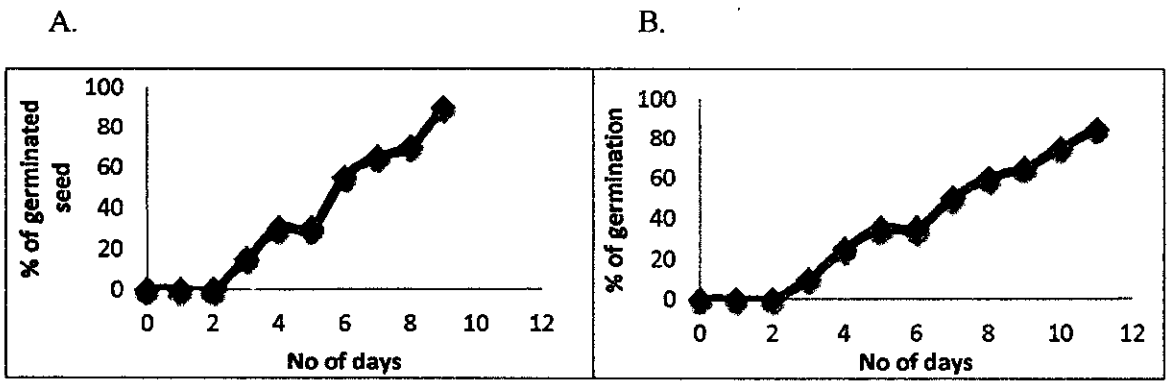


Fig 12: Germination response in soil (20°C):

A. Seed stratified for 2 days at 20°C in water soaked filter paper B. Seed stratified in 20°C for 4 days in water soaked filter paper and then sown into soil.

4.9 Effect of Stratification of seed (27°C) in soil:

Seeds were stratified in water soaked double layer filter paper in 27°C for 2 days(figure A) and 4 days in 27°C (figure B).Seeds showed 60% germination in between 5-10 days when it was stratified for 2 days in 27°C and it showed 50-60% germination between 10- 15 days.

A. B.

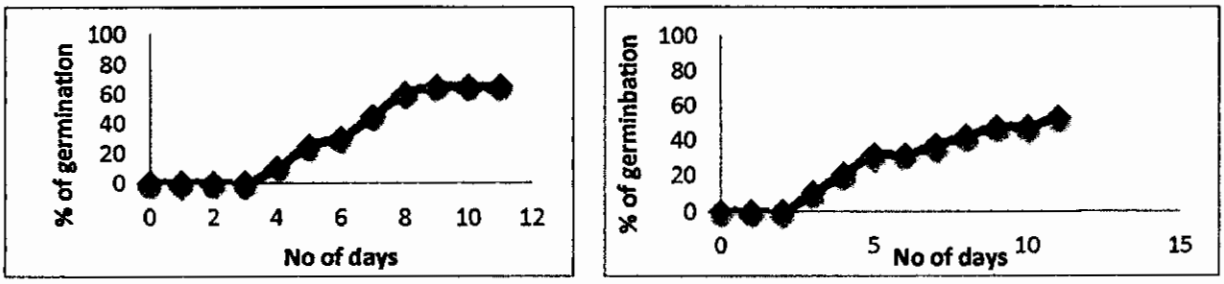


Fig.8: Germination response of seeds stratified in 27°C

A. Seed stratified for 2 days at 27°C in water soaked filter paper B. Seed stratified in 27°C for 4 days in water soaked filter paper and then sown into soil.

4.10. Combined effect of stratification at 27°C and GA3 (40mg/L) in seed germination

Sterilized seeds are stratified are stratified in water soaked double layer filter paper for 4 days at 27°C and then transferred to Ms media containing high concentration(40mg/L) of GA3(Fig.A) and in absence of GA3 (Fig:A) separately .Seeds showed 65% germination in 5 days in absence of GA3 and 90% germination showed within 5 days in presence of GA3 .

A. **B.**

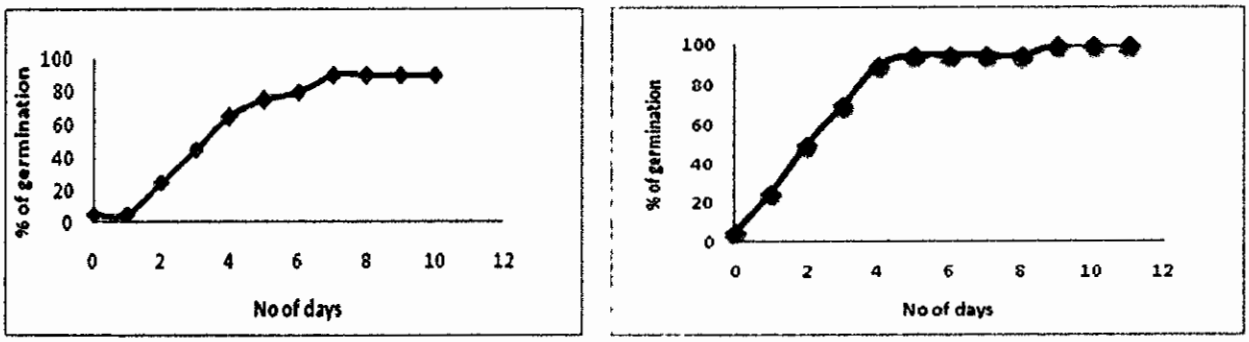


Fig.13: Germination response of seeds in combined effect of high temperature (27°C) and high concentration of GA3 (40mg/L)

A. Seed germination in absence of GA3 B. Seed germination in presence

Chapter Five

Discussion

To improve late germination of capsicum plants, especially horticultural commodities, different stages of lifecycle is highly desirable in the present day of food shortage. So, we need more methods for improving this condition by simple, un-expensive and environment friendly ways. Seed stratification is one of such type of method and gibberellic acid (GA3) have shown improved germination and growth of many crops (Basra *et al.*, 2006).

5.1. Germination slows down to stratification at 4°C:

The present study showed that cold treatment (4°C) slowed down germination and also decreased the percentage of germination compared with the untreated seeds of *Capsicum annum*. According to the graph and assay seeds stratified at 4°C for 2 days and 4 days take longer time to get germinated. Pepper seeds germinate slowly at less than optimal temperatures. Cochran (1935) reported that no peppers germinated after 45 days in a greenhouse with a temperature range between 4 and 10°C.

5.2. High temperature (27°C) and high concentration of GA3 (40mg/l) showed high germination rate:

Stratification had a significant effect on seed germination of Capsicum seeds. Non-stratified seeds gave only 33% germination, whereas seeds stratified for 4 days at 27°C days gave 90% germination (Fig. 8). Stratification might act simply to lower the rate of enzymatic reactions taking place in the seed, and might cause differential changes in enzyme concentrations or in enzyme production (Bewley and Black, 1994). Koyuncu and Sesli (2000) reported that 25 days of stratification had a significant effect on the germination percentage of *Juglans regia* L. nuts. These reports and our results show that stratification is successful in breaking seed dormancy, though the duration of treatment may vary with the species.

Pre-treatment with GA3 significantly enhanced germination of non-stratified Capsicum seeds. GA3 treatments at 40 mg/l concentrations yielded the highest germination percentage (90%; Fig.) within 5 day. Increasing the concentration of GA3 resulted in an increase in germination percentage.

GA3 has been found to be effective in increasing germination in several species and to break dormancy in dormant seeds. Pretreatment of blueberry seeds with GA3 at 100–500 mg/l accelerated germination (Ballington, 1984). *Arbutus andrachne* L. (eastern strawberry tree) seeds treated with 250 mg/l GA3 had 86% germination (Karam and Al-Salem, 2001). It has been reported that germination can be induced by gibberellic acid in *Vaccinium myrtillus* L. (Giba et al., 1993), *Vaccinium corymbosum* L. (Dweikat and Lyrene, 1988) and *Fagus sylvatica* (Nicolás et al., 1996) seeds. These results also confirm that GA3 treatment enhances seed germination.

5.3 Germination response of seeds in presence of GA3 at 4°C

Germination inhibitors isolated from papaya seeds interfere with germination (Chowand Lin, 1991). GA action in seeds can be blocked by the presence of an inhibitor; removing the inhibitor or its effect by an antagonist permits GA action (Khan, 1971). Our results also supports that the promotive action of GA3 may be blocked by inhibitory factors in the seed when the seeds are stratified at 4°C .Germination was slowed when seeds were sown into agar media containing GA3 in 4°C. It showed 60-70% germination within 10 days.

5.4 Combined effect of stratification and GA3 on Capsicum seed germination

The combined treatment with GA3 + stratification had a statistically significant effect on germination (Fig.15). Seeds which are stratified in absence of GA3 gave 65% germination, whereas seeds treated with 40 mg/l GA3 + 4 day stratification gave 90% germination. Increasing the stratification period from 0 to 12 days at 40 mg/l GA3 resulted in up to a high increase in germination. A significant interaction effect of GA3 concentration and duration of stratification was found. Seed dormancy in some species may be due to insufficient development of the embryo, chemical inhibition, or the failure of chemical reactions that make food reserves in the seed available to the developing embryo (Hilhorst and Karssen, 1992; Karam and Al-Salem, 2001). Physiological dormancy in seeds is dependent on the ratio of the levels of abscisic acid (a growth inhibitor) and GA (a growth regulator) (Hilhorst and Karssen, 1992). Serial physiological changes occur in the seed (Hartmann et al., 1997). The chilling process appears to enhance the

production of some types of growth-promoting substances such as GA (Powell, 1987). Giba et al. (1993) reported that the inhibitory effect of retardants was overcome by gibberellic acid. Treatment with GA3 + stratification was found to be successful for mahaleb (Gercekcioglu and Cekic, 1999) and plum (Ozvardar and Ozcagiran, 1991). These reports accord with our results showing that GA3 + stratification enhances germination of Capsicum seeds.

Chapter Six

Conclusion

Capsicum germinated well at 27°C and high concentration (40mg/l) of GA3 promotes germination rate. Farmers will use late summer and early spring to cultivate this crop. Colored capsicum is one of the important high value vegetable crop. Bangladesh has huge prospects of producing Capsicum and some advanced farmers started to grow this crop periodically to fulfill the local demand of city areas. The climate, environmental condition and other important attributes are available in some regions of Bangladesh for successful production of Capsicum. Late germination of Capsicum seeds is an obostacle for production of this crop. Stratification treatment of seeds at 25-30°C will helps farmers to eradicate this challenge.

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Appendix-2

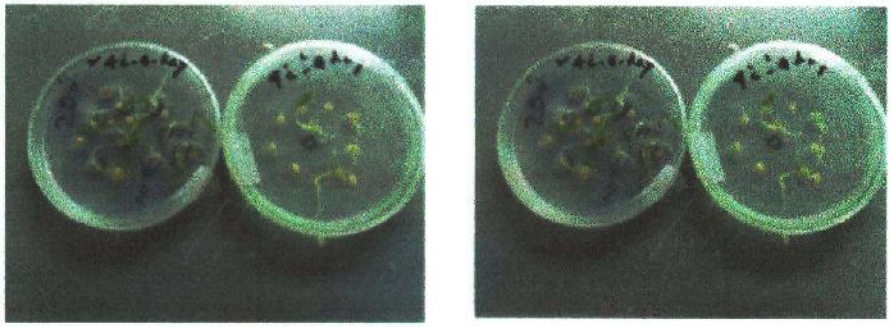
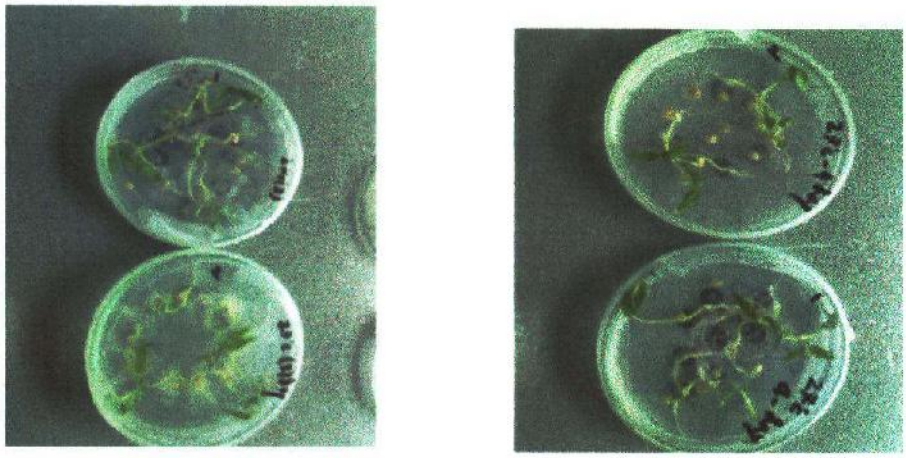


Fig:3 Germination response of seeds at 4°C for 2 days and 4 days



Germination response of seed stratified at 27°C