INVESTIGATING THE GERMINATING BEHAVIOUR OF CHICKPEA (*CICER ARIETINUM* L.)

Project report submitted to Central University of Punjab

For the award of

M.Sc. Life Sciences
(Specialization in Plant Sciences)

By
Ajay Prakash Uniyal
Registration no. 16mslsps08

Supervisor
Dr. Vinay Kumar

Department of Plant Sciences School of Basic and Applied Sciences Central University of Punjab, Bathinda-151001

May 2018

DECLARATION

I declare that project entitled "**Investigating the germinating behaviour of Chickpea (*Cicer arietinum* L.)**" has been prepared by me under the guidance of, Dr. Vinay Kumar, Department of Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this project has formed the basis for the award of any degree or fellowship previously.

Ajay Prakash Uniyal
Registration No. 16mslsps08
Department of Plant Sciences,
School of Basic and Applied Sciences,
Central University of Punjab, Bathinda-151001

Date:

CERTIFICATE

I certify that Ajay Prakash Uniyal (Reg. no. 16mslsps08) has prepared his project report entitled "**Investigating the germinating behaviour of Chickpea (*Cicer arietinum* L.)**" for the award of M.Sc. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Department of Plant Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

Dr. Vinay Kumar
Assistant Professor,
Department of Plant Sciences,
School of Basic and Applied Sciences,
Central University of Punjab, Bathinda-151001

Date:

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(Ajay Prakash Uniyal)

Student

ABSTRACT

Investigating the germinating behavior of Chickpea (*Cicer arietinum* L.)

Name of student: Ajay Prakash Uniyal
Registration number: 16mslsps08
Degree for which submitted: M.Sc. Life Science (Specialization in Plant Sciences)
Name of supervisor: Dr. Vinay Kumar
Name of Centre: Department of Plant Sciences
Name of School: School of Basic and Applied sciences

Key Words: Seed germination, Radicle protrusion, Biochemical analysis, Starch degradation, Phases of germination.

Seed germination is the crucial stage in plant development process. It commences with water imbibition and culminates with radicle protrusion. This process has been observed to be occurred in sequential three phases, phase I with rapid water uptake followed by phase II (Plateau Phase) and phase III in which radicle extrusion takes place. Water imbibition during phase I triggers the sets of events that causes some physiological and biochemical changes in seeds as starch degradation by activation of hydrolytic enzymes etc that are strongly related to plumule and radicle growth. The present investigation aims to investigate the germinating behaviour of Chickpea (Cicer arientium L.) and biochemical alteration during the three phases of germination. Phase I is found to be from 0-20 hours, Phase II from 20-32 hours and Phase III after 32 hours. During germination, both starch and proteins were found to be degraded to provide the developing embryo with sugars and free amino acids respectively and the content of reducing sugars and total sugars were found to be increased. The result indicates that the reserve seed storage mobilisation may be helpful to proceed the seedling growth.

Ajay Prakash Uniyal **Dr. Vinay Kumar**

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Full form	Abbreviations
3,5-Dinitrosalicyclic Acid	DNSA
Abscisic Acid	ABA
Bovine Serum Albumin	BSA
Degree Celsius	℃
Figure	Fig.
Gram	g
Hours	hrs
Hydrochloric acid	HCl
Indole Acetic Acid	IAA
Mercuric chloride	$HgCl_2$
Microgram	µg
Microlitre	µl
Milligram	mg
Milliliter	ml
Millimolar	mM
Nanometer	nm
Normal	N
Revolutions per minute	rpm
Ultraviolet	UV
Water	H_2O

1. Introduction

Seed links between two successive life cycles. Seed can be considered as a stress resisting structure against unfavorable periods and allowing dispersal to find appropriate place for germination. In seeds all the metabolic process are in quiescent phases to promote desiccation tolerance (Rajjou et al., 2012). Only after the seed germination the metabolic activities are resumed. Seed germination incorporates all those events starting when seed imbibe water and culminates when radicle starts emerging out from it (Hasanuzzaman et al., 2013). There are two proposed mechanism to explain the seed germination process (Nanogaki et al., 2007). First mechanism proposed the elongation of proximal embryonic axis as hypocotyl to overcome the restrains by the covering tissues while second mechanism involves weakening of the surrounded covering layers around embryo for easy radicle protrusion.

The complete process of seed germination in respect to water uptake can be divided into three phases. Phase I is the process of rapid water imbibition followed by Phase II which is identified as a plateau phase. It is the major phase as all the metabolic activites are resumed in this phase (Bewley et al., 2008) and last Phase III during which radicle protrusion takes place. Phase II is the major phase

Seed germination is complex network of signalling and gene expression (Han et al., 2015). Seed stores food reserves mainly in form of protein, lipids and carbohydrates (Jukanti et al., 2012). Water uptake by reserve materials in seed stimulate the release of phytohormones mainly Gibberlic acid that diffuses and initiates cascade of signals for synthesis of hydrolytic enzymes as alpha amylases that further hydrolyze the stored food reserve as starch into simple sugars to fuel the developing embryo with energy for forming plumule and radicle. The activation of alpha amylase by Gibberlic acid is repressed by Abscisic acid, another phytohormone that maintains the seed dormancy (Ali et al., 2017). When the appropriate signals for the germination are induced, Gibberlic acid counteract the inhibitory effect of Abscisic acid. Thus, seed germination is complex catabolic process during which storage mobilization occurs to support radicle and plumule growth and other key determining cellular processes.

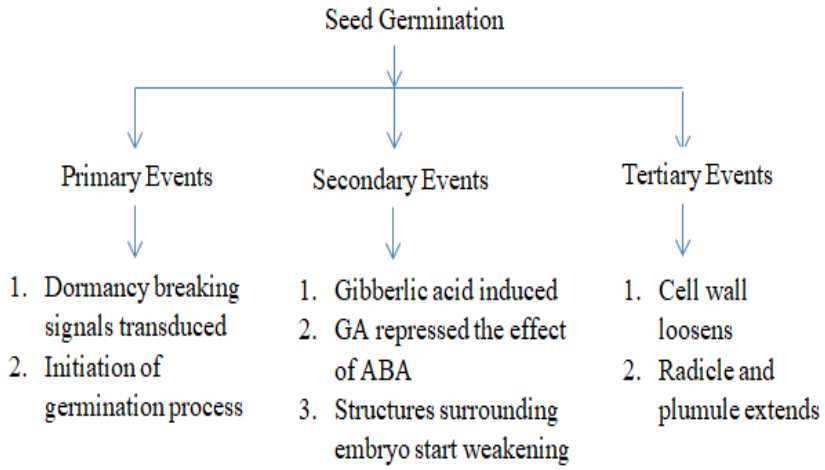

Fig 1. Overview of major events associated with seed germination.

1.2 Knowledge gap

According to my best knowledge not a single document associated with temporal changes in term of biochemical alteration during the distinct phases of seed germination in chickpea is available.

1.3 Hypothesis

Keeping the novelty of the work, a hypothesis was designed to study the morphological, spatial and temporal associated behaviour during germination in Chickpea (Cicer arietinum).

1.4 Objectives

Overall, the objectives to fulfill the proposed hypothesis, has been drafted as given below-

1. Identify the defined stages during seed germination
2. Germinated associated behaviour analysis by biochemical means

2. Review of literature

Seed germination is highly complex process of transformation of inert quiescent seeds into metabolizing system (Atici et al., 2002). This process is associated with degradation and mobilization of reserve storage food accumulated in seed during maturation. All the germinating signals for mobilization of reserve foods initiate with imbibition of water from outside through seed coat (Ali et al., 2017). The efficiency of reserve food mobilization during germination depends upon activation of set of hydrolytic enzymes. In addition to the activation of hydrolytic enzymes, germination process is regulated by hormonal interaction of hormones and environmental signals and it occurs only under favorable conditions.

2.1 Legumes

Legumes seeds are nutrients both for animal and human consumption and are good source of starch, dietary fibers, lipids, proteins and other But their nutritional values are limited by process of undesirable compounds as anti-nutritional factors that include protease inhibitors, lectins, pectins, trypsin inhibitors etc. (Donangelo et al., 1995). Germination can be considered as the suitable procedure of improving nutritional value by reducing level of the anti-nutritional factors. Among the legume, chickpea is an important crop in developing countries with comparatively larger grain size and cotyledons remain attached to the plant from germination to provide energy for metabolism.

2.2 Chickpea

Chickpea, one of the legume crop is widely grown in tropical and subtropical countries. Chickpea seeds are big in size and have average composition of 60% carbohydrates, 16-21% proteins, 5-9% lipids and 2-3% other nutrients (Jukanti et al., 2012). India ranks third in production of legumes considering chickpea as the largest importer and producer (FAO 2016). Chickpea seed contains fibres which are helpful against constipation, lowers risk of cancer, lessen the heart disease risk and stroke. (Rahman et al., 2008) Germinated seeds were also recommended against scurvy diseases and rich in nutritionally important unsaturated fatty acids as linoleic acid, oleic acid,

campestrol, stigmasterol and other sterols (Jukanti et al., 2012). Nutritional status of chickpea seed is tabulated in the table 1.

Table 1: Nutritional status of chickpea seed

Components	Varieties of Chickpea (%)	
	Kabuli	Desi
Starch	39.12	38.48
Soluble Sugars	8.43	7.53
Crude proteins	24.63	22.76
Non fibrous Carbohydrates	49.13	46.81
Tannin	0.09	0.12
Phenolic Compounds	0.27	0.26

Source: Maheri-Sis et al. (2008).

2.3 Structure of Chickpea seed

Chickpea seed contain two cotyledons joined at the adaxial surfaces, covered by seed coat. A small hypocotyl or embryonic axis and radicle are located in chickpea beak. On ventral side are hilum, funicular scar and micropyle (Wood et al., 2011). The pore controls the moisture entry into the seed. Raphe extends from bottom of hilum rin, called corona to spermotylium containing chalazal base. Embryo is enclosed by the outer protective covering layer called testa (seed coat). Seed coat of chickpea has two distinct regions, external palisade and internal parenchymatous. Color pigments are present in palisade regions. Embryo is also connected with two cotyledons that contains starch granules surrounded by proteins.

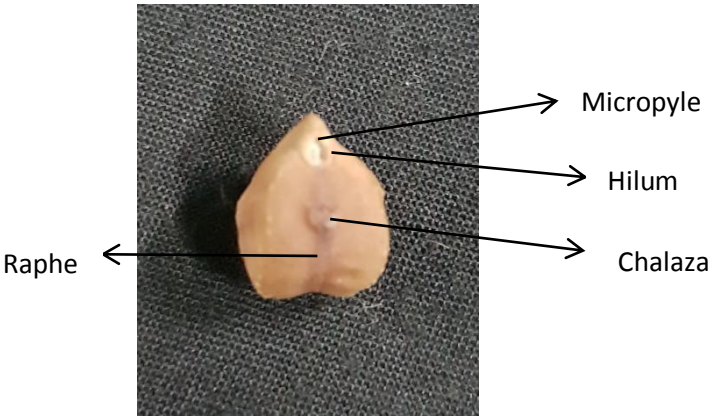

Fig 2. Structure of chickpea seed.

2.4 Seed germination process

Seeds needs the amalgamation of all the favorable signals for achieving germination (Dekkers et al., 2013). Only when the right response takes place, the seed induce germination process and germinated seedlings become mature plants. The germination is controlled by various physical cues as water, temperature, hormones, seed storage substances, light etc.

2.4.1 Seed dormancy

Seed dormancy is considered as block for the germination. The nor-dormant seed has capacity of germinating over wide range of environmental factors. This blocking mechanism has been evolved through adaptation to the environment, so that germination only initiates when the environmental conditions are favorable (Koorneef et al., 2002). Baskin and Baskin in 2004 had proposed the classification system for the seed dormancy- physiological dormancy, morphological dormancy, morph-physiological dormancy, physical and combinational dormancy (Baskin et al., 2004). Out of these, physiological dormancy is most abundant found in all angiosperm clades. It is evident that ABA plays the important role in induction and maintenance of dormancy. Overexpressing the ABA biosynthesis gene was found to fasten the seed dormancy and delay the process of germination (Bentsink et al., 2008). Dormancy can be released by shifting to biosynthesis of GA and degradation of ABA with subsequent formation of seedlings.

2.4.2 Role of seed coat in germination

Seed coat (testa) is derived from the inner, outer or both integuments (Wood et al., 2011). Seed coat not only provides structural and protective function but also has decisive role in the seed germination timing by regulating the uptake of water. Germination occurs when the constraints imposed by seed coat will be overcome (Moïse et al., 2005). Innermost part of seed coat several active enzymes as chitinases and peroxidases. Chitinases are expressed during late seed development and peroxidases are involved in suberin and lignin biosynthesis. These compounds prevents the water entry when gets accumulated. Seed coat is also regarded to play the critical role in the assimilates and nutrient transfer. It also supplies zygote with water, oxygen, minerals, phytohormones as IAA, ABA and carbon and nitrogen assimilates (Debeaujon et al., 2007). Seed coat is also site for interconversion of asparagine and aspartate to the composition adaptable for protein accumulation (Smý kal et al., 2014). The seed coat pigmentation is also related with the imbibition capability. Browning of seed is proportional to its impermeabilization in the chickpea. These pigments have beneficial effect on human health as cardio protective, anticancer and anti-inflammable (Smykal et al., 2014).

2.4.3 Initiation of germination by water

Germination begins with the uptake of water by the quiescent dry seeds. Dry seeds have low water potential that causes water influx to activate subsequent cellular processes. In relation to water uptake, the seed germination in legumes is divided into major three phases as represented in fig 3. Phase I in which seed imbibe water and initiates the metabolic process (Bewley et al., 1997), Phase II the dormant state and Phase III including resumption of water and emergence of radicle from it as reported by Finch-Savage and Leubner-Metzger (2006). After water uptake the embryo becomes too large that it bursts its outer shell and the small plantlet emerge from it. If right water condition is not achieved by seed, it remains in dormant state. With respect to the hydration in the seeds that imbibe water, different processes occur. As water content increase to 18-20% respiration rise occurs

and processes as glycolysis and kreb cycle starts. Further hydration to 45% activates maximum respiration and mitochondrial biogenesis (Bewley et al., 1997, Nicolas and Aldasoro, 1979; Salon et al., 1988). At 50-55%, transcription process is activated and hydrolysis of storage reserves is inititated. 60% hydration is characteristics of meristematic cells. The major driving force for the water uptake is the osmotic forces and sugar is the main source to create osmotic pressure.

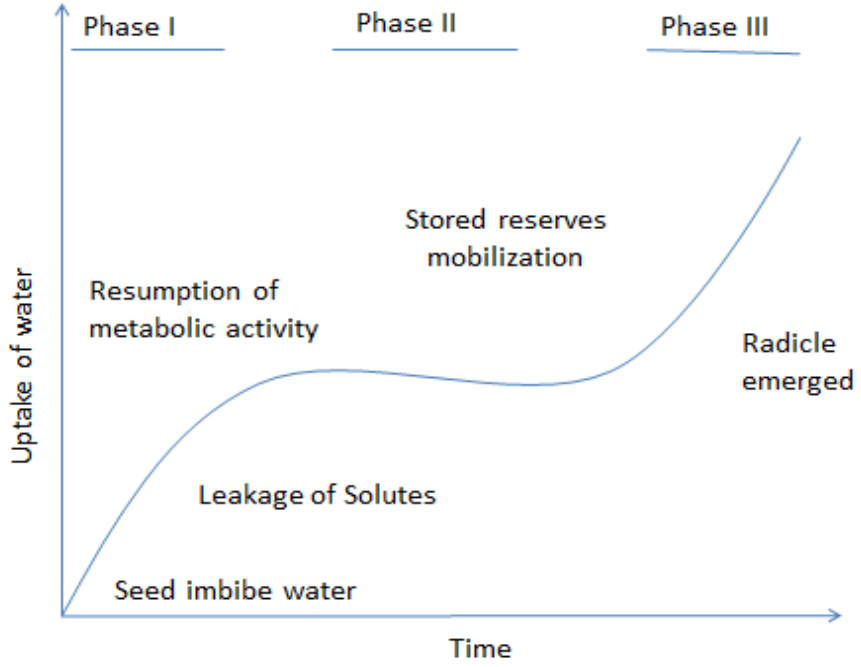

Fig 3. Three distinct phases of germination

2.4.4 Phytohormones control germination

Germination is controlled by the dynamic balance between ABA and GA (Gutierrez et al., 2007). Both ABA and GA control each other antagonistically at molecular level (Toh et al., 2008). ABA is the major factor to determine the dormancy status of seed. It inhibits transition to germination phase and its concentration decreases with the imbibition. Catabolic removal of ABA is essential for transition from dormant state to germinative one (Rodríguez-Gacio et al., 2009). On the other hand, GA is important endogenous growth regulator having profound effect on the germination process. It counteract the effect of the ABA on the germination. Release of GA stimulates the secretion

of hydrolases from the aleurone layer mobilising the stored endosperm reserves for seedling growth (Sun et al., 2008). GA also regulates seed germination and stem elongation by repressing DELLA protein effects which are involved in repressing the stem elongation and cell division (Schwechheimer et al., 2012) shown in fig 4. Synthesis of GA is initiated when phytochrome mediated signals are perceived by seed.

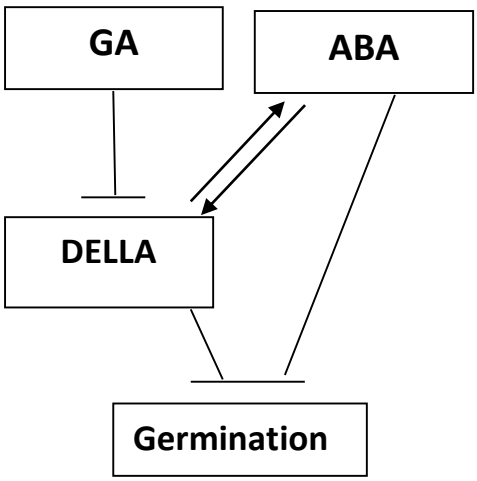

Fig 4. GA and ABA role in seed germination

2.4.5 Hydrolytic enzymes and germination

Water activates many metabolic processes such as activating the hydrolytic enzymes as amylases, proteases, hydrolases etc. These enzymes hydrolyse the starch, lipid, protein and other storage material into simple form available for growth and development of emrbyo. Amylase is synthesised denovo during seed germination that mobilise the stored starch till the initiation of photosynthesis. Amylase activity is regulated by reducing sugar. Amylase activity of cotyledons increased gradually while starch decreases during germination (Yan et al., 2014). Triglycerols are also hydrolysed by the lipase during germination to provide the energy for biosynthesis of biomolecules (Eastmond, 2009). Triglycerols are broken down into fatty acids and glycerols (Quettier et al., 2009). Fatty acids undergo beta oxidation to proceed the glyoxylate cycle. Not only this, hydrolytic enzymes also breakdown the antinutrient factors present in the plant .Phytate is one of the major antinutrient present in the legumes. The role of phytate in seed is as of antioxidant to reduce free radical generation and peroxidation (Androitis et

al., 2003). Phytate act as chelator for metal ions as zinc, calcium, iron etc. During the germination, phyate is hydrolysed by phytase releasing the metal ions as phosphate, cations, inositols etc that are utilised by the seedlings (Ali et al., 2017).

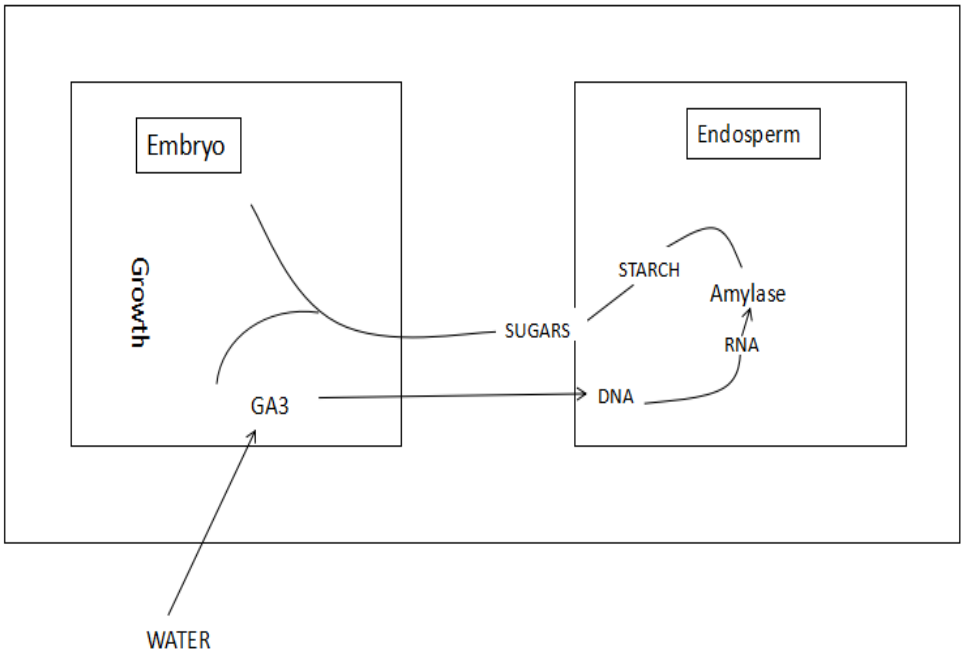

Fig 5. Major events during seed germination.

3. Materials and methods

3.1 Collection of Sample

Chickpea (C. arietinum L.); released variety ICC 4958 was used to investigate the germination behaviour. Seeds were procured from the Chickpea plant grown in the field of Central University of Punjab.

Fig 5. Dried seeds of chickpea variety ICC 4958.

3.2 Identification of defined stages during seed germination

Good and mature dry seeds were taken and their weights were noted (Calculated as weight of seed in zero hours). Then the dry seeds were washed with 0.01% HgCl2 and potassium permagnate to avoid any fungal growth. Seeds were then placed in sterilised petriplates (9 cm diameter) lined with filter paper. 10 ml of water was added to moisten the filter paper. The weights of seeds were taken after every four hours as 0, 4, 8, 16 to 52 hours. Softness and any alteration in the morphology of seed coat were also noted timely. 48 seeds were taken in triplicate with 4 seeds in each petri plates.

3.4 Biochemical analysis of seeds

Biochemical estimation of starch, reducing sugars, total sugars and proteins were undertaken in seeds harvested during 0, 8, 16, 24, 32, 40 and 48 hours after incubating with 10ml distilled water.

Harvested seeds were crushed by mortar and pestle into flour, The flour was then used for the biochemical analysis of starch, total sugars, reducing sugars and proteins.

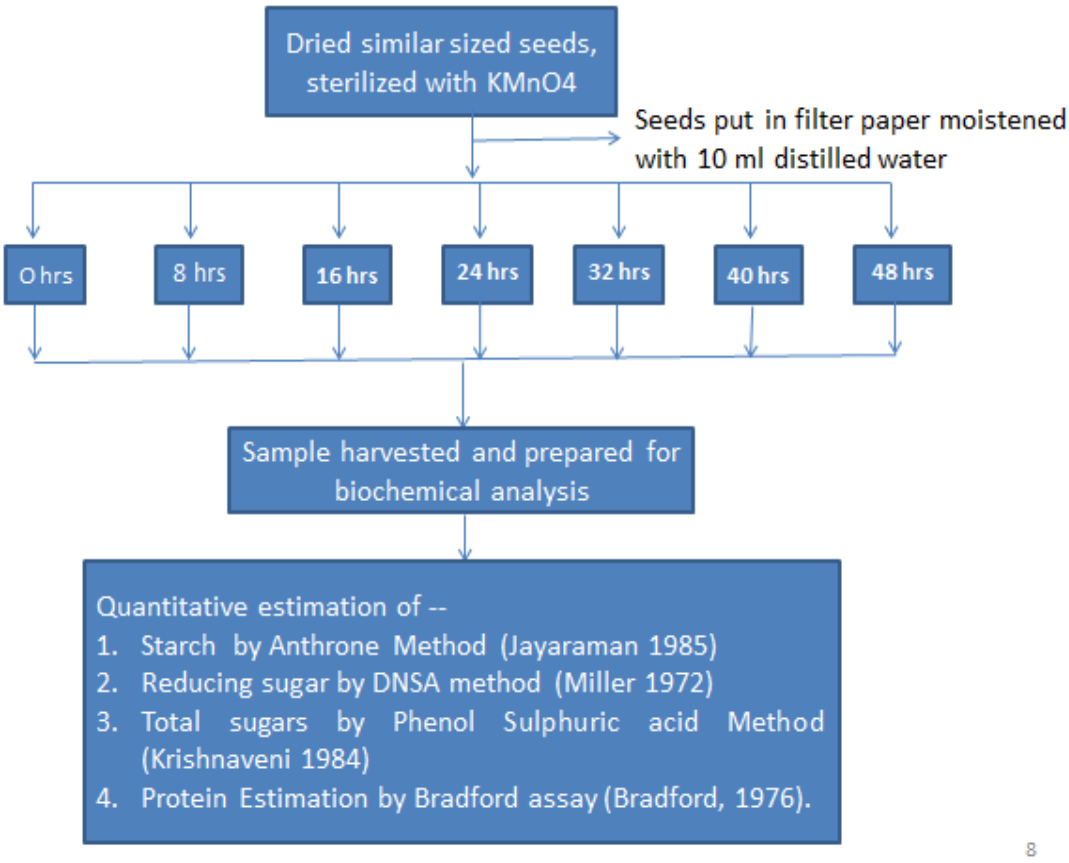

Fig 7. Workflow for biochemical analysis of seed.

3.4.1 Estimation of Starch

Starch was estimated by the anthrone method as described by Jayaraman, 1985 with little modifications.

Principle Sample is treated with 80% alcohol to remove sugars. Starch is extracted with perchloric acid. In hot acidic medium, starch gets hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This forms green color product with anthrone reagent.

Preparation of Standard Curve Working glucose solution was prepared by taking 100 mg of glucose and transferred it carefully into 100 ml with distilled water. Then 10 ml of stock standard solution were diluted in 100 ml with distilled water in volumetric flask. 0.2-1 ml of working standard solution was taken in test tube and water was added to bring volume upto 1ml. Then 4ml of anthrone reagent was added and the contents were mixed and placed in

the water bath for 10 mins. The test tube was allowed to cool and the optical density was measured using the SHIMADZU UV 160A dual beam spectrophotometer. Blank was prepared using 1ml distilled water and 4 ml anthrone reagent. The graph was constructed by plotting glucose construction on X axis and absorbance at 630 nm on y axis.

Extraction Seeds at different hours were taken and crushed in mortar and pestle separately. 100 mg of crushed sample were taken and extracted with 80% boiling ethanol (4-5ml) and then centrifuged at 10,000 rpm for 10 mins. The supernatant were reserved for estimation of sugars and pellet was used for starch extraction.

Residue extracted from the alcoholic extract was suspended in 5ml of water and starch was extracted with 6.5 ml of cold 52% perchloric acid for 20 min at 0ºC and centrifuged for 15 min at room temperature. Volume of supernatant was made to 100 ml with distilled water.

Estimation Suitable amount of aliquots were taken and make up the volume upto 1ml with distilled water. Add 4 ml of anthrone reagent and allow it to boil for 10 minutes in water bath. After letting it to cool for some minutes, optical density was noted in SHIMADZU UV 160A dual beam spectrophotometer at 630 nm. Starch was estimated from the glucose standard curve.

3.4.2 Estimation of Total Sugars

Total sugars were estimated by the Phenol sulphuric acid method as described earlier by Krishnaveni, 1984.

Principle Sulphuric acid breaks down the complex sugars into mono sugars which then dehydrated to furfural compounds. These compounds then react with phenol to give orange yellow color which is measured at 490 nm.

Preparation of Standard Curve 0.2-1ml of working glucose solution was taken in separate test tubes and added water to bring volume upto 1ml. Then 1ml of 5% phenol was added followed by 5ml of 96% sulphuric acid. The test tubes were then put in water bath for 10-20 min. After cooling the test tube the optical density was measured at 490 nm. Blank was prepared by 1ml of water with 1ml of 5% phenol and 5ml of 96% sulphuric acid.

Extraction 100 mg of crushed seed samples from each germinating stages were taken and hydrolyzed by keeping it in boiling water bath with 2 N HCl for at least 3 hours. The samples were cooled to room temperature and then the extract was filtered with whatman filter paper.

Estimation 0.2 ml of aliquot from the extract was taken. The volume was make up to 1 ml with the distilled water. Then 1 ml of 5 % Phenol solution was added and afterward 5ml of 96% sulphuric acid. After keeping for 20 minutes yellow color was formed. The optical density was noted with SHIMADZU UV 160 A dual beam spectrophotometer at 490 nm. Total sugars were estimated from standard curve of glucose.

3.4.3 Estimation of Reducing sugars

Reducing sugars were estimated by DNSA method described by Miller 1972, with some modifications.

Principle 3,5-Dinitrosalicyclic acid (DNSA) detects the presence of free carbonyl group of reducing sugars. It involves oxidation of aldehyde and ketone functional group that reduces DNSA to 3-amino-5-nitrosalicylic acid (ANSA) that is orange red in color whose optical density is noted at 540 nm.

Preparation of Standard Curve 0.2**-**1ml of working solution were taken in separate test tubes and water was added to bring volume upto 1ml. Then 3ml of DNS reagent was added and heated for 5 min at boiling water bath. Sodium potassium tartarate was added to stabilize the color. Blank was prepared by adding 1ml water and 3 ml DNS reagent. Optical density was taken at 510 nm using SHIMADZU UVA dual beam spectrophotometer.

Extraction and Estimation The supernatant during extraction of starch is used for estimation of reducing sugars. 0.2 ml of the aliquot was taken and then the volume was make upto 1 ml with distilled water. Then 3 ml of DNSA reagent was added and the test tube was kept in boiling water bath for 10 mins. Take out the test tubes and add distilled water to stop the reaction. Measure the optical density at 540 nm with SHIMADZU UV 160 A dual spectrophotometer.

3.4.4 Estimation of Protein

Total protein content was evaluated using Bradford assay (Bradford, 1976).

Principle Binding of protein molecules to Coomassie dye under acidic conditions result in change in color from brown to blue.

Extraction Seeds were powdered by mortar and pestle. 50 mg of crushed material were taken and added 0.1M NaOH (1ml for 30 mg) and mixed by vortex. The solution was then centrifuged at 15000 rpm for 7-10 mins at 4^{o}C. Supernatant was taken and soluble protein was then assayed by Bradford reagent.

Preparation of Standard Curve BSA stock solution was prepared to get the concentration of 2mg/ml .From the stock solution, prepared the BSA dilutions from 0.2 mg/ml to 1.0 mg/ml. After preparing the different sets of dilutions, 3 ml of Bradford's reagent was added and measure the optical density at 595 nm using SHIMADZU UVA dual beam spectrophotometer.

Estimation 0.2ml of the aliquot (supernatant) was taken and volume was make upto 1ml using phosphate saline buffer. Then 4-5ml of Bradford reagent was added and mixed by vortexing. The blank sample was prepared by adding 1ml of phosphate saline buffer and 4-5ml of Bradford reagent. The absorbance was measured at 595 nm after 2 min by using SHIMADZU UV 160 A dual spectrophotometer. The protein in sample was calculated from the standard graph of BSA .

4. Results

4.1 Three distinct phases of seed germination has been identified in chickpea

In chickpea, germination process is distinguished into three phases: Phase I in which there is rapid water imbibition and hence rapid increase in weight. This takes place from 0 hrs to 20 hrs in uniform selected seed samples. Phase II lasts from 20 hrs to 32 hrs, Phase III that is radicle protrusion start after 32 hrs. Weight profile of seed with germinating times is tabulated in Table No. 2.

Table 2. Weight of chickpea seeds during different hours of germination

Sr. No.	Germinating time in hours	Average Weight (grams)	Germination Phase
1	0	0.4716±0.002	Phase I
2	4	0.677±0.0008	
3	8	0.774±0.006	
4	12	0.826±0.02	
5	16	0.926±0.01	
6	20	0.95±0.01	
7	24	0.95±0.01	Phase II
8	28	0.95±0.01	
9	32	0.95±0.01	
10	36	0.99±0.006	
11	40	1±0.001	Phase III
12	44	1.03±0.04	
13	48	1.08±0.04	

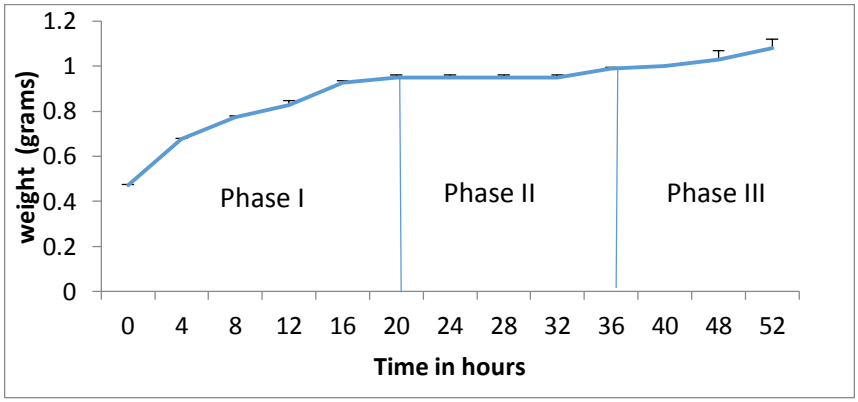

Fig 8. Seed weight pattern during different germination time interval

4.2 Degrading starch content during germination

Before imbibition, chickpea seed contain great amount of starch (~12.8mg/100mg). The decrease in starch content was seen after seeds imbibe water as tabulated in Table No. 3. Starch degrades from 12.8 mg/100 mg in 0 hrs to 7.28 mg/100 mg in 48 hrs by upregulated hydrolytic activity of amylases into glucose or others from 0 hrs sample that is used for growth and development. Reduction in starch content is seen during phase I and phase II.

Table 3: Starch content at different hours of germination

Sr.No.	Germinating time (in hours)	Average Optical density at 630 nm	Concentration (mg/100mg)
1	0	1.092	12.8±1.6
2	8	0.929	10.8±0.74
3	16	0.9003	10.5±1.6
4	24	0.8063	9.4±2.06
5	32	0.746	8.6±0.823
6	40	0.629	7.27±0.06
7	48	0.63	7.28±0.34

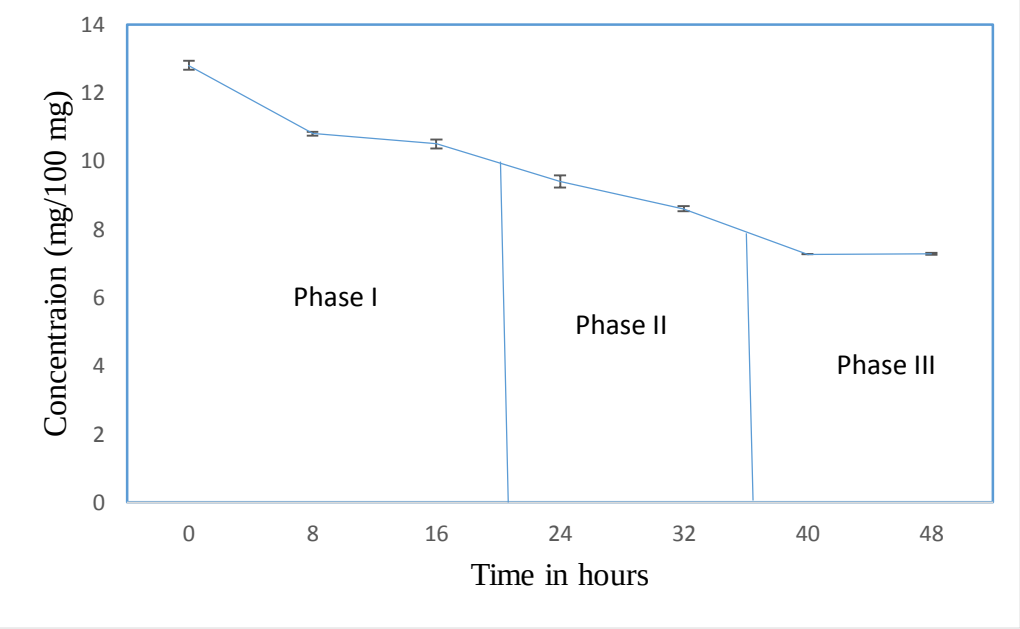

Fig 9. Degradation of starch during germination

4.3 Increased reducing sugar content during germination

With progression of germination, the content of reducing sugar was found to be increased. The reducing sugar content during different hours of germination is represented in Table No. 4. The increase in amount of reducing sugar clearly indicates reducing sugar as the primary soluble carbohydrate compound in seedling tissue. Reducing sugar content increased rapidly in [phase II and phase III of germination.

Table 4: Reducing sugars content of Chickpea seed at different hours of germination

Sr. No.	Germinating time (in hours)	Optical density at 540 nm	Concentration (mg/100mg)
1	0	0.1006	0.064±0.007
2	8	0.124	0.084±0.016
3	16	0.145	0.102±0.011
4	24	0.251	0.193±0.01
5	32	0.289	0.224±0.007
6	40	0.331	0.26±0.01
7	48	0.389	0.31±0.02

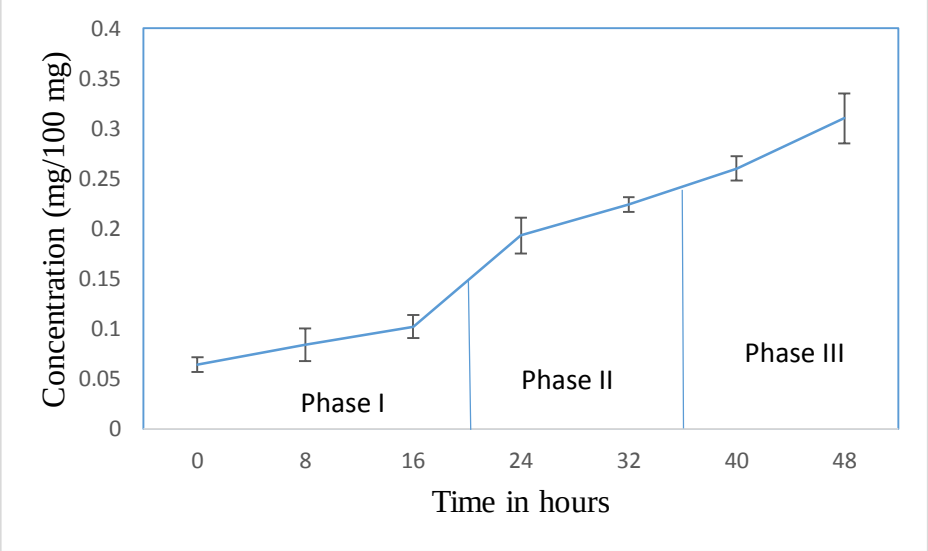

Fig 10. Increment in reducing sugars for proceeding germination

4.4 Increased total sugar content during germination process

During germination, there was increase in total soluble sugars in chickpea seeds due to energy needs of growing plant. The sugar content during different hours of germination is represented in Table No. 5. Total sugars increased rapidly during phase III of germination.

Table 5. Total sugars content of Chickpea seed at different hours of germination

Sr. No.	Germinating time (in hours)	Average Optical density at 490 nm	Concentration (mg/100mg)
1	0	0.71	0.295±0.003
2	8	0.745	0.31±0.003
3	16	0.811	0.338±0.003
4	24	0.823	0.343±0.002
5	32	0.972	0.407±1.2
6	40	1.1	0.461±0.01
7	48	1.232	0.518±0.002

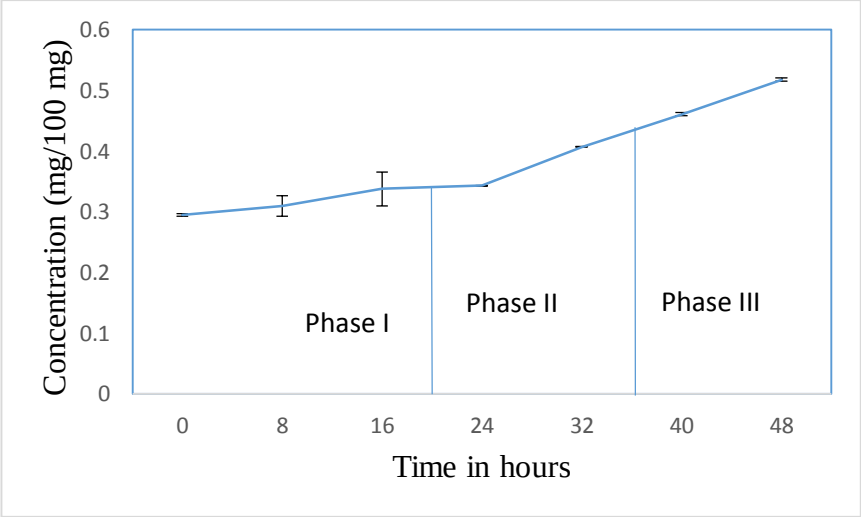

Fig 11. Total sugars concentration increase during germination

4.5 Decreased protein content during course of seed germination.

The seed protein content, estimated by Bradford assay was found to be decreased at initial stage of germination. The highest protein content was found to be in dry seeds 0.45mg/50 mg as represented in Table No. 6. The results confirmed previous findings about storage proteins that get hydrolysed and mobilized after germination. Upon germination, by action of proteinase, the peptides generated by cleavage are transported to developing plant.

Table 6. Estimated protein content at different hours of germination

Sr. No.	Germinating time (in hours)	Average Optical density at 595 nm	Concentration (mg/50mg)
1	0	0.408	0.450±0.009
2	8	0.365	0.399±0.005
3	16	0.251	0.264±0.001
4	24	0.244	0.255±0.0007
5	32	0.232	0.241±0.004
6	40	0.215	0.221±0.003
7	48	0.206	0.210±0.0001

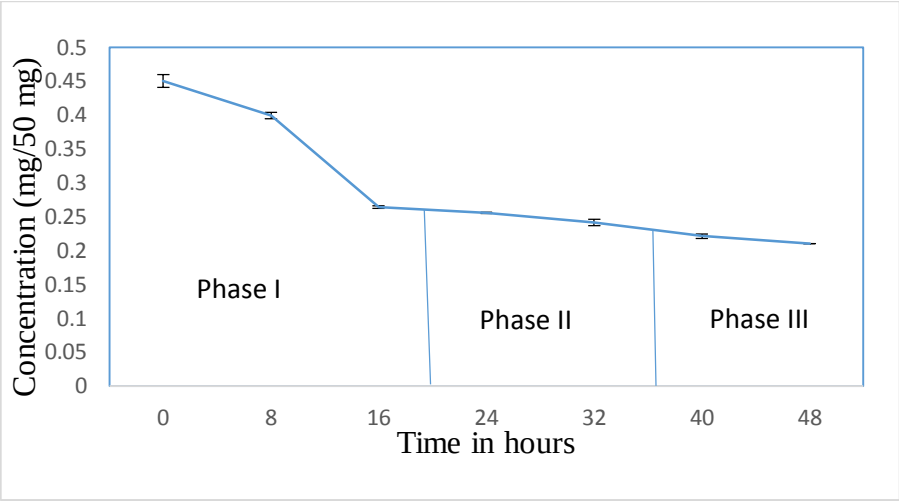

Fig 12. Decrement in the quantity of protein in the course of germination

5. Discussion

Seed germination is one of the crucial stage in the development. The biochemical changes in seed are strongly related to seedling survival rate and hence affect yield and quality. Seed germination process is complex mechanism and the process is triggered with the imbibition of water and activation of various metabolic processes including starch degradation. By imbibition the inner contents of seed increase in volume that exerts pressure on seed coat and hence gets ruptured. Thereafter, the plumule and radicle emerged.

Germination has got the profound effect on the growth of plant. Proper germination means proper seedling development. There are many hurdles to the germination process as hard seed coat, antinutrient factors present in seed and perception of improper germinating signals. Legumes The present study gives glimpse to understand the germination process and to get the idea of the minimal factors essential for the germination.

The subject of the present study is the most consumable legume in the world, Chickpea which is both economically and nutritionally important. An attempt is made to study the effect of water on the germination and breakage of dormancy and the physiological and biochemical alteration found during germination.

To investigate the germination process in chickpea, the fresh seeds were incubated in filter paper moistened with distilled water. Dry seeds have tiny opening called micropyle, through which water enter the seeds. Water brings certain changes in seeds like seed coat start becoming less hardened. With respect to the water uptake, first three different phases in the chickpea were identified and then the temporal changes in the biochemical constituents were noted. The time was chosen such that it covers all three different phases of germination and feasible too. The major biochemical storage reserves studied was starch, reducing sugars, total sugars and proteins. The importance of all these stored nutrients in germination has been reviewed in chapter 2.

5.1 Three phases of germination in chickpea

The uptake of water is triphasic. In the first phase, seed uptake rapid water that drastically increases its weight. This process takes place upto 20 hours in chickpea. Influx of water had caused temporary structural perturbation in the structure of chickpea leading to rapid solute leakage that had caused browning of surrounding solution. Second phase that is plateau phase takes place from 20 hours to 32 hours. Imbibition of water had caused the resumption of the most of the metabolic activities during this phase (Bewley et al., 2013) as major of the starch degradation and formation of total sugars and reducing sugars takes place and hence no net increase in weight of seed. This phase is of great importance as all the events causing weakening of structures surrounding embryo occurs in this phase. Phase III in which radicle protrude comes after 32 hours in the studied variety. The weight gets increased up in this phase as sugars were not consumed up. It is stored for further metabolic activities. The result had some contradiction with Vashish et al. (2007) in which phase I comes form 0 hours to 10 hours, phase II is from 10 hours to 40 hours and Phase III comes after 40 hours in studied Chickpea variety, Pusa 1053.

5.2 Biochemical analysis of seed

The reserves stored in seeds contain the nutrients necessary for the development of seedlings. Storage compounds are quickly degraded and source of energy is produced for radicle protrusion and seedling development. As the germination progress, the reserve storage material in seed mobilizes to provide the energy for biosynthetic process, building blocks for embryo development and for other processes as protein synthesis etc. Insoluble carbohydrates as starch are the main reserve food material in the chickpea (Yamasaki 2003).). Degradation of starch is essential for the process of germination. The starch content in the studied variety of Chickpea, ICC 4958 was seemed to be decreased during germination. The finding is in good agreeement with Rahman et al. (2008) and Polesi et al. (2011). Starch degradation takes place majorly during phase I and phase II indicating active metabolic activity of seeds during these phases,

Reducing sugar and Total sugar contents in the chickpea seeds are found to be increased during germination. There is gradual increase in the sugar content from 0 hours to 48 hours. The higher sugar content can be correlated with higher starch degradation as starch degrades to produce glucose. Glucose is further converted to soluble sugars that are transported to growing axis of embryo (Ali et al., 2017). Besides these functions, sugars are also efficient in protecting the membrane integrity of plant system. Concentration of these compounds increased as seed mature and hence play role in protection (Ferreira et al., 2009). Reducing sugars and total sugars concentration was less during phase I and phase II concluding the demand of these substances for radicle extension and emergence.

Total protein content during different phases and hours of germination as represented in table 6 were seemed to be decreased. From the table, it if found that the variety had significant protein content and upon germination storage proteins are degraded by peptidases to produce free amino acids that are utilised by the embryo to synthesise new proteins (Bing et al., 2003). The highest protein content is found in dry seeds (~0.450 mg/50 mg) and lowest in 48 hours (~0.210 mg/50 mg). This also indicates the vigorous activeness of proteolytic enzymes for hydrolysis of storage proteins. The result obtained contrasted with Portari et al., 2005.

The present study is much emphasized on finding the stage with higher changes in the biochemical constituents. The different stages identified has different ratio of biochemical composition that states different sets of transcriptional process and signalling. If the major or minor events are understand in transition from one phase to another, the germination time period can be modulated for easy development of seedlings.

Summary

Seed germination is a vital stage of development that starts by water imbibition, reserve storage mobilization and concludes with the radicle protrusion. The process of seed germination is triggered by many signals and one of them is water. The study on investigation of germinating behaviour of Chickpea (Cicer arietinum L.) comprises of following objectives-

1. Identification of the specially defined phases of germination.

2. Biochemical analysis of seed during different hours of germination.

The study of germination is undertaken by observing seeds showing imbibition of water and noting their weights after every 4 hours. It is conducted in sterilised glass Petridishes lined with Filter paper in 10 ml distilled water medium. On basis of increase in weight of seeds, three different germination phases has been identified. Phase I with rapid increase in weight of seeds is from 0 hours to 20 hours might be caused due to rapid uptake of water. Phase II (plateau stage) is from 20 hours to 32 hours with no increase in weight of seeds and phase III in which radicle protruse starts after 32 hours. Weights of seeds seem to be increased during phase III.

Biochemical analyses of seeds were done after every 8 hours of germination that is from 0 hours to 48 hours. Chickpea seed stores food reserves mainly in form of carbohydrates and proteins. De novo amylase synthesis during seed germination stimulates stored starch mobilization for providing the development embryo with simple sugars till photosynthesis initiate. Starch is majorly present in dry seeds (12.8±1.6 mg/100 mg) and its concentration decreases as seed germination process starts. Major portion of the starch were degraded during phase I and II. There was increase in protein contents from 0.450±0.009 mg/50 mg at 0 hours to 0.210±0.0001 mg/50 mg at 48 hours during the study. During the germination, by action of proteases the stored protein produces free amino acids which support the protein synthesis in embryo and endosperm and proceed the germination process. Reducing sugar content increase from 0.064±0.007 mg/ 100 mg at 0 hours to 0.31±0.02 mg/ 100 mg at 48 hours during the study to provide the developing embryo with carbon or energy to make the plumule and radicle. Total sugars also increased from 0.295±0.003 mg/100 mg at 0 hours to

0.518±0.002 mg/100mg at 48 hours. Major increment in the total sugars and reducing sugars were seen in phase II and phase III where major reserve mobilization has taken place. But sill, the detailed knowledge of temporal and spatial changes in gene expression at time of germination is missing. Detailed knowledge of transcriptional phase of seed germination is scarce. There is gap of understanding of powerful seed resources to gain further novel regulation and gene networks underlying germination of chickpea seed.

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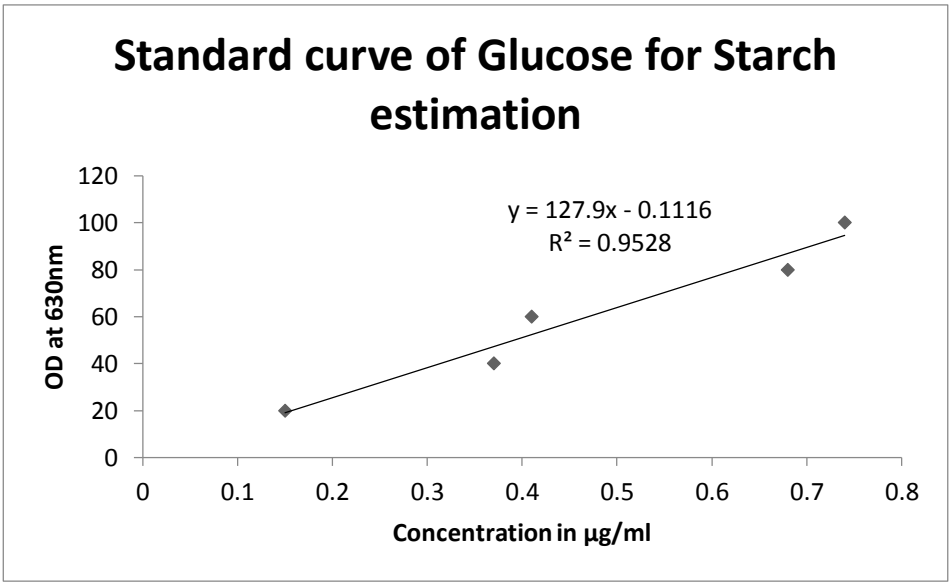
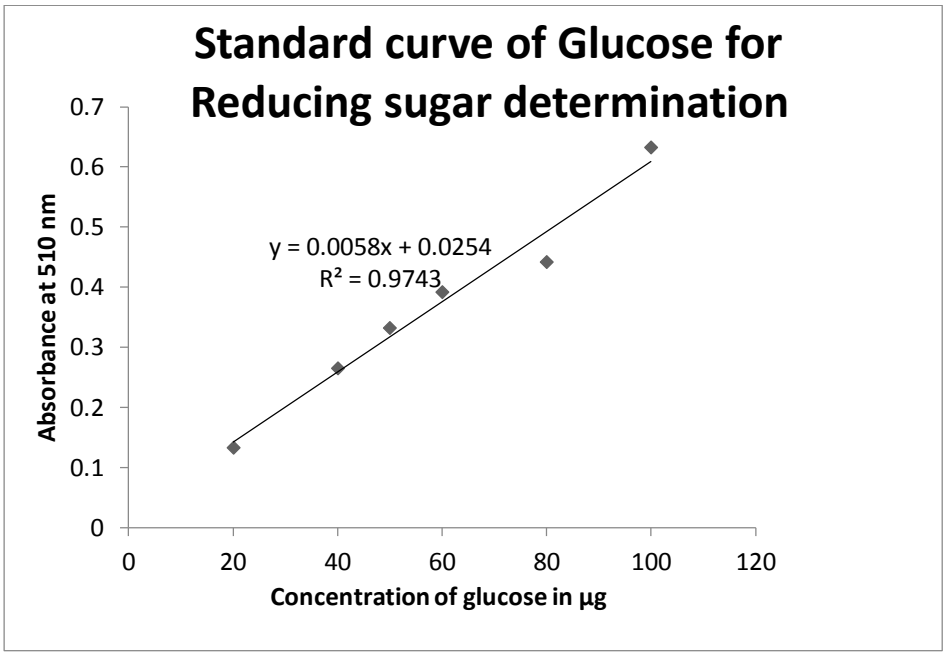
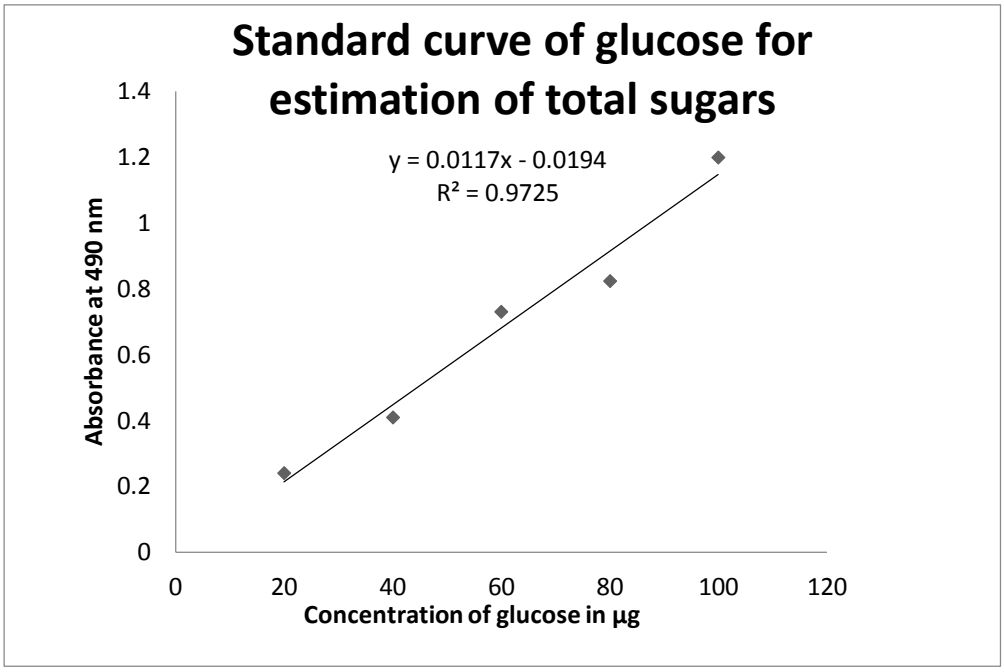
Appendix A. Preparation of reagents for biochemical tests

Sr. No.	Reagent	Preparation
1	Anthrone reagent	100mg of Anthrone reagent + 100ml concentrated H_2SO_4
2	DNSA reagent	1g 3,5 dinitrosalicyclic acid + 20ml 2M NaOH + 30g sodium potassium tartarate + make final volume of 100ml with dH_2O
3	Bradford reagent	50mg Coomassie Brilliant Blue G-250 + 25 ml 95% ethanol + 50 ml 85% phosphoric acid + volume make up to 500 ml with dH^2O

Appendix B. Standard curves






Standard curve of glucose for estimation of total sugars
y = 0.0117x - 0.0194
R² = 0.9725
Absorbance at 490 nm
Concentration of glucose in µg
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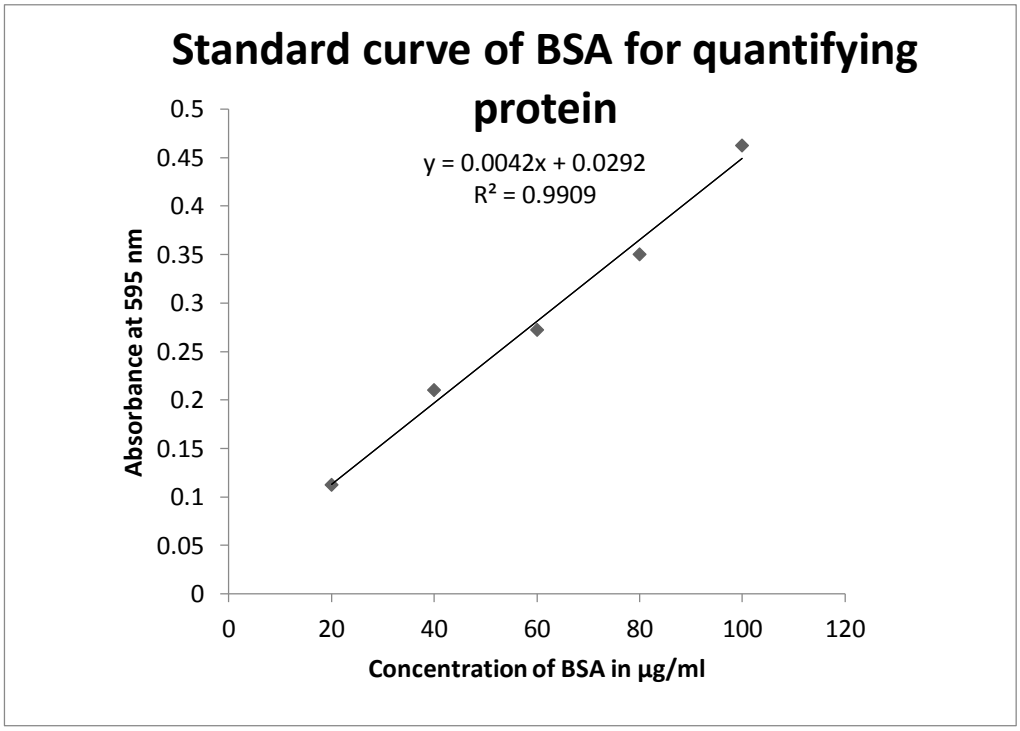
0.4
0.6
0.8
1
1.2
1.4
0
20
40
60
80
100
120


Standard curve of BSA for quantifying protein
y = 0.0042x + 0.0292
R² = 0.9909
Absorbance at 595 nm
Concentration of BSA in µg/ml
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0
20
40
60
80
100
120

Urkund Analysis Result

Analysed Document:	Thesis chickpea final report.docx (D39368076)
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