EFFECT OF TEMPERATURE ON PHYSIOLOGY OF RESERVE MOBILISATION IN *PISUM SATIVUM* L. SEEDS DURING GERMINATION

Dissertation submitted to the Faculty of Science
University of Calicut
In part fulfillment of the requirements for the degree of
Doctor of Philosophy
In Botany

SHEREENA. J

DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, 673 635
2005

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CERTIFICATE

This is to certify that the thesis entitled "**Effect of Temperature on Physiology of Reserve Mobilisation in *Pisum sativum* L. Seeds during Germination**" submitted by **Ms. Shereena. J** in part fulfillment of the requirements for the degree of **Doctor of Philosophy** in Botany, University of Calicut, is a bonafied record of research work undertaken by her in this department under my supervision during the period 2001-'05 and that no part of it has not been submitted before for the award of any degree.

Dr. Nabeesa Salim

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DECLARATION

I hereby declare that the thesis entitled "**Effect of Temperature on Physiology of Reserve Mobilisation in *Pisum sativum* L. Seeds during Germination**" submitted by me in part fulfillment of the requirements for the degree of **Doctor of Philosophy** in Botany, University of Calicut, has not been submitted before for the award of any degree.

Place: C.U.Campus
Date: 16.4.05

Shereena. J

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Shereena J

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Dedicated to
my parents and brother

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GENESIS

While conducting biochemical analyses of seed reserves in *Pisum sativum*, the dry matter determination was done by keeping the seeds at 60-70°C for prolonged period until the dry weight value became constant. During the investigation, the author incidentally observed that *P. sativum* seeds kept at 60°C and 70°C for about one week were germinable even though germination percentage was slightly reduced. This observation was the incitation to investigate the effect of higher temperature on viability, imbibition and pattern of reserve mobilization during germination of *P. sativum* seeds. Seeds were treated at various temperatures such as 35°C, 40°C, 45°C and 50°C in hot air oven for 7 days continuously and germination and reserve mobilization studies were conducted. Another interesting behaviour of *P. sativum* seeds was their ability to germinate in the chilled condition (in refrigerator).

P. sativum seeds are highly viable, and readily germinable and they rapidly germinate under favourable environmental condition (Mahler *et al*, 1988). Determining what controls the effect of temperature on seed viability requires information on seed metabolism and environmental conditions in the habitat (Baskin and Baskin, 2000). *Pisum sativum* plants are well-adapted to grow in the areas with cool and humid climatic conditions of tropics, subtropics and temperate regions with temperature ranging from 7-30°C (Davies *et al*, 1985). These plants grow as inter annual, can tolerate frost to -20°C in the seedling stage (Slinkard *et al*, 1994). Since *P. sativum* seeds are well adapted to wide variations in the environmental temperature an attempt is made in the present investigation to elucidate the physiological and biochemical aspects of temperature-treated seeds during germination at room temperature (30±2°C) and under chilled condition (Refrigerator 3±1°C).

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Introduction

Temperature is the most important environmental factor that influences seed development (Mayer and Poljakoff-Mayber, 1989; Copeland and McDonald, 1995), maturation, viability/storability (Bewley and Black, 1994; Baskin and Baskin, 2000) germination (Khan, 1978; Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1982, 1994). Similarly, moisture content of orthodox seeds occurring in the range of 5-10%, is influenced by relative humidity of storage condition and is also intimately associated with temperature. This relationship has been postulated as 'thumb rules' (Harrington, 1960).

Maturation drying is the normal terminal event in the development of orthodox seeds after which they pass into a metabolically quiescent state. Acquisition of desiccation tolerance during development is probably the consequence of morphological and physiological changes inclusive of the synthesis of specific protective substances in the later stages of seed maturation. Many seeds are usually incapable of germination unless they are dried and so it

appears that drying overcomes restraints imposed on the internal and external factors.

Several metabolic changes occur in seeds during post-harvest drying. These changes involve the appearance of two types of important metabolites that are believed to have functional significance with respect to the protection of seed tissues against rigours of desiccation. They are sugars and oligosaccharides and some specific proteins (Bewley and Black, 1994). Embryos are induced to become desiccation tolerant by the abundance of disaccharide sugars and oligosaccharide- raffinose and stachyose. These sugars play critical role in the promotion of vitrification of water and consequent protection and stabilization of lipids and proteins of cell membranes (Blackman *et al*, 1992; Gorecki *et al*, 1997). Similarly Late-Embryogenesis-Abundant proteins (LEA) which are hydrophilic due to their specific amino acid composition and stability at high temperature do not get denatured at high temperature. Their ability to attract water molecules maintains water enriched local environment inside the seed and they are thought to play an important role in protection against desiccation (Bewley, 1997).

During post-harvest drying of seeds progressive removal of water from seeds occurs. In mature dry seed water exists as bound water associated with macromolecular surfaces and this water is not readily freezable, instead they get vitrified and hence many dry seeds can withstand subzero temperature or cryopreservation (Bewley and Black, 1994; Baskin and Baskin, 2000).

Regarding dry seeds 'dry' is a relative term and does not mean that water is absent. Some water is present in dry seeds and the amount and the extent of metabolic reactions of dry seeds depend on moisture content of the seed. Respiratory reactions occur in seeds *albeit* at lower rate. In *Pisum sativum* very low rate of respiration and very little oxygen consumption occur at below 20% water content (Bewley and Black, 1994). Upon drying of seeds, slight conformational

changes occur in cell membrane (Vertrucci and Leopold, 1987; Senrathna *et al*, 1988; Bewley, 1997) and their changes include structural changes in protein (Rupley *et al*, 1983), phase changes in phospholipid (Crowe and Crowe, 1992) and vitrification of water (Bewley and Black, 1994).

Post-harvest drying of seeds leads to a reduction in moisture content by 5-10% in orthodox seeds and the concept of critical moisture content is not applicable to orthodox seeds unlike recalcitrant seeds. Nevertheless, treatment of seeds at temperature higher than ambient temperature may cause further reduction in moisture content leading to viability loss.

Even though post-harvest drying of seeds is a routine practice of seed technology of orthodox seeds to reduce their moisture content, studies on the effect of higher temperature on seed viability/germination are scanty. As mentioned earlier, *Pisum sativum* is distributed over a wide area covering tropical, subtropical and temperate region (Kay, 1979; Makasheva, 1983) and hence the seeds are adapted to a wide range of fluctuations in climatic conditions as far as survival and germination are concerned, likewise the physiological aspects of germination and reserve mobilization also are expected to be altered.

Perusal of botanical literature revealed that several aspects of seed germination inclusive of moisture content and germinability (Perry and Harrison, 1970; Ellis and Roberts, 1982; Ellis *et al*, 1988, 1989, 1990) leachate conductivity (Powell and Matthews, 1978), soaking injury (Rowland and Gusta, 1977; Prusinski and Borowska, 1996) and effect of temperature on germination (Munro *et al,* 2004) have been investigated in *P. sativum*. However, studies on pre-treatment of seed at higher temperature and the effect of these treatments on seed germination and reserve mobilisation are lacking.

Pisum sativum enjoys a unique place in the history of genetics as the plant that served Gregor Mendel with distinction during the course of his celebrated experiments.

Pea (*Pisum sativum L.*) probably originated in southwestern Asia, possibly northwestern India, Pakistan or adjacent areas of former USSR and Afghanistan and thereafter spread to the temperate zones of Europe (Kay, 1979; Makasheva, 1983). Pea was introduced into the Americas soon after Columbus and a winter type pea was introduced from Austria in 1922. Pea was taken to China in the first century (Makasheva, 1983) and was reported to be originally cultivated as a winter annual crop in the Mediterranean region (Smart, 1990).

Pea plants require a cool, relatively humid climate and are grown at higher altitudes in tropics with temperatures from 7 to 30°C and production is concentrated between the Tropics of Cancer and 50°N (Davies *et al*, 1985). As a winter annual, pea tolerates frost to –2°C in the seedling stage, although top growth may be affected at –6°C. Winter hardy peas can withstand -10°C, and with snow cover protection, tolerance can be increased to -40°C and, the optimum temperature levels for the vegetative and reproductive periods of peas were reported to be 21 and 16°C, and 16 and 10°C (day and night), respectively (Slinkard *et al,* 1994).

Effects of prolonged treatment with high temperature on radicle emergence, growth of axis, early proteolysis and protein synthesis in embryonic axis of germinating pea (*Pisum sativum*) seeds were studied by Gumilevskaya *et al* (1997). They have reported that 28°C was best for radicle emergence and further growth of axis. The highest temperature under which germination was possible was 35-38°C. Germination slowed down at 35°C, was stunted at 38°C and ceased at 40°C (Chumikina *et al,* 1993).

Incidentally, survival of *P. sativum* seeds at higher temperature and germination under chilling condition [in refrigerator (3±1°C)] were

observed while some seed technological aspects were investigated by the present author. So this study was undertaken to elucidate the minimum level of moisture content required to maintain seed viability, by treating the seeds at temperature higher than ambient temperature. Seeds treated at 35°C, 40°C, 45°C and 50°C and germination studies were conducted at room temperature as well as under chilling condition (refrigerator) in order to assess the temperature tolerance and chilling sensitivity of *P.* sativum seeds/seedlings having varying amount of moisture content. Another objective of the investigation was the comparison of physiological aspects of seed germination at different temperature and pattern of reserve mobilization during germination. Estimation of starch, sugars, protein, free amino acids and amylase assay were done in seeds/seedlings during germination of seeds treated at higher temperatures.

Organisms respond to temperature stress by producing a unique set of proteins named heat shock proteins (Vierling, 1991) and these proteins are involved in the acquisition of chilling tolerance (Collins *et al*, 1995). In order to examine the involvement of proteins in temperature treatment of *P. sativum* seeds polyacrylamide gel electrophoresis was undertaken.

Significant role of soluble carbohydrates in the promotion of water vitrification during desiccation and chilling tolerance is well established in both orthodox and recalcitrant seeds (Koster and Leopold, 1988; Blackman *et al*, 1992; Bewley and Black, 1994; Corbineau *et al*, 2000). *Pisum sativum* seeds contain 43% of starch and hence amylase assay and sugar analyses were included in the investigation to elucidate the role of sugars particularly raffinose family oligosaccharides (RFO) in the acquisition of tolerance to desiccation and chilling injury.

Histochemical localization of proteins and the insoluble polysaccharides was undertaken to pinpoint the mobilisation of these reserves from cotyledonary cells during germination. Cellular changes

occurring in the embryonic axis and testa of seeds due to temperature shock and subsequent changes during chilling were examined by anatomical studies.

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Review of literature

Majority of angiosperm seeds are orthodox and they survive desiccation. Several metabolic changes occur in seeds during desiccation. These changes involve the appearance of two types of products that are thought to have functional significance with respect to the production of seed tissue against the rigours of desiccation and these products are oligosaccharides and sugars as well as specific type of proteins (Bewley and Black, 1994).

Sucrose and oligosaccharides play a significant role in promotion of vitrification of water and facilitate the stabilization of lipid and protein in cell membranes by binding the oligosaccharides to phospholipids forming hydrogen bonding (Koster and Leopold, 1988). According to Bewley and Black (1994) disaccharide sucrose and oligosaccharides raffinose and stachyose are abundant in desiccation tolerant seeds. In soybean seeds it has been found that there is a conversion of previously existing monosaccharide to sucrose and/or oligosaccharide during desiccation and these metabolites are formed from the breakdown of starch and there is a steep drop in starch

content coinciding with the acquisition of desiccation resistance and these sugars facilitate stabilization of protein and lipids in the cell membranes during higher temperature treatment/desiccation period (Blackman *et al,* 1992). Lahuta *et al* (1998) suggested the relationship between desiccation tolerance and soluble sugars in pea seeds during germination. When 96 hour germinated seeds were subjected to fast and slow drying, nearly 10% of the epicotyl survived fast drying and 15% survived slow drying treatments. The desiccation tolerance of root tissue was lost during the first 48 hour of imbibition. The loss of desiccation tolerance in seedling was accompanied by rapid degradation of and accumulation of fructose and galactose. Aberlenc-Bertossi *et al* (2003) have studied the relationship between desiccation toleration and dry matter, water and sugar content of oil palm. According to these authors both in *planta* and *in vitro,* desiccation tolerance was associated with a fall in the monosaccharide/sucrose ratio, reduction of moisture content and accumulation of dry matter. In *planta*, survival to dehydration was related with deposition of oligosaccharides whereas *in vitro*, it was related with high sucrose accumulation. Gorecki *et al* (1997) reported that in *Lupinus luteus* highest rate of oligosaccharide accumulation appeared during seed desiccation and was correlated with the acquisition of ability to germinate.

Corbineau *et al* (2000) studied germination and carbohydrate metabolism in fresh developing *Pisum sativum* L. cv. Baccara seeds after artificial drying at 25°C at various RH 20.75 and 99% and found that the acquisition of desiccation tolerance was associated with an accumulation of raffinose and stachyose, the latter being more abundant in embryonic axis than in the cotyledon. The (raffinose + stachyose)/sucrose ratio was increased during seed development and reached 1.1 in the axis and 0.2 in the cotyledon just before the onset of desiccation tolerance. When natural acquisition of desiccation

tolerance occurred on the mother plant, artificial drying of isolated seeds induced an increase in oligosaccharide content in the cotyledons. Immature seeds, the moisture content of which was higher than 60% (fresh weight basis), did not tolerate fast drying (25°C and 20 or 75% RH). Such drying did not result in the synthesis of stachyose and induced an increase in electrolytic leakage, a decrease in the ability of seeds to convert 1-amino cyclopropane 1-carboxylic acid (ACC) to ethylene and an increase in ethane synthesis, thus indicating a deterioration of cell membrane properties and lipid peroxidation. According to Buchanan *et al* (2000) increase in the soluble non-reducing sugars occur during desiccation. In particular, accumulation of raffinose is associated with desiccation tolerance and it is important for the longevity of mature dry seeds. The ratio of sucrose to raffinose might be a determining factor and for maintenance of desiccation tolerance, the ratio in the maturing seed cells must be no greater than 20:1. Raffinose appears to prevent sucrose crystallization during cell dehydration, allowing cytoplasm to retain a stable glassy state (Buchanan *et al*, 2000; Corbineau *et al*, 2000)

Very little conformational changes occur in cell membranes upon drying of seeds (Vertucci and Leopold, 1987; Senarathna *et al*, 1988; Bewley, 1997). Nevertheless there are references on minor changes occurring in lipid bilayers due to desiccation (Seewaldt *et al,* 1981). Protein structure of seeds also is changed when most of the interstitial water is removed during drying (Rupley *et al,* 1983). According to Crowe and Crowe (1992), the membrane phospholipid component forms a gel phase during maturation drying. Notwithstanding, drying is a prerequisite for maintaining the seed quality.

According to Bewley (1997) ABA-induced protein synthesis is thought to play a role in establishing desiccation tolerance. Besides storage proteins, abscisic acid (ABA) induced genes associated with

seed maturation and acquisition of desiccation tolerance encodes small heat-shock proteins and Late-Embryogenesis-Abundant (LEA) proteins. Importance of these proteins in the seed is the protection of other proteins and membranes. LEA proteins might solvate these cellular components by forming amorphous coils, protecting cell content from disruption or damage in the desiccated stage (Bewley, 1997).

After desiccation dry seeds contain various types of water occurring as tightly bound structural part of macromolecules as thin film coated over the surface of molecule and/or as bridges over hydrophobic sites resulting in changes in the phase behavior of membrane lipids (Bewley and Black, 1994). According to these authors, it is recognized that in the dry state the cytoplasm of desiccation tolerant seeds, water exists in a glassy (vitrified) state even at physiological temperatures and this vitrification stops or slows down all chemical reactions requiring molecular diffusion.

When the seeds were subjected to higher temperature progressive removal of water occurs. Bound water, associated with macromolecules is lost first resulting in structural and functional deterioration of seeds (Bewley and Black, 1994). Higher temperature induces tighter packing of molecules and increases their structural disorder leading to loss of seed vigour and/or viability.

Hanley *et al* (2001) reported that in seeds of certain genera of Fabaceae such as *Lupinus, Gastrolobium, Hippocrepis* and *Cyclopia* higher temperature up to 120°C for short period (5min.) resulted in fast growth and increased productivity during their seedling growth. A similar study of higher temperature treatment for short duration (5 min.) on *Prosopis caldenia* showed a higher germination percentage (de Villalobos *et al,* 2002). Thomas *et al,* (2003) reported that the heat shock of 50°C, resulted in stimulated seed germination of

Dracophyllum secundum, sprengelia monticola, Gahnia sieberiana and *Kunzea ambigua* from soil seed bank.

Ellis and Roberts (1982) suggested that hardseededness is an important consequence of desiccation the highest seed moisture content at which irreversible hardseededness was induced by desiccation to 7.9%, while the proportion of seeds damaged by soaking was constant between 7.9% and 16.8% moisture content and this proportion was doubled following desiccation from 7.9% to 7.2% moisture content. According to Ellis *et al* (1990), in the seeds of *Pisum sativum*, germination was declined from 94% to 50% on slow drying from 14.8% to 3.7% moisture content (wet weight basis) at ambient temperature. However, the damage following desiccation was avoided if the moisture content of the seeds before the beginning of germination test was raised by humidification. Soaking the seeds in water for 24 hours at 20°C resulted in increased germination loss. In a similar study Mai-Hong *et al* (2003) observed that the seeds of *Peltophorum pterocarpum*, a tree legume, and the hardseededness were induced when seeds were dried to about 15% moisture content. The normal seedling growth or the germination percentage was affected by over drying. It increased the time for germination and caused abnormalities in germinating seedlings.

In *Raphanus sativus* L. var, *longipinnatus* Bailey cv. Xin Li Mei seeds on treatment with dry-heat (76°C) for 12-72 hours, at 3.2% seed moisture content, germination and vigour were maintained well after 48 hours of drying, but vigour was reduced after 60 hours and germination after 72 hours. At 9.4% seed moisture content germination was reduced significantly after 36 hours and vigour was reduced after 12 hours. The recommended 48 hours dry heat treatment at 76°C reduced seed quality (germination and/or vigour) for all seed containing moisture contents above 3.2%. These reductions of seed quality were

increased as initial seed moisture content was increased (Meng *et al,* 2003).

Information regarding the relationship between laboratory seed vigour testing and seedling field emergence is very important to estimate seed performance after sowing and helps seed producers to adopt the best procedures to improve stand establishment. In soybean [*Glycine max* (L.) Merrill] seedling field emergence result based evaluations of seed water content, standard germination, accelerated ageing, electrical conductivity, and seedling field emergence showed a close association between planting environmental conditions, seed physiological quality and seedling field emergence. The most accurate predictions were obtained for AA (Accelerated Ageing) values $\geq$ 90%, when field emergence was higher than 80% (r^2 = 0.90). Based on the results it was concluded that the AA test provided an accurate estimate of field emergence of soybean seedlings. However, as seedbed environmental conditions became less favourable, the ability of the AA test to estimate field performance significantly decreased (Torres *et al,* 2004).

When dry viable seeds imbibe water, many physical and structural changes occur during initial phase of imbibition and throughout germination. Since the water potential of mature dry seed is much lower than that of the surrounding medium, water uptake occurs. As seeds start to take up water, there is a rapid leakage of solutes such as sugar, organic acids, ions amino acids and protein. The selectively permeable membranes that normally retain solutes within the cells, lose their integrity during drying of seeds and do not act as retentive barriers during initial stages of imbibition (Bewley and Black, 1994). Powell and Matthews (1978) studied the imbibition of pea seeds and reported that rapid uptake of water by dry seeds can result in imbibitional injury and rapid early leakage was caused by physiological disruption of membranes. Imbibitional damage resulted in reduced

respiration and germination. The role of testa with regard to imbibition in the pea seeds is critical and leakage was more rapid in the seeds with damaged testa compared to that of intact seed coat.

According to Simon (1984) the leakage of solute from seeds during imbibition was due to reorganization or repair of phospholipids and proteins of membrane which adopt some other configuration under dry conditions that prevail in dry conditions. When the dry seeds of pea were placed in water, leakage of potassium was very fast at the start of imbibition and about 10% of potassium was lost by this process. The rate of leakage slowed down by 10 to 20 minutes and reached a steady rate that is maintained for the next half an hour or more and finally coming to half after a day of soaking. In *Brassica* seeds the conductivity of leachate was significantly related with germination and was clearly higher for samples with lower germination (Thornton, 1990). Doijode (1988) had reported in 11 *Capsicum* cultivars that there was a relation between electrical conductivity and ageing of seeds. Electrical conductivity and soluble sugar content in leachate were high in the leachate of aged seeds.

In *Zea mays* ageing the kernel for 18 days at 40°C and a moisture content of about 15%, increased leakage during imbibition (Bruggink *et al,* 1991). According to Dell' Aquila (1999) in lens seeds the germination percentage and the physiological parameters such as electrical conductivity of leachate are closely related.

Lee *et al,* (1995) suggested a relationship of viability to leaching of sugars from leek, onion and cabbage seeds. According to these authors the sugar content of dry seeds was relatively unchanged by seed ageing (treating seeds at 45°C), while the percentage of total sugars in leachate after a 24 hour soaking increased as seed germinability declined in all seeds. Glucose, fructose, sucrose and stachyose were detected in leachate from non-germinable treatment

(treatment at 45°C) of all seeds, while raffinose was found in detectable quantities in leek and cabbage only. Thus, dry, non-aged seeds of all the three crops contained similar amounts of sugars. There were qualitative and quantitative differences in leakage of sugar between the three lots as influenced by ageing. Non-germinable cabbage seeds leaked the greatest amount sugars compared to other lots.

According to Bewley (1997), the germination commences with the uptake of water by the dry seed (imbibition) and is completed when the radicle protrude to outside. During the early phase of imbibition most of the water taken up is presumably used to rehydrate desiccated organic molecules, such as those of nucleic acids and proteins. Later, free water begins to accumulate in cytoplasmic vacuoles and it is at this stage that the cells appear to become susceptible to drying (Sutcliffe and Pate, 1977). Imbibition of water is followed by a general activation of seed metabolism.

Increased respiration is one of the earliest biochemical events to be detected in imbibed seeds. This is followed closely by the release of hydrolytic enzymes that digest and mobilize the stored reserve and renew cell division and cell enlargement in embryonic axis (Bewley and Black, 1985).

Mobilisation of the stored reserve in seeds has been studied in many cereal grains and legume seeds (Khan, 1978; Bewley and Black, 1983, 1985 and 1994; Mayer and Poljakoff Mayber, 1989; Copeland and McDonald, 1995). Starch is the carbohydrate most commonly found in seeds. It is stored in two related forms, amylase and amylopectin. Sucrose, the sugar translocated from the mother plant to the seed is the substrate for starch formation.

During germination, carbohydrates, the principal reserve materials, are degraded in the cotyledon or endosperm and the products are translocated to the developing axis (Bewley and Black,

1983 and 1985, Mayer and Poljakoff-Mayber, 1989). In garden pea, 35-40% of the seed dry weight is attributable to starch, (Bewley and Black, 1985). Hydrolysis of reserves in intact legume cotyledon commences after emergence and elongation of the radicle. The depletion of starchy reserve from the cotyledon of Pea is biphasic, showing an initial slow rate that lasts for 5-6 days being followed by rapid decline. The free sugar and dextrin released by starch hydrolysis are rapidly translocated to the growing axis.

Mayer and Poljakoff-Mayber (1989) suggested that most of the enzymes involved in the breakdown and interconversion of carbohydrates become active during germination and are synthesized *de novo*. Douglass *et al*, (1993) reported a positive correlation between starch and germination whereas negative correlation between sugar content and germination from sweet corn seedling emergence in cold soil. The positive correlation could be associated with its insolubility of starch in water. The water insoluble starch remains available to the embryo to be metabolized and used as energy source for germination, emergence and seedling growth. According to Issa *et al*, (1994) during germination of cowpea, the seed starch and non-reducing sugars were decreased and were followed by an increase in reducing sugars. Sucrose content was highest after 2 days of germination and protein mobilization was slightly increased.

Raffinose oligosaccharides are major soluble carbohydrates in pea (*Pisum sativum* L.) seeds. Their biosynthesis proceeds by stepwise addition of galactose units to sucrose, which are provided by the unusual donor galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol). Chain elongation also proceeds by transfer of galactose units between raffinose oligosaccharides. The protein, a member of family 36 of glycoside hydrolases, catalyzes the synthesis of stachyose, the tetrasaccharide of the raffinose series, by galactosyl transfer from galactinol to raffinose. It also mediates the synthesis of the

pentasaccharide verbascose by galactosyl transfer from galactinol to stachyose as well as by self-transfer of the terminal galactose residue from one stachyose molecule to another. These activities show optima at pH 7.0. The enzyme also catalyzes hydrolysis of the terminal galactose residue of its substrates, but is unable to initiate the synthesis of raffinose oligosaccharides by galactosyl transfer from galactinol to sucrose (Peterbauer *et al.* 2002).

Swain and Dekker (1969) have shown that the total activity of pea cotyledonary α-amylase and its specific activity increased between 7 to 10 days of germination in darkness. The tests to detect the activity of inhibitors on α-amylase activity during the early days of germination have shown that the α-amylase was synthesized newly during germination. In *Zea mays* three classes of starch degrading enzymes have been identified: α-amylase β-amylase and phosphorylase. α and β-amylase have been shown to be controlled by independent loci, each locus consisting of two codominant alleles (Chao and Scandalios, 1975).

Davis (1979) reported from pea seeds that the activity of α-amylase reached a peak at 7^{th} day of germination. The activity of β-amylase remained the same while that of α-amylase increased. The increase in activity of α-amylase showed corresponding decrease in starch. Similar studies by Sumathi *et al,* (1995) reported that the ungerminated seeds of some legumes exhibited very low amylase activity and the activity was increased on progressive germination. The seeds showed high amylase activity after 48 hours of germination.

In rice seeds the drying treatments had not made any decrease in germination and viability of seeds (Shephard *et al,* 1995). The α-amylase activity has shown a close correlation with radicle extension of seeds. The radicle elongation was delayed after ageing and also artificial ageing delayed the onset of α-amylase activity from 36 to 48

hours in seeds harvested earlier. The ungerminated seeds with shorter radicle showed low α-amylase activity.

In addition to α-amylase, proteolytic enzymes (protease), β-amylase and other starch degrading enzymes are involved in mobilization of endosperm reserve (Hopkins and Hüner, 2003). Yamasaki (2003) have isolated β-amylase from germinating millet (*Panicum miliaceum* L.) and the enzyme hydrolyzed amylose, amylopectin and soluble starch. While the enzyme showed some activity against native starch by itself, the starch dehydration was accelerated 2.5-fold using α-amylase, pullulanase and α-glucosidase.

Earlier Murray (1984) suggested that in pea cotyledons, the initial break down of starch must involve starch phosphorylase alone, since β-amylase can act only on soluble substrates and α-amylase is absent.

In the non endospermic seeds such as legumes (pea, beans) the initial stage of radicle elongation depends on stored reserve in the cotyledon and these reserves are mobilized/transported to the elongating embryonic axis. The cotyledon forms the major site of storage of reserves in a pea seed (Sutcliffe and Pate, 1977). Like other legumes peas are rich in protein and vitamins. Fully grown ripe pea seeds contain sugar (5.9 - 4.1%), starch (32.9-43.4%) & protein (22.9%) (Anonymous, 1969). In *Pisum sativum* cotyledons, the protein bodies are clumped together and vacuolated indicating their breakdown during germination (Ashton, 1976). Bewley and Black (1985) have described systematically the characteristics of seed storage protein. They are usually deposited within special cellular organelles called protein bodies. These range in diameter from 0.1-25μm and are surrounded at least during development by a single membrane. Some protein bodies are simple in that they consist of a protein matrix surrounded by limiting membrane.

Juliano and Varner (1969) reported from germinating pea seeds that there was a slow decrease in protein content of cotyledon up to 5 days when the seeds are germinated in the dark. Thereafter it decreases rapidly and is only 7% of the original content by fifteenth day. According to Bewley and Black (1985) cotyledons of mature dry seed contain little proteinase activity in garden pea (*Pisum sativum*) but show an increase in this enzyme after several days associated with the mobilization of major protein reserves-vicilin and legumin. This enzymatic degradation of stored protein leads to an accumulation of free amino acids in the cotyledon but these decrease after about 3 days as they are transported into the growing axis. In the garden pea and broad bean, legumin is degraded earlier and faster than vicilin. By studying the state and properties of ribosome in the cotyledonary cells of dry seeds and the activation of genome and translation machinery during germination Gumilevskaya *et al* (1995) reported translational and transcriptional activities in storage parenchyma cells of germinating pea seeds from the onset of imbibition until radicle emergence.

The plants and other organism respond to high temperature stress with the synthesis of heat shock proteins (Lindquist and Craig, 1988; Vierling 1991) and a number of them are known to function as molecular chaperons (Georgopoulos and Weich, 1993). During temperature stress molecular chaperons are believed to act in preventing irreversible protein denaturation that would otherwise be detrimental to the cells (Parsell and Lindquist, 1993). Gumilevskaya *et al,* (1996) reported the synthesis of high molecular weight and low molecular weight heat shock proteins in pea seeds on elevating the temperature to 38-40°C for 2-4 hours from 28°C in which the seeds have been germinated. In higher plants heat shock proteins are generally induced by a short exposure to a temperature of 38-40°C (Iba, 2002).

According to Lafuente *et al,* (1991) cucumber seed cotyledons on exposure to 37 or 42°C for 6 hours were induced to produce five HSPs with molecular mass of 25, 38, 50, 70 and 80KDa and upon treatment at 12.5 or 25°C for 4 days and the synthesis of three proteins with molecular mass of 14, 17 and 43 KDa were reduced.

According to Brodl and Ho, (1991) in barley aleurone cells of seeds, the heat shock causes the suppression of the synthesis of some natural proteins, while the syntheses of some other proteins are not affected. The seeds of sorghum have been reported to synthesise heat shock protein after temperature treatments and the seedlings have shown maximal thermotolerance after the full development of HSPs. But to withstand the lethal temperature the seeds required a treatment with lower temperature so as to induce HSP for tolerating the effect of high temperature (Howarth and Skot, 1994).

Seedlings of soybean, pea, sunflower, wheat, rice, maize and pearl millet have been reported to show the accumulation of heat shock protein to significant levels after three hours of heat shock but the proteins were characterised by considerable heterogeneity in molecular weight, isoelectric point, stainability and radiolabel incorporation (Mansfield and Key, 1987). Most of the proteins appeared to be synthesized only during heat shock and some were detectable at low levels in control tissue. *Zea mays* seedlings produce heat shock protein of molecular weight of 45 KDa on exposure to soil drying and temperature stress (Ristic *et al,* 1991).

Howarth (1990) reported that in sorghum and pearl millet seeds the heat shock did not show any affect in normal protein synthesis and the synthesis of HSP showed restricted levels.

According to Dell' Aquila *et al* (1998) normal germination protein synthesis in wheat and barley was slightly modified by age, but following heat shock treatment a general reduction of most of the

control polypeptide occurred and heat shock response resulted in the production of several HSPs with different molecular weight like 17-14.2 KDa.

According to Helm *et al* (1993) in *Pisum sativum* and *Glycine max* low molecular weight heat shock proteins of PsHSP 22.7 and GmHSP 22.0 respectively are synthesized after 3 hours of heat stress at 37°C and these two HSPs are observed in higher molecular weight structures with apparent masses between 80 and 240 KDa. DeRocher *et al* (1991) showed that the accumulation of HSP 18.1 and immunodetected protein was proportional to the severity of the heat stress.

Downie and Bewley (2000) reported that in white spruce (*Picea glauca* (Moench.) Voss.) seeds, the raffinose family oligosaccharides provide carbon reserve for the early stages of germination prior to radicle protrusion. Desiccated seeds contained sucrose and the first three members of raffinose family oligosaccharides, raffinose, stachyose and verbascose. Upon radicle protrusion at 25°C the raffinose, stachyose and verbascose were decreased to low amounts and the sucrose was mobilized to fructose and glucose.

According to Matheson (1984) sucrose is the predominant form of transport carbohydrate in majority of seeds during germination. The supply of sucrose to phloem cells in cotyledon is derived initially from the reserves of free sugars which are often substantiated as in the cotyledons of wrinkle seeded pea cultivars. This supply of sucrose for export can be maintained through polysaccharide breakdown.

During germination there is considerable interconversion of amino acids, necessitated by other metabolic events, the requirement of transport system and difference between amino acid compositions of reserves compared with new cytoplasmic proteins. The amino acid released from storage protein can contribute directly to protein

synthesis in growing parts, but labeling studies have shown that considerable interconversion and metabolism of amino acid carbon occur in storage tissue during germination. Even during the period of massive protein breakdown in the cotyledons some amino acids were reincorporated into the soluble fraction. Amino acid may contribute large amount of substrate carbon to the respiratory system, and even to sugar synthesis in gluconeogenic seeds such as castor bean (Lea and Joy, 1983).

The embryonic axis is known to influence the mobilization of starch reserves through the development of amylase activity in cotyledon of dicot seeds. In the absence of the axis the amylase activity declined, remained unaltered or even increased in various culture of pea (Morohashi and Ueno (1980) and beans (Morohashi, 1982).

According to Horvath *et al* (1993), 2-3 weeks germinated seedlings of *Arabidopsis thaliana* L. (Heyn.) in controlled environment at 21°C when transferred to 3°C for 24h have shown changes in gene expression due to cold acclimation. In a similar study Gilmour *et al* (1988) have reported that *Arabidopsis thaliana* L. (Heyn.) Columbia and *Landberg erecta* on cold acclimation at 4°C induce changes in population of mRNA and a marked increase in the level of four polypeptides having molecular weight of about 47KDa.

Moynihan *et al* (1995) reported that the 6-8 week seedlings of potato, pea, cucumber, lima beans, egg plant, tomato, cotton, soybean and *Arabidopsis thaliana* on exposure to chilling temperature showed increased heat evolution and it was due to the increased capacity to produce respiratory heat after exposure to chilling temperature. Lång *et al.,* (1994) reported that in *Arabidopsis thaliana* exposed to cold temperature (2°C) for 4h. have increased ABA level and according to them ABA was indirectly required for the development of full freezing

tolerance. According to Nykiforn and Johnson-Flanagan (1999) in *Brassica napus* L. var.*oleifera* cv. Westar seedling growth at 10°C was lower in comparison to seedling grown at 22°C. Seed at 6°C displayed slow and incomplete germination and poor seedling growth as a result of both thermal and developmental effects.

EFFECT OF TEMPERATURE ON PHYSIOLOGY OF RESERVE MOBILISATION IN *PISUM SATIVUM* L. SEEDS DURING GERMINATION

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In Botany

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Materials and methods

Garden pea or green pea (*Pisum sativum* L. Cv. Bonni villa) seeds were purchased from National Seed Corporation, Thiruvananthapuram. Healthy and good quality seeds were selected by hand picking method and deformed seeds with broken testa were discarded. The seed samples were tested for viability using tetrazolium test.

Temperature treatment

Healthy seeds were selected and kept in hot air oven at 35°C for 7 days. After 7 days, seeds were taken for germination studies. Another set of seeds were kept in hot air oven at 40°C, for 7 days, and germination studies were carried out. Similar treatments were given to separate seed samples at 45°C and 50°C. Seeds without any treatment were considered as control.

1. Germination studies

Germination studies were carried out at room temperature (30 ± 2°C) and in refrigerator (3 ± 1°C). Thirty seeds each in five replicates were selected, surface sterilized with 0.1% (w/v) mercuric chloride solution and washed thoroughly with double distilled water. These seeds were used for germination studies.

1.1. Germination under laboratory condition

1.1.1. Germination at room temperature

Germination studies, at room temperature (30 ± 1°C), of control and the seed treated at 35°C, 40°C, 45°C and 50°C were carried out in sterilized Petri dishes lined with whatman No.1 filter paper. Seeds were soaked in double distilled water for 4-5 hours and water containing leachate was decanted and kept for germination in the dark condition. The germination rate, percentage and Seed Vigor Index were calculated by observing daily count of germination.

1.1.2. Germination in the refrigerator (3±1°C)

Germination studies of control and treated seeds of 35°C, 40°C, 45°C and 50°C were carried out in sterilized Petri dishes lined with whatman No.1 filter paper. The seeds were soaked in double distilled water for 4-5 hours and water containing leachate was decanted and kept for germination in the refrigerator. The germination rate, percentage and Seed Vigor Index were calculated by observing daily count.

1.2. Germination under field condition

For the field study seeds of control and treatments of 35°C, 40°C, 45°C and 50°C were sown in garden pots filled with garden soil, sand and dried powered cow dung mixed in 2:1:1 ratio. Hundred and twenty seeds of five replicates from control and each treatment were sowed in pots and kept for germination. Daily counts of germinated seeds were taken until germination was completed.

Germination percentage

Germination percentage of seeds subjected to treatment and control was calculated using the formula:

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds kept for germination}} \times 100$$

Field Emergence Index

The field emergence index for each experiment was calculated based on the procedure used by Egli & TeKrony (1995) according to the following equation:

$$FEI = \frac{FE}{G} \times 100,$$

Where: FEI = Field Emergence Index; FE = Mean Seedling Field Emergence of all seed lots; G = Mean Standard Germination of all seed lots. In this index, the higher the value, the better the field conditions until FEI = 1.0, so that FE = Standard germination (SG) (ideal).

Seed Vigour Index

The Seed Vigor Index (SVI) was calculated (Copeland and McDonald, 1995), using the formula given below:

$$SVI = \frac{\text{No. of seeds germinated}}{\text{Days of first count}} + \cdots + \frac{\text{No. of seeds germinated}}{\text{Days of last count}}$$

Seedling vigour

The seeds of control and all the treatments were sown in garden pots as described earlier. Twelve days old seedlings of control and experimentals were uprooted and washed with tap water until all the sand particles were washed off. The seedlings along with cotyledons were blotted with filter paper and weighed using electronic balance (Shimadzu Ax 120 Electronic balance). Then cotyledon was detached carefully and weight was determined. The weighed samples were kept in hot air oven at 100°C for 1 hour and kept at 60°C until constant weight was obtained. Seedling vigour was calculated based on mg dry weight/seedling.

1.3. Moisture content determination

Thirty seeds from each of the treatment and control were taken and brought to room temperature by keeping in a desiccator. Fresh weights of these seeds were determined using Shimadzu Ax 120 Electronic balance. Then the weighed seeds were kept in hot air oven at 103°C for 10 hour (ISTA, 1985) and again weighed. Drying and weighing were repeated until constant weight was obtained. Percentage of moisture content was calculated as explained by ISTA (1985).

$$\text{M. C. \%} = \frac{\text{Fresh weight of seeds} - \text{Dry weight of seeds}}{\text{Fresh weight of seeds}} \times 100$$

1.4. Imbibition studies

Weight of individual seeds (20 seeds in 5 replicates) treatment and control were determined. Then the seeds were kept in petridishes lined with filter paper and were soaked in double distilled water. The positions of individual seeds in Petri dishes were marked. Individual seeds were again weighed after blotting with filter paper at the intervals of 1hour upto 12 hour and 24 hour using electronic balance.

1.5. Conductivity studies

The electrical conductivity of leachate was considered as a measure of electrolytes leached out the seeds during the imbibition and germination. In the present study, the conductivity of the leachate was measured using Toshniwale TCM 15 Autoranging conductivity and TDS meter by cumulative and non-cumulative methods.

1.5.1. Cumulative conductivity measurement

Fifty seeds each in five replicates of all treatments and control were selected weighed and soaked separately in 250 ml of de-ionized water and incubated at room temperature. After 1hour, electrical

conductivity of steep water was measured. The seeds were replaced in the same steep water and conductivity measurements were repeated at an interval of 1hour upto 12 hour and at 24 hours.

1.5.2. Non-cumulative conductivity measurement

Fifty seeds each in five replicates of control and all the treatments were soaked in 50 ml de-ionized water. After 1hour steep water was completely drained off and collected in a container and conductivity measurements of the steep water were taken. Same volume of de-ionized water drained off was again added to the seeds for further imbibition and conductivity measurement was taken at an interval of 1 hour upto 12 hour and also at 24^{th} hour.

In all the conductivity experiments, the conductivity of de-ionized water was subtracted from measurements of steep water and all the measurements were taken at room temperature. Conductivity was calculated based on per gram seed weight and expressed as micro mho cm^{-2}/gram of seeds.

1.6. Estimation of sugar in leachate

For the estimation of sugars in leachate, the steep water at the interval of 1, 2, 3 up to 12 and at 24 hours were collected and after every removal, same volume of redistilled water was added to the seeds for further imbibition. To a known volume of the leachate from control and all the treatments 95% ethanol was added and was centrifuged, so as to sediment the particles if any. The supernatant was decanted to evaporating dishes and was evaporated to dryness. The precipitated sugars were dissolved in 3 ml redistilled water.

Estimation of sugar was done according to Montgomery (1957). To 1 ml of the supernatant 0.1ml 80% (w/v) phenol was added and shaken well. Five milliliter of concentrated sulfuric acid was added quickly from a burette and allowed to cool. The optical density of solution was measured colorimetrically (No. 4 green filter) using Systronics colorimeter. Glucose procured from Merck chemical company was used as standard.

2. BIOCHEMICAL STUDIES

Biochemical analysis of metabolites was carried out using dry and germinating seeds of both control and treatments.

Germination: The germination was carried out in Petri dishes lined with Whatman No.1 filter paper. Seeds of control and treatments were germinated at room temperature (30±2°C) and in refrigerator (3±1°C). However, the seeds germinated at room temperature and in refrigerator showed differences in the duration of germination, hence sample collection was carried out at regular intervals of one day up to 12^{th} day of germination in the case of seeds germinated at room temperature and on 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} day of germination in the case of seeds germinated in refrigerator. This type of sample collection was followed on the basis of morphological similarities between the seeds germinated at room temperature and in the cold because the seeds germination in the refrigerator showed very slow growth. The seeds germinated in the refrigerator on 10^{th} day of germination showed almost equal growth rate to that of 1^{st} day of germination at room temperature. Seeds/seedling samples collected at the regular intervals were de-coated and used for the analyses of different metabolites and amylase assay.

2.1. Dry weight

Dry weight of seeds and seedlings which contain large amount of moisture content was determined by hot air oven method. Weighed samples were kept in hot air oven at 100°C for 1 hour followed by at 60°C for 24 hours and the materials were brought to room temperature by keeping in a desiccator. Drying and weighing were repeated until constant weight was obtained.

2.2. Starch estimation

The method of Pucher *et al* (1948) described by Whelan (1955) was used to estimate the starch in seed samples. Two hundred

milligram of tissue was weighed and homogenized in a mortar and pestle in double distilled water and transferred into a test tube. The homogenate was then centrifuged for 5minutes and the supernatant was collected using 30%(v/v) perchloric acid. The residue was again washed 3-4 times with 30% (v/v) perchloric acid, centrifuged and the supernatant was collected. The final volume of combined supernatant was noted. A known volume of extract was pipetted from combined supernatant and an equal volume of freshly prepared iodine-potassium- iodide reagent was added. After 10 minutes it was centrifuged for 10 minutes. The supernatant was decanted off. The precipitate was then washed with alcoholic sodium chloride to remove excess iodine reagent. After centrifugation the blue precipitate was treated with alcoholic sodium hydroxide till blue color was discharged and again washed with alcoholic sodium chloride. The precipitate was dissolved in a known volume of 10% (v/v) sulfuric acid by heating in a bath of boiling water and centrifuged for 10 min.

Estimation of starch was done according to Montgomery (1957). To 1 ml of the supernatant 0.1ml 80% (w/v) phenol was added and shaken well. Five milliliter of concentrated sulfuric acid was added quickly from a burette and allowed to cool. The optical density of solution was measured at colorimetrically (No. 4 green filter) using Systronics colorimeter. Soluble starch procured from Merck chemical company was used as standard

2.3. Estimation of sugars

The qualitative and quantitative analyses of sugars were done using Thin- layer Chromatography. The silica gel impregnated with 0.02 M sodium acetate was used for resolution. Thin layer plates of 250μ thickness were prepared.

Preparation of sample: One gram tissue of control and treated seeds were ground with 80% ethyl alcohol in mortar and pestle and reflexed for 4 hours. The homogenate was centrifuged and supernatant was collected and the residue was again homogenated 4-times with 80%

alcohol and after each centrifugation the supernatants were combined. The combined extract was evaporated about to dryness over a boiling water bath. The precipitated sugars were then dissolved in 3 ml redistilled water.

Separation and estimation of sugar by TLC: One micro litre of the extract was applied on TLC plates and resolved. The samples were resolved using Chloroform-Methanol (60:40) solvent system. Visualization of sugars was carried out using Anisaldihyde- Sulfuric acid reagent of Stahl (1969).

Preparation of anisaldihyde-sulfuric acid reagent: One ml concentrated sulfuric acid was added to a solution of 0.5ml anisaldehyde in 50ml acetic acid. The reagent was prepared freshly before use.

Treatment after spraying Anisaldihyde- Sulfuric acid reagent: The plates were heated at 100-105°C until the spot attain maximum colour intensity. The pink background was bleached by exposure to steam (water bath).

Quantitative analysis: The algebraic method of Purdy and Truter (1962) was used for quantitative analysis. In this method, the solution containing the unknown sample was diluted precisely. The solution containing the standard sugar and diluted and undiluted sample solutions were chromatographed on the same layer and the spot area was measured. The results were calculated using the equation:

$$\log W = \log W_s \frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_d} - \sqrt{A}} \times \log d$$

where,

d = dilution factor.

W = amount of the analysis material.

Ws = amount of the standard applied.

As = spot area of the standard.

A = spot area of the material for analysis.

Ad = spot area of diluted material for analysis.

2.4. Amylase assay

One gram tissue each of control and treatments were homogenized in 10 ml, cold, 50mM potassium phosphate buffer (p^H 7.5), using a pre-chilled glass mortar and pestle. The homogenate was centrifuged in refrigerated centrifuge (Plasocraft ROTA R4R V/FM) at 10,000 rpm for 25 minutes. The supernatant was used for amylase assay.

Dinitrosalicylic acid method as explained by Bernfeld (1955) was followed to estimate amylase activity.

Preparation of Dinitrosalicylic acid: One gram of 3,5-Dinitrosalicylic acid was dissolved in 20ml of 2N NaOH and 5ml of water at room temperature and stirred slowly, as to avoid capturing of carbon dioxide. Thirty grams of Rochelle salt added, dissolved and made up to 100ml. The solution was kept protected from light and Carbon dioxide.

Assay: Two hundred microlitre of 10% (w/v) homogenate , 0.5 ml of 100mM sodium phosphate buffer of optimum p^H and 0.2ml of 4% substrate (soluble starch procured from Merck) were incubated for 30 minutes at optimum temperature. The enzyme reaction was ceased by the addition of 1ml of dinitrosalicylic acid reagent at the 30^{th} minute. The tubes containing this mixture was heated for 5 minutes in boiling water bath and then cooled. It was made up to 10 ml by adding double distilled water. The optical density of solution containing brown reduction product was measured using Shimadzu (UV-1601) UV-Visible spectrophotometer at 540nm. Maltose procured from Merck chemical company was used as standard. Unit activity and specific activity were calculated.

Unit activity: Unit activity as mg maltose/g tissue formed during 30 minutes at 37°C was calculated.

Specific activity: The amount of protein in enzyme solution was determined by Lowry's method. The specific activity of amylase activity was calculated by dividing unit activity by mg protein present in the tissue.

2.4.1. p^H Optimum: The optimum p^H for enzyme activity was determined by incubating enzyme assay system for 30 minutes at 37°C with substrate, in buffers of p^H ranging from 4-8 at an interval of 0.5 p^H, in a water bath. The buffer p^H in which the enzyme showed highest activity was taken as p^H optimum.

2.4.2. Temperature Optimum: The temperature optimum of enzyme activity was determined by incubating the assay system for 30 minutes at temperature ranging from 20°C to 40°C at an interval of 5°C with substrate and buffer having optimum p^H. The temperature at which the enzyme shows highest activity was considered as temperature optimum.

2.4.3 Enzyme proportionality range: The enzyme proportionality range for enzyme activity was determined by incubating the assay system for 30 min. at optimum temperature with optimum p^H, 200 µl of 4% (w/v) soluble starch and 10% (w/v) enzyme extract ranging from 50-400 µl.

2.4.4. Substrate saturation: The substrate saturation for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature with optimum p^H, 200 µl of 10% (w/v) enzyme extract and different concentration of 4% (w/v) soluble starch ranging from 100-800 µg/ml.

2.4.5. Confirmatory test for α and β- amylase

α-amylase: Confirmatory test for α-amylase was carried out using the method of Kneen *et al* (1943). The enzyme preparation was held at 70°C for 15 minutes in the presence of 0.2% (w/v) calcium acetate. The

suspension was cooled, centrifuged in cold at 10,000 rpm for 10 minutes and the supernatant was used for the assay. α-amylase withstand heat treatment in the presence of calcium acetate and β-amylase was destroyed.

β-amylase: To the enzyme preparation, cold 0.1N HCl was added slowly with constant stirring until p^H lowers to 3.3. The suspension was held at 4-5°C for 18 hours and centrifuged in cold at 10,000 rpm for 10 min. The p^H of the suspension was raised to 4.6 by cautious addition of 0.1N NaOH. The resulting suspension was again centrifuged in cold at 10,000rpm for 10 minutes. The supernatant was used for the assay. β-amylase will withstand the treatment.

2.5. Analysis of protein

2.5.1. Total protein estimation

The method of Lowry *et al* (1951) was followed to estimate the total protein, as modified by Khanna *et al* (1969).

Two hundred milligrams of the tissue was ground in cold double distilled water using mortar and pestle and the homogenate was collected. A known volume from the homogenate was pipetted and mixed with equal volume of cold 10% (w/v) trichloroacetic acid and kept for flocculation for 30 minutes in an ice bath. The protein precipitate was collected by centrifugation for 10 minutes and the supernatant was decanted off. The residue was washed twice with cold 2% (w/v) trichloroacetic acid, followed by washing with 80% (v/v) acetone and then with anhydrous acetone, to remove pigments. The precipitate obtained after centrifugation was digested in known volume of 0.1 N NaOH and heated in a bath of boiling water for five minutes. The resulting suspension was clarified by centrifugation. To 1 ml of supernatant 5 ml of alkaline copper reagent was added and shaken well. After 10 minutes, 0.5 ml of 1N Folin-Ciocalteau reagent was added, immediately shaken well and kept in dark for 30 minutes. The optical density was read at 750 nm using Shimadzu (UV- 1601) UV-

Visible spectrophotometer. Bovine Serum Albumin fraction V, procured from Merck chemical company, was used as standard.

2.5.2. Soluble protein estimation

The method of Lowry et al (1951) was followed to estimate the soluble protein, as modified by Khanna *et al* (1969).

Two hundred milligrams of the tissue was ground in 100mM 7.5 phosphate buffer using chilled mortar and pestle. The suspension was centrifuged and the supernatant was collected, measured the volume and used for the analysis of soluble protein. A known volume from the supernatant was pipetted and mixed with equal the volume of cold 10% (w/v) trichloroacetic acid and the precipitate was allowed for flocculation for 30 minutes in an ice bath. The protein precipitate was collected by centrifugation for 10 minutes and the supernatant decanted off. The residue was washed twice with cold 2% (w/v) trichloroacetic acid, followed by washing with 80% (v/v) acetone and then with anhydrous acetone, for removing pigments. The precipitate obtained after centrifugation was digested in known volume of 0.1 N NaOH by heating in a bath of boiling water for five minutes. The resulting suspension clarified by centrifugation. To 1 ml of the supernatant 5 ml of alkaline copper reagent was added and shaken well. After 10 minutes, 0.5 ml 1N Folin-Ciocalteau reagent was added, immediately shaken well and kept in dark for 30 minutes. The optical density was read at 750 nm using Shimadzu (UV- 1601) UV-Visible spectrophotometer. Bovine Serum Albumin (BSA) fraction V, procured from Merck chemical company, was used as standard.

2.5.3. Electrophoretic studies of protein profile

One gram of the samples was ground in pre-chilled mortar and pestle in sodium phosphate buffer of p^H 7.5. The homogenate was centrifuged at 10,000 rpm for 20 min using plastocraft-model ROTA R4R V/FM refrigerated centrifuge and the supernatant was collected. The protein sample was dissociated into its polypeptide subunits by

adding an equal volume of a solution containing stacking buffer, 10% SDS, 2-Mercaptomethanol, Glycerol and 0.1% Bromophenol blue in Tris.Cl (p^H 6.8) buffer and was heated for 2 minutes (Laemmli, 1970). The subunits were separated electrophoretically in SDS-PAGE slab gel having 12% separating gel and 4% stacking gel. The gel after electrophoresis was stained with Coomassie brilliant blue and the bands were compared with known molecular weight marker protein (PMW-M) procured from GENEI chemical company.

Composition for the preparation of separating gel (12%):

30% acrylamide/ 8% bis acrylamide	6ml
4X Tris.Cl (p^H 8.8)	3.75ml
10% SDS	3.75ml
Redistilled water	5.25ml
10% (w/v) ammonium per sulphate	0.05ml
TEMED	0.01ml

Composition for the preparation of stacking gel (4%):

30% acrylamide/ 8% bis acrylamide	0.65ml
4X Tris. Cl (p^H 6.8)	1.25ml
10% SDS	1.25ml
Redistilled water	3.05ml
10% (w/v) ammonium per sulphate	0.025ml
TEMED	0.05ml

The sample was electrophoresed using Tris (0.25M) – Glycine (1.9M) running buffer of p^H 8.3.

2.6. Estimation of amino acids

The qualitative and quantitative analysis of amino acids were carried out using 2-dimensional Thin- layer Chromatography. Thin layer plates of 250μ thickness were prepared.

Preparation of sample: One gram tissue of control and treated seeds were ground with 80% ethyl alcohol in mortar and pestle and reflexed for 2 hours. The suspension was centrifuged and supernatant was collected and re-extraction was done 4-times and supernatants were combined. The combined extract was evaporated about to dryness over a boiling water bath. The precipitated amino acids were then dissolved in 3 ml 10% n-propanol and centrifuged.

Separation and estimation of amino acids by TLC: One micro litre of the extract was applied on TLC plates and resolved. The samples were resolved using 2-dimensional TLC. After resolving the 1st direction using the solvent *n*-butanol-acetic acid-water (80:20:20) the thin layer plates were dried well and the 2nd direction was resolved using the solvent chloroform-methanol-17% ammonia (40:40:20). Visualization of amino acids were carried out using Ninhydrin reagent of Stahl (1969).

Preparation of Ninhydrin reagent: Two hundred milli gram Ninhydrin is dissolved in 100ml ethanol.

Treatment after spraying Ninhydrin reagent: The plates were heated at 110°C until color development was reached.

Quantitative analysis: The algebraic method of Purdy and Truter (1962) was used for quantitative analysis. In this method, the solution containing the unknown sample was diluted precisely. The solution

containing the standard amino acids and diluted and undiluted sample solutions were chromatographed on the same layer and the spot area was measured. The results were calculated using the equation:

$$\log W = \log W_s \frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_d} - \sqrt{A}} \times \log d$$

Where,

d = dilution factor.

W = amount of the analysis material.

Ws = amount of the standard applied.

As = spot area of the standard.

A = spot area of the material for analysis.

Ad = spot area of diluted material for analysis.

3. HISTOCHEMICAL/ANATOMICAL STUDIES

Histochemical/anatomical studies on embryonic axis, cotyledon and testa were carried out.

3.1. Tissue preparation

3.1.1. Cotyledon

Cotyledon of control and seeds treated at 35°C, 40°C, 45°C and 50°C germinated at the room temperature after 1, 5 and 10 days of germination and in the refrigerator after 10, 30 and 50 days of germination were collected for histochemical studies. The samples were fixed in FAA and dehydrated through alcohol-TBA series and embedded in paraffin wax. Using a rotary microtome the individual

blocks were cut at 11μ thickness. The sections were deparaffinised, hydrated and stained.

3.1.2. Embryonic axis

Embryonic axis of control seeds and treatments germinated at the room temperature were used for anatomical study. After 24 hours of germination, radicle tip were collected. Similarly radicle tip of the seeds germinated in the refrigerator were collected after 10 days. The samples were fixed in Carnoy's fluid for 2 hours and were transferred to 70% alcohol-TBA series. The samples were dehydrated through alcohol-TBA series and embedded in paraffin wax. Using a rotary microtome the individual blocks were cut at 11μ thickness. The sections were deparaffinised, hydrated and stained.

3.1.3. Testa

Control and seeds treated at 50°C were germinated at the room temperature and after 24 hours, testa were separated and collected for anatomical studies. The samples were fixed in FAA and treated with 25% hydrofluric acid for softening. The samples were dehydrated through alcohol- TBA series and embedded in paraffin wax. Using a rotary microtome the individual blocks were cut at 11μ thickness. The sections were deparaffinised, hydrated and stained.

3.2. Staining

Staining was carried out for the histochemical/anatomical studies of cotyledon, embryonic axis and testa

3.2.1. Histochemical study of cotyledon

Histochemical staining was carried out for total protein and insoluble polysaccharide reserves in the cotyledon.

3.2.1.1 Localization of protein

For the localization of total protein sections were stained with mercuric bromophenol blue according to Mazia *et al* (1953) as explained by Berlyn and Miksche (1976).

The hydrated sections were placed in bromophenol blue stain for 15 minutes and then in 0.5% acetic acid for 20min. The sections were then placed in Sorenson's buffer of pH 6.8. The sections were dehydrated through alcohol-TBA series, cleared in xylene and were mounted in DPX.

Preparation of mercuric bromophenol blue stain: Ten gram of mercuric chloride and 0.1g of bromophenol blue were dissolved in 100ml distilled water and filtered.

3.2.1.2. Localization of insoluble polysaccharides

The staining procedure for localization of insoluble polysaccharides was carried out as explained by Berlyn and Miksche (1976) using Periodic acid-Schiff's reagent.

The hydrated sections were placed in 0.5% (w/v) Periodic acid solution at 23°C for 15 minutes and the sections were washed using running tap water for 10 minutes. The sections were then placed in Schiff's reagent for 10 minutes at 3±1°C and washed in tap water for 20 seconds. After washing, sections were placed in 2% sodium bisulfite for 2 minutes and washed again in tap water for 5-10 minutes. The sections were dehydrated through alcohol series, cleared in xylene and mounted in DPX.

Preparation of Schiff's reagent: Two gram of Basic Fuchsin and 3.8g of potassium metabisulfite were added to 200ml of 0.15 N HCl in a flask and shaken on a mechanical shaker for 2hours. One gram of activated charcoal was added to the mixture and stirred for 15 minutes, so as to decolorize the solution. The mixture was filtered using a

Buchner funnel, side arm flask, and vacuum pump using Whatman No.1 filter paper. The stain was kept at 4°C.

3.2.2. Anatomical study of embryonic axis and testa

3.2.2.1. Embryonic axis

The deparaffinised sections were hydrated through alcohol series and were immersed in Delafeild's hematoxylin for 30 minutes. The stained sections were washed in water and dehydrated through alcohol series. The clearing was carried out in xylene and was mounted in DPX mountant.

3.2.2.2. Testa

The deparaffinised sections were hydrated through alcohol series, treated with 1% periodic acid for 5 minutes and rinsed with water. Then the sections were stained with a mixture of Azure II-Methylene blue at 60°C for 1 minute (Khasim, 2002). The stained sections were rinsed with water and dried using hair dryer and mounted in DPX mountant. The lignified walls and tannin appeared green after staining, the cell wall appeared reddish purple and all the other components appeared in shades of blue.

Preparation of Azure II-Methylene blue stain: The preparation of stain was done by mixing equal volume of 1% aqueous Azure II and 1% Methylene blue in 1% sodium borate solution prepared freshly.

The photographs of the stained sections were taken using Nikon microscope and Nikon Model Eclipse E-500 camera and Image analyzer.

Statistical Analysis of data: All experiments were repeated minimum five times. Data obtained were analysed for standard deviation and test of significance was done using't' test.

EFFECT OF TEMPERATURE ON PHYSIOLOGY OF RESERVE MOBILISATION IN *PISUM SATIVUM* L. SEEDS DURING GERMINATION

Dissertation submitted to the Faculty of Science
University of Calicut
In part fulfillment of the requirements for the degree of
Doctor of Philosophy
In Botany

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Results

Seeds of *Pisum sativum* L. were readily germinating and the seeds did not show any type of dormancy. The germination of seeds was hypogeal

1. Germination studies

1.1. Germination at room temperature

Under laboratory conditions in Petri dishes, the radicle emergence was observed on 24th hour of soaking and the emergence of plumule on 2nd day. The development of secondary root was noticed from 6th day of germination in normal seedlings.

1.2. Effect of temperature treatment of seed on germination

Control seeds and the seeds treated at 35°C and 40°C showed 100% imbibition and 100% germination. The treatment at 45°C resulted in 100% imbibition but only 93% of them were germinated. The remaining seeds were found to be dead. Seeds treated at 50°C showed only 93% imbibition and the remaining 7% were hard seeds. Out of 93% imbibed seeds only 78% were germinated and the

remaining seeds were non-viable (Table 1). In order to test the viability of the hard seeds, they were subjected to mechanical scarification by making a small incision on the testa with blade, all of them were imbibed but none of them were germinated.

1.3. Moisture content of seeds

Moisture content of control seeds [seeds stored at room temperature (28 ± 4°C)] was 12.3%. All temperature treatments up to 50°C given to *P. sativum* seeds resulted in continuous as well as gradual reduction in the moisture content compared to control seeds (Table 2). Similarly moisture content was continuously but gradually reduced as the temperature increased and even after 60 °C, the moisture content reduction was significant up to 70 °C. In other words, dry weight value never become constant by raising temperature up to 70 °C.

The seeds treated at 35°C for 7 days showed moisture content of 10.3%. The seeds treated at 40°C, 45°C and 50°C showed the moisture content of 9.2%, 7.7% and 6.5% respectively. (Table 2).

1.4. Seed germination rate/Seed Vigour Index

Table 3 shows the seed vigour index and germination rate of control and temperature treated seeds under field condition. The seed vigour index values of control and the seeds treated at 35°C were maximum and almost equal. But in other treatments, the SVI was decreased gradually with increase in temperature. At 40°C, 45°C and 50°C the germination rate of seeds were very slow and a corresponding reduction was observed in SVI values (Fig. 2).

Control seeds and seeds treated at 35°C exhibited similar pattern of germination and the germination was completed by 4 days. On 2nd and 3rd day almost equal numbers of seeds were germinated. In

Table 1: Effect of temperature treatment on germination in *Pisum sativum* seeds

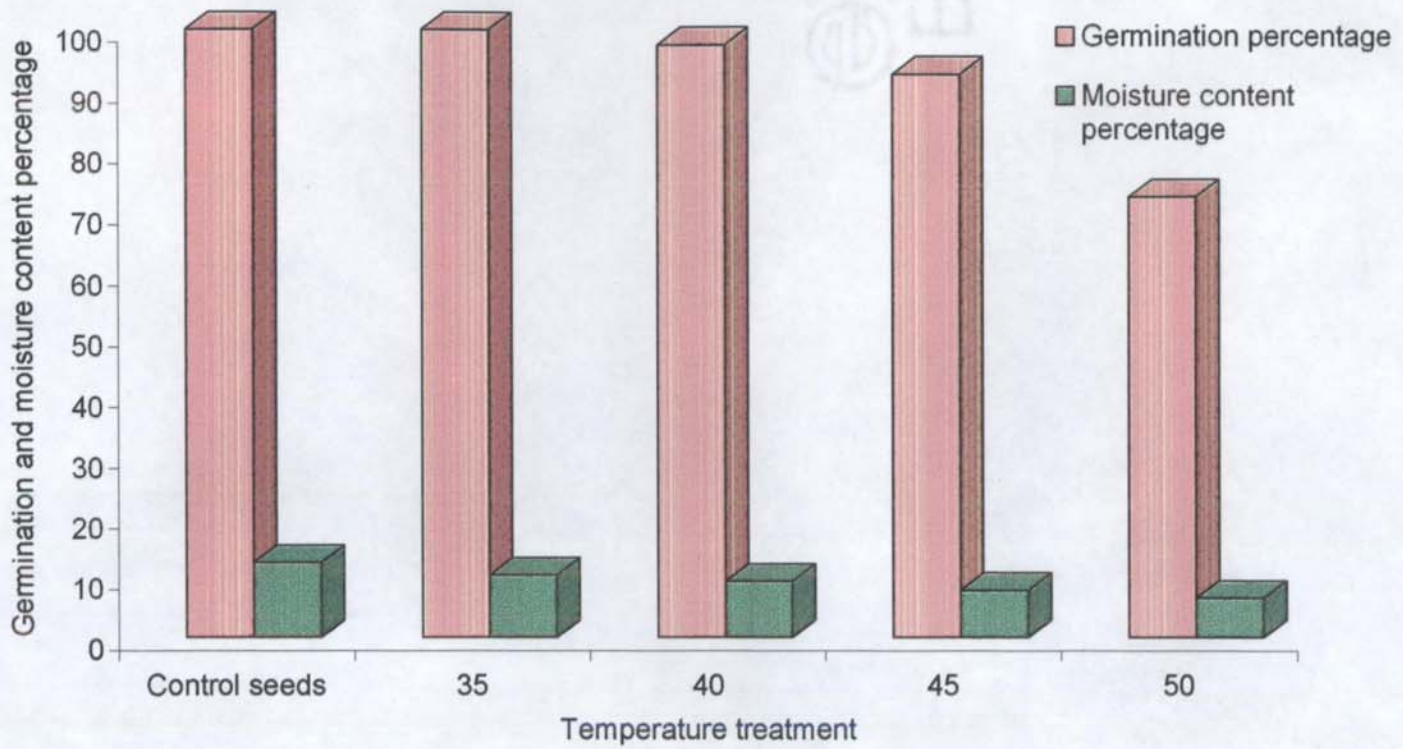
Temperature °C	Germination %	Imbibition %	Hard seed %
Control	100 ± 0	100 ± 0	0
35	100 ± 0	100 ± 0	0
40	100 ± 0	100 ± 0	0
45	93 ± 2	100 ± 0	0
50	78 ± 0.26	93 ± 0.86	*7 ± 0.9
55	60 ± 0.29	33 ± 0.60	*7 ± 0.6
60	53 ± 0.80	37 ± 0.20	*10 ± 0.89
65	23 ± 0.30	65 ± 0.50	*12 ± 0.20
70	3.0 ± 0.42	4 ± 0.19	*23 ± 0.50

*Hard seeds imbibed after scarification

Table 2: Effect of temperature on moisture content in *Pisum sativum* seeds

Temperature °C	Moisture content of seeds	
	M.C %	M.C/seed
Control	12.3 ± 0.70	2.92 ± 0.31
35	10.3 ± 0.23	2.51 ± 0.56
40	9.2 ± 0.51	2.20 ± 0.80
45	7.7 ± 0.54	1.84 ± 0.41
50	6.5 ± 0.88	1.55 ± 0.22
55	4.72 ± 1.80	0.89 ± 0.27
60	2.89 ± 0.92	0.58 ± 0.21
65	2.01 ± 0.90	0.41 ± 0.13
70	1.67 ± 0.30	0.33± 0.09

Figure 1: Effect of temperature treatment on germination and moisture content percentage in *Pisum sativum* seeds during germination at room temperature



the case of seeds treated at 40°C also, the germination was completed in 4 days but maximum numbers of seeds were germinated on 3rd day. The seeds treated at 45°C required 5 days for the completion of germination and almost equal numbers of seeds were germinated on 2nd and 3rd days. Seven days were taken by the seeds treated at 50°C for the completion of germination and almost equal numbers were germinated on 3rd, 4th and 5th days. In control seeds and treatments at 35°C, 40°C and 45°C the germination was started on 2nd day, whereas in the case of seeds treated at 50°C germination was started only on 3rd day (Fig. 2).

1.5. Field Emergence Index (FEI)

Table 3 shows the FEI of *P. sativum* seeds during germination. The control seeds and treatment at 35°C shows FEI=100, showing ideal germination. In treatments at 40°C, 45°C and 50°C FEI is less than 100.

1.6. Seedling vigour

Considerable difference in morphological character between control and treatments was observed during germination. The seedlings of control seeds and treatment at 35°C did not show any difference. The seedling of treatment at 40°C was slightly shorter in size than control and treatment at 35°C. The seedlings of treatment at 45°C and 50°C were showed stunted growth. The radicle was short and stout (Plate 1).

Total biomass (seedling vigour) in mg dry weight/seedling showed a reduction during the increase in temperature. The seedlings of control and treatments at 35°C and 40°C showed a gradual reduction corresponding to the increasing temperature. A sudden decrease in biomass/seedling was observed in the case of treatment at 45°C and 50°C (Table 4).

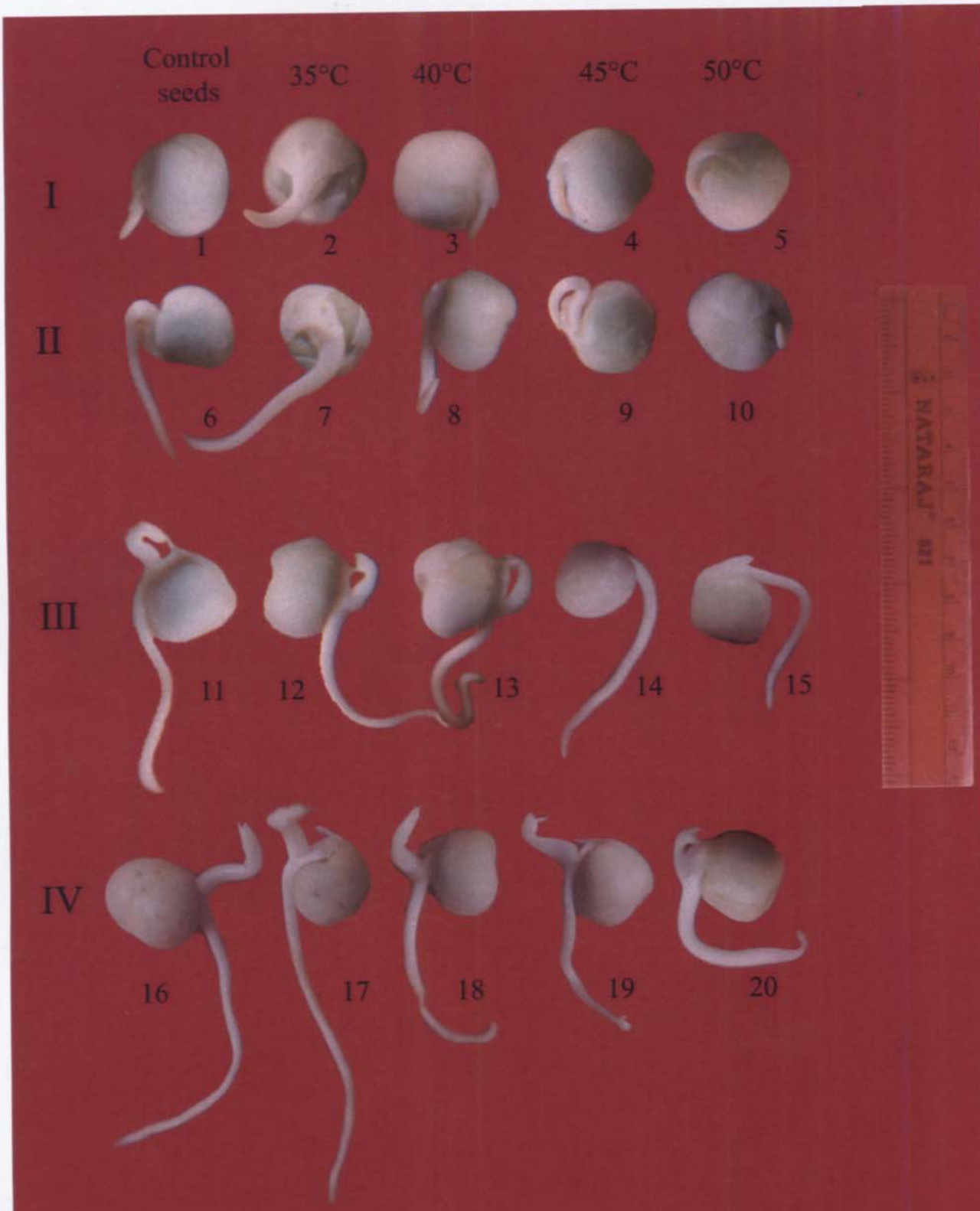


Plate 1: Morphology of *Pisum sativum* seeds during germination at room temperature

I. First day
II. Second day
III. Third day
IV. Fourth day

Table 3: Effect of temperature treatment on seed germination rate and vigour index in *Pisum sativum* seeds

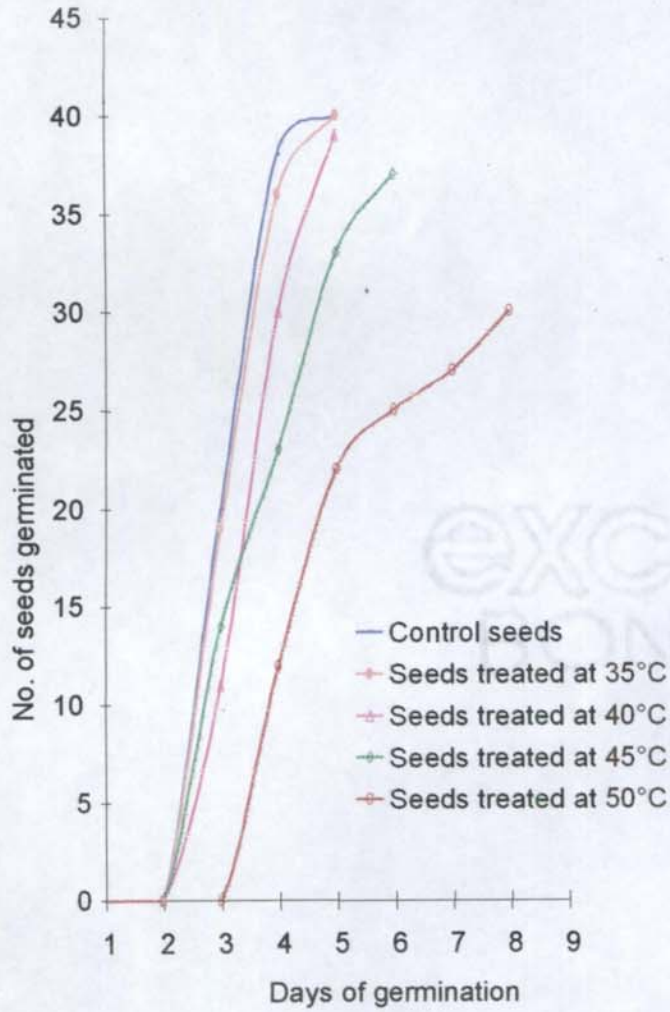
Temperature (°C)	Days of germination							*SVI	*FEI
	1	2	3	4	5	6	7		
	Number of seeds germinated								
Control	0	20 ± 2.3	18 ± 1.3	2 ± 1.2	-	-	-	16.29 ± 1.1 (40.29 ± 3.8)	100
35	0	19 ± 2.8	17 ± 1.4	4 ± 0.63	-	-	-	16.16 ± 0.09 (40.18 ± 2.6)	100
40	0	11 ± 1.6	19 ± 1.9	9 ± 1.0	-	-	-	14.60 ± 0.37 (36.4 ± 0.67)	97.5 ± 1.26
45	0	14 ± 2.6	9 ± 1.9	10 ± 0.87	4 ± 1.0	-	-	13.30 ± 0.11 (33.16 ± 2.2)	99.4 ± 0.63
50	0	0	12 ± 2.3	10 ± 2.0	3 ± 0.2	2 ± 0.6	3 ± 0.1	9.96 ±1.8 (24.33 ± 1.8)	96.1 ± 1.88

*Values in parenthesis are SVI percentage
*SVI= Seed Vigour Index
*FEI= Field Emergence Index

44B

16

Figure 2: Effect of temperature treatment on seed germination rate in *Pisum sativum* seeds at room temperature



1.7. Rate of imbibition

In control seeds and the seeds treated at 35°C, imbibition rate was fast during the initial 2hours ($P< 0.01$ each). Between 2^{nd} and 4^{th} hour significant increase ($P<0.02$ each) was occurred and afterwards the increase in weight was negligible up to 12^{th} hour (Table 5). The seeds treated at 40°C also showed almost similar pattern of imbibition. But a seed treated at 45°C, slight difference in the rate of imbibitions was observed compared to the control seeds. After 2^{nd} hour, more increase in weight was shown at all intervals in comparison with control and other treatments. In the case of seeds treated at 50°C a doubling of weight was observed during the initial 2 hours of imbibition and afterwards significant increase in weight was noticed at 4^{th} hour ($P<0.01$) and 6^{th} hour ($P<0.01$) and later the imbibition rate remained unchanged up to 12^{th} hour (Fig. 3).

1.8. Conductivity of leachate

1.8.1. Cumulative conductivity

During 1^{st} hour of soaking, control seeds and treatments, did not show any significant difference in leachate conductivity (Table 6a). After 2^{nd} hour of soaking also there was no significant difference. The conductivity values were almost equal in the control seeds and the seeds treated at 35°C and 40°C on 3^{rd} hour. At this interval the seeds treated at 45°C and 50°C showed higher values than the other samples. The seeds treated at 45°C and 50°C showed an increased leachate conductance than the others on 3^{rd} hour. On 4^{th} hour, the control seeds and the seeds treated at 35°C and 40°C showed only negligible difference and the seeds treated at 45°C and 50°C, the leachate conductance was higher compared to other seeds ($P<0.01$ and 0.01 respectively). Samples of 5^{th} hour also showed a similar pattern of leachate conductivity (Fig. 4a). But 6th day onwards there

Table 4: Effect of temperature treatment on seedling vigour in *Pisum sativum* seeds

Temperature of treatment (°C)	mg dry weight/seedling
Control	72.9 ± 8.6
35	60.3 ± 7.1
40	54.8 ± 4.6
45	32.6 ± 10.3
50	28.7 ± 9.9

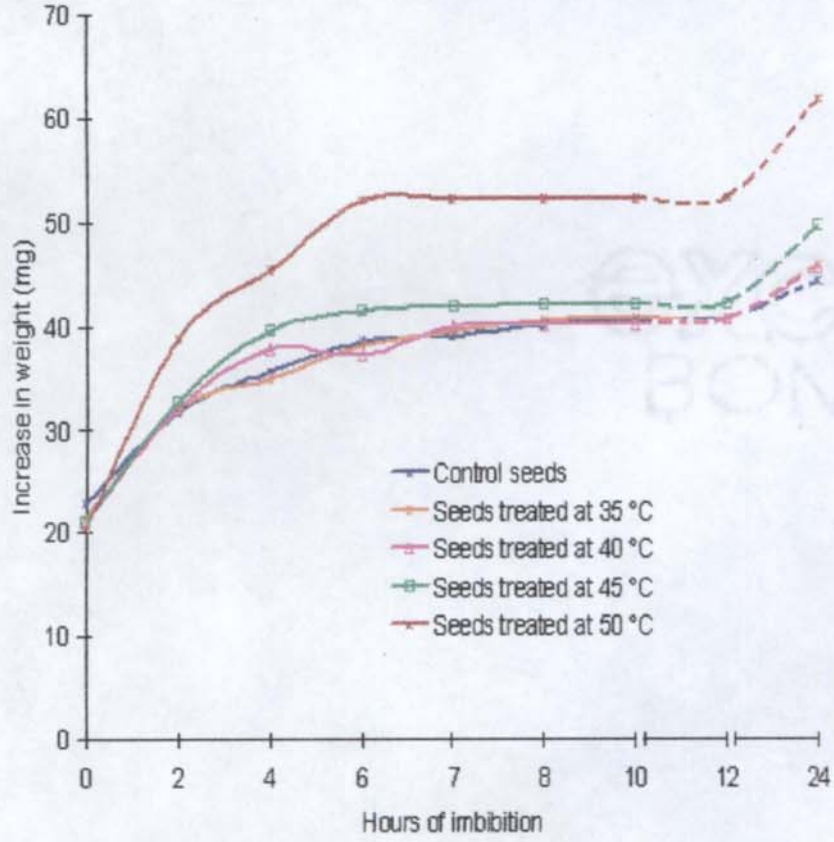
Table 5: Effect of temperature treatment on the imbibition rate in *Pisum sativum* seeds

Time (hours)	Increase in weigh (mg)				
	Control seeds	Temperature (°C)			
		35	40	45	50
0	22.80 ± 1.2	21.34 ± 1.5	20.93 ± 1.2	21.00 ± 1.1	20.32 ± 1.5
2	30.82 ±1.8	31.10 ±1.0	32.16 ± 0.9	32.81 ±1.3	38.85 ± 1.2
4	36.69 ± 0.1	35.00 ± 0.6	37.87 ± 0.6	39.56 ± 0.8	45.32 ± 0.5
6	38.59 ± 0.9	38.20 ± 0.8	37.24 ± 0.8	41.47 ± 0.7	52.01 ± 0.2
7	39.03 ± 0.4	39.50 ± 0.5	40.00 ± 0.9	41.89 ± 0.6	52.11 ± 0.7
8	40.11 ± 0.3	40.50 ± 0.5	40.03 ± 0.5	41.99 ± 0.8	52.14 ± 0.6
10	40.55 ± 0.7	40.69 ± 0.8	40.16 ± 0.6	42.06 ± 0.7	52.19 ± 0.4
12	40.76 ± 0.5	40.72 ± 0.8	40.59 ± 0.9	42.12 ± 0.9	52.26 ± 0.2
24	44.22 ±1.3	45.81 ± 0.5	45.62 ± 0.9	49.59 ±1.0	61.82 ±1.3

45 P

19

Figure 3: Effect of temperature treatment on imbibition rate in *Pisum sativum* seeds during germination at room temperature



was no significant difference in the conductivity of control and treatment up to 9th hour when a comparison is made between the samples and between the intervals. However, significant increase in leachate conductivity was observed in seeds treated at 45°C ($P<0.01$) and 50°C ($P<0.05$) at 10th hour. Samples of 12th and 24th hour also showed almost similar rate of conductivity (Table 6a). On 12th hour all the seeds showed a slight increase in conductivity of leachate. During 24th hour of soaking, even though the testa was broken the increase in conductance was negligible (Fig. 4a).

1.8.2. Non-Cumulative conductivity

When a comparison is made between the control seeds and the seeds treated at 35°C, 40°C, 45°C and 50°C during 1st hour of soaking all the samples did not show any difference (Table 6b). After 2 hours of soaking the control seeds and all the treatments showed a sharp increase in leachate conductivity and among the treatments there was no difference. On 3rd hour of soaking the seeds treated at 35°C, 40°C and the control seeds remained as such but the treatment at 45°C and 50°C showed an increase ($P < 0.02$ for each sample). From 3rd hour onwards conductivity was decreasing slowly (Table 7) up to 7th hour in control seeds and treatment at 35°C and 40°C but it was up to 8th hour in the treatment of 45°C and up to 9th hour in the case of 50°C treated seeds. In control seeds and the treatment at 35°C and 40°C, 7th hour of soaking onwards, the leachate conductivity showed no significant difference whereas, in treatment at 45°C and 50°C it was from 8th and 9th hour respectively (Fig. 4b).

1.9. Effect of temperature on sugar content in the leachate

Table 7 shows data on leachate analysis. During 1st hour of imbibition there was only slight leaching of sugar content and no

Table 6a: Effect of temperature treatment on conductivity of electrolytic leakage in *Pisum sativum* seeds during imbibition at room temperature (cumulative)

Time (hours)	Conductivity in μ mho cm^{-2}/g				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	4.3 ± 0.9	4.0 ± 0.8	4.2 ± 1.2	4.5 ± 0.5	4.5 ± 0.4
2	30.5 ± 1.8	31.8 ± 1.5	32.6 ± 1.9	33.5 ± 0.4	33.9 ± 1.3
3	40.6 ± 1.6	40.9 ± 1.2	41.0 ± 1.6	43.8 ± 1.3	45.2 ± 1.9
4	46.4 ± 0.4	46.1 ± 0.2	46.7 ± 0.5	52.5 ± 1.6	55.8 ± 0.8
5	56.8 ± 0.8	55.9 ± 1.3	56.3 ± 1.9	60.4 ± 0.8	62.0 ± 1.8
6	61.7 ± 1.6	60.2 ± 1.9	61.2 ± 0.7	63.4 ± 0.7	65.1 ± 1.9
7	61.1 ±1.6	60.6 ± 1.3	61.8 ± 1.8	63.5 ± 1.2	65.2 ± 1.6
8	62.6 ±1.2	62.3 ± 1.1	61.8 ± 1.6	63.7 ± 1.7	65.9 ± 1.3
9	62.6 ± 1.8	62.1 ± 1.6	62.0 ± 0.2	63.7 ± 0.4	65.9 ± 1.6
10	62.6 ± 1.2	62.1 ± 1.2	62.0 ± 1.4	71.4 ± 1.8	72.3 ± 1.9
12	70.1 ± 0.7	70.4 ± 0.8	70.8 ± 0.8	74.1 ± 1.2	76.2 ± 1.3
24	72.1 ± 1.2	72.8 ± 1.9	72.6 ± 1.3	76.7 ± 0.5	79.6 ± 1.4

Table 6b: Effect of temperature treatment on conductivity of electrolytic leakage in *Pisum sativum* seeds during imbibition at room temperature (non-cumulative)

Time (hours)	Conductivity in μ mho cm^{-2}/g				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	3.3 ± 1.3	3.8 ± 0.8	4.9 ± 1.4	5.0 ± 1.8	5.7 ± 1.7
2	19.9 ±1.7	19.5 ± 1.5	20.3 ± 1.9	21.8 ± 0.1	21.9 ± 1.3
3	23.2 ±1.3	23.2 ± 1.2	24.3 ± 1.6	26.2 ± 1.4	28.3 ± 1.8
4	14.5 ± 0.7	15.2 ± 0.9	15.7 ± 1.2	18.3 ± 1.0	20.5 ± 0.3
5	9.3 ± 0.8	9.9 ± 1.3	9.6 ± 1.1	15.3 ± 0.8	17.7 ± 1.0
6	4.8 ± 1.1	4.2 ± 1.9	4.8 ± 0.5	8.6 ± 0.7	10.9 ± 0.9
7	2.2 ± 1.4	2.3 ± 1.3	2.0 ± 0.1	3.9 ± 0.2	4.3 ± 0.4
8	1.7 ± 1.2	1.8 ± 1.1	1.7 ± 0.1	1.9 ± 0.6	3.1 ± 0.3
9	1.0 ± 1.5	1.2 ± 1.2	1.7 ± 0.7	1.6 ± 0.4	1.3 ± 0.5
10	1.1 ± 1.2	1.7 ± 1.6	1.6 ± 1.8	1.3 ± 1.8	1.6 ± 1.0
12	1.2 ± 0.5	1.5 ± 0.8	1.5 ± 0.4	1.1 ± 0.2	1.2 ± 1.0

Figure 4a: Effect of temperature treatment on electrolytic leakage in *Pisum sativum* seeds during imbibition (cumulative) at room temperature

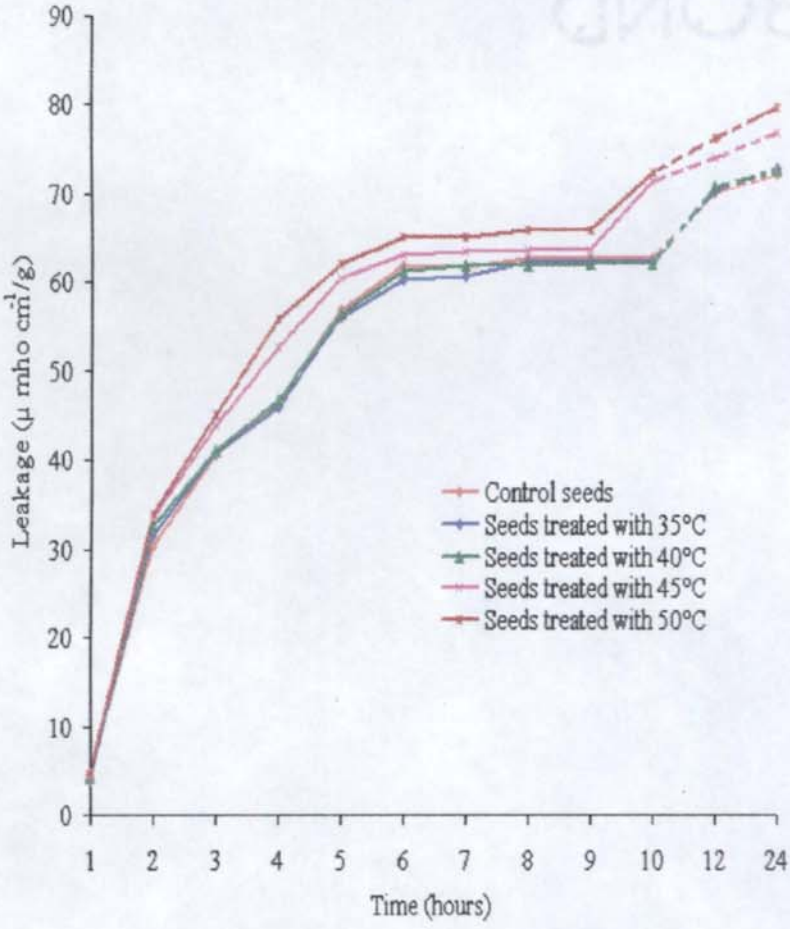
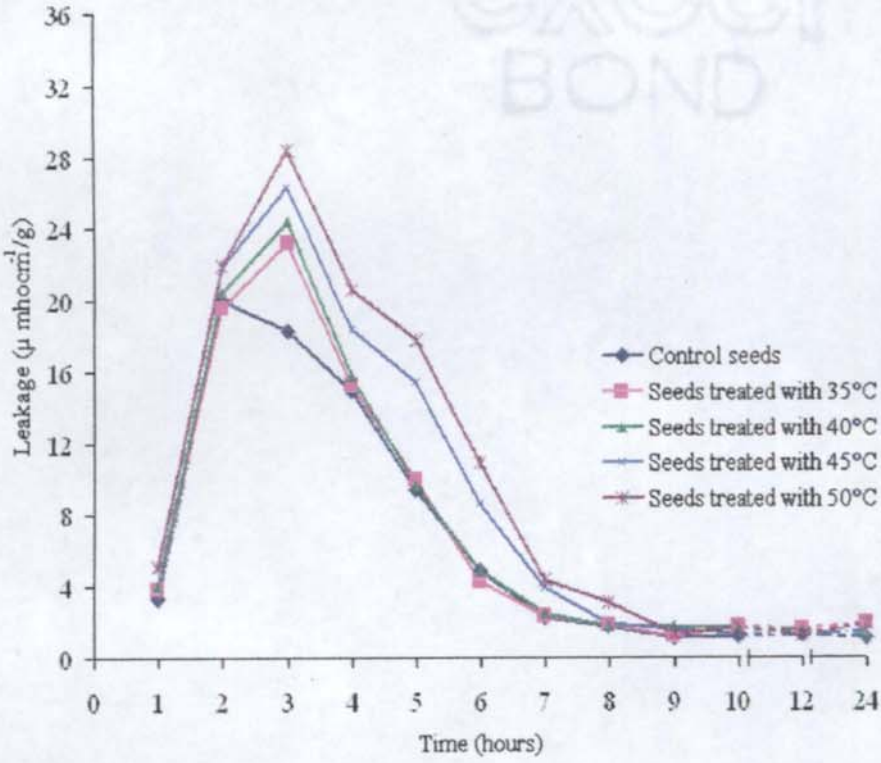


Figure 4b: Effect of temperature treatment on electrolytic leakage in *Pisum sativum* seeds during imbibition (non-cumulative)



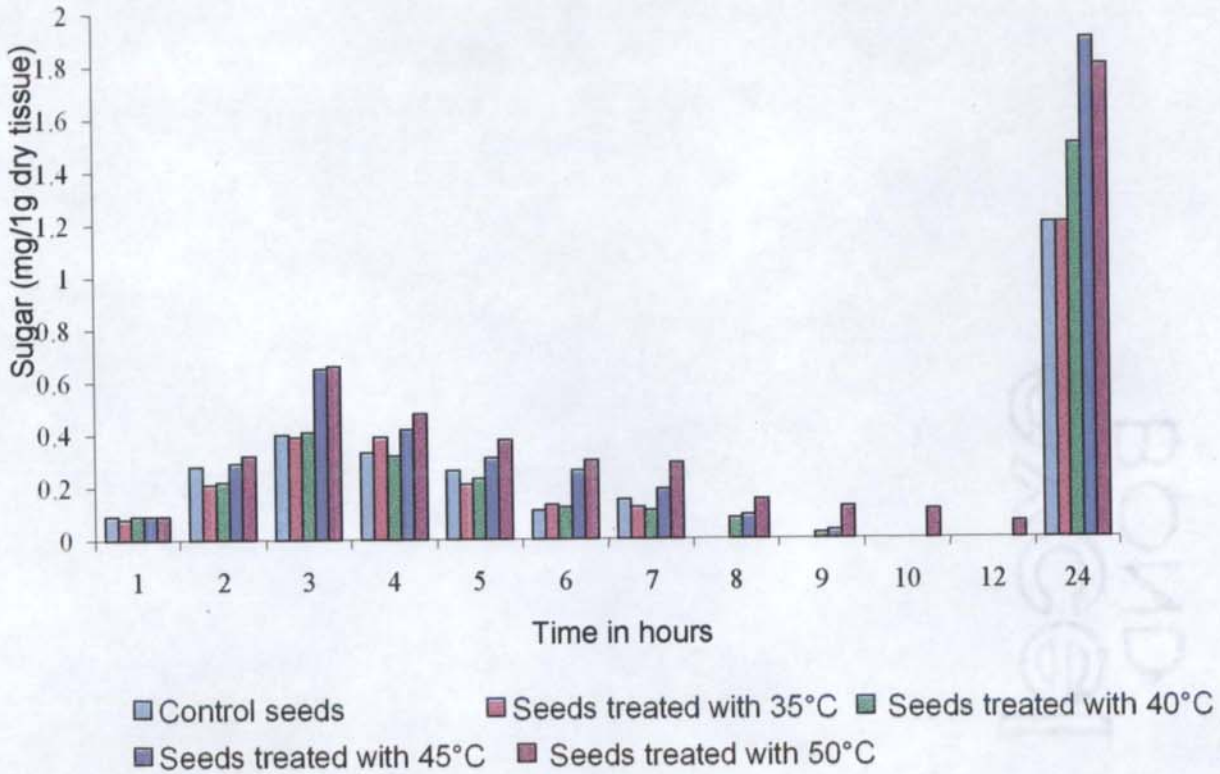
significant difference was seen among control and seeds treated at 35°C, 40°C, 45°C and 50°C. After 2nd hour of imbibition, sugar content was increased more than 2-fold compared to 1st hour in both control and experimental samples. The difference in sugar content between control and treated seeds was negligible. Seeds treated at 45°C and 50°C showed higher amount of sugars than that of control and experimental seeds at 35°C and 40°C after 3 hour of imbibition. The sugar content of control and 35°C and 40°C treated seeds was almost equal, and also showed only a slight increase when compared to that of 2nd hour (Table 7). Maximum amount of sugar content was present in the leachate of 3rd hour in all seed lots. Seeds treated at 45°C and 50°C showed significant increase ($P<0.01$ and 0.01respectively) compared to other treatments and control after 3 hours. Thereafter it was decreased. During 3rd and 4th hour of imbibition, the seeds treated at 45°C and 50°C showed considerable reduction in the amount of sugars and the difference among other treatments and control were negligible. The 5th hour sample also showed uniform reduction of sugar content in the leachate compared to 4th hour. After 6th hour the seeds of control and treatments at 35°C, 40°C, 45°C and 50°C exhibited negligible reduction (Table 7). Seventh hour sample showed the sugar content in control seeds and seeds treated at 35°C, 40°C, 45°C and 50°C which was remained unchanged when compared with 6th hour. During 8th and 9th hour of soaking the leachate contained only trace amount of sugars in control and seeds treated at 35°C but in treatment at 40°C, 45°C and 50°C onwards only slight amount of sugar content was present. Seeds of control and all the treatments except 50°C showed only trace amount of sugar and the 50°C sample showed further reduction during 10th and 12th hour of imbibition (Fig. 5). After 24 hours of soaking, since the seeds were germinated, the sugar content

Table 7: Effect of temperature treatment on the leachate sugar content in *Pisum sativum* seeds during imbibition at room temperature

Time (hour)	Sugar (mg/1g dry tissue)				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	0.09 ± 0.03	0.08 ± 0.01	0.09 ± 0.02	0.09 ± 0.04	0.09 ± 0.03
2	0.28 ± 0.07	0.21 ± 0.01	0.22 ± 0.01	0.29 ± 0.04	0.32 ± 0.02
3	0.40 ± 0.08	0.39 ± 0.01	0.41 ± 0.07	0.65 ± 0.03	0.66 ± 0.02
4	0.33 ± 0.06	0.39 ± 0.04	0.32 ± 0.02	0.42 ± 0.01	0.48 ± 0.05
5	0.26 ± 0.07	0.21 ± 0.06	0.23 ± 0.04	0.31 ± 0.03	0.38 ± 0.01
6	0.11 ± 0.06	0.13 ± 0.05	0.12 ± 0.08	0.26 ± 0.03	0.30 ± 0.01
7	0.15 ± 0.02	0.12 ± 0.01	0.11 ± 0.04	0.19 ± 0.09	0.29 ± 0.05
8	t	t	0.08 ± 0.01	0.09 ± 0.03	0.15 ± 0.04
9	t	t	0.02 ± 0.05	0.03 ± 0.01	0.12 ± 0.03
10	t	t	t	t	0.11 ± 0.02
12	t	t	t	t	0.06 ± 0.05
24	1.2 ± 0.5	1.2 ± 0.2	1.5 ± 0.6	1.9 ± 0.2	1.8 ± 0.5

*t means trace amount

Figure 5: Effect of temperature treatment on sugar leakage in *Pisum sativum* seeds During germination at room temperature



of the leachate showed more than 10-fold increase compared to other intervals in all treatments and control and among themselves there were no significant difference in the distribution of sugars among treatments and between the control and the experimental.

1.10. Germination studies under cold condition

1.10.1 Germination

Germination rate and seedling growth in refrigerator condition were very slow compared to that of room temperature and hence, a chronological comparison can not be made between the morphological and/or physiological characters of seeds germinated at room temperature and in refrigerator. In the refrigerator radicle emergence was observed on 9^{th} day of soaking and the emergence of plumule was occurred on 30^{th} day (Plate 2 & 3).

1.10.2. Effect of temperature treatment of seed on germination

The control seeds and seeds treated at 35°C, 40°C and 45°C when germinated in refrigerator, showed 100% germination. The seeds treated at 50°C showed only 93% germination and the rest were imbibed but not germinated (Table 8).

1.10.3. Germination rate

In control seeds and the seeds treated at 35°C the germination was started on 9^{th} day and was completed by 11^{th} day in refrigerator condition. The Seed Vigour Index of control and the seeds treated at 35°C did not show any difference between them. In the seeds treated at 40°C and 45°C the germination started from 10^{th} day and was over by 13^{th} and 14^{th} day respectively. Germination of seeds treated at 50°C started from 11^{th} day and ended on 15^{th} day. Seeds Vigour Index of samples treated at 40°C, 45°C and 50°C was decreased with increase

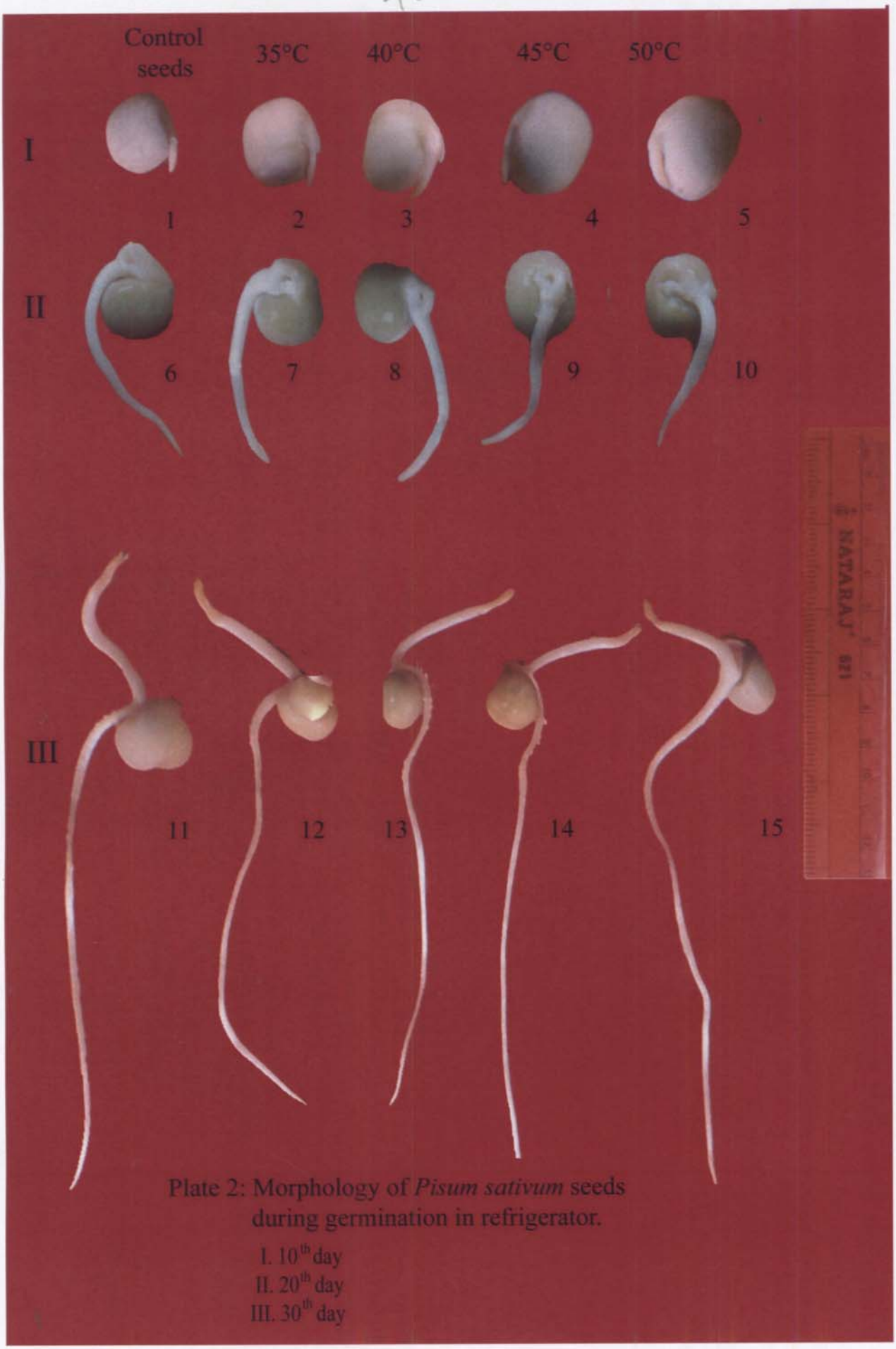


Plate 2: Morphology of *Pisum sativum* seeds during germination in refrigerator.

I. 10th day
II. 20th day
III. 30th day

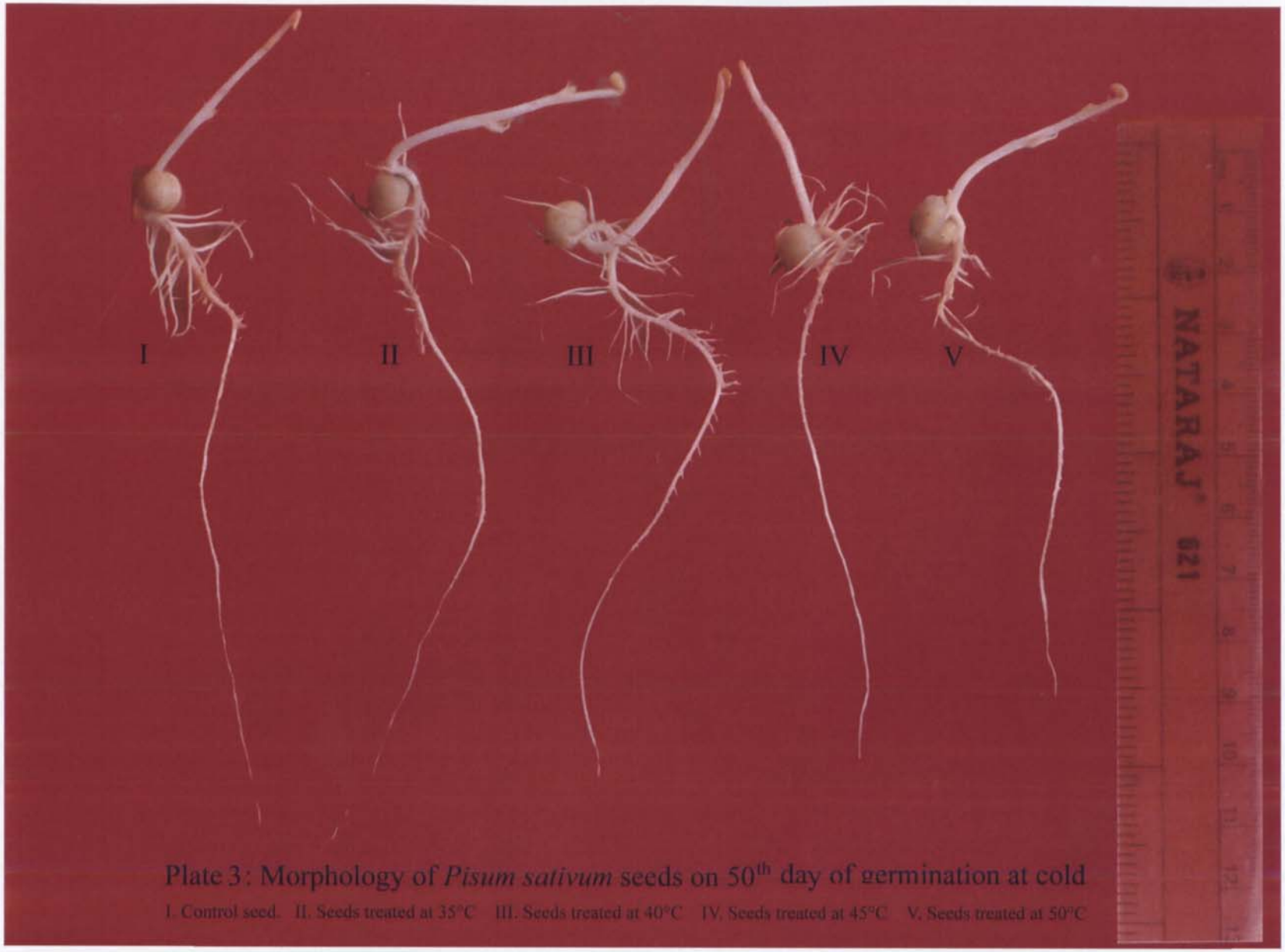


Plate 3: Morphology of *Pisum sativum* seeds on 50th day of germination at cold

I. Control seed. II. Seeds treated at 35°C III. Seeds treated at 40°C IV. Seeds treated at 45°C V. Seeds treated at 50°C

in temperature (Table 8) showing more reduction in seeds treated at 50°C.

1.10.4. Seedling vigour

There was no considerable difference in total biomass (seedling vigour) in mg dry weight/seedling and morphological character between control and treatments when germinated in refrigerator (Table 8).

1.11. Rate of imbibition

On germination in refrigerator the seeds of control and treatments showed similar pattern of imbibition (Table 9) but the seeds treated at 50°C showed a higher rate in terms of mg H_2o/g fresh tissue. During 1st hour of soaking the control seeds and all the treatments showed a significant increase in weight ($P<0.01$). Similar pattern of imbibition was noticed during 2nd hour of soaking ($P<0.01$). The increase in weight on 3rd and 4th hour of soaking was negligible in control seeds and treatment at 35°C, and 40°C and 50°C the weight was remained constant up to 9th hour of imbibition. On 5th hour all the sample except treatment at 45°C and 50°C showed a significant increase in weight and again remained unchanged up to 7th hour (Table 9). A significant increase was noticed on 8th and 9th hour ($P<0.01$ at each hour) and from 9th day onwards it was remained as such. In general there was no linearity in the imbibition pattern during germination in refrigerator

1.12. Conductivity

1.12.1. Cumulative Conductivity

When the seeds of control and treatments at 35°C, 40°C, 45°C and 50°C were germinated in refrigerator during 1st hour of soaking there was no considerable difference ($P>0.2$) between the conductivity

Table 8: Effect of temperature treatment on seed vigour index in *Pisum sativum* seeds germinated in refrigerator

Temperature (°C)	Days of germination										SVI	Germination percentage	Seedling vigour (mg/dry weight/seedling)
	6	7	8	9	10	11	12	13	14	15			
	Number of seeds germinated per day												
C	0	0	0	10 ± 0.45	15 ± 0.81	15 ± 0.32	-	-	-	-	3.97 ± 0.62	100 ± 0	56.3 ± 8.8
35	0	0	0	10 ±	16 ± 0.33	14 ± 0.28	-	-	-	-	3.98 ± 0.67	100 ± 0	59.4 ± 9.3
40	0	0	0	0	9 ± 0.96	12 ± 0.47	17 ± 0.19	2 ± 0.11	-	-	3.56 ± 0.84	100 ± 0	58.2 ± 7.5
45	0	0	0	0	8 ± 0.60	10 ± 0.81	14 ± 0.88	4 ± 0.46	4 ± 0.54	-	3.47 ± 0.89	100 ± 0	56.5 ± 4.6
50	0	0	0	0	0	6 ± 0.38	14 ± 0.86	10 ± 0.91	5 ± 0.52	2 ± 0.12	2.97 ± 0.54	92.5 ± 0.5	54.7 ± 6.9

Figure 6: Effect of temperature treatment on Seed Vigour Index in *Pisum sativum* seeds during germination in refrigerator

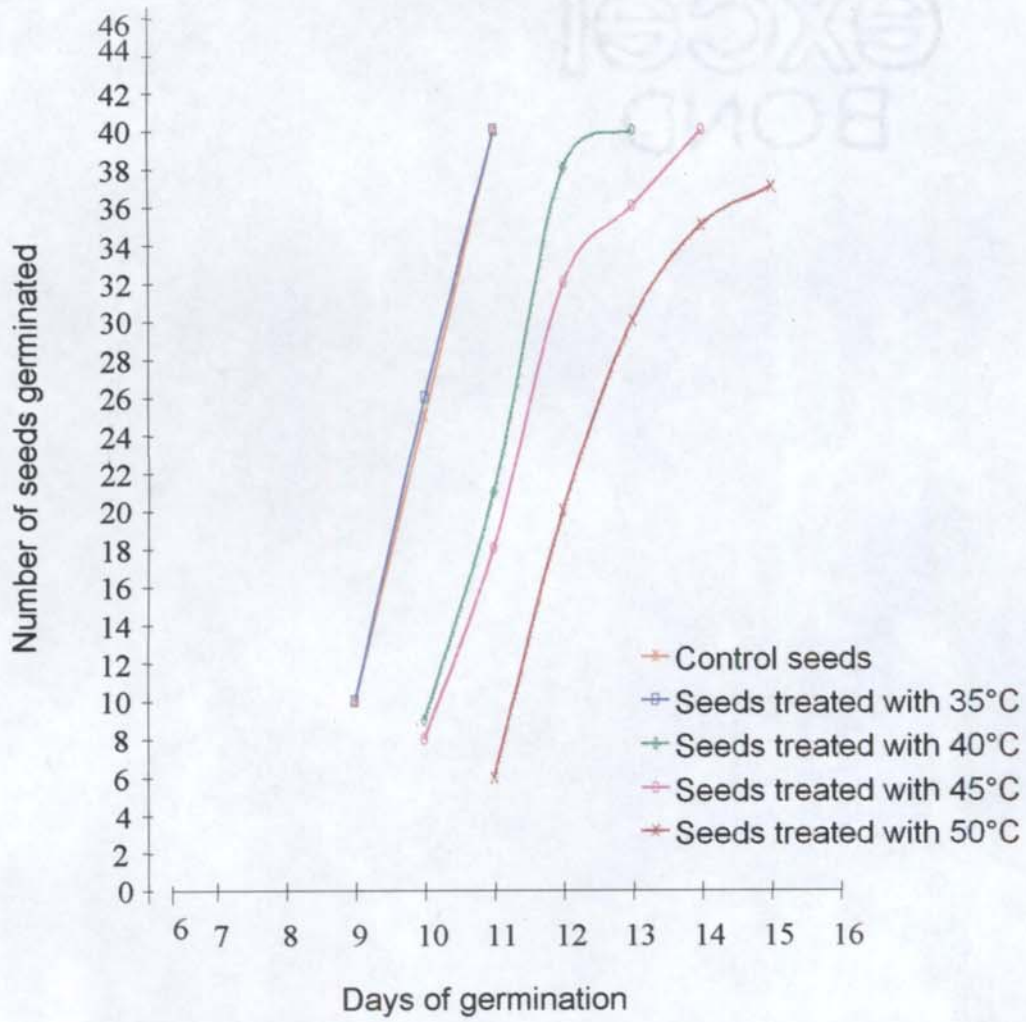


Table 9: Effect of temperature treatment on the imbibition rate in *Pisum sativum* seed during germination in refrigerator

Time (hours)	Weigh (mg)				
	Control seeds	Temperature (°C)			
		35	40	45	50
0	22.8 ± 1.9	23.9 ± 1.6	22.4 ± 2.1	22.8 ± 2.3	20.3 ± 1.8
1	27.8 ± 2.2	29.3 ± 1.8	26.4 ± 3.6	30.2 ± 1.2	33.9 ±1.0
2	31.1 ± 0.9	33.2 ± 1.3	30.7 ± 1.0	34.6 ± 2.1	40.8 ± 1.1
3	32.7 ± 2.8	35.6 ± 3.1	32.5 ± 2.2	36.4 ± 1.3	41.7 ± 1.2
4	34.5 ± 1.3	37.0 ± 1.2	34.4 ± 1.3	37.0 ±1.2	41.9 ± 1.6
5	38.4 ± 1.6	41.8 ± 0.7	38.9 ± 0.6	38.3 ± 1.2	42.6 ± 0.8
6	38.7 ± 1.0	41.8 ± 0.9	38.9 ± 0.5	38.6 ± 1.6	42.6 ± 1.1
7	38.9 ± 0.6	41.8 ± 0.8	38.9 ± 0.9	38.6 ± 1.6	42.4 ± 1.0
8	43.1 ± 1.1	46.2 ± 1.6	43.7 ± 0.8	38.3 ± 0.9	42.6 ± 0.8
9	47.0 ± 1.2	50.6 ± 1.8	47.1 ± 0.9	38.4 ± 0.9	42.6 ± 0.8
10.	47.0 ± 1.2	50.6 ± 1.8	47.1 ± 0.9	38.6 ± 0.9	42.0 ± 0.3
11	47.0 ± 1.2	50.6 ± 1.5	47.1 ± 0.9	42.0 ± 1.1	46.8 ± 1.2
12	47.0 ± 1.2	50.6 ± 1.8	47.1 ± 0.9	42.0 ±1.1	46.8 ± 1.2
24	47.0 ± 1.2	50.6 ± 1.8	47.1 ± 0.9	42.0 ± 1.1	46.8 ± 1.2

of control and treatments (Table 10a). On 2nd hour of soaking the conductivity was showed a sharp increase (P<0.01) and increased by more than double. When a comparison is made between the control and treatments, the control seeds and the treatments at 35°C, 40°C and 45°C showed no significant difference whereas the conductivity seeds treated at 50°C was comparatively higher (P<0.01). During 3rd hour of soaking all the samples showed an increase (Table 10a). From 3rd hour onwards up to 9th hour conductivity increased gradually and 9th day onwards it was retained as such. In between control and treatments, the seeds treated at 50°C showed an increased conductance only up to 6th day, thereafter the difference was negligible.

The seeds on germination in refrigerator showed a higher conductivity than the seeds germinated at room temperature.

1.12.2. Non-cumulative Conductivity

Table 10b shows the non-cumulative conductivity of electrolytic leakage of control seeds and treatment at 35°C, 40°C, 45°C and 50°C during germination in refrigerator. During 1st hour of soaking the treatment at 50°C showed an increased leachate (P<0.01) than the others and the difference between the others were negligible. A sharp increase in conductivity of the entire sample was noticed on 2nd hour of soaking and on 2nd hour the treatment at 50°C did not show an increased conductance (P<0.2) than the others. On 3rd hour all the samples showed a decrease in leachate conductance and the seeds treated at 50°C showed a higher (P<0.01) conductance than the others (Table 10b). Third hour to 7th hour leachate samples of all the seeds showed equal amount of conductance but the seeds treated at 50°C showed a higher leakage than the others (Table 10b). In 50°C treated seeds also the conductivity was equal at all stages from 3rd to 7th day of

Table 10a: Effect of temperature treatment on conductivity of electrolytic leakage in *Pisum sativum* seeds during imbibition in refrigerator (cumulative)

Time (hours)	Conductivity in μ mho cm^{-2}/g				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	27.4 ± 3.7	28.6 ± 4.1	25.4 ± 3.2	29.1 ± 2.6	33.8 ± 3.8
2	71.3 ± 3.6	70.6 ± 4.6	72.3 ± 5.8	72.8 ± 2.4	88.7 ± 4.2
3	98.6 ± 3.9	99.1 ± 5.9	90.8 ± 6.2	92.6 ± 1.8	11.6 ± 2.3
4	109.6 ± 1.8	112.7 ± 5.4	117.9 ± 2.8	116.4 ± 1.7	127.8 ± 2.6
5	124.7 ± 1.6	120.7 ± 4.2	128.1 ± 2.1	120.4 ± 6.6	133.9 ± 1:1
6	131.2 ± 5.7	133.0 ± 3.2	136.5 ± 2.1	139.7 ± 2.8	145.3 ± 3.3
7	152.5 ± 6.4	155.3 ± 4.4	157.1 ± 2.8	156.9 ± 3.9	159.1 ± 4.9
8	174.7 ± 4.8	170.6 ± 2.1	172.3 ± 3.5	179.0 ± 4.6	180.2 ± 3.6
9	187.2 ± 4.4	187.2 ± 4.8	188.6 ± 5.5	189.5 ± 4.8	188.7 ± 3.2
10	188.6 ± 2.3	187.2 ± 5.0	189.2 ± 4.6	189.6 ± 3.8	189.2 ± 2.6
12	189.0 ± 2.1	189.6 ± 3.9	189.3 ± 4.0	189.8 ± 5.1	189.5 ± 2.2

Table 10b: Effect of temperature treatment on conductivity of electrolytic leakage in *Pisum sativum* seeds during imbibition in refrigerator (non-cumulative)

Time (hours)	Conductivity in μ mho cm^{-2}/g				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	22.6 ± 2.2	23.3 ± 2.6	22.9 ± 3.1	22.3 ± 1.8	39.9 ± 2.4
2	48.6 ± 2.6	48.3 ± 2.7	47.9 ± 2.9	49.1 ± 2.9	53.6 ± 2.5
3	31.7 ± 2.4	32.5 ± 2.2	31.6 ± 2.0	33.0 ± 3.1	47.8 ± 3.0
4	32.8 ± 3.1	34.6 ± 3.1	32.8 ± 2.2	33.9 ± 3.0	48.5 ± 3.9
5	34.2 ± 1.8	34.8 ± 2.6	35.7 ± 2.6	38.6 ± 1.8	50.2 ± 4.8
6	35.1 ± 1.9	39.9 ± 2.9	37.6 ± 2.5	39.9 ± 1.6	46.1 ± 6.2
7	39.2 ± 2.4	38.6 ± 2.0	39.7 ± 2.4	40.3 ± 2.9	41.7 ± 4.8
8	10.7 ± 1.8	10.8 ± 2.1	11.3 ± 2.1	11.6 ± 1.8	12.7 ± 1.9
9	1.5 ± 0.8	1.6 ± 0.2	1.9 ± 0.3	1.9 ± 0.2	2.2 ± 0.3
10	1.3 ± 0.9	1.3 ± 0.3	1.4 ± 0.4	1.7 ± 0.2	1.8 ± 0.5
12	1.6 ± 0.9	1.7 ± 0.4	1.5 ± 0.1	1.6 ± 0.3	1.3 ± 0.1

soaking. 7th day onwards the leachate conductivity showed a decrease.

1.12.3. Effect of temperature on sugar content in the leachate

The treatments and control seeds during germination in refrigerator did not show any difference in sugar content in leachate. On 2nd hour of imbibition the sugar content leached from all the samples were almost equal to the previous hour. There after it was decreased but the decrease was not significant. The sugar content was noticed only up to 6th hour in all the samples and up to 9th hour control seeds and treatments showed only trace amount of sugar in leachate. Tenth day onwards it was totally absent In all the samples (Table 10c).

2. Carbohydrate changes

2.1. Starch

During germination at room temperature the control seeds and seeds treated at 35°C and 40°C showed almost similar pattern of starch distribution. Similarly the seeds of treatment at 45°C and 50°C also showed more or less similar pattern of starch distribution (Table 11a).

The seeds of control and treatments at 35°C and 40°C showed a slow and gradual reduction of starch content during earlier 5 days of germination. The reduction of starch content occurred was not significant compared to that of previous days. The starch content was decreased significantly from 5th to 6th day ($P<0.01$) and again decreased to half on 7th day. The same rate of reduction was occurred everyday up to 10th day (Fig. 7a). From 10th day onwards the reduction was again slow and gradual but was insignificant ($P<0.1$ and 0.2).

In the case of seeds treated at 45°C and 50°C starch content showed a gradual and insignificant reduction upto 3rd day of germination (Table 11a). On 4th day the reduction in starch content was

Table 10c: Effect of temperature treatment on the leachate sugar content in *Pisum sativum* seeds during imbibition in refrigerator

Time (hour)	Sugar (mg/1g dry tissue)				
	Control seeds	Temperature (°C)			
		35	40	45	50
1	0.54 ± 0.05	0.59 ± 0.08	0.62 ± 0.02	0.66 ± 0.04	0.69 ± 0.06
2	073 ± 0.17	0.77 ± 0.12	0.84 ± 0.08	0.79 ± 0.03	0.71 ± 0.03
3	0.32 ± 0.11	0.39 ± 0.10	0.44 ± 0.01	0.41 ± 0.01	0.46 ± 0.01
4	0.11 ± 0.01	0.09 ± 0.02	0.12 ± 0.01	0.15 ± 0.01	0.25 ± 0.01
5	0.29 ± 0.03	0.23 ± 0.05	0.14 ± 0.04	0.18 ± 0.08	0.23 ± 0.04
6	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.9 ± 0.01	0.07 ± 0.01
7	t	t	t	t	t
8	t	t	t	t	t
9	t	t	t	t	t
10	N.D	N.D	N.D	N.D	N.D
12	N.D	N.D	N.D	N.D	N.D
24	N.D	N.D	N.D	N.D	N.D

*t means trace amount

decreased to about half on 5th day. Again significant reduction of starch content was observed on 6th day. From 7th to 9th day the starch content was decreased about to half on each day. Thereafter, from 9th day onwards the decrease was again gradual on each day but these reductions were not significant (Fig. 7a).

During germination in refrigerator, the seeds of control and treatments at 35°C, 40°C, 45°C and 50°C showed almost similar pattern of starch distribution. But there occurred slight but insignificant reduction in starch content as the temperature was increased (Table 11b). When the starch content was compared between control and treatments during germination in refrigerator, the starch content was decreased when temperature was increased (Table 11b). During germination up to 30th day the entire sample showed a fast and significant reduction. From 30th day onwards the starch content was decreased to half on each sampling day.

The control seeds germinated in refrigerator showed gradual but insignificant reduction up to 20 days and thereafter sudden as well as significant reduction from interval to interval was occurred during 30, 40 and 50 days retaining only 13% on 50th day (Table 11b). In all treatments also same trend was observed in starch distribution. But there occurred a slight but insignificant reduction in starch content of all treatments as the temperature of treatment was increased (Fig. 7b).

2.2. Sugars

In dry seeds of control and all the treatments no quantitative difference in sugar content was observed and all the samples showed fructose, glucose, maltose, raffinose, rhamnose, sucrose stachyose and verbascose.

Table 11a: Effect of temperature treatment on starch in *Pisum sativum* during seed germination at room temperature

Days of germination	Starch (mg/g dry tissue)				
	Control seeds	Temperature of treatment			
		35	40	45	50
0	432.5 ± 6.7	435.2 ± 4.4	439.7 ± 5.9	435.5 ± 3.9	438.3 ± 3.2
1	411.5 ± 2.5	409.8 ± 2.6	416.8 ± 2.6	417.3 ± 2.7	415.6 ± 2.1
2	400.2 ± 3.2	403.4 ± 4.6	402.1 ± 2.7	399.1 ± 1.2	395.3 ± 1.8
3	389.4 ± 4.9	388.1 ± 3.3	387.3 ± 4.5	357.9 ± 4.0	350.9 ± 5.9
4	350.9 ± 3.2	362.6 ± 1.9	360.0 ± 9.9	297.6 ± 1.5	292.6 ± 3.3
5	327.2 ± 7.0	319.9 ± 1.8	320.1 ± 3.2	156.7 ± 3.2	158.9 ± 1.7
6	289.5 ± 1.2	288.2 ± 2.7	281.2 ± 3.4	108.1 ± 1.9	107.1 ± 1.8
7	137.5 ± 2.4	135.3 ± 1.0	132.6 ± 2.4	78.9 ± 3.4	77.3 ± 8.3
8	88.3 ± 2.7	86.7 ± 3.9	88.9 ± 4.5	36.1 ± 2.8	36.9 ± 5.1
9	46.4 ± 2.4	47.9 ± 2.7	45.1 ± 2.9	22.9 ± 4.1	21.9 ± 3.5
10	20.67± 1.9	23.2 ± 2.9	22.1 ± 2.1	15.3 ± 3.7	15.4 ± 3.2
11	15.8 ± 1.7	16.1 ± 1.9	14.9 ± 2.0	10.9 ± 3.1	10.3 ± 3.9
12	12.7 ± 1.8	12.3 ± 2.8	13.1 ± 3.2	9.3 ± 3.2	9.8 ± 2.4

Table 11b: Effect of temperature treatment on starch in *Pisum sativum* during seed germination in refrigerator

Days of germination	Starch (mg/g dry tissue)				
	Control seeds	Temperature of treatment			
		35	40	45	50
0	432.5 ± 2.7	435.2 ± 2.4	439.8 ± 1.9	435.5 ± 2.9	438.3 ± 3.2
10	400.9 ± 3.1	405.3 ± 7.4	378.9 ± 2.7	358.8 ± 2.0	353.7 ± 4.3
20	323.7 ± 1.8	30.4 ± 3.2	300.1 ± 2.5	278.2 ± 4.0	271.3 ± 8.2
30	202.4 ± 3.3	199.9 ± 1.9	170.6 ± 7.6	161.2 ± 2.7	157.8 ± 1.9
40	99.8 ± 1.7	95.9 ± 2.2	87.3 ± 2.6	80.6 ± 1.5	77.6 ± 2.7
50	59.3 ± 6.2	58.5 ± 7.1	49.5 ± 41.3	41.8 ± 8.2	40.8 ± 6.4

Figure 7a: Effect of temperature treatment on starch in *Pisum sativum* seed during germination at room temperature

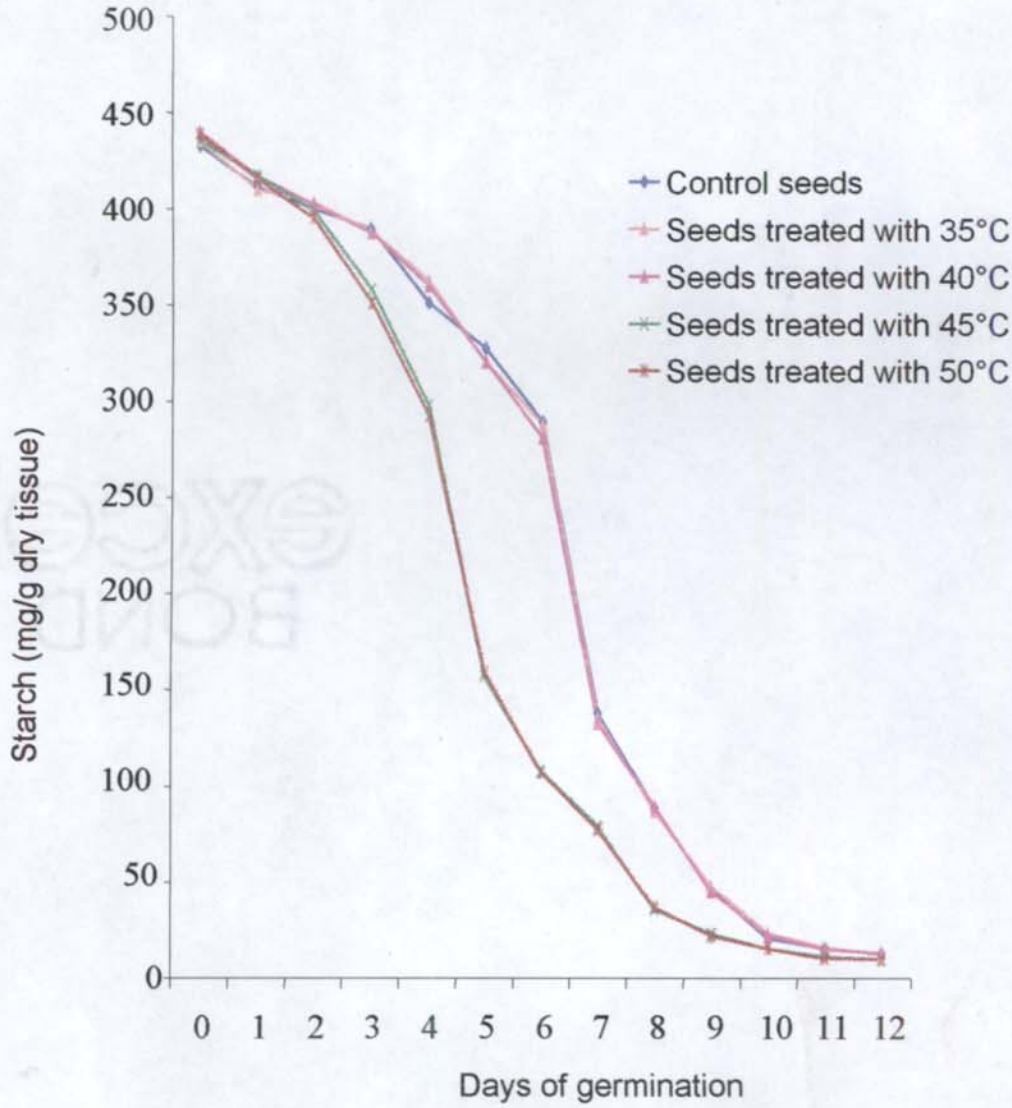
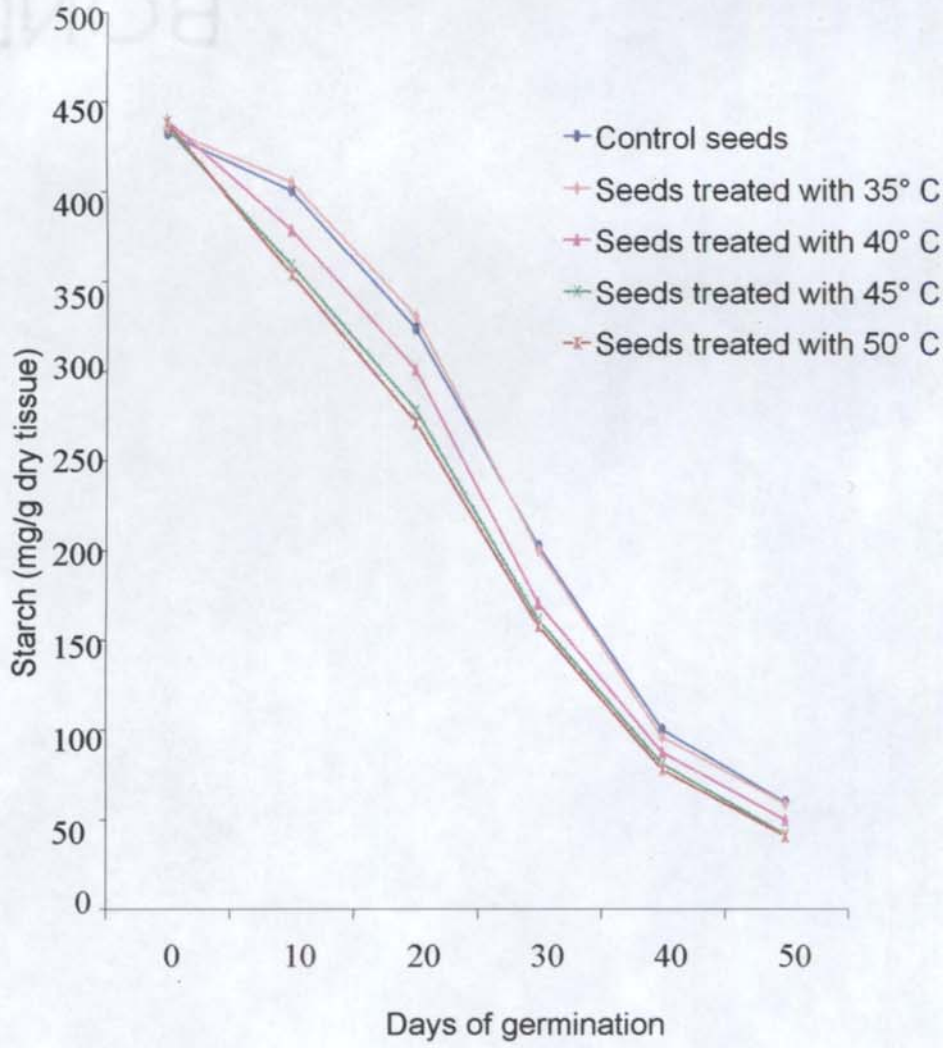


Figure 7b: Effect of temperature treatment on starch in *Pisum sativum* seed during germination in refrigerator



During germination at room temperature up to 12 day, the seeds of control and treatment at 35°C showed similar pattern of sugar distribution (Table 12a and 12b respectively). On 1st day of germination all the sugars except rhamnose were present. Fructose showed a very slight increase whereas, glucose, maltose, raffinose, sucrose, stachyose and verbascose were reduced to half the amount compared to that of dry seeds. Samples of 2nd day showed the presence of only maltose and sucrose. The maltose and sucrose content were reduced to less than half compared to that of the 1st day samples of both control and treatment of 35°C. As germination advanced, up to 6th day there was no qualitative difference in the sugar distribution both in control and treatment of 35°C. However the maltose content showed a gradual but negligible increase in both control and experimental but the increase in sucrose was significant. Fructose and glucose were reappeared in 6th day and 7th day samples respectively in control and experimental sample and sucrose and maltose content was increased. From 7th day onwards fructose and glucose were showed a slight increase in both control and the seeds treated at 35°C, whereas maltose and sucrose showed were retained as such from 3rd day onwards.

Table 12c shows the sugar distribution of the seeds treated at 40°C. The seeds treated at 40°C during 1st day of germination all the sugars were present except rhamnose. Fructose showed a slight increase, glucose showed a slight decrease and all the other sugars maltose, raffinose, sucrose, stachyose and verbascose were reduced to half the amount on 1st day of germination. During 2nd day the maltose and sucrose were showed a reduction again. From 3rd day to 8th day both maltose and sucrose showed a gradual increase and from 9th day onwards both were quantitatively unchanged. Fructose was

Table 12a : Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination at room temperature (Control seeds)

Sugar	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Sugar (mg/g dry tissue)												
Fructose	2.39 ± 0.25	3.68 ± 0.08	N.D	N.D	N.D	N.D	1.5 ± 0.29	1.9 ± 0.08	2.2 ± 0.02	2.4 ± 0.02	2.7 ± 0.11	3.1 ± 0.16	3.3 ± 0 .23
Glucose	5.88 ± 0.11	4.94 ± 0.16	N.D	N.D	N.D	N.D	N.D	1.93 ± 0.02	2.44 ± 0.04	2.59 ± 0.08	2.73 ± 0.32	3.96 ± 0.23	4.58 ± 0.01
Maltose	7.53 ± 0.17	4.44 ± 0.16	1.9 ± 0.05	2.33 ± 0.12	2.45 ± 0.17	2.68 ± 0.32	3.92 ± 0.29	4.58 ± 0.10	5.99 ± 0.06	6.35 ± 0.25	6.4 ± 0.31	6.58 ± 0.01	6.33 ± 0.09
Raffinose	1.22 ± 0.20	0.69 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rhamnose	7.61 ± 0.16	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Sucrose	10.49 ± 0.20	5.36 ± 0.14	2.3 ± 0.07	2.49 ± 0.09	3.62 ± 0.18	4.54 ± 0.36	4.96 ± 0.20	5.24 ± 0.22	5.52 ± 0.18	5.5 ± 0.08	5.33 ± 0.11	5.28 ± 0.07	5.1 ± 0 .09
Stachyose	1.23 ± 0.20	0.62 ± 0.03	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Verbascose	3.96 ± 0.33	1.95 ± 0.14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total	40.31 ± 0.20	21.68 ± 0.13	4.24 ± 0.11	4.82 ± 0.60	6.07 ± 0.17	7.22 ± 0.34	10.38 ± 0.29	13.65 ± 0.14	16.15 ± 0.78	16.84 ± 0.41	17.16 ± 0.21	18.92 ± 0.11	19.31 ± 0.1

Table 12b: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination at room temperature (35°C treated seeds)

Sugar	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Sugar (mg/g dry tissue)												
Fructose	2.67 ± 0.13	3.66 ± 0.17	N.D	N.D	N.D	N.D	1.6 ± 0.33	1.9 ± 0.78	2.1 ± 0.14	2.3 ± 0.15	2.6 ± 0.18	3.1 ± 0.19	3.5 ± 0.35
Glucose	5.69 ± 0.14	4.65 ± 0.17	N.D	N.D	N.D	N.D	N.D	1.99 ± 0.62	2.52± 0.21	2.62 ± 0.19	2.67 ± 016	3.99 ± 0.01	4.53 ± 0.22
Maltose	7.01 ± 0.09	4.53 ± 0.08	1.95 ± 0.32	2.35 ± 0.11	2.48 ± 0.14	2.67 ± 018	3.89 ± 0.12	4.55 ± 0.78	5.86 ± 0.14	6.37 ± 0.19	6.44 ± 0.22	6.52 ± 0.06	6.29 ± 0.04
Raffinose	1.09 ± 0.33	0.55 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rhamnose	7.91 ± 0.07	N.Δ	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Sucrose	10.53 ± 0.23	5.56 ± 0.07	2.29 ± 0.45	2.33 ± 0.32	2.48 ± 0.05	2.72 ± 0.05	3.79 ± 0.06	4.6 ± 0.09	5.89 ± 0.12	6.3 ± 0.03	6.41 ± 0.02	6.55 ± 0.13	6.2 ± 0.14
Stachyose	1.71 ± 0.10	0.89 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Verbascose	3.85 ± 0.12	1.91 ± 0.03	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total	40.46 ± 0.15	21.75 ± 0.12	4.24 ± 0.38	4.68 ± 0.22	4.96 ± 0.09	5.39 ± 0.11	9.25 ± 0.18	13.04 ± 0.56	16.37 ± 0.15	17.82 ± 0.14	17.89 ± 0.14	20.16 ± 0.01	20.52 ± 0.75

Table 12c: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination at room temperature (40°C treated seeds)

Sugar	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Sugar (mg/g dry tissue)												
Fructose	2.78 ± 0.09	3.7 ± 0.13	N.D	N.D	N.D	N.D	N.D	0.65 ± 0.05	0.97 ± 0.11	1.23 ± 0.15	1.56± 0.17	1.99 ± 0.13	2.54 ± 0.23
Glucose	5.49 ± 0.03	4.92 ± 0.04	N.D	N.D	N.D	N.D	N.D	N.D	N.D	1.23 ± 0.06	2.18 ± 0.18	2.34 ± 0.24	2.78 ± 0.15
Maltose	7.37 ± 0.05	4.61 ± 0.03	2.03 ± 0.06	2.38 ± 0.08	2.44 ± 0.05	2.7 ± 0.15	3.82 ± 0.05	4.58 ± 0.22	5.76 ± 0.07	6.33 ± 0.05	6.47 ± 0.04	6.55 ± 0.08	6.3 ± 0.09
Raffinose	1.03 ± 0.04	0.54 ± 0.02	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rhamnose	7.55 ± 0.06	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Sucrose	10.38 ± 0.03	5.47 ± 0.06	1.34 ± 0.11	2.35 ± 0.15	2.42 ± 0.06	2.69 ± 0.08	3.88 ± 0.07	4.65 ± 0.08	5.88 ± 0.09	6.32 ± 0.05	6.48 ± 0.09	6.55 ± 0.14	6.31 ± 0.13
Stachyose	1.71 ± 0.02	0.88 ± 0.04	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Verbascose	3.99 ± 0.08	1.88 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total	40.57 ± 0.15	22.0 ± 0.19	3.37 ± 0.85	4.73 ± 0.12	4.89 ± 0.05	5.39 ± 0.11	7.70 ± 0.06	9.88 ± 0.12	12.16 ± 0.09	15.11 ± 0.83	16.63 ± 0.12	17.43 ± 0.15	17.93 ± 0.14

reappeared and was gradually increasing when germination proceeds. Glucose showed a reappearance and gradual increase from 9th day onwards.

The seeds treated at 45°C (Table 12d) and 50°C (Table 12e) also showed the presence of fructose, glucose, maltose, raffinose, rhamnose, sucrose stachyose and verbascose in dry and imbibed (1st day) seeds. Second day onwards up to 7th day only maltose and sucrose were present. The quantity of sucrose and maltose were significantly reduced on 2nd day. But there was a doubling in both maltose and sucrose on 3rd day and thereafter both the sugars were retained as such. Rhamnose and fructose were reappeared on 7th day and 8th day respectively. The quantity of rhamnose and fructose were remained unchanged up to 12th day. Glucose was reappeared on 11th day and showed increase from 11th to 12th day ($P<0.01$).

During germination in refrigerator, the seeds of control and the treatments at 35°C and 40°C showed similar pattern of sugar distribution (Table 13a to 13c). On 10th day fructose, glucose, maltose, raffinose, rhamnose and stachyose showed a reduction compared to the dry seeds. Sucrose was increased significantly ($P<0.01$) and the verbascose was not detected. On 20th day of germination fructose showed a slight increase ($P<0.01$), whereas maltose and raffinose were decreased significantly and sucrose was reduced significantly. Rhamnose, stachyose and verbascose were not detected on 20th day. Samples of 30th day showed the reappearance of stachyose and rhamnose and verbascose was absent. From 30th day onwards fructose, glucose, maltose, raffinose and sucrose showed no quantitative differences but stachyose was significantly reduced ($P<0.01$). During 50th day of germination the stachyose was again disappeared and rhamnose was reappeared.

Table12d: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination at room temperature (45°C treated seeds)

Sugar	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Sugar (mg/g dry tissue)												
Fructose	2.92 ± 0.18	3.76 ± 0.07	N.D	N.D	N.D	N.D	N.D	N.D	0.66 ± 0.05	0.85 ± 0.03	0.96 ± 0.05	1.22 ± 0.06	1.50 ± 0.07
Glucose	5.37 ± 0.17	4.85 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	1.82 ± 0.15	2.85 ± 0.23
Maltose	7.38 ± 0.06	4.59 ± 0.06	1.98 ± 0.18	2.01 ± 0.14	2.22 ± 0.17	2.36 ± 0.18	2.45 ± 0.19	2.48 ± 0.22	2.45 ± 0.14	2.47 ± 0.09	2.44 ± 0.14	2.56 ± 0.07	2.58 ± 0.09
Raffinose	1.05 ± 0.19	0.82 ± 0.07	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rhamnose	7.62 ± 0.09	N.Δ	N.D	N.D	N.D	N.D	N.D	0.68 ± 0.04	1.32 ± 0.08	1.22 ± 0.08	1.65 ± 0.22	1.58 ± 0.16	1.44 ± 0.10
Sucrose	10.65 ± 0.06	5.59 ± 0.17	1.32 ± 0.05	2.22 ± 0.06	3.65 ± 0.05	3.58 ± 0.05	3.44 ± 0.18	3.54 ± 0.22	3.58 ± 0.06	3.59 ± 0.19	3.61 ± 0.24	3.65 ± 0.18	3.33 ± 0.19
Stachyose	1.84 ± 0.09	0.26 ± 0.05	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Verbascose	3.76 ± 0.06	1.82 ± 0.03	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total	40.83 ± 0.12	21.69 ± 0.11	3.30 ± 0.11	4.23 ± 0.10	5.87 ± 0.11	5.94 ± 0.11	5.89 ± 0.18	6.70 ± 0.22	8.01 ± 0.83	8.12 ± 0.10	8.66 ± 0.16	10.83 ± 0.12	11.7 ± 0.94

Table 12e Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination at room temperature (50°C treated seeds)

Sugar	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Sugar in mg/g dry tissue												
Fructose	3.12 ± 0.22	3.85 ± 0.02	N.D	N.D	N.D	N.D	N.D	N.D	0.67 ± 0.02	0.82 ± 0.33	0.99 ± 0.11	1.03 ± 0.47	1.54 ± 0.15
Glucose	5.46 ± 0.14	4.95 ± 0.02	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	1.91 ± 0.05	2.83 ± 0.09
Maltose	7.37 ± 0.32	4.43 ± 0.44	1.99 ± 0.24	2.02 ± 0.25	2.23 ± 0.21	2.39 ± 0.01	2.42 ± 0.04	2.45 ± 0.18	2.42 ± 0.45	2.44 ± 0.33	2.48 ± 0.20	2.49 ± 0.05	2.52 ± 0.03
Raffinose	0.88 ± 0.24	0.52 ± 0.25	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rhamnose	8.81 ± 0.08	N.Δ	N.D	N.D	N.D	N.D	N.D	0.65 ± 0.04	1.38 ± 0.08	1.44 ± 0.08	1.73 ± 0.22	1.32 ± 0.16	1.10 ± 0.10
Sucrose	10.02 ± 0.17	5.62 ± 0.18	1.33 ± 0.17	2.29 ± 0.38	3.58 ± 0.04	3.62 ± 0.07	3.51 ± 0.11	3.47 ± 0.01	3.56 ± 0.11	3.55 ± 0.13	3.57 ± 0.15	3.52 ± 0.14	3.21 ± 0.18
Stachyose	0.32 ± 0.19	0.06 ± 0.21	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Verbascose	4.1 ± 0.04	2.33 ± 0.77	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Total	40.08 ± 0.15	21.74 ± 1.19	3.32 ± 0.21	4.31 ± 0.13	5.81 ± 0.03	6.01 ± 0.04	5.93 ± 0.18	6.57 ± 0.05	8.03 ± 0.17	8.25 ± 0.21	8.77 ± 0.17	10.27 ± 0.11	11.2 ± 0.12

The seeds treated at 45°C (Table 13d) and 50°C (Table 13e), on 10^{th} day of germination in refrigerator fructose, glucose, maltose, raffinose, rhamnose and stachyose were showed a significant reduction compared to the dry seeds. Sucrose was significantly increased ($P<0.01$) and verbascose was not detected. On 20^{th} day of germination fructose showed slight increase, whereas maltose, sucrose and raffinose were decreased significantly ($P<0.01$). Rhamnose, stachyose and verbascose were not detected. The maltose and sucrose content of 20^{th} day samples were quantitatively lesser than the control seeds and the seeds treated at 35°C and 40°C but the other sugars showed no difference. Twentieth day onwards glucose was retained as such. On 30^{th} day fructose, maltose and glucose showed no quantitative differences. Rhamnose, stachyose and verbascose were absent. From 30^{th} day onwards fructose, maltose and glucose showed no quantitative change. Sucrose showed an increase from 40^{th} day onwards. The reappearance of stachyose was observed on 40^{th} day but rhamnose and verbascose were absent. On 50^{th} day rhamnose was reappeared and stachyose showed no quantitative difference but verbascose was still absent.

2.3. Amylase assay

When the assay for amylase activity of seeds during germination at room temperature and in refrigerator was carried out, the enzyme activity was increased as germination proceeded.

2.3.1. p^H optimum for α-amylase activity

The enzyme extract of seeds germinated at room temperature and in refrigerator, assayed at different p^H ranging from 4 to 8 at interval of p^H 0.2 (Fig. 8a), and maximum activity was obtained at p^H 6.8 (Fig. 8a). Even though another small peak was obtained at p^H 5.6, confirmatory test for α-amylase activity was negative.

The seeds treated at 45°C (Table 13d) and 50°C (Table 13e), on 10th day of germination in refrigerator fructose, glucose, maltose, raffinose, rhamnose and stachyose were showed a significant reduction compared to the dry seeds. Sucrose was significantly increased ($P<0.01$) and verbascose was not detected. On 20th day of germination fructose showed slight increase, whereas maltose, sucrose and raffinose were decreased significantly ($P<0.01$). Rhamnose, stachyose and verbascose were not detected. The maltose and sucrose content of 20th day samples were quantitatively lesser than the control seeds and the seeds treated at 35°C and 40°C but the other sugars showed no difference. Twentieth day onwards glucose was retained as such. On 30th day fructose, maltose and glucose showed no quantitative differences. Rhamnose, stachyose and verbascose were absent. From 30th day onwards fructose, maltose and glucose showed no quantitative change. Sucrose showed an increase from 40th day onwards. The reappearance of stachyose was observed on 40th day but rhamnose and verbascose were absent. On 50th day rhamnose was reappeared and stachyose showed no quantitative difference but verbascose was still absent.

2.3. Amylase assay

When the assay for amylase activity of seeds during germination at room temperature and in refrigerator was carried out, the enzyme activity was increased as germination proceeded.

2.3.1. p^{H} optimum for α-amylase activity

The enzyme extract of seeds germinated at room temperature and in refrigerator, assayed at different p^{H} ranging from 4 to 8 at interval of p^{H} 0.2 (Fig. 8a), and maximum activity was obtained at p^{H} 6.8 (Fig. 8a). Even though another small peak was obtained at p^{H} 5.6, confirmatory test for α-amylase activity was negative.

Table 13a: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination in refrigerator (Control seeds)

Sugar	Days of germination					
	0	10	20	30	40	50
	Sugar (mg/g dry tissue)					
Fructose	2.39 ± 0.25	1.86 ± 0.01	2.29 ± 0.01	2.58 ± 0.15	2.40 ± 0.18	2.62 ± 0.17
Glucose	5.88 ± 0.11	3.22 ± 0.08	3.02 ± 0.07	2.98 ± 0.04	2.99 ± 0.25	2.89 ± 0.33
Maltose	7.53 ± 0.17	5.69 ± 0.07	3.24 ± 0.12	3.22 ± 0.13	3.00 ± 0.16	2.96 ± 0.18
Raffinose	1.22 ± 0.20	0.32 ± 0.09	0.11 ± 0.12	0.56 ± 0.14	0.48 ± 0.18	0.11 ± 0.17
Rhamnose	7.61 ± 0.16	1.88 ± 0.04	N.D	N.D	N.D	0.99 ± 0.13
Sucrose	10.49 ± 0.20	13.11 ± 0.13	3.02 ± 0 .08	3.88 ± 0 .05	3.98 ± 0 .01	3.79 ± 0.13
Stachyose	1.23 ± 0.20	0.15 ± 0.11	N.D	0.33 ± 0.22	0.12 ± 0.32	N.D
Verbascose	3.96 ± 0.33	N.D	N.D	N.D	N.D	N.D
Total	40.31 ± 0.20	26.23 ± 0.06	11.68 ± 0.56	13.55 ± 0.10	12.97 ± 0.18	13.36 ± 0.19

Table 13b: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination in refrigerator (35°C treated seeds)

Sugar	Days of germination					
	0	10	20	30	40	50
	Sugar (mg/g dry tissue)					
Fructose	2.67 ± 0.13	1.79 ± 0.33	2.32 ± 0.31	2.63 ± 0.15	2.58 ± 0.19	2.71 ± 0.25
Glucose	5.69 ± 0.14	3.42 ± 0.15	3.05 ± 0.12	3.00 ± 0.05	2.96 ± 0.15	2.88 ± 0.12
Maltose	7.01 ± 0.09	5.44 ± 0.03	3.56 ± 0.24	3.40 ± 0.08	3.35 ± 0.01	3.03 ± 0.13
Raffinose	1.09 ± 0.33	0.57 ± 0.18	0.14 ± 0.16	0.55 ± 0.04	0.50 ± 0.18	0.09 ± 0.17
Rhamnose	7.91 ± 0.07	1.64 ± 0.08	N.D	N.D	N.D	0.99 ± 0.16
Sucrose	10.53 ± 0.23	13.19 ± 0.11	3.11 ± 0.15	2.90 ± 0.08	2.82 ± 0.06	2.76 ± 0.26
Stachyose	1.71 ± 0.10	0.11 ± 0.06	N.D	0.35 ± 0.01	0.12 ± 0.30	N.D
Verbascose	3.85 ± 0.12	N.D	N.D	N.D	N.D	N.D
Total	40.46 ± 0.15	26.16 ± 0.13	12.18 ± 0.19	12.28 ± 0.68	12.37 ± 0.15	12.46 ± 0.15

Table 13d: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination in refrigerator (45°C treated seeds)

Sugar	Days of germination					
	0	10	20	30	40	50
	Sugar (mg/g dry tissue)					
Fructose	2.92 ± 0.18	1.87 ± 0.10	2.46 ± 0.17	2.69 ± 0.19	2.64 ± 0.08	2.78 ± 0.33
Glucose	5.37 ± 0.17	3.24 ± 0.14	3.15 ± 0.16	3.00 ± 0.15	2.90 ± 0.02	2.96 ± 0.37
Maltose	7.38 ± 0.06	5.64 ± 0 .28	2.40 ± 0 .14	2.21 ± 0 .18	2.56 ± 0 .15	2.59 ± 0 .15
Raffinose	1.05 ± 0.19	0.54 ± 0.29	0.18 ± 0.03	0.62 ± 0.12	0.60 ± 0.09	0.18 ± 0.07
Rhamnose	7.62 ± 0 .09	1.81 ± 0 .20	N.D	N.D	N.D	1.05 ± 0.06
Sucrose	10.65 ± 0.06	13.19 ± 0.14	1.88 ± 0.24	1.92 ± 0 .29	2.56 ± 0.15	2.75 ± 0.03
Stachyose	1.84 ± 0.09	0.19 ± 0.03	N.D	N.D	0.80 ± 0.34	0.90 ± 0.28
Verbascose	3.76 ± 0.06	N.D	N.D	N.D	N.D	N.D
Total	40.83 ± 0.12	26.29 ± 0.16	10.07 ± 0.14	10.44 ± 0.19	12.06 ± 0.13	13.29 ± 0.17

Table 13e: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination in refrigerator (50°C treated seeds)

Sugar	Days of germination					
	0	10	20	30	40	50
	Sugar in mg/g dry tissue					
Fructose	3.12 ± 0.22	1.85 ± 0.05	2.50 ± 0.11	2.72 ± 0.13	2.60 ± 0.07	2.76 ± 0.27
Glucose	5.46 ± 0.14	3.30 ± 0 .14	3.15 ± 0.13	3.01 ± 0.25	2.92 ± 0.24	2.94 ± 0.29
Maltose	7.37 ± 0.32	5.59 ± 0.22	2.39 ± 0.10	2.24 ± 0 .22	2.62 ± 0 .14	2.54 ± 0 .13
Raffinose	0.88 ± 0.24	0.34 ± 0 .18	0.15 ± 0.01	0.72 ± 0.40	0.66 ± 0.07	0.10 ± 0.08
Rhamnose	8.81 ± 0.08	1.87 ± 0 .19	N.D	N.D	N.D	1.07 ± 0.03
Sucrose	10.02 ± 0.17	13.24 ± 0.16	1.91 ± 0.16	1.96 ± 0.25	2.56 ± 0.03	2.81 ± 0.22
Stachyose	0.32 ± 0.19	0.11 ± 0 .18	N.D	N.D	0.82 ± 0.26	0.97 ± 0.03
Verbascose	4.1 ± 0.04	N.D	N.D	N.D	N.D	N.D
Total	40.08 ± 0.15	26.3 ± 0 .16	10.1 ± 0 .10	10.65 ± 0.25	12.18 ± 0.12	13.17 ± 0.15

In order to confirm the specificity of amylase the enzyme extract was subjected to heat treatment. Since the extract withstand heat treatment in the presence of calcium acetate and hence the enzyme was confirmed to be α-amylase. When the assay for β-amylase was carried out in the presence of 0.1N HCl, the extract did not show any activity and hence the enzyme was confirmed as the α-amylase activity.

2.3.2. Temperature optimum for α-amylase activity

When assay was conducted at the optimum p^H at different temperature ranging from 20°C to 40°C, higher activity was shown at 37°C. Hence the optimum temperature for the α-amylase assay was confirmed as 37°C (Fig. 8b).

2.3.3. Enzyme proportionality range for α-amylase activity

The assay was conducted at optimum p^H (6.8) and optimum temperature (37°C) using different volumes of enzyme extract (10% w/v) ranging from 50 – 400 µl, the assay system showed optimum activity at 200 µl enzyme extract. Hence the optimum enzyme concentration for the α-amylase assay was confirmed as 200 µl of 10% (w/v) enzyme extract (Fig. 8c).

2.3.4. Substrate saturation for α-amylase activity

When the assay was conducted at optimum p^H, optimum temperature and optimum enzyme and at different quantities of substrate (4% soluble starch) ranging from 100–800 µg/ml, the assay system showed optimum activity at 400-µg/ml substrate (Fig. 8d).

There was no significant difference between the control and the seeds treated at 35°C, 40°C, 45°C and 50°C in optimum p^H, temperature, substrate and in enzyme proportionality range for the seeds germinated at room temperature and in refrigerator.

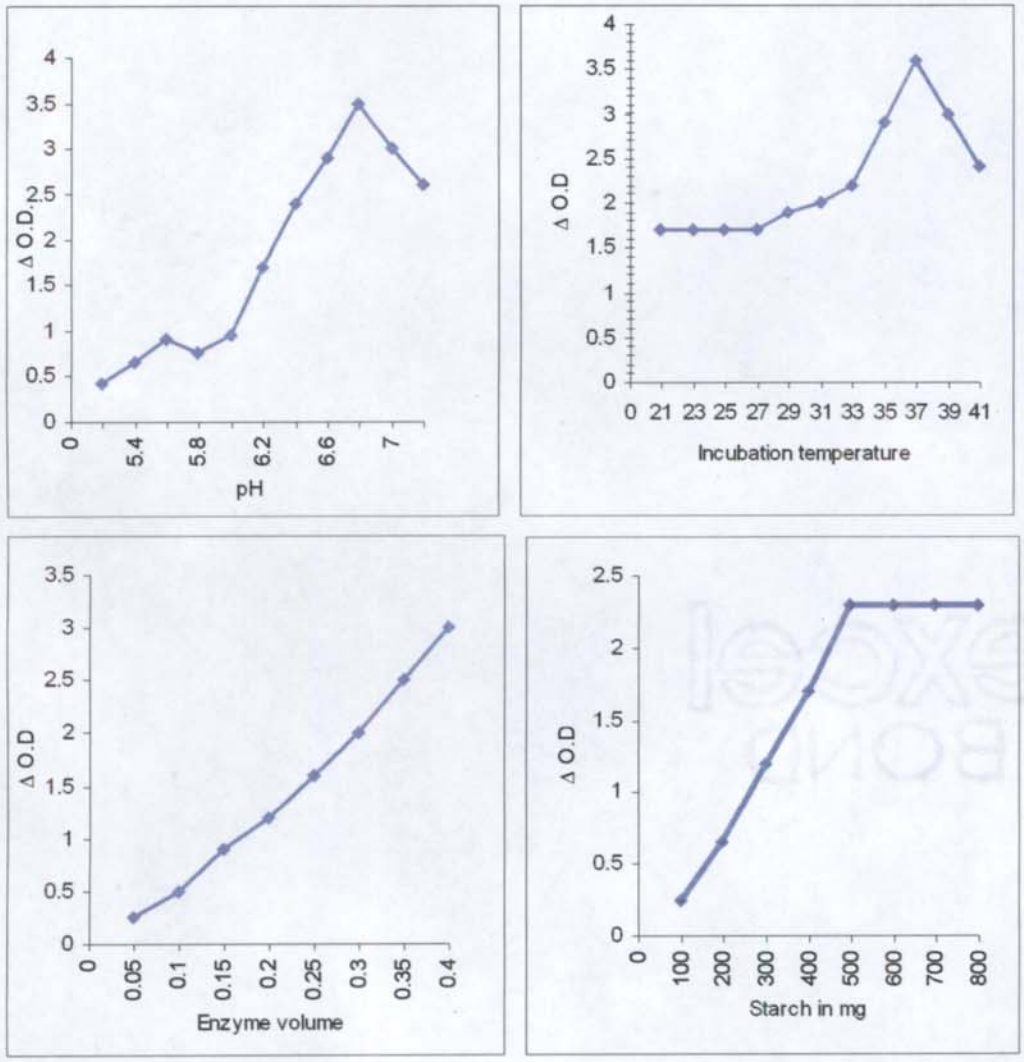


Figure 8 a: p^H optimum for α-amylase activity
b: Temperature optimum for α-amylase activity
c: Enzyme volume for optimum α-amylase activity
d: Substrate saturation for α-amylase activity

Table 13c: Effect of temperature treatment on Sugar in *Pisum sativum* seed during germination in refrigerator (40°C treated seeds)

Sugar	Days of germination					
	0	10	20	30	40	50
	Sugar in mg/g dry tissue					
Fructose	2.78 ± 0.09	1.88 ± 0.23	2.44 ± 0.11	2.69 ± 0.15	2.60 ± 0.28	2.74 ± 0.31
Glucose	5.49 ± 0.03	3.14 ± 0.06	3.10 ± 0.12	3.05 ± 0.14	2.85 ± 0.27	2.90 ± 0.30
Maltose	7.37 ± 0.05	5.55 ± 0.09	3.28 ± 0.14	3.29 ± 0.21	3.48 ± 0.14	3.00 ± 0.24
Raffinose	1.03 ± 0.04	0.42 ± 0.25	0.17 ± 0.06	0.59 ± 0.04	0.58 ± 0.01	0.10 ± 0.01
Rhamnose	7.55 ± 0.06	1.70 ± 0.06	N.D	N.D	N.D	1.00 ± 0.01
Sucrose	10.38 ± 0.03	13.11 ± 0.07	3.20 ± 0.13	3.01 ± 0.21	2.88 ± 0.09	2.68 ± 0.06
Stachyose	1.71 ± 0.02	0.11 ± 0.04	N.D	0.38 ± 0.04	0.14 ± 0.02	N.D
Verbascose	3.99 ± 0.08	N.D	N.D	N.D	N.D	N.D
Total	40.57 ± 0.15	25.91 ± 0.13	12.19 ± 0.09	12.63 ± 0.38	12.39 ± 0.10	12.42 ± 0.13

2.3.5. Unit activity

The seeds of control and all the treatments germinated at room temperature showed very low activity up to 3 days of germination (Table 14a). The unit activity of α-amylase in control seeds and treatments at 35°C and 40°C was increased gradually showing maximum value on 10^{th} day of germination, whereas in the case of seeds treated at 45°C and 50°C increase was occurred but the peak value was noticed on 9^{th} day of germination. Thereafter it was decreased. (Fig. 9a). The differences in unit activity between control and treatments at 35°C and 40°C on one hand and difference between 45°C and 50°C on the other hand were negligible.

In the case of seeds germinated in refrigerator, the control seeds and treatment at 35°C, showed negligible increase in total activity were up to 10^{th} day of germination and the difference among them were negligible (Table 14b). From 10^{th} and 20^{th} day of germination the seeds of control and all the treatments showed a slight increase in activity and from 20^{th} to 30^{th} day the increase in activity was significant ($P<0.01$). During 30^{th} to 40^{th} day of germination a sudden increase in activity was shown by all the samples. From 40^{th} to 50^{th} day an increase in activity was noticed in all the treatments and control but the increase in activity was not significant ($P<0.05$ in control and treatment at 35°C, $P<0.1$ each in treatment at 40°C, 45°C and 50°C). Generally the seeds treated at 45°C and 50°C showed lesser activity than the other treatments and control seeds during 10^{th} to 50^{th} day of germination (Fig. 10a).

2.3.6. Specific activity

Dry seeds of *Pisum sativum* did not show any activity during early 5 days of germination at room temperature, the seeds of control

and all treatments showed very slow increase in the specific activity of α-amylase up to 7th day (Table 14a). Thereafter the seed of control and all the treatments showed a significant increase. When a comparison was made, the control seeds and treatments at 35°C and 40°C exhibited almost similar pattern in activity but it was significantly higher than the other treatment at 45°C and 50°C (Fig. 9b).

In the case of control seeds and treatments at 35°C, 40°C, 45°C and 50°C when germinated in refrigerator, seeds did not show any change in activity up to 10th day (Table 14b). Control seeds and the treatment at 35°C and 40°C showed a 2-fold increase from 10th to 20th day and the same pattern was followed from 20th to 30th day of germination. The treatments at 45°C and 50°C showed more activity compared to the control and other treatments (35°C and 40°C) from 10th to 20th day. But the pattern of increase was similar to control and treatments at 35°C and 40°C (Table 14b). From 20th to 30th day, the seeds treated at 45°C and 50°C showed a doubling in activity. From 30th to 40th day of germination the control and treatments at 35°C and 40°C showed a 2-fold increase, whereas, the treatments at 45°C and 50°C showed a slight decrease in specific activity, resembling almost similar activity in all treatments and control. Thereafter, from 40th to 50th day of germination control and all the treatments showed a remarkable increase in activity (Fig. 10b).

3. Protein metabolism

3.1. Total protein

In the case of seeds germinated at room temperature, control seeds showed a decrease in protein content during initial four days of germination ($P<0.01$ up to 3rd day and 0.02 in between 3rd and 4th day) (Table 15a). On 5th day the protein content was slightly higher than that

Table 14a: Effect of temperature treatment on amylase activity and mg Maltose/30 minutes in *Pisum sativum* seeds during germination at room temperature

Days	Unit activity (mg maltose/g dry tissue) and specific activity (mg maltose/mg protein/30minutes)				
	Control seeds	Temperature treatment			
		35	40	45	50
0	16.15 ± 0.34 (0.108 ± 0.005)	16.70 ± 1.3 (0.106 ± 0.04)	16.00 ± 1.5 (0.105 ± 0.002)	16.70 ± 1.3 (0.108 ± 0.003)	16.70 ± 1.9 (0.109 ± 0.001)
1	16.08 ± 2.2 (0.110 ± 0.001)	16.21 ± 1.1 (0.113 ± 0.002)	16.16 ± 1.3 (0.111 ± 0.001)	16.33 ± 2.1 (0.117 ± 0.004)	16.81 ± 1.5 (0..129 ± 0.005)
2	16.32 ± 3.4 (0.123 ± 0.002)	16.43 ± 3.1 (0.124 ± 0.05)	16.19 ± 1.5 (0.123 ± 0.003)	18.03 ± 2.3 (0.145 ± 0.005)	18.43 ± 1.0 (0.150 ± 0.004)
3	16.36 ± 1.3 (0.135 ± 0.004)	16.22± 0.34 (0.133 ± 0.001)	16.33 ± (0.126 ± 0.006)	16.30 ± 2.1 (0.135 ± 0.004)	16.43 ± 1.4 (0.136 ± 0.006)
4	18.00 ± 1.8 (0.161 ± 0.001)	18.00 ± 2.3 (0.1615 ± 0.02)	18.00 ± 1.5 (0.151 ± 0.006)	22.91± 1.5 (0.146 ± 0.004)	20.02 ± 1.3 (0.144 ± 0.001)
5	20.36± 0.89 (0.196 ± 0.006)	21.00 ± 2.2 (0.193 ± 0.003)	20.91 ± 2.0 (0.193 ± 0.005)	26.72 ± 1.6 (0.194 ± 0.002)	29.23 ± 1.7 (0.150 ± 0.006)
6	38.43 ± 2.8 (0.423 ± 0.002)	34.46 ± 2.1 (0.369 ± 0.004)	36.11 ± 1.6 (0.369 ± 0.007)	43.22 ± 1.9 (0.372 ± 0.008)	40.71 ± 1.9 (0.38 ± 0.002)
7	50.63 ± 1.8 (0.595 ± 0.003)	53.03 ± 1.2 (0.626 ± 0.006)	51.24± 0.34 (0.635 ± 0.003)	71.62 ± 1.9 (0.65 ± 0.002)	73.64 ± 2.0 (0.68 ± 0.004)
8	83.95 ± 1.7 (1.68 ± 0.007)	84.21 ± 0.3 (1.23 ± 0.004)	86.11 ± 2.4 (1.37 ± 0.006)	103.19 ± 2.2 (1.24 ± 0.004)	104.21 ± 2.2 (1.35 ± 0.001)
9	129.56 ± 1.9 (3.45 ± 0.003)	127.41± 1.2 (3.60 ± 0.005)	131.28 ± 2.0 (2.92 ± 0.004)	105.46 ± 1.8 (2.11 ± 0.006)	107.24 ± 1.1 (2.10 ± 0.004)
10	185.60 ± 1.3 (4.03 ± 0.001)	183.55 ± 1.6 (4.22 ± 0.004)	180.10 ± 1.4 (4.01 ± 0.001)	107.35 ± 2.5 (3.55 ± 0.003)	106.60 ± 3.1 (3.52± 0.004)
11	153.94 ± 1.2 (6.22 ± 0.002)	159.04 ± 1.9 (6.04 ± 0.001)	156.13 ± 0.9 (6.11 ± 0.004)	93.22 ± 1.7 (4.56 ± 0.001)	90.96 ± 1.8 (4.84 ± 0.005)
12	111.72 ± 1.8 (7.87 ± 0.005)	112.00 ± 2.0 (7.06 ± 0.001)	109.25 ± 1.3 (7.12 ± 0.001)	81.23 ± 1.8 (7.51 ± 0.003)	85.50 ± 1.0 (7.43 ± 0.006)

Table 14b: Effect of temperature treatment on amylase activity and mg Maltose/30 minutes in *Pisum sativum* seeds during germination in refrigerator

Days	Unit activity (mg maltose/g dry tissue) and specific activity (mg maltose/mg protein/30minutes)				
	Control seeds	Temperature treatment			
		35	40	45	50
0	16.17 ± 1.3 (0.106 ± 0.004)	16.15 ± 0.34 (0.108 ± 0.005)	16.00 ± 1.5 (0.105 ± 0.002)	16.70 ± 1.3 (0.108 ± 0.003)	16.70 ± 1.9 (0.109 ± 0.001)
10	16.89 ± 1.1 (0.106 ± 0.005)	16.88 ± 0.8 (0.106 ± 0.003)	16.98 ± 1.0 (0.114 ± 0.002)	16.54 ± 0.55 (0.136 ± 0.003)	16.87 ± 1.7 (0.140 ± 0.008)
20	29.88 ± 1.8 (0.220 ± 0.008)	25.38 ± 1.6 (0.207 ± 0.001)	25.96 ± 1.5 (0.293 ± 0.005)	20.38± 1.0 (0.515 ± 0.001)	20.88± 1.1 (0.799 ± 0.001)
30	45.84 ± 0.85 (0.592 ± 0.005)	42.11 ± 0.31 (0.506 ± 0.003)	44.96 ± 1.9 (0.798 ± 0.001)	42.11 ± 0.40 (1.416 ± 0.008)	44.84± 1.4 (1.611 ± 0.002)
40	75.21 ± 5.4 (1.163 ± 0.001)	72.87 ± 3.7 (1.131 ± 0.003)	68.57 ± 2.3 (1.153 ± 0.003)	65.87± 4.8 (1.105 ± 0.005)	66.21± 4.9 (1.308 ± 0.007)
50	85.26 ± 4.2 (4.800 ± 0.006)	83.48 ± 6.6 (4.987 ± 0.004)	78.99 ± 4.7 (4.965 ± 0.001)	72.48± 3.0 (3.044 ± 0.007)	73.26± 3.7 (3.546 ± 0.005)

*values in parenthesis are specific activity

58B

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Figure 9a: Effect of temperature treatment on unit activity in *Pisum sativum* during germination at room temperature

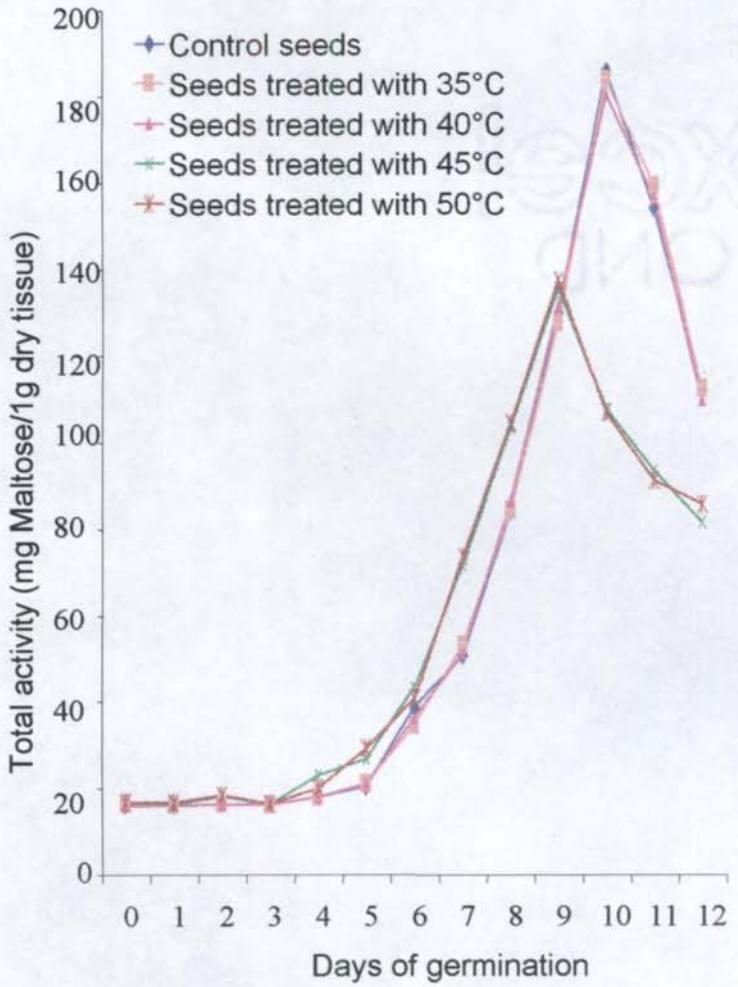


Figure 9b: Effect of temperature treatment on specific activity in *Pisum sativum* during germination at room temperature

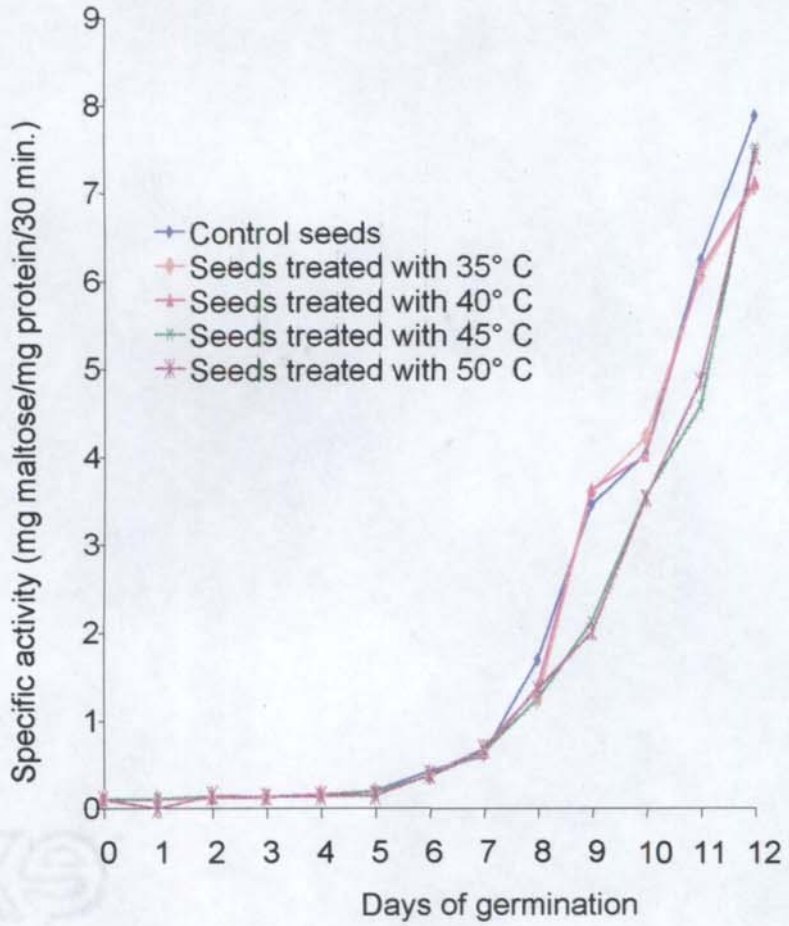


Figure 10a: Effect of temperature treatment on unit activity in *Pisum sativum* during germination in refrigerator

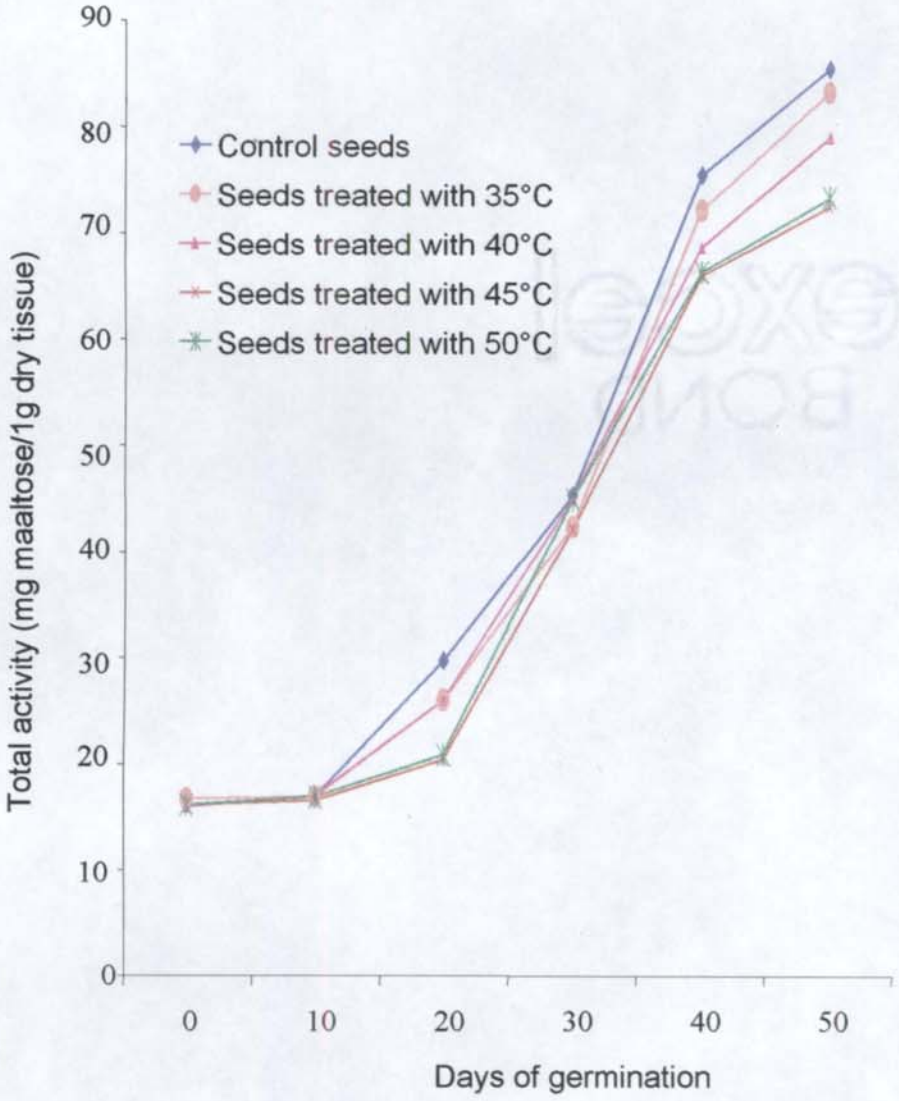
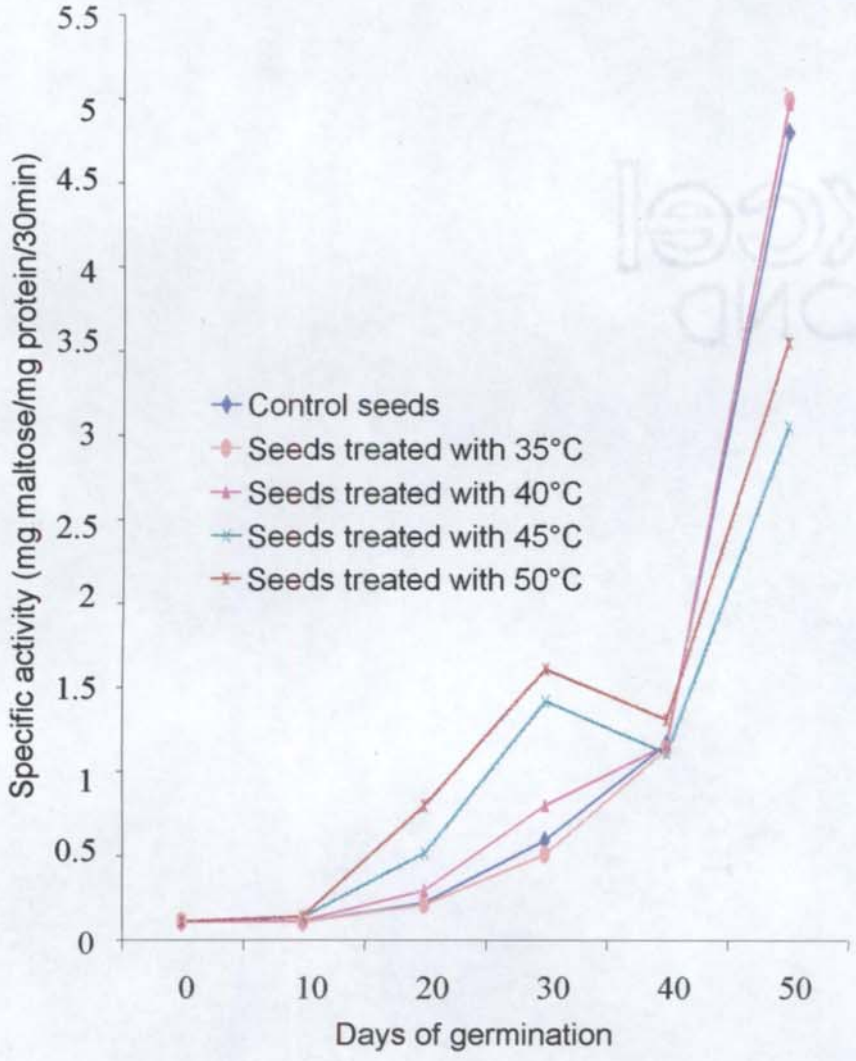


Figure 10b: Effect of temperature treatment on specific activity in *Pisum sativum* during germination in refrigerator



of 4th day ($P<0.01$). Thereafter a gradual decrease (significant at all intervals of sampling) in protein content was observed in the germinating seeds and on 12th day of germination only 28% of the initial protein content was retained. The total protein distribution during germination of control and seeds treated at 35°C, 40°C, 45°C and 50°C when germinated at room temperature did not show any significant difference between the control and experimental as well as among each treatment (Fig. 11a).

When the seeds were germinated (control and experimentals) in refrigerator the protein mobilization was very slow in the control and treatments (Table 15b). Nevertheless, at each interval the reduction was significant up to 40 days. In control seeds and seeds treated at 35°C, 40°C, 45°C and 50°C the protein content was significantly increased ($P<0.01$) on 10th day at cold condition. Tenth day onwards the decrease was gradual up to 40th day. But the 20th day protein content was higher than that of dry seeds. In the case of control, 35°C and 40°C experimental samples, a gradual decrease in protein content was noticed from 10th day germinated seeds to 40th day of germination. The seeds treated at 45°C and 50°C showed much faster rate of reduction in protein content than that of control and treatment at 35°C. During germination in refrigerator the reduction of total protein during each interval was significant ($P<0.01$ in all sample) when a comparison is made between the treatment and between the samples of each treatment (Fig. 11b).

3.2. Soluble protein

When the soluble protein was analyzed, a gradual reduction of protein content was observed both in control and experimental during germination at room temperature (Table 16a). Soluble protein distribution of control and the seeds treated at 35°C and 40°C was

Table15a: Effect of temperature treatment on total protein in *Pisum sativum* during seed germination at room temperature

Days of germination	Protein in mg/g dry tissue				
	Control seeds	Temperature of treatment			
		35	40	45	50
0	294.3 ± 3.5	282.5 ± 1.1	289.3 ± 8.3	294.8 ± 5.2	288.6 ± 2.8
1	260.7 ± 5.0	256.7 ± 3.8	266.6 ± 1.2	242.2 ± 1.8	251.3 ± 3.9
2	226.0 ± 1.1	226.1 ± 1.1	228.1 ± 2.4	224.0 ± 9.1	228.6 ± 8.0
3	206.0 ± 3.0	202.7 ± 1.8	204.2 ± 1.8	205.9 ± 1.6	209.1 ± 1.9
4	198.4 ± 2.4	190.1 ± 2.2	193.1 ± 0.9	196.8 ± 2.6	195.2 ± 1.6
5	217.5 ± 2.0	219.5 ± 1.9	211.8 ± 1.8	220.1 ± 1.3	218.1 ± 2.3
6	182.9 ± 3.2	189.8 ± 5.1	192.5 ± 1.3	187.9 ± 1.2	195.2 ± 2.0
7	163.1 ± 1.1	168.8 ± 1.6	172.0 ± 1.8	173.7 ± 3.0	180.2 ± 1.9
8	142.4 ± 1.9	150.3 ± 0.9	152.4 ± 1.9	152.6 ± 1.9	140.8 ± 1.8
9	120.6 ± 4.0	126.7 ± 2.5	123.9 ± 6.4	130.6 ± 1.7	133.8 ± 1.7
10	104.7 ± 3.2	106.9 ± 3.2	110.7 ± 2.3	108.6 ± 2.5	107.8 ± 8.0
11	90.3 ± 0.9	93.8 ± 0.2	99.6 ± 2.0	91.2 ± 2.7	90.5 ± 0.4
12	82.5 ± 0.8	88.3 ± 1.9	81.6 ± 3.1	89.5 ± 0.7	83.4 ± 0.2

Table 15b: Effect of temperature treatment on total protein in *Pisum sativum* during seed germination in refrigerator

Days of germination	Protein (mg/g dry tissue)				
	Control seeds	Temperature treatment			
		35	40	45	50
0	294.3 ± 3.5	282.5 ± 1.1	289.3 ± 0.8	294.8 ± 0.5	288.6 ± 2.8
10	368.5 ± 2.6	366.8 ± 3.1	342.3 ± 1.8	331.8 ± 1.0	324.6 ± 3.2
20	337.7 ± 2.2	339.3 ± 3.2	333.9 ± 1.8	324.1 ± 1.3	316.7 ± 2.3
30	216.6 ± 2.1	212.3 ± 4.0	218.2 ± 0.3	202.2 ± 1.4	201.9 ± 2.3
40	196.4 ± 2.3	195.1 ± 1.2	181.4 ± 1.1	165.6 ± 0.1	132.3 ± 1.6
50	90.3 ± 1.2	95.6 ± 1.8	86.2 ± 0.8	71.0 ± 2.2	63.8 ± 1.7

Figure 11a: Effect of temperature treatment on total protein in *Pisum sativum* during seed germination at room temperature

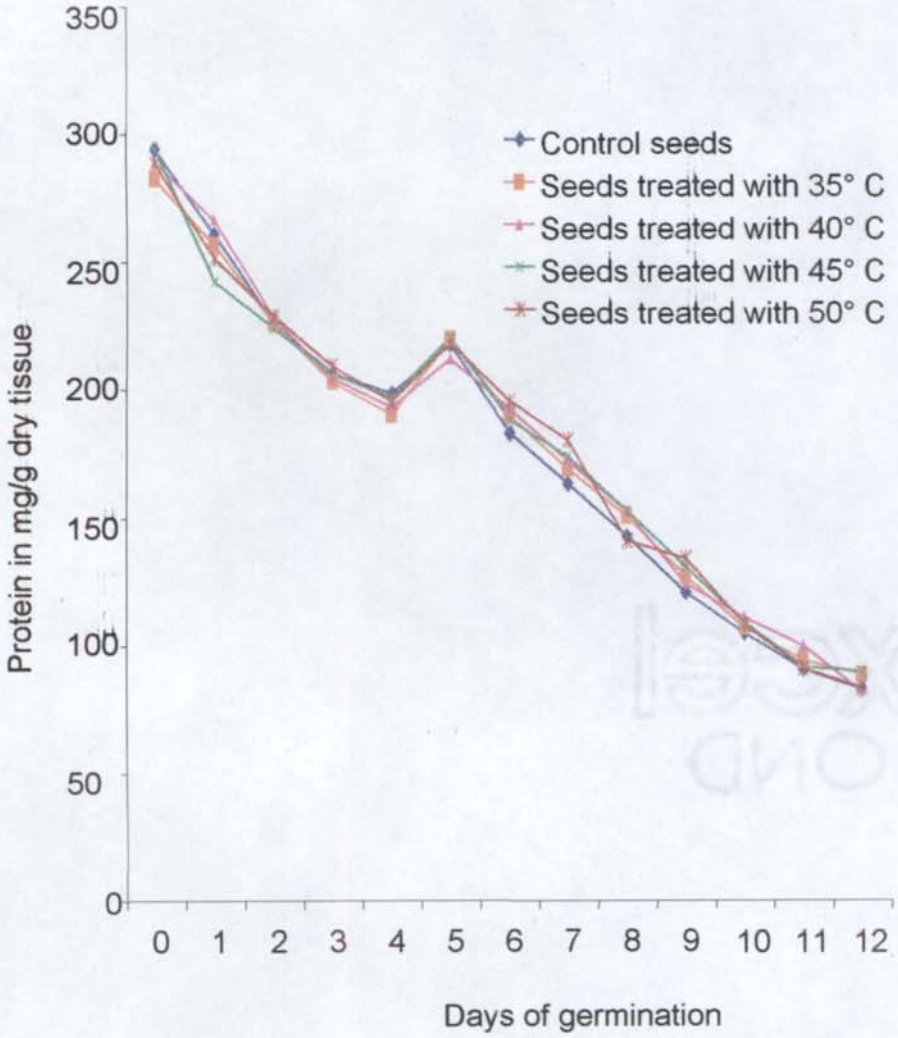
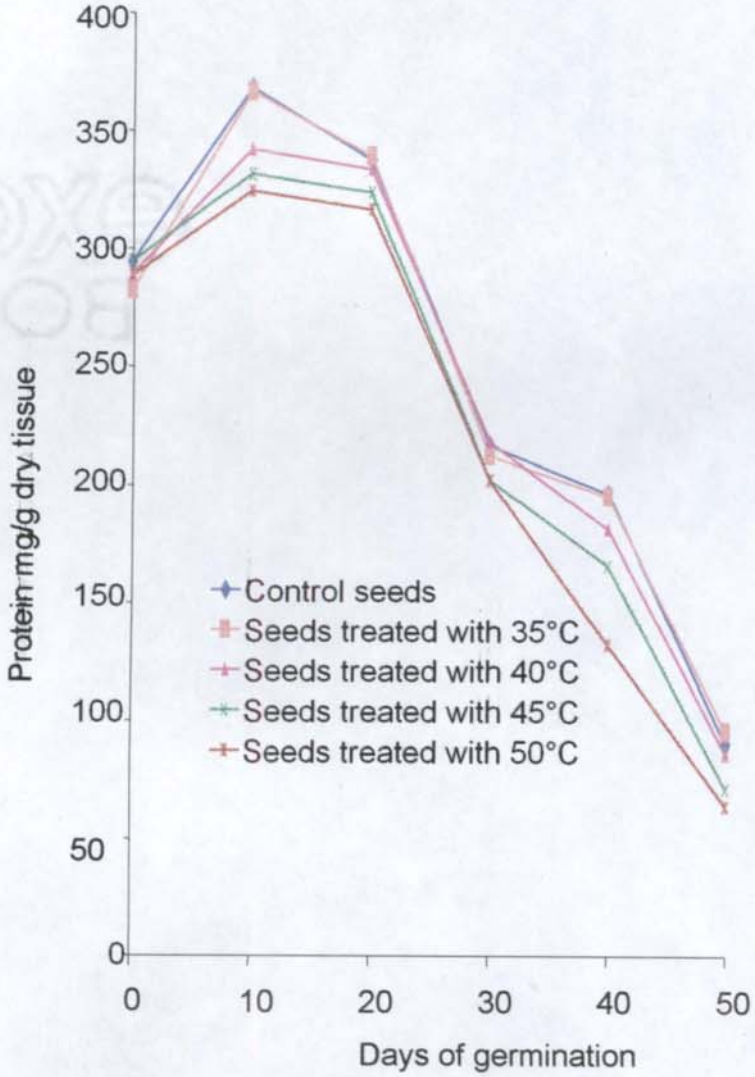


Figure 11b: Effect of temperature treatment on total protein in *Pisum sativum* during seed germination in refrigerator



almost similar at all stages of germination. In seeds treated at 45°C and 50°C the amount of protein content was slightly less than that of control and treatments at 35°C and 40°C during all days of germination at room temperature but the pattern of distribution was almost similar to the control and treatments of 35°C and 40°C (Fig. 12a).

When the germination is conducted out in refrigerator, the soluble protein content also showed an increase on 10^{th} day. But the 20^{th} day content of control and treatments at 35°C and 40°C was higher than that of dry seeds whereas in seeds treated at 45°C and 50°C it was almost same. On 20^{th} day the seeds treated at 50°C showed slightly higher amount ($P<0.01$) of soluble protein when compared with other seeds. Control seeds and treatment at 35°C and 40°C showed almost equal content on 30^{th} day (Table 16b).

When germination proceeds the reduction rate was found to be significant ($P<0.01$ each) in all cases. In the case of seeds treated at 45°C and 50°C showed fast decrease in protein content than that of control and treatments at 35°C and 40°C. The difference in protein content between the control and treatment at 35°C was negligible and the seed treated at 40 °C was slightly less than that of control and seeds treated at 35°C. The difference between the seeds treated at 45°C and 50°C was negligible (Fig. 12b)

3.3. Protein profile by gel electrophoresis

One-dimensional SDS-PAGE resolved polypeptides of molecular mass 97.4 to 14.0 KDa. The dry seeds of control and treatments have resolved 15 bands, of these, 3 bands were darkly stained (one polypeptide each at 66.0 KDa and 43.0 KDa and one in between 66.0 KDa and 43.0 KDa), 5 bands were moderately stained (one polypeptide in between 43.0 KDa and 29.0 KDa, one polypeptide in

Table 16a: Effect of temperature treatment on soluble protein in *Pisum sativum* during seed germination at room temperature

Days of germination	Protein in mg/g dry tissue				
	Control seeds	Temperature of treatment			
		35	40	45	50
0	168.8 ± 4.1	169.1 ± 2.8	163.9 ± 4.9	167.3 ± 8.5	168.9 ±4.7
1	147.9 ± 1.2	142.5 ± 5.8	144.9 ± 1.9	139.0 ± 1.9	130.2 ± 2.6
2	132.6 ± 2.6	132.5 ± 2.5	130.9 ± 4.2	124.1 ± 2.6	122.5 ± 1.3
3	121.5 ± 2.1	121.5 ± 6.8	129.0 ± 3.8	117.6 ± 2.3	113.4 ± 2.5
4	111.6 ± 6.9	111.4 ± 1.3	119.2 ± 8.9	107.5 ± 2.5	105.5 ± 1.7
5	103.4 ± 1.9	108.5 ± 1.9	107.9 ± 4.3	95.4 ± 3.1	91.1 ± 1.3
6	90.8 ± 2.1	93.2 ± 3.2	97.8 ± 1.9	85.4 ± 1.9	83.5 ± 2.4
7	84.6 ± 2.5	84.9 ± 1.9	80.6 ± 2.4	78.2 ± 2.8	73.3 ± 1.3
8	67.8 ± 7.0	68.1 ± 2.3	62.8 ± 2.1	56.4 ± 1.7	55.0 ± 1.9
9	49.9 ± 3.2	49.1 ± 6.1	44.9 ± 2.5	33.2 ± 2.6	34.0 ± 3.0
10	38.0 ± 1.2	37.9 ± 7.3	33.6 ± 1.9	28.9 ± 2.1	27.4 ± 1.7
11	22.3 ± 1.7	20.2 ± 2.1	23.1 ± 3.0	19.6 ± 1.9	19.6 ± 1.6
12	14.8 ± 1.2	14.5 ± 2.6	14.2 ± 2.1	10.9 ± 1.5	10.7 ± 1.8

Table 16b: Effect of temperature treatment on soluble protein in *Pisum sativum* during seed germination in refrigerator

Days of germination	Protein in mg/g dry tissue				
	Control seeds	Temperature of treatment			
		35	40	45	50
0	168.8 ± 4.1	169.1 ± 2.8	163.9 ± 4.9	167.3 ± 8.5	168.9 ±4.7
10	226.5 ± 2.8	229.5 ±1.2	228.7 ± 1.5	223.9 ± 2.1	220.5 ± 1.3
20	198.0 ± 2.2	194.6 ± 0.9	188.6 ± 0.7	164.7 ± 2.7	162.1 ± 2.5
30	174.4 ± 1.6	176.9 ± 0.3	169.4 ± 1.3	144.3 ± 0.6	140.5 ± 3.2
40	53.5 ± 0.9	56.7 ± 0.2	45.3 ± 1.8	20.2 ± 1.2	22.8 ± 0.9
50	24.3 ± 0.9	22.3 ± 0.5	19.9 ± 1.2	9.2 ± 1.0	8.9 ± 1.7

60B

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Figure 12a: Effect of temperature treatment on soluble protein in *Pisum sativum* during seed germination at room temperature

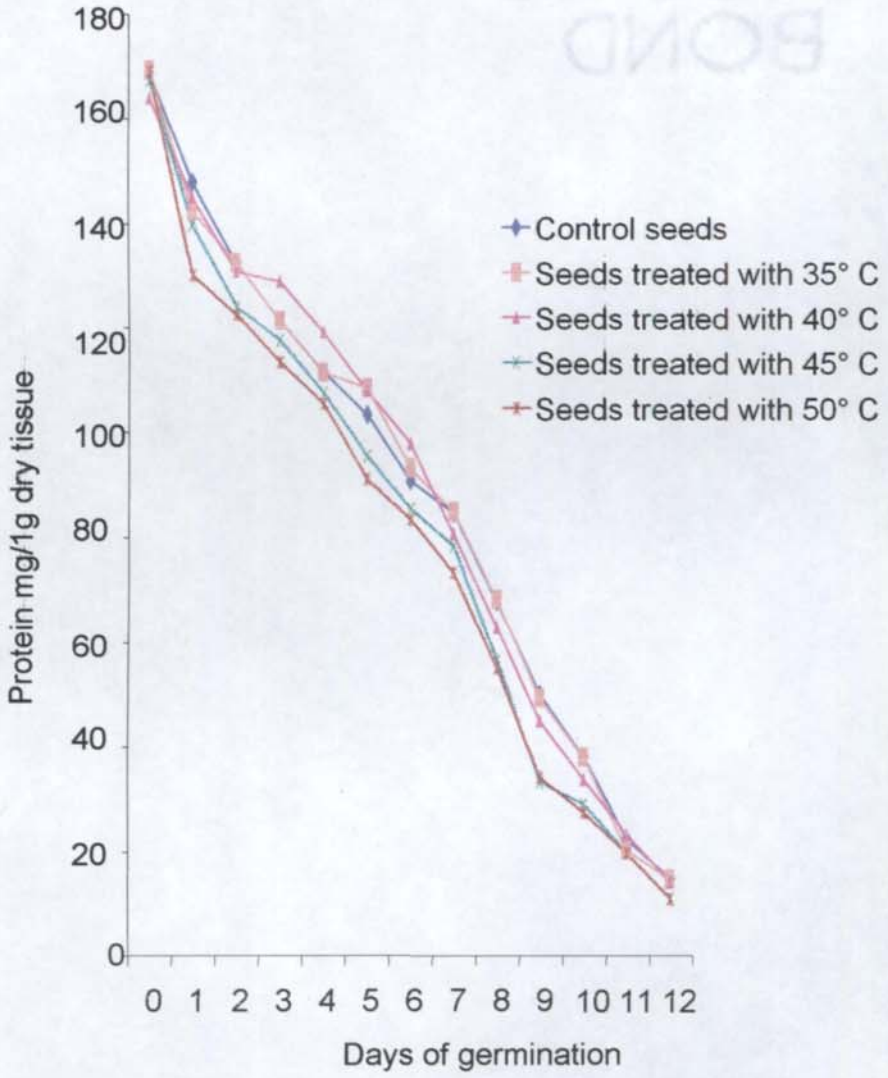
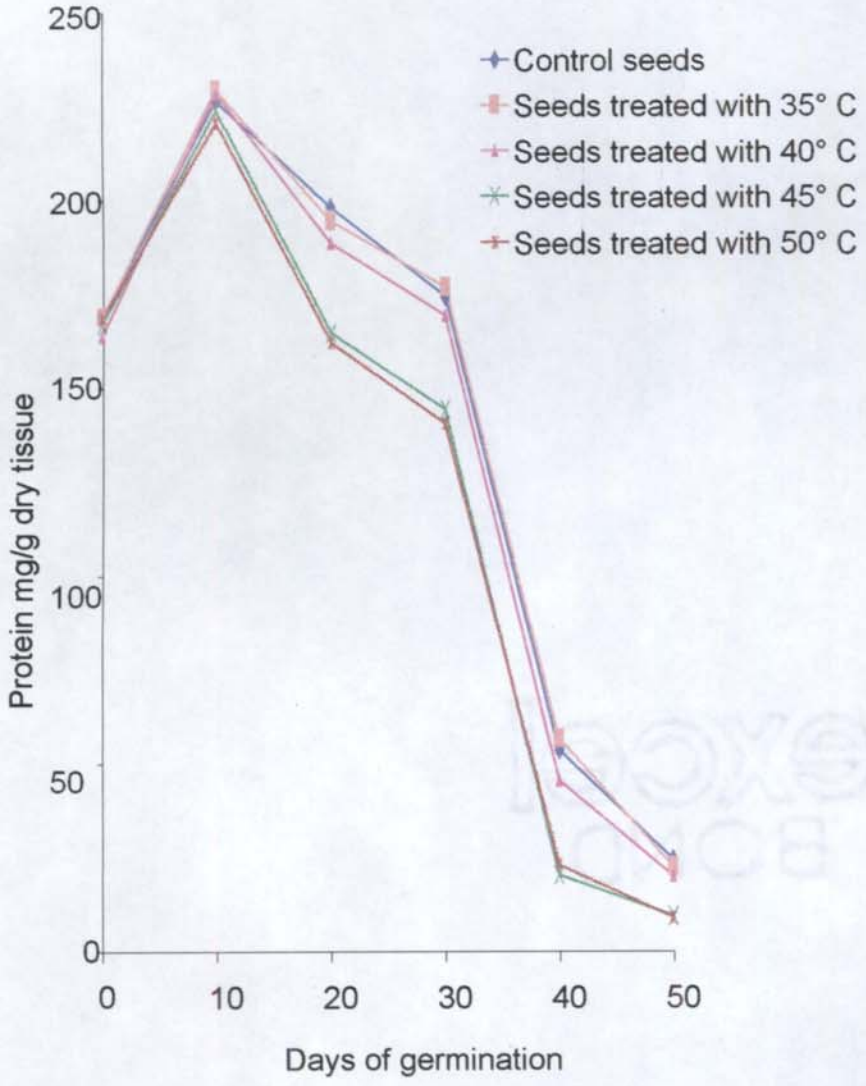


Figure 12b: Effect of temperature treatment on soluble protein in *Pisum sativum* during seed germination in refrigerator



between 29.0 KDa and 20.1 KDa and one each at 29.0 KDa, 20.1 KDa and 14.0 KDa), and 7 minor bands. There was no considerable difference in the protein profile between the dry seeds of control and treatments (Plate 4, Fig. a).

Among the 16 bands from the seeds of control and treatments germinated at the room temperature, 3 polypeptides were darkly stained (one polypeptide each at 66.0 KDa and 43.0 KDa and one in between 66.0 KDa and 43.0 KDa), 5 bands were moderately stained (one band each at 97.4 KDa, 20.1 KDa and 14.0 KDa, one bands inbetween 43.0 KDa to 29.0 KDa and 29.0 KDa to 20.1 KDa) and 8 minor bands. In the case of seeds treated at 50 °C, 16 polypeptides were resolved and the additional polypeptide was present in between 66.0 KDa and 43.0 KDa (Plate 4, Fig. b).

The control seeds germinated in refrigerator have resolved 21 bands, of these 2 bands were darkly stained (one polypeptide each at 66.0 KDa and 43.0 KDa), 6 bands were moderately stained (two bands at 97.4 KDa, two bands at 29.0 KDa, one band in between 43.0 KDa to 29.0 KDa and one band at 14.0 KDa) and 13 minor bands.

The control seeds and the treatment at 35°C, 40°C, 45°C and 50°C did not show any difference in number of polypeptides between each other when germination was carried out in refrigerator (Plate 4c).

6. Amino acids

The control seeds and treatment at 35°C, 40°C, 45°C and 50°C during germination at room temperature and in refrigerator showed the presence of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine and γ-amino butyric acid (Table 17 a to e).

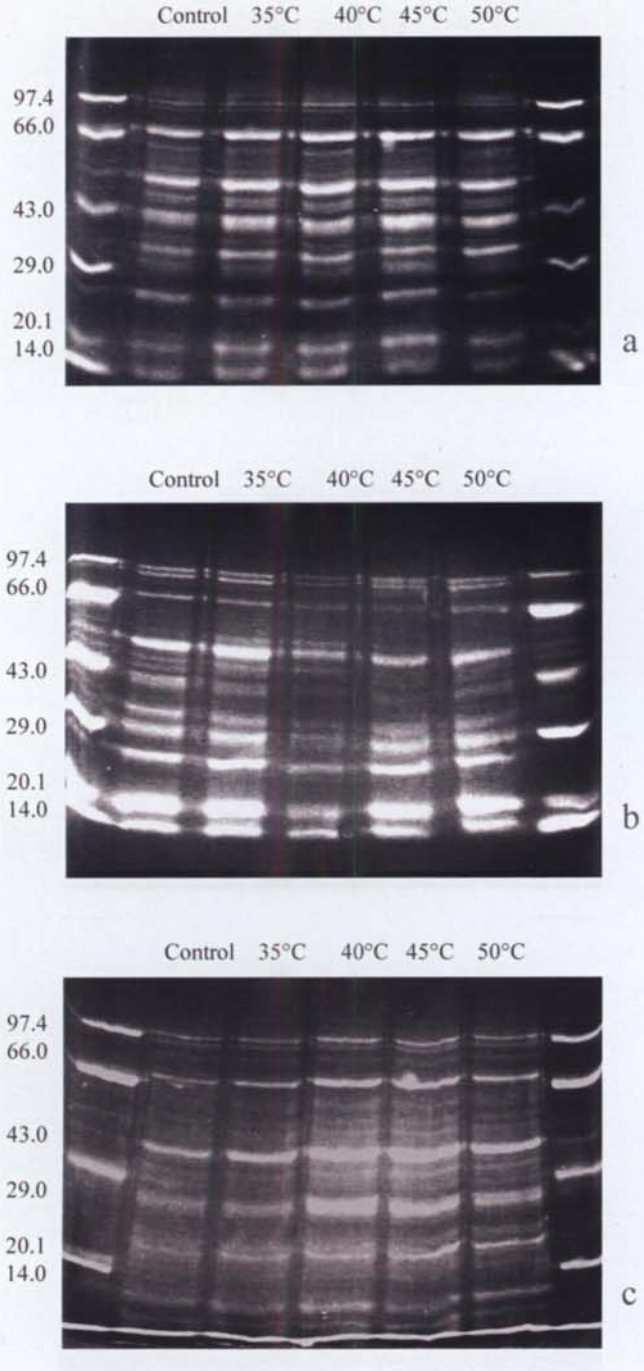


Plate **4**: Effect of temperature on protein profile in *Pisum sativum* seeds.

a. Dry seeds.

b. Seeds germinated for 24 hours at room temperature.

c. Seeds germinated for 10 days at cold.

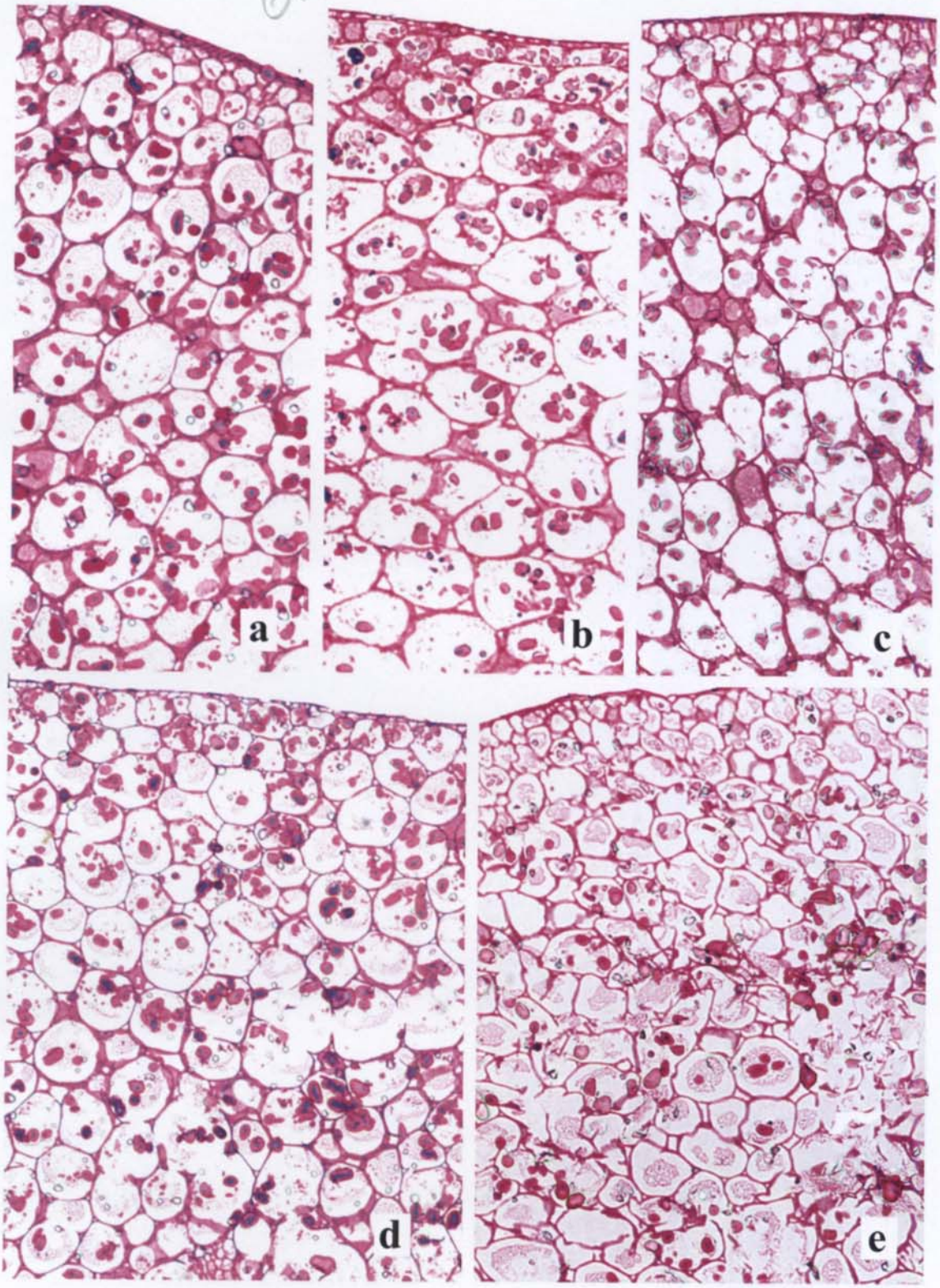


Plate 8: Starch distribution in the cotyledon of *Pisum sativum* seedling after 5 th day of germination at room temperature.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

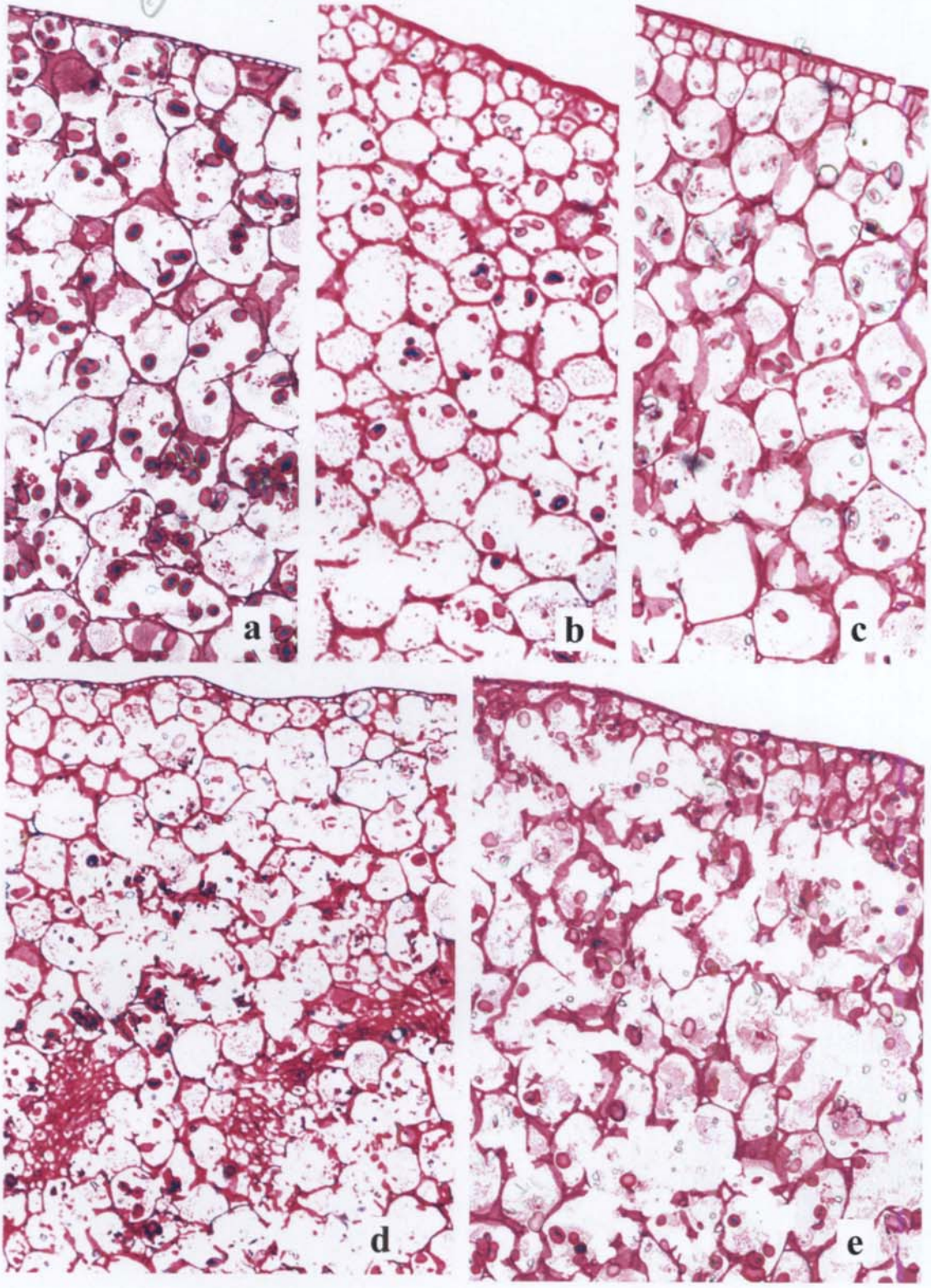


Plate 9: Starch distribution in the cotyledon of *Pisum sativum* seedling after 10th day of germination at room temperature.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

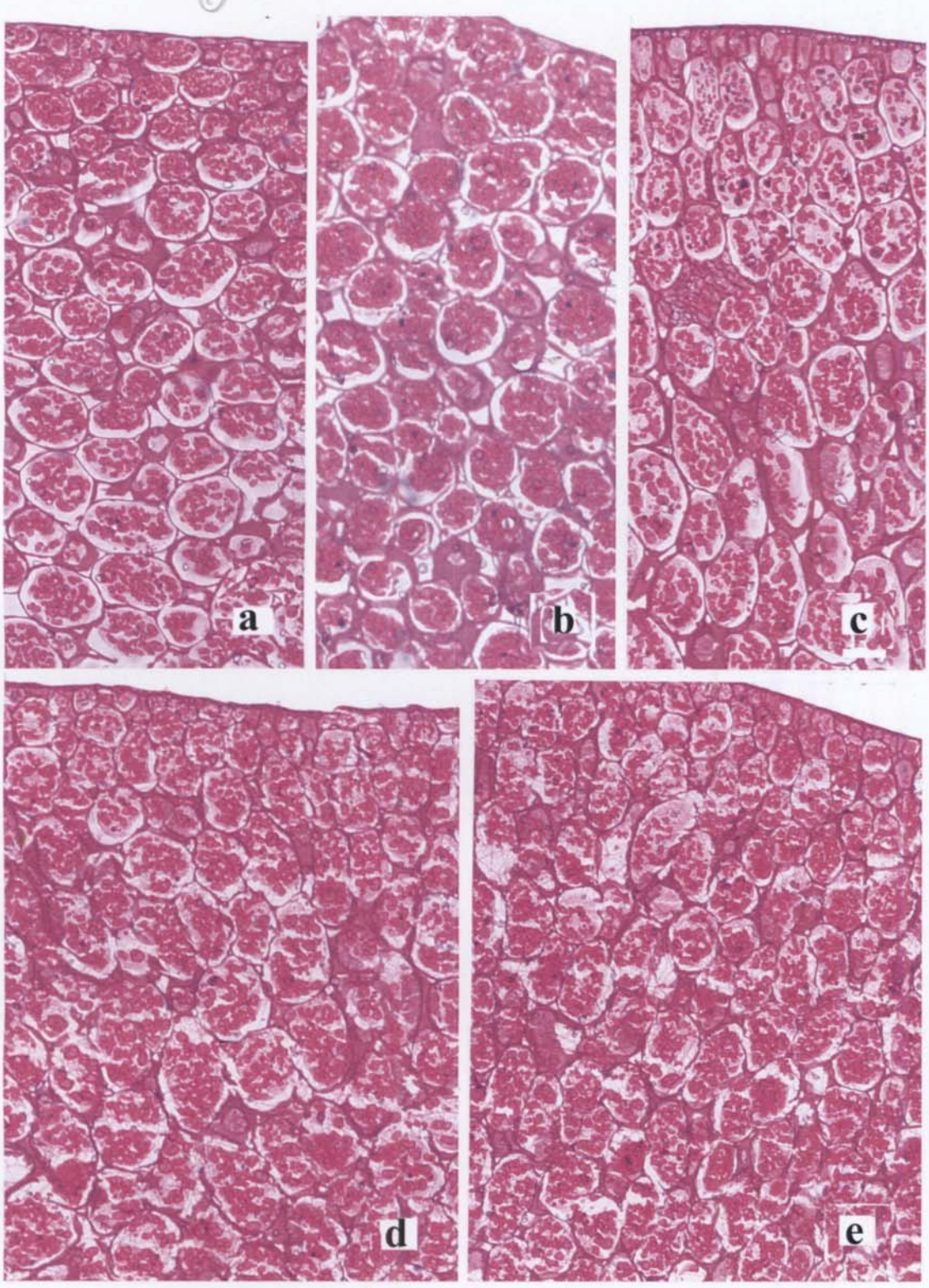


Plate 10: Starch distribution in the cotyledon of *Pisum sativum* seedling after 10th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C
c. Seeds treated at 40°C **d.** Seeds treated at 45°C
e. Seeds treated at 50°C

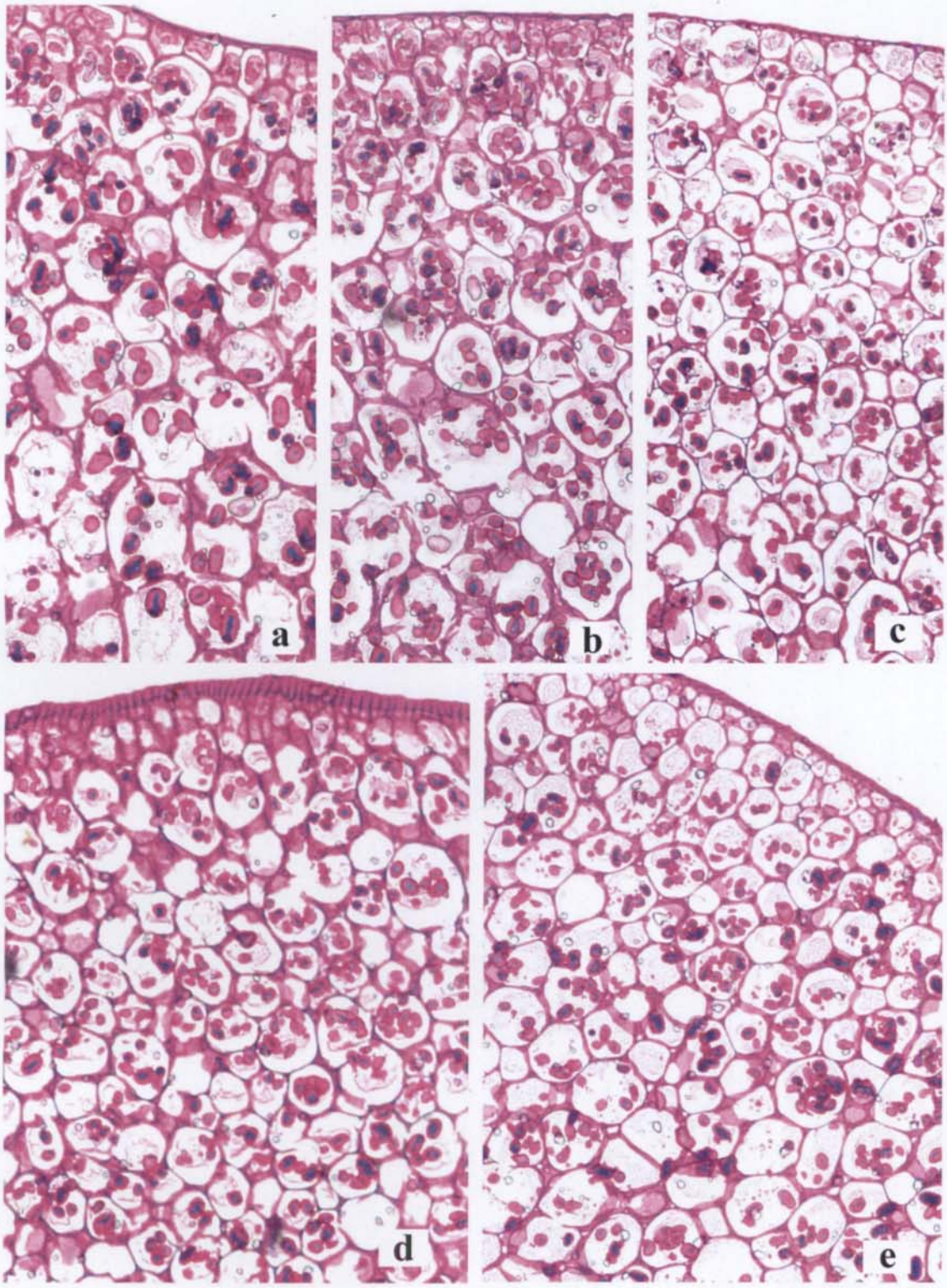


Plate 11 : Starch distribution in the cotyledon of *Pisum sativum* seedling after 30th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C
c. Seeds treated at 40°C **d.** Seeds treated at 45°C
e. Seeds treated at 50°C

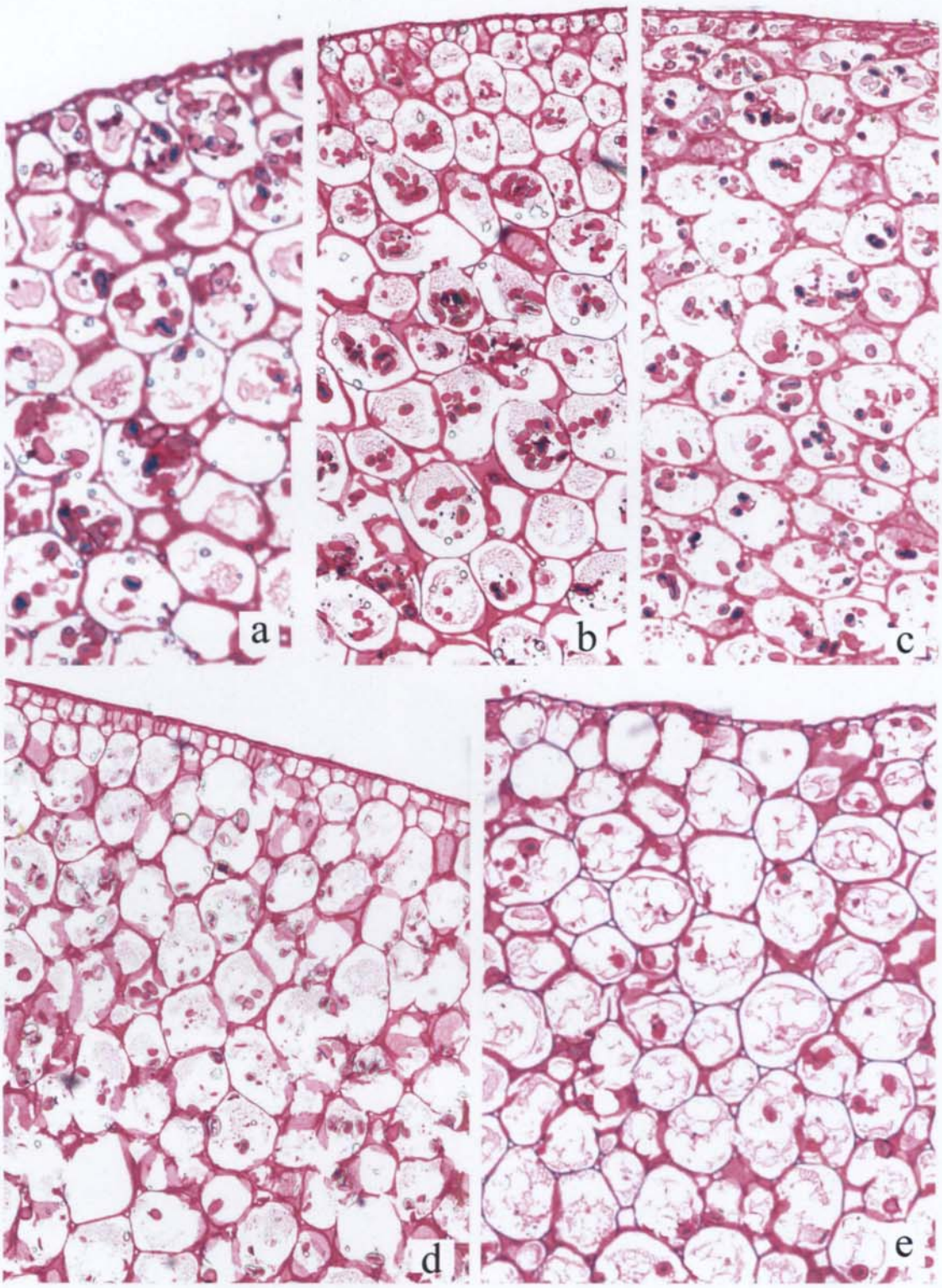


Plate 12: Starch distribution in the cotyledon of *Pisum sativum* seedling after 50th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C
c. Seeds treated at 40°C **d.** Seeds treated at 45°C
e. Seeds treated at 50°C

In ungerminated seeds of control and treatments at 35°C, 40°C, 45°C and 50°C glutamic acid was the most abundant amino acid followed by proline, alanine, histidine, methionine and lysine. Aspartic acid, glycine, serine, threonine, leucine, tyrosine and arginine were present in lesser amounts. γ-amino butyric amino acid, valine and isoleucine were detected comparatively in higher amount. Phenyl alanine was totally absent

In control seeds and treatment at 35°C during germination at room temperature arginine was gradually and significantly increasing from 1st to 3rd day and a remarkable reduction was occurred on 4th day and afterwards a doubling was noticed and remained unchanged during later days of germination (Table 17a & 17b). Aspartic acid was gradually increasing up to 5th day and from 5th to 6th day the increase was 4-fold. From 6th day onwards it was again gradually increasing. Glutamic acid showed a slow and gradual increase from 1st to 12th day of germination in which significant increase ($P< 0.01$) was observed between 4th and 5th day of germination. Isoleucine was detected only up to 2nd day and was reappeared from 9th day onwards and the quantity was remained unchanged up to 12th day. Leucine content was doubled during imbibition and almost same quantity was present up to 12th day. Methionine showed a negligible increase up to 3rd day and a decrease was noticed from 3rd to 4th day. From 5th day onwards methionine was not observed. Phenylalanine was not present up to 2nd day and 3rd day onwards it was slowly and gradually increasing showing a significant increase ($P< 0.01$) between 8th and 9th days. Proline was slowly increasing up to 5th day and a significant increase ($P< 0.01$) was noticed on 6th day. From 6th day onwards it showed quantitatively no difference. Serine was slowly increasing up to 4th day. From 4th to 5th

Table 17a: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination at room temperature (Control seeds)

Amino acids	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Amino acids (mg/g dry tissue)												
Ala	0.99 ± 0.11	1.61 ± 0.15	1.64 ± 0.02	1.79 ± 0.06	1.83 ± 0.02	1.94 ± 0.09	2.65 ± 0.03	2.83 ± 0.12	3.04 ± 0.14	3.49 ± 0.11	3.63 ± 0.10	3.78 ± 0.13	3.90 ± 0.07
Arg	0.22 ± 0.12	0.41 ± 0.14	0.94 ± 0.18	1.83 ± 0.16	0.56 ± 0.10	0.83 ± 0.19	1.95 ± 0.11	2.11 ± 0.12	2.36 ± 0.14	3.22 ± 0.11	3.33 ± 0.23	3.47 ± 0.09	3.62 ± 0.22
Asp	0.15 ± 0.01	0.18 ± 0.01	0.25 ± 0.06	0.36 ± 0.02	0.45 ± 0.01	0.49 ± 0.02	2.01 ± 0.13	4.58 ± 0.09	5.39 ± 0.08	6.78 ± 0.04	7.14 ± 0.04	7.52 ± 0.03	7.85 ± 0.04
Glu	1.10 ± 0.08	2.04 ± 0.12	2.41 ± 0.16	2.63 ± 0.10	3.89 ± 0.18	5.11 ± 0.13	6.02 ± 0.14	7.39 ± 0.10	8.19 ± 0.12	10.2 ± 0.17	10.7 ± 0.12	11.2 ± 0.15	11.8 ± 0.15
Gly	0.21 ± 0.08	0.54 ± 0.03	0.59 ± 0.06	0.65 ± 0.06	0.82 ± 0.05	0.94 ± 0.04	2.00 ± 0.14	2.69 ± 0.18	3.82 ± 0.16	3.74 ± 0.17	3.84 ± 0.09	4.03 ± 0.11	4.21 ± 0.12
His	0.92 ± 0.10	1.92 ± 0.04	1.85 ± 0.12	2.16 ± 0.19	2.37 ± 0.08	2.44 ± 0.06	2.68 ± 0.01	2.82 ± 0.02	2.95 ± 0.09	3.05 ± 0.08	3.63 ± 0.08	3.72 ± 0.05	3.95 ± 0.01
Ile	0.41 ± 0.15	0.60 ± 0.12	0.71 ± 0.17	N.D	N.D	N.D	N.D	N.D	N.D	0.98 ± 0.13	1.02 ± 0.10	1.17 ± 0.10	1.30 ± 0.10
Leu	0.21 ± 0.22	0.53 ± 0.19	0.60 ± 0.13	0.69 ± 0.16	0.71 ± 0.13	0.52 ± 0.10	0.40 ± 0.12	0.30 ± 0.17	0.35 ± 0.18	0.31 ± 0.19	0.35 ± 0.12	0.36 ± 0.12	0.38 ± 0.13
Lys	0.86 ± 0.11	0.91 ± 0.11	1.23 ± 0.15	1.91 ± 0.16	2.01 ± 0.17	2.36 ± 0.12	3.21 ± 0.18	3.86 ± 0.19	3.92 ± 0.12	4.16 ± 0.17	4.23 ± 0.11	4.37 ± 0.19	4.90 ± 0.11
Met	0.82 ± 0.11	0.91 ± 0.18	1.22 ± 0.14	1.85 ± 0.16	0.66 ± 0.18	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	N.D	0.21 ± 0.04	0.31 ± 03	0.65 ± 0.11	0.78 ± 0.15	0.96 ± 0.12	1.20 ± 0.07	2.26 ± 0.02	2.45 ± 0.03	2.54 ± 0.05	2.68 ± 0.09
Pro	0.95 ±0.08	1.04 ± 0.03	1.06 ± 0.06	1.25 ± 0.04	1.68 ± 0.02	1.59 ± 0.07	2.32 ± 0.02	2.97 ± 0.23	2.32 ± 0.01	2.23 ± 0.02	2.21 ± 0.01	2.30 ± 0.01	2.39 ± 0.01
Ser	0.24 ± 0.22	1.21 ± 0.18	2.58 ± 0.13	3.54 ± 0.25	4.11 ± 0.14	3.68 ± 0.12	8.15 ± 0.23	5.21 ± 0.15	3.00 ± 0.33	5.92 ± 0.19	6.13 ± 0.07	6.39 ± 0.10	6.67 ± 0.13
Thr	0.23 ± 0.12	0.39 ± 0.14	0.47 ± 0.20	0.52 ± 0.20	0.65 ± 0.2	0.47 ± 0.29	1.85 ± 0.11	2.96 ± 0.23	4.65 ± 0.22	6.87 ± 0.23	8.14 ± 0.14	9.32 ± 0.19	12.36 ± 0.18
Tyr	0.19 ± 0.01	0.34 ± 04	0.41 ± 0.02	0.45 ± 0.07	0.56 ± 0.05	0.83 ± 0.09	1.95 ± 0.01	2.11 ± 0.08	2.36 ± 0.07	3.22 ± 0.07	3.33 ± 0.07	3.47 ± 0.01	3.66 ± 0.02
Val	0.39 ± 0.13	1.15 ± 0.09	1.86 ± 0.22	2.07 ± 0.19	2.09 ± 0.18	2.20 ± 0.02	2.53 ± 0.04	2.86 ± 0.18	3.22 ± 0.11	4.77 ± 0.13	4.96 ± 0.13	5.17 ± 0.06	5.39 ± 0.17
Gaba	0.51 ± 0.12	0.90 ± 0.13	1.17 ± 0.02	1.28 ± 0.08	1.30 ± 0.04	1.60 ± 0.11	2.17 ± 0.12	2.76 ± 0.09	2.11 ± 0.21	2.24 ± 0.32	2.42 ± 0.23	2.59 ± 0.09	2.78 ± 0.01
Total	8.4 ± 0.56	14.23 ± 0.92	18.99 ± 0.25	23.7 ± 0.58	24.0 ± 0.98	25.65 ± 0.15	40.67 ±0.78	46.41 ± 0.21	48.9 ± 0.55	53.28 ±0.17	67.5 ± 0.87	71.4 ±0.76	77.7 ± 0.88

Table 17b: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination at room (seeds treated at 35°C)

Amino acids	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Amino acids (mg/g dry tissue)												
Ala	0.93± 0.16	1.61 ± 0.17	1.62 ± 0.18	1.69 ± 0.16	1.80 ± 0.10	1.95 ± 0.12	2.62 ± 0.09	2.80 ± 0.13	3.04 ± 0.14	3.47 ± 0.23	3.66 ± 0.21	3.75 ± 0.17	3.92 ± 0.19
Arg	0.22 ± 0.03	0.42 ± 0.11	0.95 ± 0.15	1.85 ± 0.08	0.57 ± 0.02	0.83 ± 0.07	1.94 ± 0.05	2.12 ± 0.08	2.32 ± 0.11	3.21 ± 0.04	3.34 ± 0.01	3.46 ± 0.03	3.73 ± 0.09
Asp	0.15 ± 0.15	0.18 ± 0.11	0.25 ± 0.01	0.36 ± 0.09	0.45 ± 0.06	0.49 ± 0.08	2.01 ± 0.11	4.58 ± 0.33	5.39 ± 0.49	6.78 ± 0.11	7.14 ± 0.21	7.52 ± 0.03	7.85 ± 0.18
Glu	1.11 ± 0.13	2.02 ± 0.11	2.47 ± 0.16	2.62 ± 0.19	3.86 ± 0.28	5.21 ± 0.19	6.12 ± 0.24	7.40 ± 0.43	8.18 ± 0.29	10.3 ± 0.11	10.7 ± 0.13	11.3 ± 0.06	11.9 ± 0.10
Gly	0.24 ± 0.06	0.51 ± 0.17	0.57 ± 0.12	0.62 ± 0.11	0.85 ± 0.11	0.62 ± 0.13	2.02 ± 0.15	2.70 ± 0.18	3.82 ± 0.13	3.72 ± 0.12	3.82 ± 0.12	4.03 ± 0.11	4.21 ± 0.19
His	0.93 ± 0.08	1.86 ± 0.05	2.16 ± 0.03	2.39 ± 0.06	2.42 ± 0.08	2.67 ± 0.04	2.80 ± 0.07	2.96 ± 0.08	3.04 ± 0.11	3.62 ± 0.16	3.75 ± 0.18	3.95 ± 0.10	4.06 ± 0.19
Ile	0.40 ± 0.22	0.61 ± 0.32	0.70 ± 0.28	N.D	N.D	N.D	N.D	N.D	N.D	0.99 ± 0.11	1.02 ± 0.31	1.17 ± 0.35	1.30 ± 0.52
Leu	0.20 ± 0.04	0.57 ± 0.07	0.63 ± 0.08	0.69 ± 0.09	0.72 ± 0.11	0.51 ± 0.16	0.42 ± 0.21	0.32 ± 0.29	0.33 ± 0.11	0.33 ± 0.19	0.35 ± 0.20	0.36 ± 0.22	0.38 ± 0.17
Lys	0.87 ± 0.09	0.90 ± 0.11	1.24 ± 0.11	1.91 ± 0.18	2.02 ± 0.17	2.35 ± 0.15	3.20 ± 0.11	3.86 ± 0.10	3.90 ± 0.12	4.16 ± 0.17	4.24 ± 0.12	4.39 ± 0.16	4.74 ± 0.14
Met	0.80 ± 0.13	0.96 ± 0.11	1.23 ± 0.14	1.84 ± 0.09	0.65 ± 0.03	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	0.22 ± 0.11	0.33 ± 0.10	0.65 ± 0.12	0.78 ± 0.18	0.95 ± 0.13	1.21 ± 0.12	2.26 ± 0.16	2.45 ± 0.19	2.53 ± 0.15	2.69 ± 0.18	2.72 ± 0.16
Pro	0.92 ± 0.07	1.02 ± 0.13	1.16 ± 0.13	1.26 ± 0.04	1.69 ± 0.07	1.52 ± 0.08	2.33 ± 0.09	2.95 ± 0.06	2.32 ± 0.04	2.24 ± 0.02	2.21 ± 0.07	2.31 ± 0.08	2.40 ± 0.05
Ser	0.24 ± 0.18	1.22 ± 0.19	2.56 ± 0.15	3.56 ± 0.33	4.12 ± 0.25	3.66 ± 0.20	8.16 ± 0.44	5.32 ± 0.08	3.04 ± 0.12	5.89 ± 0.11	6.15 ± 0.19	6.41 ± 0.16	6.72 ± 0.17
Thr	0.25 ± 0.03	0.35 ± 0.02	0.44 ± 0.07	0.55 ± 0.06	0.66 ± 0.11	0.42 ± 0.15	1.83 ± 0.22	2.90 ± 0.26	4.54 ± 0.30	6.75 ± 0.25	8.16 ± 0.28	9.45 ± 0.24	12.39 ± 0.84
Tyr	0.19 ± 0.01	0.36 ± 0.13	0.41 ± 0.10	0.45 ± 0.12	0.57 ± 0.15	0.83 ± 0.02	1.95 ± 0.19	2.12 ± 0.17	2.35 ± 0.10	3.20 ± 0.03	3.34 ± 0.04	3.48 ± 0.07	3.65 ± 0.01
Val	0.38 ± 0.04	1.15 ± 0.07	1.89 ± 0.09	2.05 ± 0.17	2.08 ± 0.12	2.21 ± 0.16	2.55 ± 0.19	2.85 ± 0.13	2.23 ± 0.17	2.77 ± 0.15	2.95 ± 0.19	2.17 ± 0.01	2.38 ± 0.03
Gaba	0.55 ± 0.02	0.92 ± 0.04	1.16 ± 0.05	1.27 ± 0.11	1.31 ± 0.17	1.62 ± 0.09	2.18 ± 0.07	2.78 ± 0.42	2.11 ± 0.11	2.28 ± 0.15	2.41 ± 0.19	2.56 ± 0.16	2.76 ± 0.22
Total	8.38 ± 0.13	14.5 ± 0.12	19.6 ± 0.99	24.67 ± 0.11	23.7 ± 0.15	25.7 ± 0.14	41.1 ± 0.16	46.8 ± 0.22	51.1 ± 0.18	62.16 ± 0.13	65.7 ± 0.22	69.0 ± 0.11	75.1 ± 0.10

day it showed a slight increase followed by a significant increase on 6th day. Thereafter, up to 8th day it was significantly decreasing. From 9th day onwards only negligible increase was observed. The distribution pattern of glycine, alanine, valine, lysine, tyrosine and histidine was almost similar during germination and their quantity was increasing from 1st day onwards. Threonine also showed almost equal quantity up to 5th day. From 5th to 6th day it was increased significantly ($p<0.02$) and 6th day onwards upto 8th day the quantity of threonine was getting doubled daily. Thereafter the increase was significant. This amino acid become most abundant compared with all others on 12th day. γ-aminobutyric acid showed very slow increase up to 6th day and thereafter there was no quantitative difference.

During germination of seeds treated at 40°C (Table 17c), arginine was steadily increasing with a doubling on each day up to 3rd day and on 4th day it was decreased to less than half. It was retained as such on 5th day. On 6th day onwards arginine showed a significant increase ($P<0.01$) followed by a slow increase up to 12th day of germination. Aspartic acid showed a slow and gradual increase up to 4th day. From 4th to 5th day there was no quantitative difference. On 6th day a 5-fold increase was occurred and a significant increase was observed on 8th and 9th day ($P<0.05$ and 0.01 respectively). Later, aspartic acid showed only gradual but negligible increase. Glutamic acid showed a significant increase on 3rd day. On 4th day it was decreased slowly followed by a steady increase on every day up to 12th day of germination. Glycine, alanine, valine, tyrosine, lysine and histidine were gradually increasing from 1st to 12th day of germination. Leucine did not show any considerable increase during germination. Methionine showed only negligible increase up to 3rd day and on 4th day it was decreased to less than half. From 5th day onwards

Table 17c: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination at room (seeds treated at 40°C)

Amino acids	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Amino acids (mg/g dry tissue)												
Ala	0.94 ± 0.16	1.61 ± 0.12	1.64 ± 0.15	1.68 ± 0.11	1.82 ± 0.11	1.97 ± 0.17	2.63 ± 0.09	2.83 ± 0.02	3.12 ± 0.21	3.56 ± 0.19	3.70 ± 0.09	3.89 ± 0.11	3.93 ± 0.10
Arg	0.24 ± 0.03	0.44 ± 0.02	0.96 ± 0.10	1.89 ± 0.18	0.58 ± 0.01	0.80 ± 0.33	1.97 ± 0.08	2.16 ± 0.03	2.33 ± 0.09	3.22 ± 0.07	3.38 ± 0.01	3.55 ± 0.09	3.63 ± 0.09
Asp	0.12 ± 0.01	0.17 ± 0.01	0.21 ± 0.02	0.29 ± 0.02	0.49 ± 0.01	0.48 ± 0.01	2.33 ± 0.16	4.65 ± 0.22	5.44 ± 0.19	6.99 ± 0.09	7.21 ± 0.11	7.65 ± 0.13	7.99 ± 0.16
Glu	1.12 ± 0.09	2.14 ± 0.09	2.52 ± 0.08	6.85 ± 0.16	4.12 ± 0.17	5.23 ± 0.12	6.05 ± 0.19	7.35 ± 0.36	8.15 ± 0.13	9.42 ± 0.06	9.74 ± 0.09	9.66 ± 0.11	9.73 ± 0.10
Gly	0.23 ± 0.01	0.51 ± 0.04	0.60 ± 0.01	0.63 ± 0.03	0.84 ± 0.08	0.63 ± 0.09	2.02 ± 0.15	2.66 ± 0.11	3.85 ± 0.33	3.71 ± 0.28	3.88 ± 0.20	4.12 ± 0.16	4.56 ± 0.45
His	0.94 ± 0.08	1.84 ± 0.11	2.18± 0.12	2.37 ± 0.11	2.45 ± 0.10	2.64 ± 0.11	2.81 ± 0.14	2.97 ± 0.14	3.06 ± 0.18	3.64 ± 0.16	3.74 ± 0.19	3.94 ± 0.09	4.08 ± 0.02
Ile	0.42 ± 0.07	0.62 ± 0.04	0.72 ± 0.07	N.D	N.D	N.D	N.D	N.D	N.D	0.97 ± 0.12	1.04 ± 0.01	1.17 ± 0.08	1.30 ± 0.27
Leu	0.23 ± 0.04	0.58 ± 0.05	0.66 ± 0.03	0.69 ± 0.08	0.74 ± 0.08	0.53 ± 0.07	0.40 ± 0.02	0.32 ± 0.01	0.30 ± 0.08	0.34 ± 0.05	0.35 ± 0.02	0.38 ± 0.01	0.38 ± 0.02
Lys	0.88 ± 0.15	0.92 ± 0.02	1.25 ± 0.07	1.92 ± 0.07	2.03 ± 0.12	2.37 ± 0.12	3.23 ± 0.19	3.89 ± 0.08	3.93 ± 0.19	4.17 ± 0.22	4.24 ± 0.21	4.41 ± 0.07	4.75 ± 0.15
Met	0.84 ± 0.07	0.95 ± 0.08	1.20 ± 0.13	1.82 ± 0.17	0.62 ± 0.15	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	0.24 ± 0.02	0.34 ± 0.04	0.65 ± 0.09	0.78 ± 0.01	0.97 ± 0.02	1.22 ± 0.06	2.27 ± 0.09	2.44 ± 0.03	2.52 ± 0.01	2.67 ± 0.09	2.73 ± 0.06
Pro	0.96 ± 0.04	1.85 ± 0.12	2.12 ± 0.16	2.22 ± 0.19	2.24 ± 0.27	2.25 ± 0.25	2.34 ± 0.29	2.98 ± 0.09	2.34 ± 0.21	2.25 ± 0.17	2.22 ± 0.18	2.35 ± 012	2.42 ± 0.19
Ser	0.26 ± 0.02	1.35 ± 0.11	2.98 ± 0.08	3.65 ± 0.12	4.52 ± 0.21	3.85 ± 0.34	8.33 ± 0.36	5.46 ± 0.27	3.22 ± 0.19	5.66 ± 0.07	6.22 ± 0.12	6.54 ± 0.12	6.71 ± 0.10
Thr	0.35 ± 0.09	0.47 ± 0.14	0.52 ± 009	0.59 ± 0.07	0.72 ± 0.06	0.85 ± 0.01	1.88 ± 0.12	3.25 ± 0.16	5.01 ± 0.19	7.22 ± 0.13	8.98 ± 0.14	10.0 ± 0.18	13.2 ± 0.18
Tyr	0.19 ± 0.03	0.37 ± 0.03	0.43± 0.06	0.45 ± 0.02	0.58 ± 0.08	0.80 ± 0.01	1.97 ± 0.07	2.15 ± 0.11	2.35 ± 0.00	3.25 ± 0.12	3.38 ± 0.16	3.51 ± 0.11	3.64 ± 0.10
Val	0.37 ± 0.09	1.16 ± 0.10	1.85 ± 0.09	2.04 ± 0.10	2.09 ± 0.15	2.24 ± 0.13	2.56 ± 0.13	2.87 ± 0.14	3.25 ± 0.17	4.77 ± 0.17	5.02 ± 0.20	5.19 ± 0.19	5.45 ± 0.13
Gaba	0.52 ± 0.14	0.97 ± 0.18	1.18 ± 0.09	1.26 ± 0.07	1.35 ± 0.02	1.85 ± 0.08	2.59 ± 0.13	2.87 ± 016	2.36 ± 0.17	2.32 ± 0.18	2.49 ± 0.17	2.62 ± 0.12	3.83 ± 0.10
Total	8.61 ± 0.11	15.9 ± 0.15	21.3 ± 0.41	28.6 ± 0.11	25.84 ±0.28	27.3 ± 0.14	42.1 ± 0.21	47.6 ± 0.10	509 ± 0.17	63.9 ± 0.11	68.9 ± 0.21	7106 ± 0.12	80.5 ± 0.20

methionine was not detected. Phenylalanine was not detected in dry seeds and 1st day imbibed seed samples. On 2nd day phenylalanine was detected and afterwards it was slowly but significantly increasing. Proline showed a 2-fold increase on 1st day and a slow increase up to 2nd day. Thereafter, there was no quantitative difference. Remarkable increase was shown by serine on 1st day and gradual increase up to 4th day. On 5th day a slight decrease was noticed. From 5th to 6th day of germination, the increase was more than 2-fold. Thereafter it was slowly decreasing up to 8th day and from 8th to 9th day, an increase was observed. Ninth day onwards serine content remained almost constant. Threonine was slowly and gradually increasing from 1st day up to 5th day. Sixth day onwards-significant increase was observed at each interval and on 12th day threonine was the most abundant amino acid. γ-aminobutyric acid was slowly increasing up to 5th day and on 6th day the increase was significant ($P<0.01$). There was no quantitative difference after 6th day.

In seeds treated at 45°C and 50°C (Table 17d & 17e respectively) during germination at room temperature arginine content was increased on 1st and 2nd day ($P<0.01$) and on 3rd day it was increased by 2-fold. On 4th day it was decreased to less than one third. From 5th to 6th day arginine content was doubled and afterwards only negligible changes occurred. Aspartic acid showed a slow increase up to 4th day and on 5th day there was no quantitative difference. On 6th day 5-fold increase was noticed and from 6th day to 7th day again a significant increase ($P<0.01$) was observed. Later, gradual increase was occurred but the day-to-day increases were significant. Glutamic acid was the abundant amino acid in ungerminated seeds of treatment at 45°C and 50°C. Glutamic acid showed a doubling on 1st day and up to 4th day there was no change in quantity. But 5th day sample showed

an increase ($P<0.01$) and later much change were not observed. From 3rd day onwards glutamic acid was quantitatively lesser than other treatments and control seeds. Glycine showed an increase on 1st day and not changed up to 5th day. On 6th day it was increased by 3-fold. Thereafter from 7th day to 8th day the increase was significant ($P< 0.01$) and later, only slight changes were noticed. Histidine content was almost similar in ungerminated seeds and 1st day sample. On 2nd day it showed 3-fold increase followed by a negligible increase up to 7th day. From 8th day onwards it was gradually decreased but the reduction was negligible. Isoleucine was detected only up to 2nd day and on 1st day it was significantly increased compared to that of the dry seeds ($P< 0.01$). On 2nd day the quantitative difference was negligible. Leucine showed a slow increase up to 4th day followed by slow decrease leading to no quantitative difference up to 12th day. Lysine showed a gradual increase during 1st to 5th day of germination. On 6th day the increase was significant ($P<0.01$) and afterwards no increase was noticed. Methionine showed an increase up to 3rd day ($P<0.01$). On 4th day it was decreased to less than half. From 5th day onwards methionine was not detected. Phenylalanine was not detected in ungerminated and 1st day imbibed samples. From 2nd day onwards it was present and was gradually increasing. Proline also was present in considerable quantity in dry seeds. Later it showed a slow and gradual increase but no significant difference was observed. Almost similar pattern of distribution was shown by threonine as that of aspartic acid but quantitatively threonine was more up to 4th day and afterwards slight increase was noticed compared to the aspartic acid. Fifth day onwards there was considerable increase up to 8th day and much change was not occurred. Tyrosine showed a significant increase ($P<0.05$) on 1st day and up to 5th day there was no difference.

Table 17d: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination at room (seeds treated at 45°C)

Amino acids	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Amino acids in (mg/g dry tissue)												
Ala	0.95 ± 0.07	1.61 ± 0.10	1.64 ± 0.12	1.75 ± 0.01	1.84 ± 0.02	1.93 ± 0.05	2.69 ± 0.02	2.98 ± 0.09	3.05 ± 0.21	3.50 ± 0.10	3.62 ± 0.05	3.78 ± 0.09	3.94 ± 0.09
Arg	0.23 ± 0.01	0.45 ± 0.03	0.93 ± 0.11	1.88 ± 0.18	0.58 ± 0.06	0.83 ± 0.09	1.94 ± 0.11	2.16 ± 0.07	2.25 ± 0.03	2.53 ± 0.12	2.78 ± 0.08	2.67 ± 0.04	2.84 ± 0.08
Asp	0.11 ± 0.01	0.15 ± 0.01	0.25 ± 0.01	0.35 ± 0.01	0.57 ± 0.01	0.49 ± 0.01	2.14 ± 0.16	3.65 ± 0.08	3.99 ± 0.02	3.87 ± 0.10	4.35 ± 0.16	4.85 ± 0.11	5.02 ± 0.05
Glu	1.10 ± 0.16	2.05 ± 0.19	2.48 ± 0.19	2.60 ± 0.12	2.44 ± 0.22	3.56 ± 0.11	3.71 ± 0.08	3.85 ± 0.06	3.62 ± 0.04	4.40 ± 0.23	4.70 ± 0.18	5.31 ± 0.17	5.62 ± 0.10
Gly	0.20 ± 0.05	0.53 ± 0.03	0.58 ± 0.01	0.64 ± 0.04	0.88 ± 0.05	0.65 ± 0.02	2.01 ± 0.13	2.68 ± 0.18	3.81 ± 0.01	3.70 ± 0.05	3.87 ± 0.03	4.14 ± 0.09	4.63 ± 0.24
His	0.95 ± 0.09	0.83 ± 0.07	2.19 ± 0.04	2.36 ± 0.08	2.47 ± 0.09	2.67 ± 0.08	2.84 ± 0.05	2.98 ± 0.08	2.10 ± 0.06	2.15 ± 0.03	2.06 ± 0.02	1.88 ± 0.05	1.65 ± 0.05
Ile	0.44 ± 0.02	0.66 ± 0.01	0.73 ± 0.02	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Leu	0.24 ± 0.01	0.56 ± 0.05	0.62 ± 0.03	0.69 ± 0.01	0.76± 0.08	0.543	0.41 ± 0.03	0.33 ± 0.05	0.32 ± 0.02	0.34 ± 0.01	0.34 ± 0.01	0.36 ± 0.01	0.38 ± 0.01
Lys	0.89 ± 0.09	0.92 ± 0.04	1.26 ± 0.19	1.93 ± 0.12	2.04 ± 0.08	2.36 ± 0.06	3.24 ± 0.21	3.88 ± 0.01	3.94 ± 0.05	4.18 ± 0.08	4.25 ± 0.07	4.32 ± 0.09	4.95 ± 0.23
Met	0.85 ± 0.05	0.98 ± 0.09	1.24 ± 0.07	1.83 ± 0.05	0.63 ± 0.01	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	0.22 ± 0.01	0.30 ± 0.03	0.65 ± 0.05	0.78 ± 0.08	0.97 ± 0.04	1.20 ± 0.11	2.25 ± 0.07	2.43 ± 0.09	2.53 ± 0.05	2.69 ± 0.07	2.74± 0.01
Pro	0.93 ± 0.06	1.89 ± 0.04	2.11 ± 0.09	2.21 ± 0.13	2.28 ± 0.19	2.26 ± 0.15	2.35 ± 0.28	2.99 ± 0.45	2.34 ± 0.33	2.21 ± 0.17	2.20 ± 0.09	2.33 ± 0.22	2.45 ± 0.18
Ser	0.25 ± 0.07	1.33 ± 0.17	2.60 ± 0.34	3.57 ± 0.21	4.13 ± 0.07	3.77 ± 0.12	3.56 ± 0.07	3.58 ± 0.09	3.56 ± 0.07	5.45 ± 0.14	5.23 ± 0.08	4.56 ± 0.18	4.78 ± 0.23
Thr	0.23 ± 0.09	0.36 ± 0.04	0.48 ± 0.08	0.52 ± 0.03	0.66 ± 0.07	0.49 ± 0.07	1.96 ± 0.11	3.22 ± 0.45	4.11 ± 0.22	4.56 ± 0.17	5.23 ± 0.34	6.20 ± 0.36	5.56 ± 0.39
Tyr	0.19 ± 0.07	0.37 ± 0.02	0.44 ± 0.01	0.45 ± 0.05	0.58 ± 0.03	0.83 ± 0.09	1.95 ± 0.07	2.15 ± 0.06	2.34 ± 0.08	3.24 ± 0.07	2.99 ± 0.09	2.64 ± 0.03	2.33 ± 0.09
Val	0.39 ± 0.06	1.12 ± 0.11	1.83 ± 0.15	2.08 ± 0.16	2.07 ± 0.18	2.22 ± 0.23	2.57 ± 0.19	2.89 ± 0.36	2.26 ± 0.26	2.77 ± 0.28	2.04 ± 0.07	2.25 ± 0.09	2.47 ± 0.20
Gaba	0.55 ± 0.01	0.96 ± 0.09	1.19 ± 0.04	1.28 ± 0.01	1.36 ± 0.04	1.86 ± 0.07	2.53 ± 0.10	2.92 ± 0.12	3.52 ± 0.19	4.33 ± 0.02	4.49 ± 0.05	4.61 ± 0.06	5.84 ± 0.35
Total	8.5 ± 0.87	14.7 ± 0.25	20.7 ± 0.14	24.3 ± 0.10	23.9 ± 0.25	25.2 ± 0.12	34.8 ± 0.18	41.4 ± 0.16	43.5 ± 0.41	49.4 ± 0.10	50.6 ± 0.12	52.6 ± 0.10	55.2 ± 0.13

Table 17e: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination at room (seeds treated at 50°C)

Amino acids	Days of germination												
	0	1	2	3	4	5	6	7	8	9	10	11	12
	Amino acids (mg/g dry tissue)												
Ala	0.94 ± 0.08	1.61 ± 0.10	1.60 ± 0.11	1.76 ± 0.06	1.83 ± 0.09	1.91 ± 0.07	2.71 ± 0.03	2.96 ± 0.06	3.09 ± 0.08	3.58 ± 0.07	3.62 ± 0.04	3.79 ± 0.02	3.98 ± 0.02
Arg	0.23 ± 0.01	0.46 ± 0.03	0.97 ± 0.05	1.87 ± 0.10	0.58 ± 0.02	0.82 ± 0.11	1.97 ± 0.09	2.16 ± 0.06	2.25 ± 0.08	2.53 ± 0.06	2.78 ± 0.15	2.67 ± 0.11	2.84 ± 0.20
Asp	0.13 ± 0.01	0.16 ± 0.01	0.28 ± 0.01	0.35 ± 0.01	0.59 ± 0.01	0.48 ± 0.01	2.21 ± 0.17	3.85 ± 0.08	3.99 ± 0.25	4.89 ± 0.06	4.54 ± 0.24	4.99 ± 0.34	4.32 ± 0.10
Glu	1.10 ± 0.12	2.08 ± 0.10	2.47 ± 0.05	2.66 ± 0.08	2.45 ± 0.03	3.86 ± 0.09	3.77 ± 0.07	3.77 ± 0.05	3.85 ± 0.10	4.40 ± 0.17	4.70 ± 0.14	5.64 ± 0.12	5.83 ± 0.12
Gly	0.25 ± 0.02	0.51 ± 0.03	0.57 ± 0.01	0.64 ± 0.04	0.89 ± 0.03	0.94 ± 0.05	2.04 ± 0.07	2.62 ± 0.22	3.83 ± 0.09	3.76 ± 0.08	3.87 ± 0.05	4.15 ± 0.15	4.69 ± 0.23
His	0.95 ± 0.10	1.83 ± 0.13	2.19 ± 0.11	2.36 ± 0.10	2.46 ± 0.16	2.66 ± 0.20	2.83 ± 0.19	2.98 ± 0.21	2.17 ± 0.11	2.16 ± 0.09	2.09 ± 0.04	1.76 ± 0.01	1.66 ± 0.03
Ile	0.41 ± 0.02	0.65 ± 0.08	0.73 ± 0.03	0.72 ± 0.07	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Leu	0.22 ± 0.05	0.59 ± 0.02	0.63 ± 0.01	0.69 ± 0.04	0.76 ± 0.01	0.56 ± 0.02	0.43 ± 0.01	0.32 ± 0.02	0.32 ± 0.01	0.33 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	0.38 ± 0.01
Lys	0.89 ± 0.06	0.91 ± 0.08	1.25 ± 0.11	1.93 ± 0.02	2.04 ± 0.08	2.38 ± 0.21	3.21 ± 0.18	3.86 ± 0.09	3.95 ± 0.03	4.19 ± 0.07	4.25 ± 0.05	4.32 ± 0.06	4.98 ± 0.11
Met	0.82 ± 0.02	0.92 ± 0.02	1.25 ± 0.14	1.88 ± 0.18	0.61 ± 0.01	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	0.21 ± 0.01	0.32 ± 0.01	0.65 ± 0.01	0.78 ± 0.04	0.96 ± 0.03	1.20 ± 0.12	2.26 ± 0.11	2.46 ± 0.16	2.53 ± 0.13	2.68 ± 0.16	2.75 ± 0.10
Pro	0.97 ± 0.18	1.87 ± 0.11	2.15 ± 0.02	2.23 ± 0.15	2.25 ± 0.12	2.27 ± 0.13	2.33 ± 0.11	2.97 ± 0.18	2.35 ± 0.21	2.23 ± 0.19	2.25 ± 0.15	2.34 ± 0.18	2.48 ± 0.10
Ser	0.24 ± 0.01	1.40 ± 0.12	2.60 ± 0.22	3.56 ± 0.18	4.19 ± 0.09	3.99 ± 0.03	3.86 ± 0.02	3.74 ± 0.07	3.96 ± 0.01	5.52 ± 0.25	5.21 ± 0.20	4.59 ± 0.18	4.77 ± 0.10
Thr	0.28 ± 0.01	0.36 ± 0.02	0.48 ± 0.04	0.52 ± 0.06	0.60 ± 0.05	0.49 ± 0.01	1.95 ± 0.10	3.26 ± 0.13	4.12 ± 0.18	4.76 ± 0.09	5.22 ± 0.19	6.91 ± 0.21	5.68 ± 0.10
Tyr	0.19 ± 0.01	0.37 ± 0.02	0.41 ± 0.03	0.45 ± 0.05	0.58 ± 0.08	0.82 ± 0.03	1.97 ± 0.09	2.16 ± 0.06	2.35 ± 0.03	3.28	2.98 ± 0.08	2.57 ± 0.02	2.29 ± 0.01
Val	0.35 ± 0.03	1.15 ± 0.10	1.82 ± 0.03	2.09 ± 0.08	2.06 ± 0.11	2.25 ± 0.11	2.58 ± 0.09	2.89 ± 0.18	2.27 ± 0.11	2.77 ± 0.10	2.04 ± 0.14	2.28 ± 0.19	2.49 ± 0.18
Gaba	0.50 ± 0.02	0.98 ± 0.01	1.19 ± 0.03	1.27 ± 0.01	1.38 ± 0.02	1.86 ± 0.10	2.55 ± 0.20	2.95 ± 0.18	3.55 ± 0.23	4.33 ± 0.03	4.49 ± 0.08	4.63 ± 0.09	4.88 ± 0.01
Total	8.47 ± 0.11	15.8 ± 0.11	20.2 ± 0.18	25.3 ± 0.14	23.9 ± 0.19	26.7 ± 0.21	35.3 ± 0.18	41.7 ± 0.15	44.3 ± 0.10	47.9 ± 0.13	50.9 ± 0.15	53.6 ± 0.17	53.9 ± 0.12

On 6th day it showed a 2-fold increase followed by a gradual but insignificant increase upto 9th day. Afterwards it was slowly decreased. Valine content was remarkably increased during 1st day of imbibition and afterwards only slight but gradual increase was observed up to 7th day. Thereafter it remained unchanged until 12th day of germination. A significant increase ($P<0.01$) was shown by imbibed seeds in the case of γ-amino butyric acid and slow and gradual increase was occurred during germination. The amount of γ-amino butyric acid was higher than that of control and other treatments during all stages of germination.

During germination in refrigerator, the seeds of control and the treatments at 35°C and 40°C on one hand and the treatments at 45°C and 50°C on the other hand showed almost similar pattern of amino acid distribution.

In control seeds and treatments at 35°C and 40°C (Table 18a, b & c respectively) during germination alanine, γ-amino butyric acid and arginine were gradually but significantly increasing. Aspartic acid was increasing very slowly up to 30th day and from 30th to 40th day it showed a 5-fold increase and in between 40th to 50th day the increase was significant ($P<0.01$). Glutamic acid content was doubled on 10th day and was remained unchanged on 20th day. Thereafter on 30th day it was again increased by 2-fold. Fortieth day sample showed a slight increase followed by a significant increase on 50th day. Glycine showed a gradual increase up to 30th day. On 40th day it was significantly increased ($P<0.01$). The increase on 50th day was 2-fold. A gradual increase was noticed in the case of histidine up to 40th day followed by a doubling on 50th day. Isoleucine was detected only up to 20th day and the quantitative difference was negligible. Leucine content was retained as such during the germination and the quantitative differences

between the intervals were negligible. Lysine showed no quantitative difference up to 20th day followed by a 3-fold increase on 30th day. Thereafter the increase was gradual. Phenylalanine was not detected up to 20th day and in between 30th and 40th day there was no quantitative difference in control seeds. But it was detected from 20th day in treatment at 35°C and 40°C showing gradual increase up to 40th day and was increased significantly on 50th day. Proline content was gradually increased up to 20th day and thereafter it was maintained as such. Serine content was doubled upto 40th day followed by decrease on 50th day of germination. Threonine content was retained almost uniform up to 30th day. On 40th day it showed a 10-fold increase in control whereas as in treatment at 35°C and 40°C it was doubled. In control seeds 40th to 50th day increase was 3-fold. But the treatment of 35°C and 40°C showed a 4-fold increase. Tyrosine showed a gradual increase up to 40th day and was not detected in 50th day samples in the case of control seeds and was present in treatments of 35°C and 40°C. Tyrosine content was gradually increasing up to 20th day and thereafter the increase was highly significant. Valine showed gradual increase up to 30th day and there was no quantitative change on 40th day. The 50th day sample showed a significant increase in valine content ($P<0.01$). γ-aminobutyric acid showed a slow increase up to 40th day and was unchanged on 50th day.

The seeds treated at 45°C (Table 18d) and 50°C (Table 18e) showed a gradual but significant increase was noticed in the case of alanine and glutamic acid from 10th day onwards. Arginine showed a doubling in quantity up to 20th day. Arginine in 30th day sample was equal to 20th day sample. Fortieth day onwards the increase in arginine was highly significant. Aspartic acid showed slow increase up to 30th day followed by a 5-fold increase on 40th day and on 50th day the

Table 18a: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination in refrigerator (control seeds)

Amino acids	Days of germination					
	0	10	20	30	40	50
	Amino acids (mg/g dry tissue)					
Ala	0.99 ± 0.11	1.66 ± 0.08	1.62 ± 0.04	1.82 ± 0.01	2.73 ± 0.11	3.57 ± 0.12
Arg	0.22 ± 0.12	0.45 ± 0.02	0.94 ± 0.06	0.83 ± 0.02	1.75 ± 0.09	2.10 ± 0.07
Asp	0.15 ± 0.01	0.19 ± 0.01	0.25 ± 0.01	0.42 ± 0.03	2.08 ± 0.05	7.34 ± 0.03
Glu	1.10 ± 0.08	2.54 ± 0.11	2.47 ± 0.15	4.99 ± 0.09	5.71 ± 0.19	11.68 ± 0.33
Gly	0.21 ± 0.08	0.58 ± 0.03	0.57 ± 0.08	0.89 ± 0.08	2.87 ± 0.09	4.78 ± 0.25
His	0.92 ± 0.10	0.98 ± 0.04	1.85 ± 0.06	1.79 ± 0.01	2.84 ± 0.04	4.22 ± 0.08
Ile	0.41 ± 0.15	0.60 ± 0.06	0.71 ± 0.06	N.D	N.D	N.D
Leu	0.21 ± 0.22	0.53 ± 0.12	0.60 ± 0.08	0.49 ± 0.01	0.45 ± 0.01	0.38 ± 0.01
Lys	0.86 ± 0.11	0.95 ± 0.05	1.23 ± 0.05	3.10 ± 0.13	4.34 ± 0.04	4.68 ± 0.08
Met	0.82 ± 0.11	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	N.D	0.65 ± 0.18	0.78 ± 0.15	2.68 ± 0.28
Pro	0.95 ± 0.08	1.70 ± 0.24	2.07 ± 0.08	2.29 ± 0.01	2.43 ± 0.04	2.39 ± 0.09
Ser	0.24 ± 0.22	1.10 ± 0.02	2.56 ± 0.03	3.77 ± 0.07	7.59 ± 0.38	5.47 ± 0.18
Thr	0.23 ± 0.12	0.35 ± 0.02	0.47 ± 0.07	0.45 ± 0.06	4.26 ± 0.29	12.25 ± 0.40
Tyr	0.19 ± 0.01	0.34 ± 0.08	0.41 ± 0.04	0.88 ± 0.01	1.73 ± 0.08	N.D
Val	0.39 ± 0.13	1.27 ± 0.03	1.89 ± 0.08	2.23 ± 0.13	2.58 ± 0.02	6.00 ± 0.88
Gaba	0.51 ± 0.12	0.92 ± 0.01	1.17 ± 0.08	1.64 ± 0.04	1.96 ± 0.09	2.02 ± 0.08
Total	8.4 ± 0.56	13.8 ± 0.17	18.8 ± 0.12	26.2 ± 0.21	44.1 ± 0.10	69.6 ± 0.12

Table 18b: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination in refrigerator (seeds treated at 35°C)

Amino acids	Days of germination					
	0	10	20	30	40	50
	Amino acids (mg/g dry tissue)					
Ala	0.93 ± 0.16	1.77 ± 0.09	1.64 ± 0.22	2.62 ± 0.21	2.80 ± 0.19	3.92 ± 0.43
Arg	0.22 ± 0.03	0.57 ± 0.11	0.95 ± 0.12	1.94 ± 0.08	2.12 ± 0.03	3.73 ± 0.23
Asp	0.15 ± 0.15	0.16 ± 0.03	0.25 ± 0.11	2.01 ± 0.22	4.58 ± 0.19	7.62 ± 0.19
Glu	1.11 ± 0.13	1.99 ± 0.29	2.41 ± 0.16	6.12 ± 0.40	7.40 ± 0.38	11.92 ± 0.09
Gly	0.24 ± 0.06	0.53 ± 0.07	0.59 ± 0.09	2.02 ± 0.12	2.70 ± 0.15	4.21 ± 0.11
His	0.93 ± 0.08	1.90 ± 0.05	2.16 ± 0.14	2.80 ± 0.18	2.96 ± 0.10	4.06 ± 0.17
Ile	0.40 ± 0.22	0.66 ± 0.03	0.70 ± 0.09	N.D	N.D	N.D
Leu	0.20 ± 0.04	0.46 ± 0.02	0.63 ± 0.06	0.42± 0.03	0.32 ± 0.03	0.38 ± 0.01
Lys	0.87 ± 0.09	1.00 ± 0.07	1.24 ± 0.13	3.20 ± 0.26	3.86 ± 0.13	4.74 ± 0.50
Met	0.80 ± 0.13	N.D	N.D	N.D	N.D	N.D
Phe	N.D	N.D	0.22 ± 0.01	0.95 ± 0.01	1.21 ± 0.09	2.72 ± 0.11
Pro	0.92 ± 0.07	1.65 ± 0.22	2.06 ± 0.13	2.33 ± 0.06	2.95 ± 0.09	2.40 ± 0.18
Ser	0.24 ± 0.18	1.32 ± 0.29	2.58 ± 0.18	8.16 ± 0.67	5.32 ± 0.38	6.72 ± 0.68
Thr	0.25 ± 0.03	0.40 ± 0.03	0.44 ± 0.09	1.83± 0.09	2.90± 0.01	12.39 ± 0.09
Tyr	0.19 ± 0.01	0.40 ± 0.02	0.81 ± 0.06	1.95 ± 0.12	2.12 ± 0.15	3.65 ± 0.17
Val	0.38 ± 0.04	1.24 ± 0.18	1.86 ± 0.03	2.55 ± 0.13	2.85 ± 0.19	5.38 ± 0.27
Gaba	0.55 ± 0.02	0.93 ± 0.10	1.16 ± 0.01	2.18 ± 0.12	2.78 ± 0.28	2.76 ± 0.30
Total	8.38 ± 0.13	14.9 ± 0.12	19.7 ± 0.23	32.1 ± 0.13	47.8 ± 0.18	76.6 ± 0.15

Table 18c: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination in refrigerator (seeds treated at 40°C)

Amino acids	Days of germination					
	0	10	20	30	40	50
	Amino acids (mg/g dry tissue)					
Ala	0.94 ± 0.16	1.61 ± 0.09	1.64 ± 003	1.97 ± 0.09	2.63 ± 0.12	3.93 ± 0.11
Arg	0.24 ± 0.03	0.44 ±0.03	0.96 ± 0.06	0.80 ± 0.09	1.97 ± 0.14	3.63 ± 0.18
Asp	0.12 ± 0.01	0.17 ± 0.01	0.21 ± 0.05	0.43 ± 0.06	2.33 ± 0.16	7.99 ± 0.27
Glu	1.12 ± 0.09	2.14 ± 0.10	2.52 ± 0.06	5.25 ± 0.21	6.75 ± 0.32	9.73 ± 0.35
Gly	0.23 ± 0.01	0.51 ± 0.04	0.60 ± 0.02	0.63 ± 0.01	2.02 ± 0.11	4.56 ± 0.22
His	0.94 ± 0.08	1.84 ± 0.13	2.18 ± 0.02	2.64 ± 0.08	2.81 ± 0.01	4.08 ± 0.16
Ile	0.42 ± 0.07	0.62 ± 0.10	0.72 ± 0.23	N.D	N.D	1.30 ± 0.38
Leu	0.23 ± 0.04	0.58 ± 0.04	0.66 ± 0.08	0.53 ± 0.03	0.40 ± 0.05	0.38 ± 0.09
Lys	0.88 ± 0.15	0.92	1.25	2.37	3.23	4.75
Met	0.84 ± 0.07	-	-	-	2.03 ± 0.16	-
Phe	N.D	-	0.24 ± 0.16	0.78 ± 0.10	0.97 ± 0.12	2.73 ± 0.20
Pro	0.96 ± 0.04	1.85 ± 0.11	2.12 ± 0.13	2.23 ± 0.02	2.34 ± 0.09	2.42 ± 0.09
Ser	0.26 ± 0.02	1.35 ± 0.15	2.98 ± 0.16	3.89 ± 0.19	8.33 ± 0.33	6.71 ± 0.45
Thr	0.35 ± 0.09	0.47 ± 0.01	0.52 ± 0.08	0.64 ± 0.08	1.88 ± 0.07	13.2 ± 0.10
Tyr	0.19 ± 0.03	0.37 ± 0.02	0.43 ± 0.04	0.80 ± 0.01	1.97 ± 0.16	3.64 ± 0.27
Val	0.37 ± 0.09	1.16 ± 0.08	1.85 ± 0.03	2.24 ± 0.17	2.56 ± 0.10	5.45 ± 0.28
Gaba	0.52 ± 0.14	0.97 ± 0.06	1.18 ± 0.02	1.85 ± 0.12	2.59 ± 0.18	3.83 ± 0.19
Total	8.61 ± 0.11	15.0 ± 0.13	20.1 ± 0.15	30.9 ± 0.17	44.8 ± 0.15	78.3 ± 0.19

increase was significant ($P< 0.01$). Considerable increase was observed in glycine only on 40^{th} and 50^{th} days. Before that there were only negligible changes. Histidine showed a gradual increase up to 20^{th} day. Afterward the increase was negligible. Isoleucine was almost equal in quantity during 10^{th} and 20^{th} day. It was absent in 30^{th} and 40^{th} day samples. The 50^{th} day sample showed the reappearance of isoleucine and was almost double than that of 20^{th} day. Leucine did not show any considerable change in quantity during germination. Lysine and tyrosine showed a gradual increase during germination. Methionine was present only in dry seeds and 20^{th} day samples and was quantitatively increased on 20^{th} day ($P<0.01$). Phenylalanine was absent in dry seeds and 10^{th} day samples and 20^{th} day onwards it was present. It showed a slow increase up to 40^{th} day followed by a 3-fold increase on 50^{th} day. Proline showed a doubling in amount from dry seeds to 20^{th} day samples. Thereafter the changes were negligible. Serine was showing a significant increase ($P< 0.01$) on 10^{th} day followed by a 3-fold increase on 20^{th} day. Up to 40^{th} day the quantity of serine was retained as such. Thereafter 50^{th} day content showed an increase ($P<0.02$). Threonine did not show any significant change up to 30^{th} day and in between 30^{th} to 40^{th} day the increase was significant. The 50^{th} day sample contained 5-fold higher threonine than that of 40^{th} day. Valine showed a significant increase on 10^{th} day ($P< 0.01$) and was retained as such on 20^{th} day. Later it showed a 2-fold increase on 30^{th} day and thereafter there was no significant change. γ-aminobutyric acid was significantly increased ($P<0.01$) on 10^{th} and 20^{th} days and on 30^{th} day it was retained as such. Fortieth day onwards the increase was gradual and significant.

Table 18d: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination in refrigerator (seeds treated at 45°C)

Amino acids	Days of germination					
	0	10	20	30	40	50
	Amino acids (mg/g dry tissue)					
Ala	0.95 ± 0.07	1.61 ± 0.09	1.64 ± 0.05	1.91 ± 0.11	2.69 ± 0.34	3.96 ± 0.33
Arg	0.23 ± 0.01	0.43 ± 0.01	0.99 ± 0.06	0.83 ± 0.17	1.88 ± 0.16	2.62 ± 0.25
Asp	0.11 ± 0.01	0.16 ± 0.07	0.25 ± 0.08	0.42 ± 0.02	2.21 ± 0.10	5.01 ± 0.41
Glu	1.10 ± 0.16	2.18 ± 0.15	2.58 ± 0.18	3.86 ± 0.23	3.77 ± 0.18	5.69 ± 0.14
Gly	0.20 ± 0.05	0.51 ± 0.02	0.58 ± 0.03	0.64 ± 0.09	2.01 ± 0.17	4.71 ± 0.19
His	0.95 ± 0.09	1.87 ± 0.16	2.16 ± 0.11	2.52 ± 0.06	2.79 ± 0.09	2.78 ± 0.07
Ile	0.44 ± 0.02	0.66 ± 0.02	0.73 ± 0.05	N.D	N.D	1.32 ± 0.11
Leu	0.24 ± 0.01	0.56 ± 0.04	0.62 ± 0.04	0.56 ± 0.02	0.41 ± 0.05	0.38 ± 0.01
Lys	0.89 ± 0.09	0.93 ± 0.07	1.22 ± 0.10	2.36 ± 0.15	3.33 ± 0.22	4.72 ± 0.27
Met	0.85 ± 0.05	N.D	1.22 ± 0.01	N.D	N.D	N.D
Phe	N.D	N.D	0.22 ± 0.01	0.78 ± 0.08	0.97 ± 0.01	2.71 ± 0.23
Pro	0.93 ± 0.06	1.87 ± 0.13	2.11 ± 0.01	2.27 ± 0.09	2.33 ± 0.03	2.50 ± 0.08
Ser	0.25 ± 0.07	1.40 ± 0.02	3.80 ± 0.13	3.99 ± 0.12	3.86 ± 0.18	4.68 ± 0.21
Thr	0.23 ± 0.09	0.49 ± 0.06	0.58 ± 0.09	0.69 ± 0.07	1.95 ± 0.16	5.62 ± 0.19
Tyr	0.19 ± 0.07	0.37 ± 0.02	0.44 ± 0.01	0.83 ± 0.03	1.95 ± 0.10	2.37 ± 0.11
Val	0.39 ± 0.06	1.15 ± 0.03	1.83 ± 0.15	2.25 ± 0.15	2.57 ± 0.16	2.52 ± 0.19
Gaba	0.55 ± 0.01	0.98 ± 0.01	2.10 ± 0.08	2.32 ± 0.07	3.75 ± 0.30	5.88 ± 0.29
Total	8.5 ± 0.87	15.1 ± 0.10	21.8 ± 0.14	26.3 ± 0.22	36.4 ± 0.17	57.4 ± 0.14

Table 18e: Effect of temperature treatment on amino acids in *Pisum sativum* during seed germination in refrigerator (seeds treated at 50°C)

Amino acids	Days of germination					
	0	10	20	30	40	50
	Amino acids (mg/g dry tissue)					
Ala	0.94 ± 0.08	1.62 ± 0.11	1.60 ± 015	1.93 ± 0.13	2.71 ± 0.19	3.67 ± 0.19
Arg	0.23 ± 0.01	0.46 ± 0.01	0.97 ± 0.08	0.82 ± 0.13	1.97 ± 0.07	2.62 ± 0.05
Asp	0.13 ± 0.01	0.15 ± 0.01	0.28 ± 0.01	0.48 ± 0.01	2.14 ± 0.13	4.35 ± 0.24
Glu	1.10 ± 0.12	2.15 ± 0.08	2.57 ± 0.12	3.56 ± 0.09	3.71 ± 0.07	5.92 ± 0.18
Gly	0.25 ± 0.02	0.53 ± 0.02	0.57 ± 0.01	0.65 ± 0.01	2.04 ± 0.14	4.55 ± 0.20
His	0.95 ± 0.10	1.83 ± 0.08	2.19 ± 0.05	2.66 ± 0.03	2.83 ± 0.05	2.95 ± 0.04
Ile	0.41 ± 0.02	0.65 ± 0.02	0.73 ± 0.04	N.D	N.D	1.35 ± 0.17
Leu	0.22 ± 0.05	0.59 ± 0.02	0.63 ± 0.01	0.54 ± 0.02	0.43 ± 0.04	0.38 ± 0.01
Lys	0.89 ± 0.06	0.91 ± 0.05	1.25 ± 0.15	2.38 ± 0.16	3.21 ± 0.23	4.67 ± 0.29
Met	0.82 ± 0.02	N.D	1.25 ± 0.17	N.D	N.D	N.D
Phe	N.D	N.D	0.21 ± 0.01	0.78 ± 0.06	0.96 ± 0.02	2.78 ± 0.17
Pro	0.97 ± 0.18	1.80 ± 0.16	2.16 ± 0.09	2.26 ± 0.05	2.35 ± 0.02	2.56 ± 0.07
Ser	0.24 ± 0.01	1.38 ± 0.10	3.81 ± 0.12	3.77 ± 0.14	3.56 ± 0.14	4.70 ± 0.18
Thr	0.28 ± 0.01	0.49 ± 0.05	0.59 ± 0.06	0.69 ± 0.03	1.96 ± 0.23	5.63 ± 0.47
Tyr	0.19 ± 0.01	0.37 ± 0.01	0.41 ± 0.01	0.82 ± 0.01	1.97 ± 0.02	2.30 ± 0.01
Val	0.35 ± 0.03	1.12 ± 0.09	1.82 ± 0.10	2.22 ± 0.15	2.58 ± 0.16	2.62 ± 0.17
Gaba	0.50 ± 0.02	0.96 ± 0.05	2.01 ± 0.12	2.66 ± 0.23	3.88 ± 0.67	5.23 ± 0.92
Total	8.47 ± 0.11	15.0 ± 0.14	23.1 ± 0.19	26.2 ± 0.18	36.3 ± 0.17	56.3 ± 0.22

4. Histochemistry/Anatomy

4.1. Root apex

When the germination was conducted at room temperature the root apex after one day of germination showed the characters of a typical root (Plate 5, Fig. a) consisting of root cap and differentiating provascular strands. The control seeds and the treatment showed many differences between them. In the case of seeds treated at 45°C and 50°C (Plate 5, Fig. d & e respectively) there was significant difference compared to the control and other treatments. The root cap cells were more intact and the cell wall breakage and feeble staining were very distinct. Differentiation of provascular strands also were not clear and intense staining masked cellular structure.

In the root apex after 10 days of germination in refrigerator an important observation is the absence of any breakage in the root apex of seeds treated at 45°C and 50°C compared to seedling germinated at room temperature. There was no significant difference between the control and the treatments (Plate 6, Fig. a, b, c, d & e)

4.2. Cotyledon

4.2.1. Insoluble polysaccharides

Periodic acid-Schiff's (PAS) reagent specifically stains insoluble polysaccharides. The sections of cotyledon stained with the PAS showed magenta coloured moderately stained cell walls and densely stained starch grains (Plate 7, Fig. a, b, c, d & e). The cell lumen of each cell was almost filled with starch grains. Majority of starch grains are elliptical or round in shape. The hilum in the center of the starch grains was stained darker and appeared as blue coloured spots.

When germination was done at room temperature, in the case of control seeds and treatments after 1st day of germination cell lumen was almost with the starch grains (Plate 7, Fig. a, b, c, d & e). There

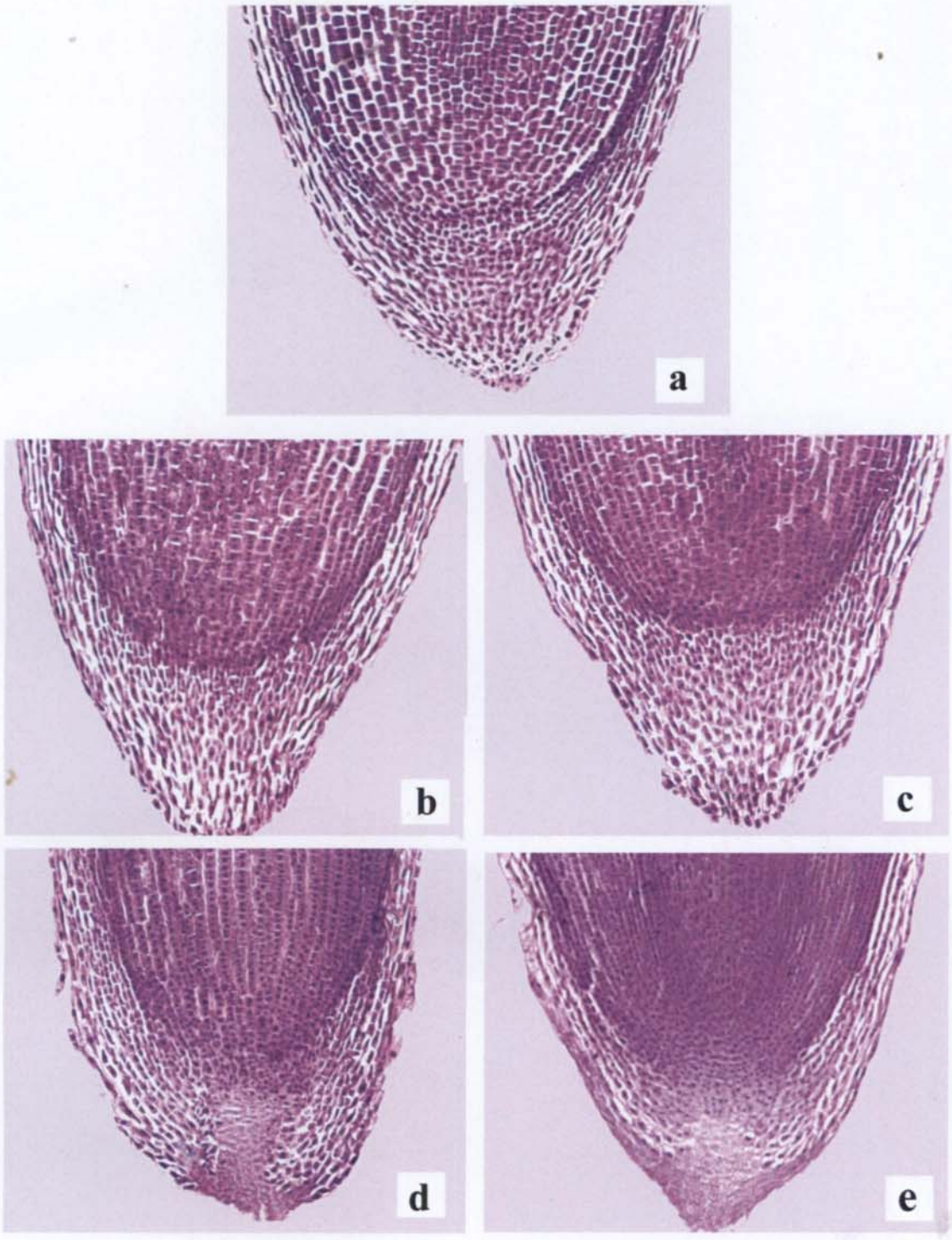


Plate 5 : Radicle apex of *Pisum sativum* seedling after 1st day of germination at room temperature.

a. Control seeds

b. Seeds treated at 35°C

c. Seeds treated at 40°C

d. Seeds treated at 45°C

e. Seeds treated at 50°C

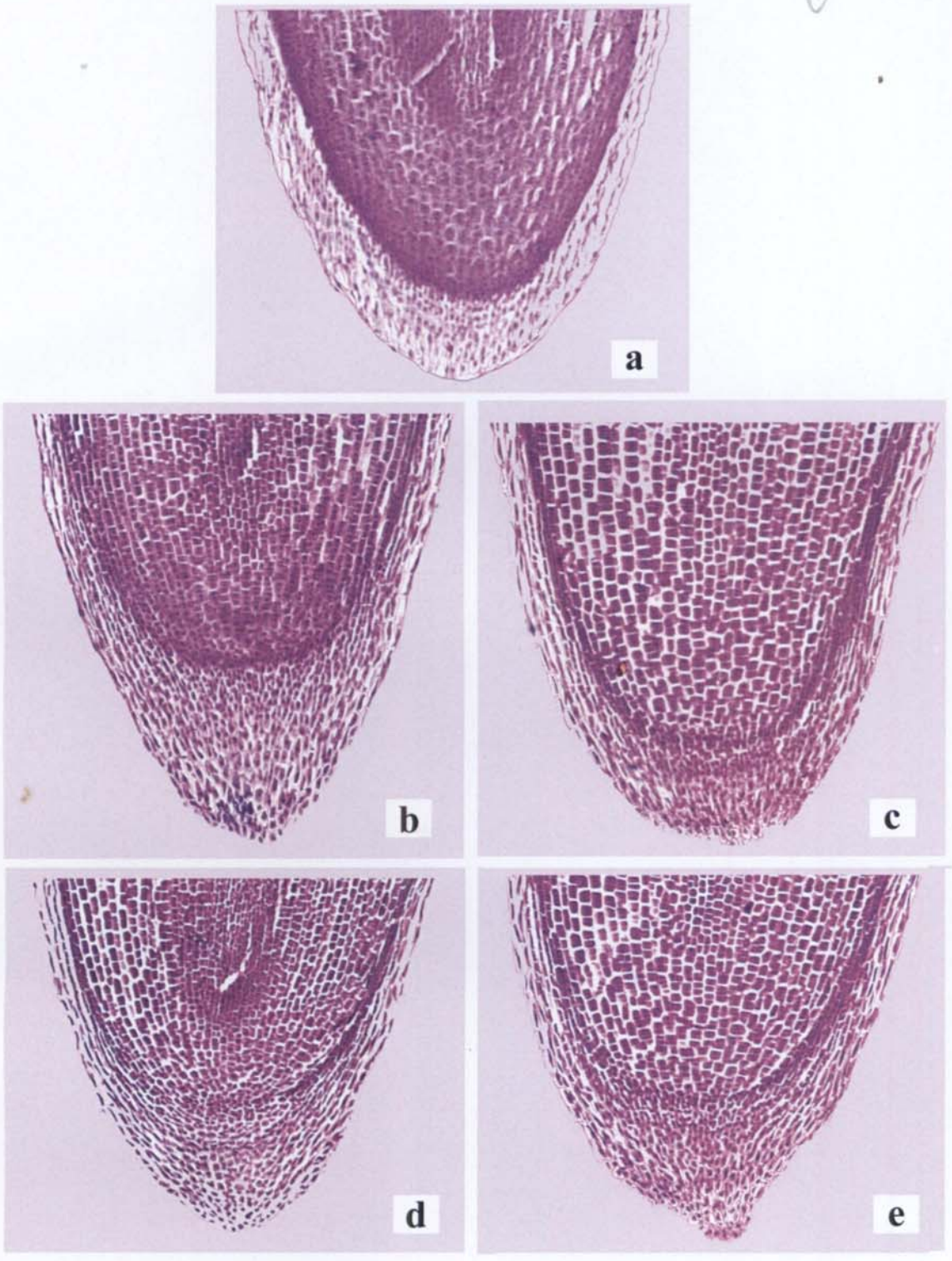


Plate 6 : Radicle apex of *Pisum sativum* seedling after 10 th day of germination at cold.

a. Control seeds
b. Seeds treated at 35°C
c. Seeds treated at 40°C
d. Seeds treated at 45°C
e. Seeds treated at 50°C

was a noticeable increase in the size of starch grain from the abaxial side to the inner region of cotyledons. After one day germination the cotyledonary cell of seeds treated at 50°C were small in size, and was shrunken and uneven in appearance (Plate 7, Fig. a, b, c, d & e) compared to the control and other treatments starch grain were appeared smaller in size and shape.

On 5th day of germination, there was remarkable reduction in the number of starch grains (Plate 8, Fig. a, b, c, d & e). All the treatments except 35°C and control showed similar distribution of starch grains. The cells of cotyledon treated at 45°C and 50°C showed broken cell wall (Plate 8, Fig. d & e respectively). The seeds treated at 50°C showed shrunken cells (Plate 8, Fig. e).

Starch grain of the cotyledonary cells reduced further than the 5th day sample both in the control and the experimentals (Plate 9, Fig. a, b, c, d & e). There was no reduction in size of starch grains. The cell lysis was clearly seen in seeds treated at 45°C and 50°C (Plate 9, Fig. d & e respectively) since the cell wall was broken, starch grain was found scattered or dislocated over the cell in the experimentals of 50°C (Plate 9, Fig. d).

When germination was conducted in refrigerator, after 10 days, the control and all experimentals exhibited more or less similar pattern of starch distribution and the shape of cells as well as starch grains were not much distinct compared to the equivalent samples of (one day old seedling) of room temperature (Plate 10, Fig. a, b, c, d & e).

After 30th day of germination, the control seeds and treatments at 35°C, 40°C, 45°C and 50°C (Plate 11, Fig. a, b, c, d & e) showed distinct cell shape and intact cell wall. Starch grain shape and distribution also were very clearly seen in comparison with the previous stage as well as the equivalent sample of room temperature.

On 50th day of germination in the cold condition control and all the treatments showed distinct cell stage and the cell lumen was very clear due to the reduction of starch grain. Slight reduction in the number and shape of the starch grain also was observed. The reduction in the number of starch grains was significant in the case of seeds treated at 45°C and 50°C (Plate 12, Fig. d & e respectively).

4.2.2. Protein

Sections of cotyledon of pea seeds stained with mercuric bromophenol blue were observed to detect the protein content. Blue stained masses occupy almost more than three fourth of each cotyledonary cell and throughout the cell the intensity of staining was very high and varied form sample to sample. Many unstained granular structures (starch) were found embedded in the protein mass

On 1st day of germination at room temperature no significant difference in the distribution and staining intensity between the control and treatments at 35°C and 40°C were observed. But the cells of treatment at 45°C and 50°C were smaller in size and protein bodies were considerably reduced and hence colourless granules (starch) were clearly seen embedded in the protein masses. In others the cells were larger in size (Plate 13, Fig. a, b, c, d & e).

The 5th day germinated samples showed a significant reduction in protein content (Plate 14, Fig. a, b, c, d & e). The treatments at 35°C and 40°C showed almost similar pattern of protein distribution and the protein bodies become more distinct compared to the control (Plate 14, Fig. a, b and c). The cells of treatments at 45°C and 50°C showed lysis of cell wall and the protein masses were reduced considerably and unevenly distributed (Plate 14, Fig. d and e respectively).

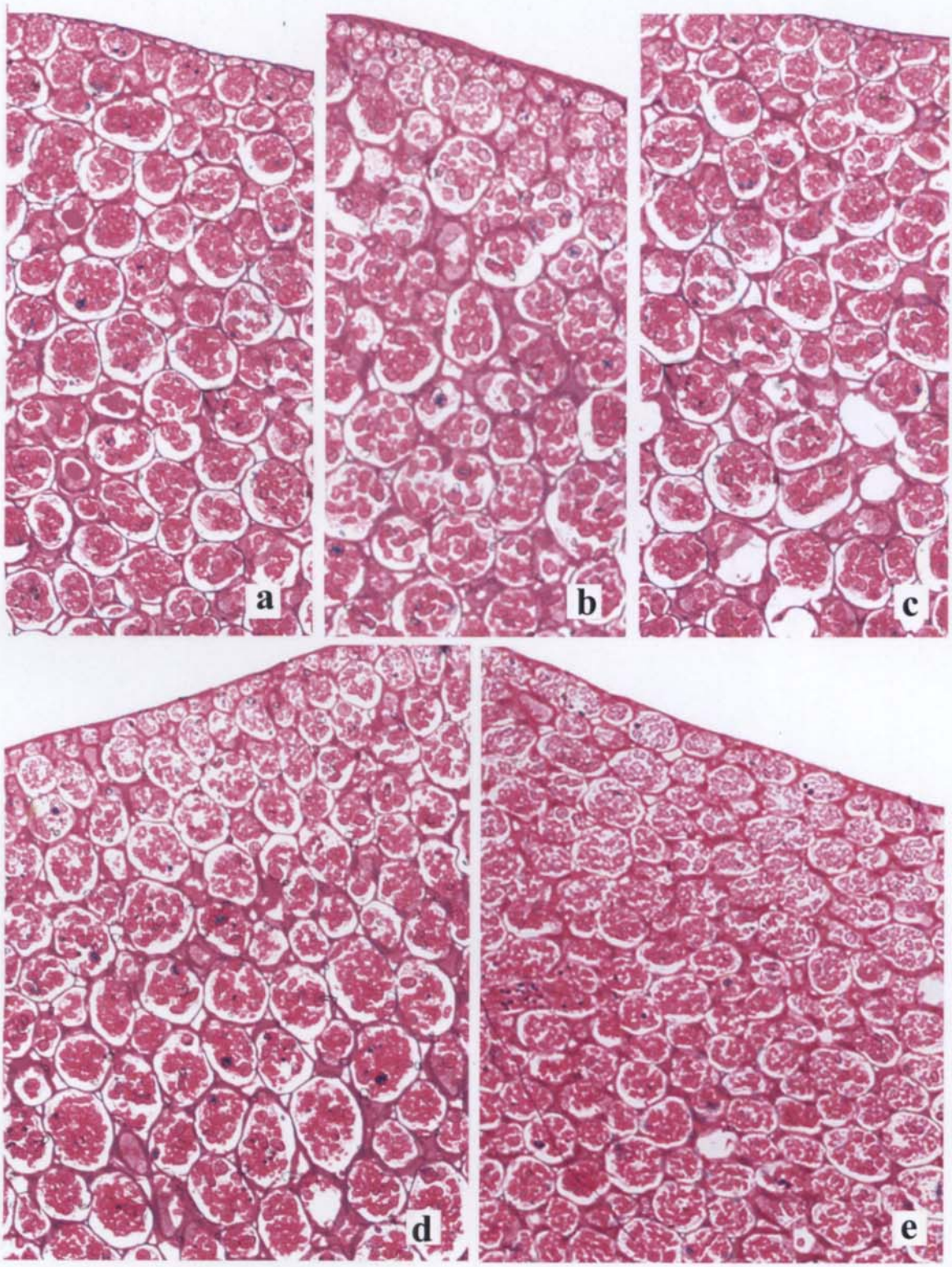


Plate 7 : Starch distribution in the cotyledon of *Pisum sativum* seedling after 1st day of germination at room temperature.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

On 10^{th} day germination at room temperature further reduction in protein content was observed and the size and sample of protein bodies were not distinct (Plate 15, Fig. a, b, c, d & e). The cell wall lysis was higher in seeds treated at 45^0C and 50^0C (Plate 15, Fig. d and e respectively). compared to control and treatments

There was no considerable difference in cell size and protein distribution between control and treatments at 35^0C and 40^0C of 10^{th} day sample seeds germinated in refrigerator (Plate 16, Fig. a, b, c, d & e). But protein bodies of seeds treated at 45^0C and 50^0C were reduced and clearly seen compared to control and other treatments.

On 30^{th} day of germination in refrigerator the cell size of all the treatments were almost similar and slightly bigger than the control cells. There was only slight reduction in protein mass on 30^{th} day when compared with 10^{th} day (Plate 17, Fig. a, b, c, d & e). The cells of seeds treated at 45^0C and 50^0C slightly swollen and cell wall appeared irregular (Plate 17, Fig. d and e respectively).

The control seeds and treatment at 35^0C, 40^0C, 45^0C and 50^0C on 50^{th} day of germination showed reduced amount of protein bodies compared to the previous sample (Plate 18, Fig. a, b, c, d & e). The cells of seeds treated at 45^0C and 50^0C were slightly broken and protein bodies become irregular (Plate 18 Fig. d and e respectively) where as in others the cells did not show lysis (Plate 18, Fig. a, b and c)

4.3. Testa

The testa of fresh seeds (Plate 19, Fig. a) was intact when compared to the seeds treated at 50^0C (Plate 19, Fig. b). Anatomical structures of fresh pea seed testa consist of an outer most layer of

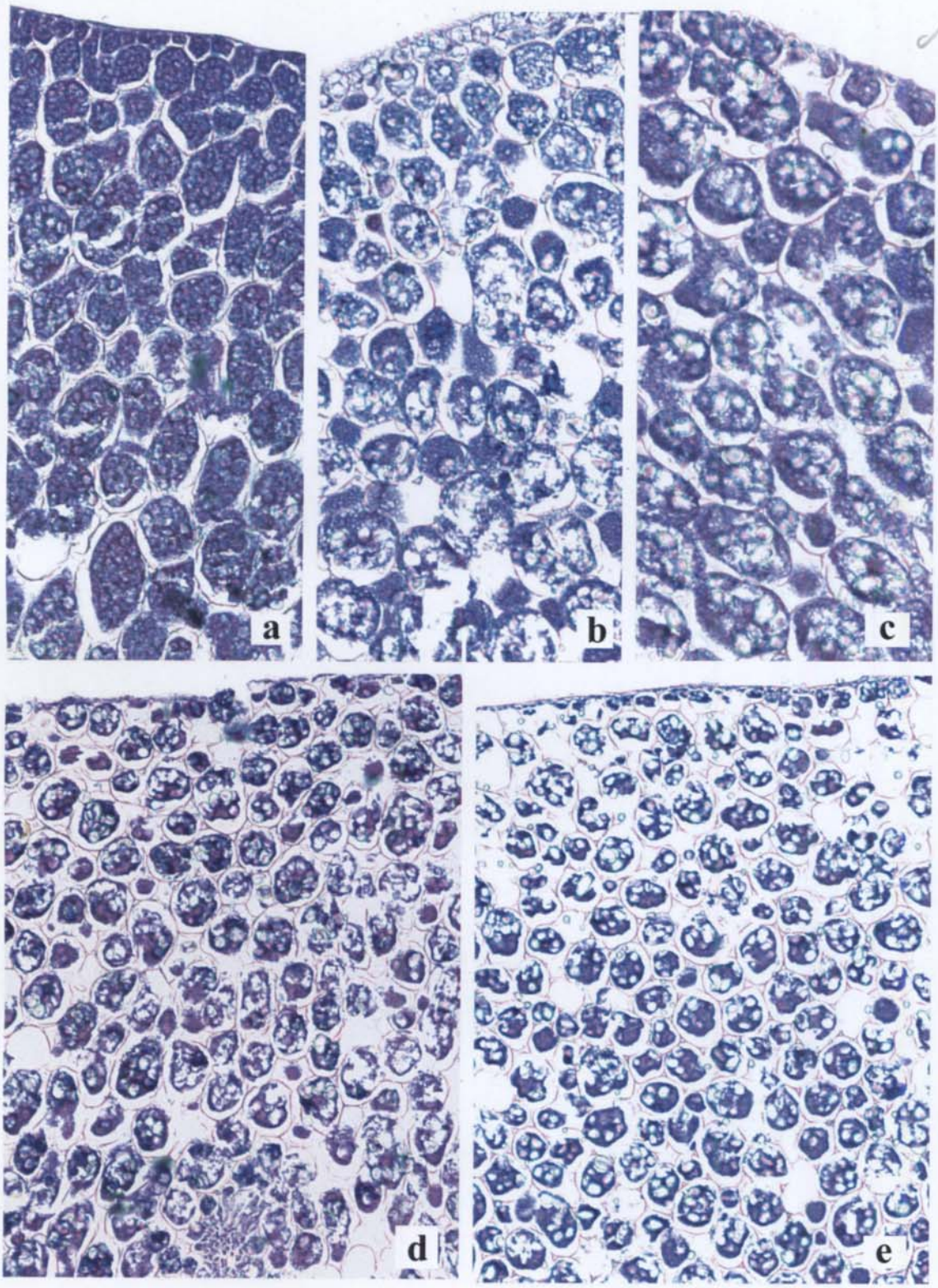


Plate 13: Protein distribution in the cotyledon of *Pisum sativum* seedling after 1st day of germination at room temperature.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

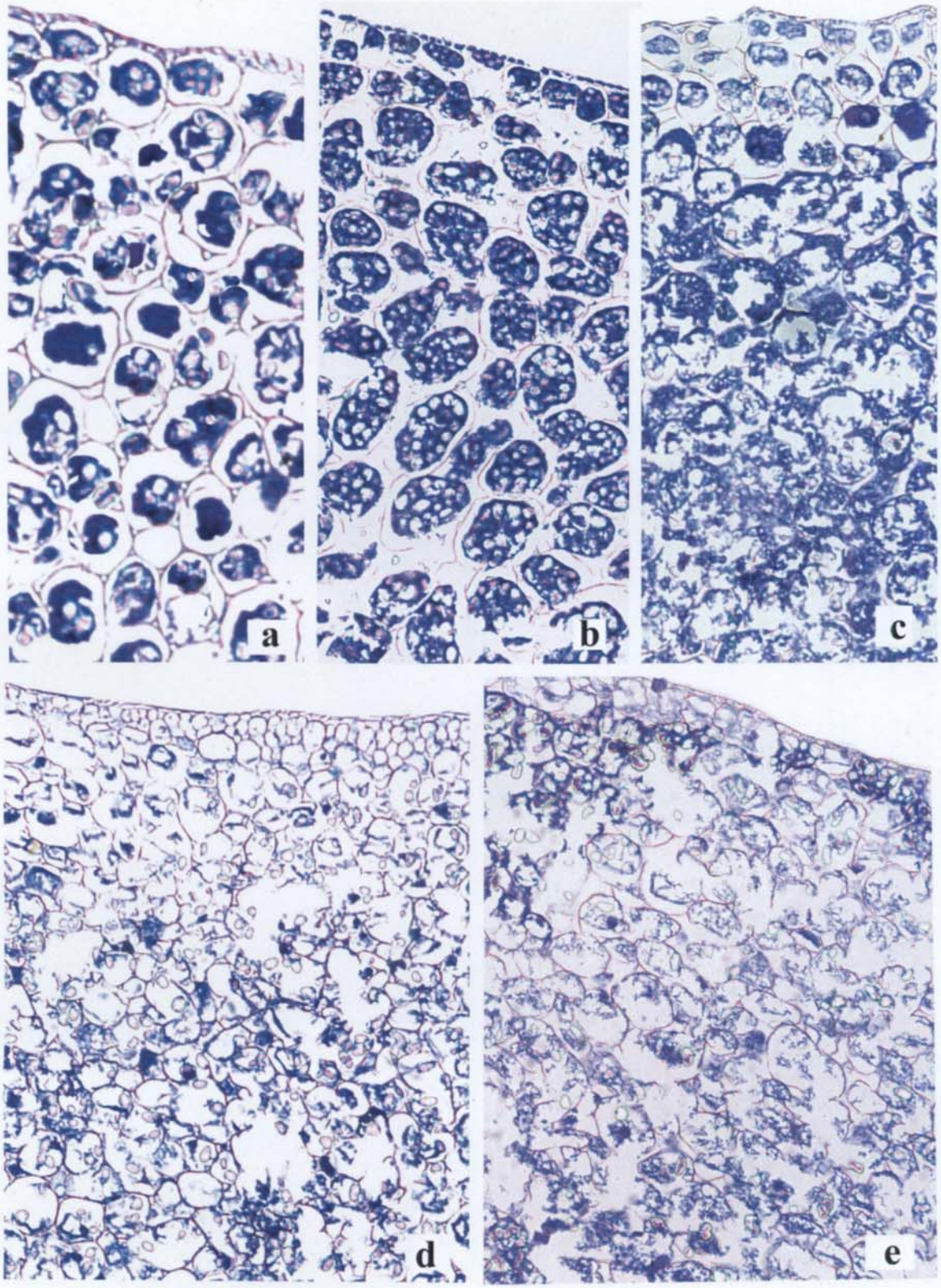


Plate 14: Protein distribution in the cotyledon of *Pisum sativum* seedling after 5 th day of germination at room temperature.

a. Control seeds
b. Seeds treated at 35°C
c. Seeds treated at 40°C
d. Seeds treated at 45°C
e. Seeds treated at 50°C

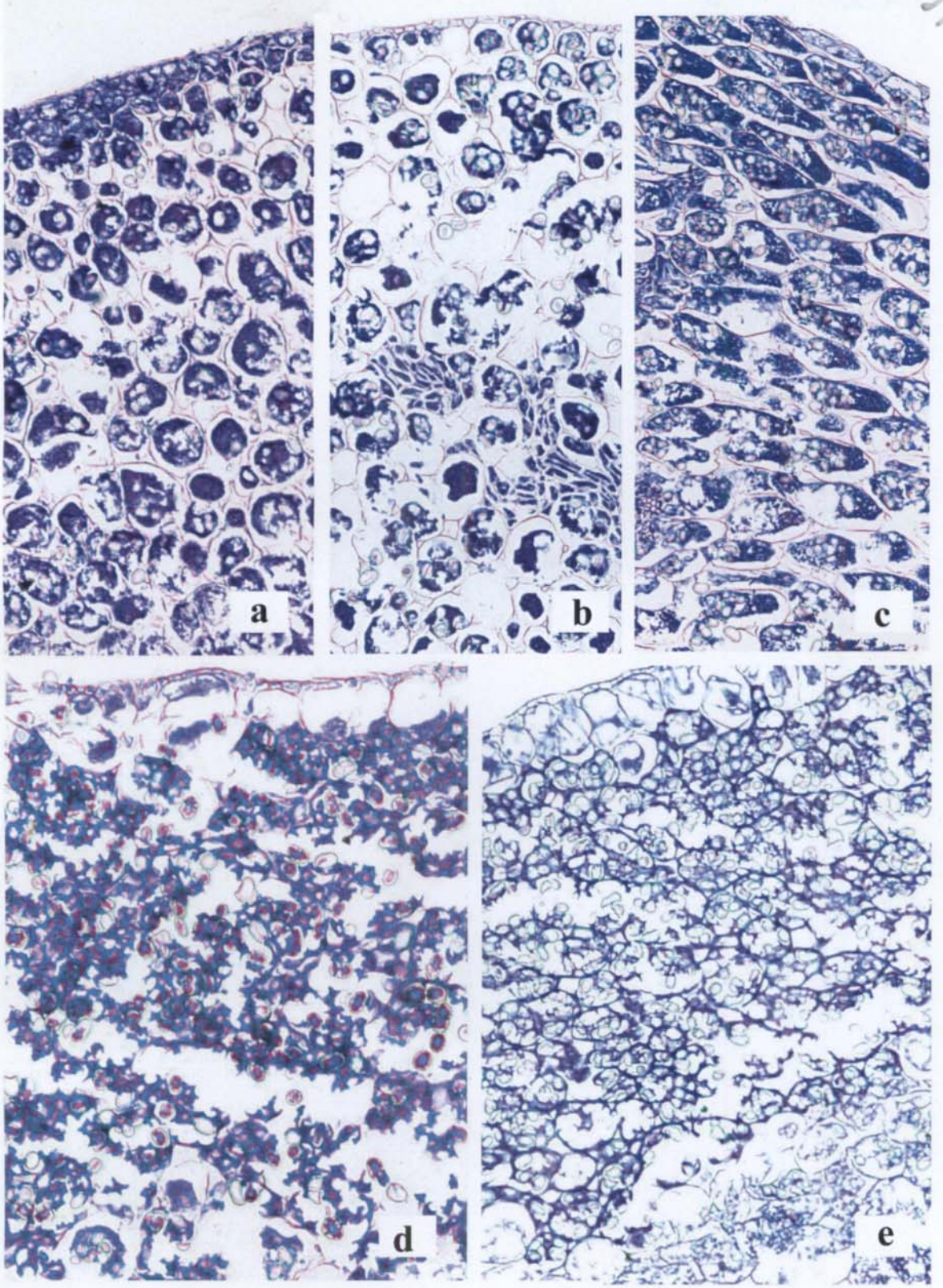


Plate 15 : Protein distribution in the cotyledon of *Pisum sativum* seedling after 10th day of germination at room temperature.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

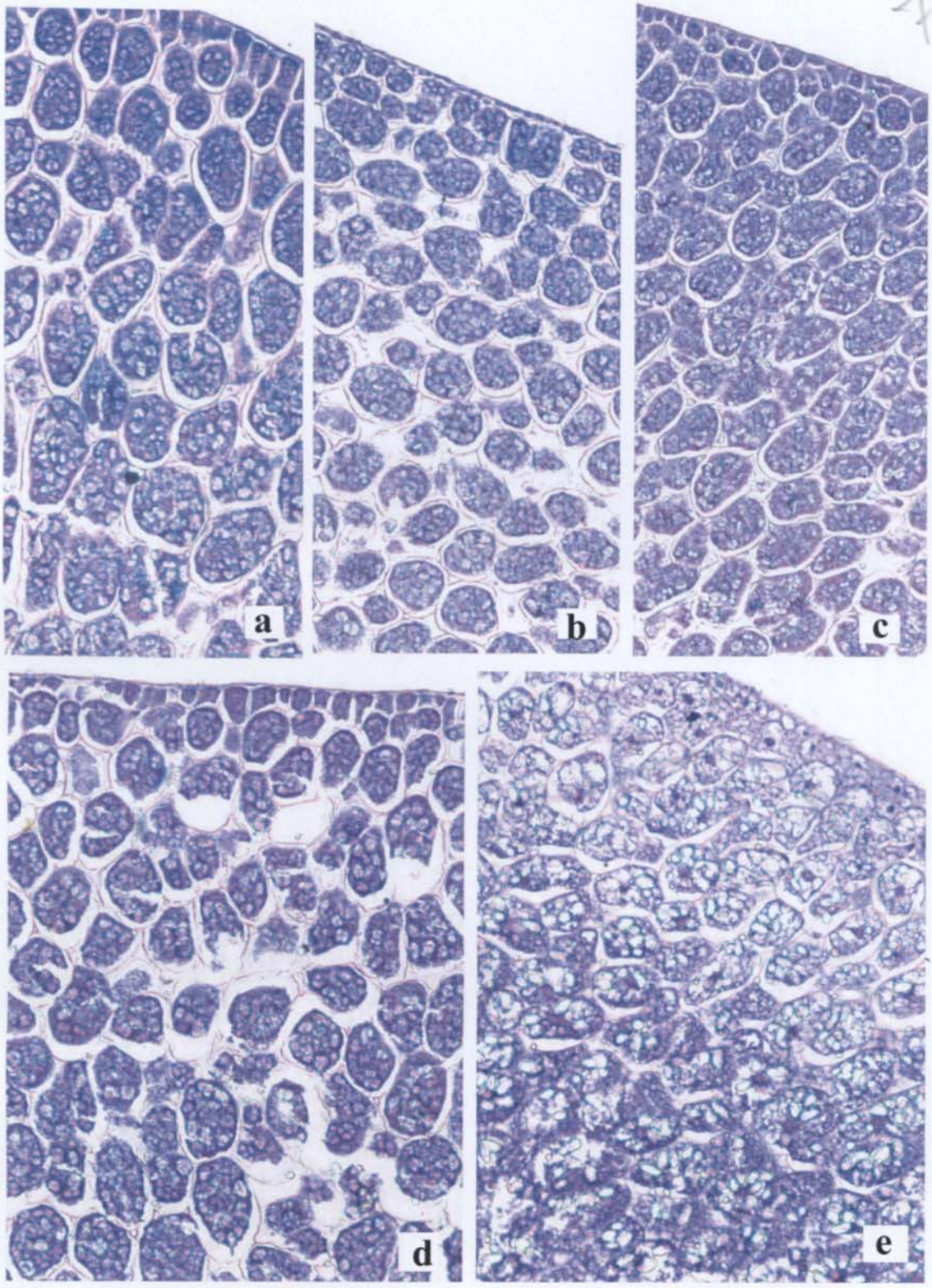


Plate 16: Protein distribution in the cotyledon of *Pisum sativum* seedling after 10th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C
c. Seeds treated at 40°C **d.** Seeds treated at 45°C
e. Seeds treated at 50°C

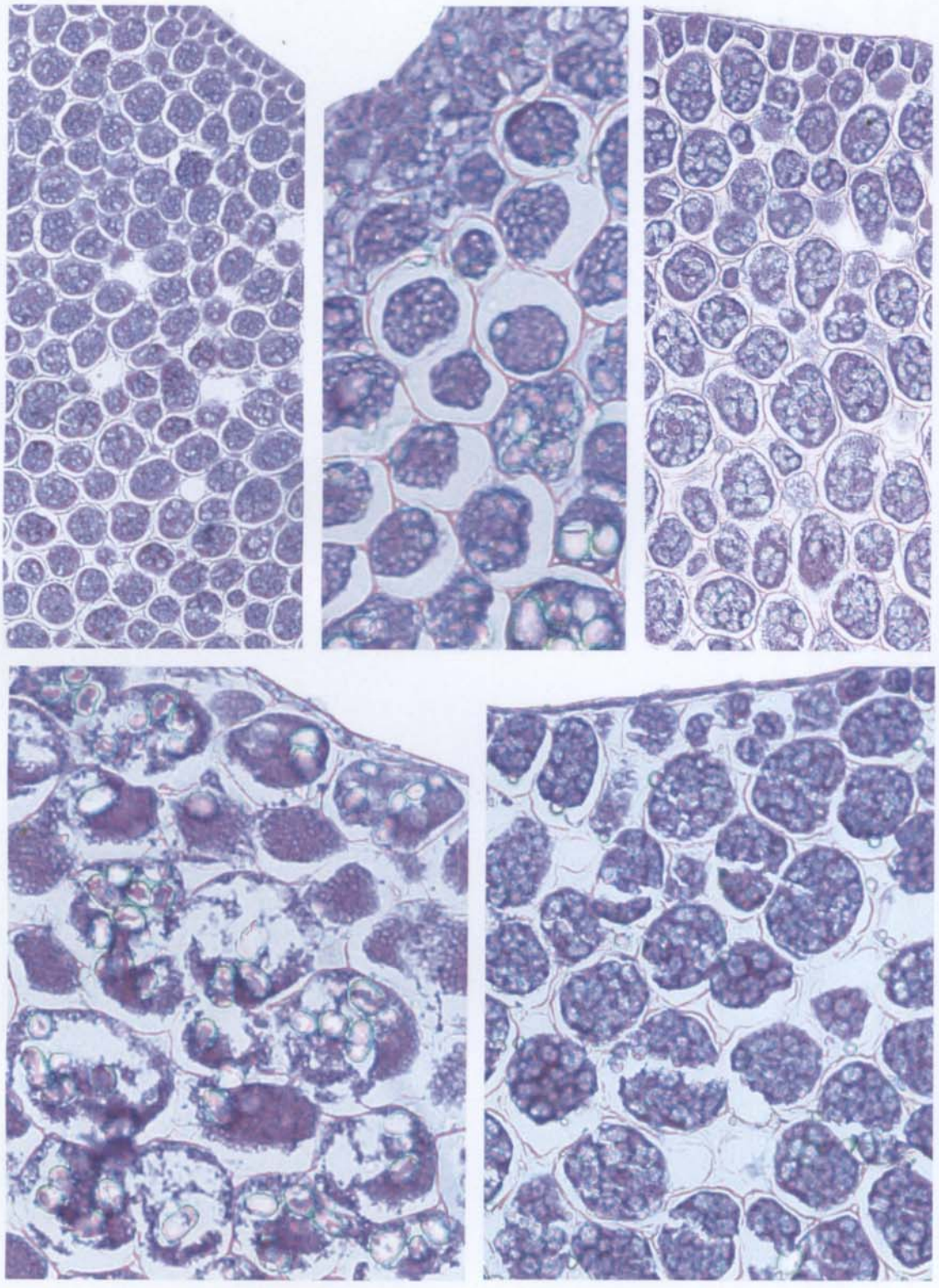


Plate 17: Protein distribution in the cotyledon of *Pisum sativum* seedling after 30th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C
c. Seeds treated at 40°C **d.** Seeds treated at 45°C
e. Seeds treated at 50°C

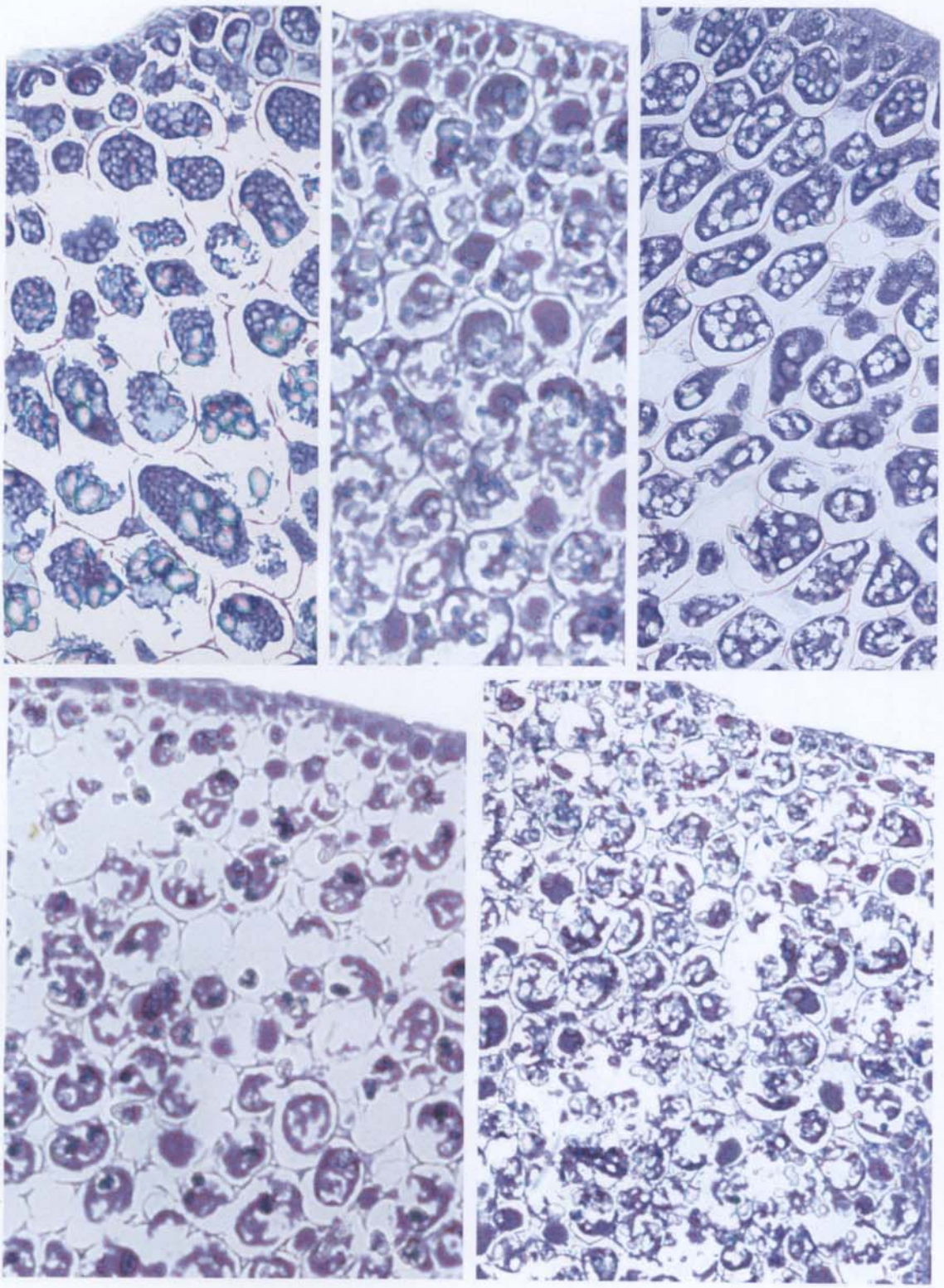


Plate 18: Protein distribution in the cotyledon of *Pisum sativum* seedling after 50th day of germination at cold.

a. Control seeds **b.** Seeds treated at 35°C

c. Seeds treated at 40°C **d.** Seeds treated at 45°C

e. Seeds treated at 50°C

palisade cells (macrosclerieds). These cells were closely packed with a pointed outer end. This outer end had a characteristic thick wall forming terminal cap. The outer region of the macroscleried was with a thick coating of suberin. Inner to this macrosclerieds were the hourglass shaped uniformly arranged Osteoscleried cells. Beneath the osteoscleried was parenchymatous layer of about 12-13 cells thick (Plate 19, Fig. a).

The testa of seeds treated at 50^0C (Plate 19, Fig. b) showed numerous broken areas in the outermost palisade cell layer. The macroclerieds cells were broken and were arranged in bundles making gap in between the bundle of cells. The osteosclerieds were unevenly distributed and some of them were broken. The parenchymatous region showed complete lysis of cells and number of cell layer also was decreased (Plate 19, Fig. b).

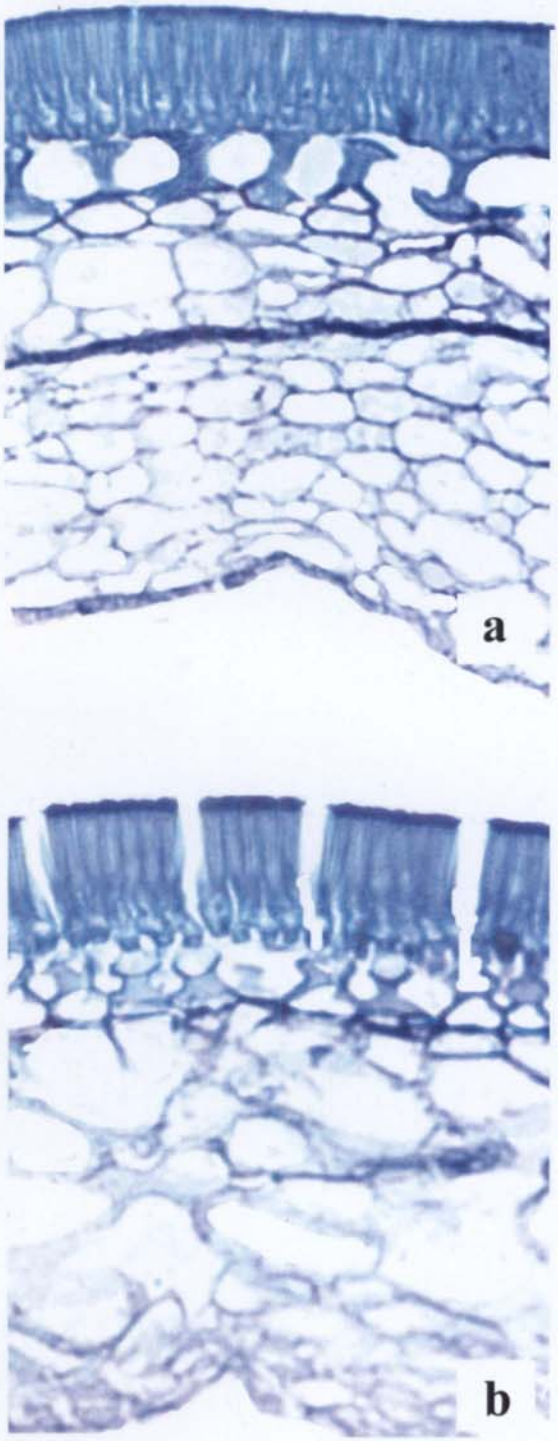


Plate 19: Seed coat (testa) of *Pisum sativum* seeds

a. Control seeds

b. seeds treated at 50 C

EFFECT OF TEMPERATURE ON PHYSIOLOGY OF RESERVE MOBILISATION IN *PISUM SATIVUM* L. SEEDS DURING GERMINATION

Dissertation submitted to the Faculty of Science
University of Calicut
In part fulfillment of the requirements for the degree of
Doctor of Philosophy
In Botany

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Discussion

Appropriate harvesting and proper post harvest technology are essential to ensure minimal deterioration during storage and to obtain seeds of maximum viability. Majority of orthodox seeds retain their viability when dried and hence seeds are stored in a dry state with low moisture content. In general, the higher the temperature-the higher the seed moisture content, the more rapidly is the viability lost (Roberts, 1973).

A number of studies have shown limits to the beneficial effects of drying on orthodox seeds due to detrimental effects of seed survival in storage condition when critical moisture content is overcome (Ellis *et al,* 1989). Drying of seeds before storage may cause damage to seeds to certain extent and the lower moisture content of seeds also may result in additional damage and these damages interfere with germination (Dell'Aquila, 1999). Drying injury results in deterioration and changes in seed vigour/seedling vigour, which in turn is dependent on reduced moisture content (Bewley and Black, 1994).

Water content of orthodox seeds declines steadily during post-developmental period and water loss becomes maximum on maturation drying or desiccation on the mother plant. The loss of water occurring during maturation drying probably commences when the funicular region joining the seed to the parent plant starts to shrivel thus cutting the supply of water and then water evaporation occurs through the desiccating outer structure such as pod wall, testa etc. With the progressive removal of water from seeds during drying, moisture content of seeds becomes significantly reduced.

Conformational changes occur in cell membranes upon drying of seeds (Seewaldt *et al,* 1981; Vertucci and Leopold, 1987; Senarathna *et al*, 1988; Bewley, 1997). Protein structure of seeds also is changed when most of the interstitial water is removed during drying (Rupley *et al,* 1983). According to Crowe and Crowe (1992), the membrane phospholipid component forms a gel phase during maturation drying. Notwithstanding, drying is a prerequisite for maintaining the seed longevity and quality.

Recently Bryant *et al* (2001) suggested that a common feature of desiccation tolerant orthodox seeds is the membrane phase behaviour during dehydration and rehydration. According to these authors, as a result of dehydration, the surfaces of membranes are brought into close approach, which causes physical stresses that can lead to a variety of effects including demixing of membrane components and fluid to gel phase transition of membrane lipids.

The vitrification of water during dehydration of orthodox seeds by natural drying or overdrying is explained as the glassy state depending upon the species/variety of seeds and during prolonged storage the glassy state may gradually decompose and/or will be

affected by temperature extremes resulting in loss of viability (Bewley and Black, 1994; Bryant *et al*, 2001).

Pisum sativum seeds, which are used in the present study, obtained from National Seed Corporation contained 12.3% of moisture content after air-drying at room temperature (Table 2). Being a member of leguminoseae *Pisum sativum* seeds are included under orthodox group since they survive desiccation.

Seeds of *Pisum sativum* are readily germinable and seeds having 12.3%-9.2% moisture content show 100% germination (Table 1). A characteristic feature of *Pisum sativum* seed is very slow and gradual decrease of moisture content and a concomitant retardation of germinability at higher temperature. Even when seeds are treated upto 70°C germinability is retained at significant level (Table 2). It is found that storage at high temperature (100°C) for prolonged period is required to obtain the constant value of dry matter.

In the present study *Pisum sativum* seeds show cent percent germination in control seeds and the seeds treated at 35°C and 40°C when their moisture content was 12.3%, 10.3% and 9.2% respectively (Table 2) . Several studies on seed germination in *P. sativum* are available pertaining to the effect of moisture content on germination and longevity (Perry and Harrison, 1970; Ellis and Roberts, 1982; Ellis *et al*, 1988, 1989, 1990), leachate constituents and conductivity during imbibition (Powell and Matthews, 1978), soaking injury and seed quality (Rowland and Gusta, 1977; Prusinski and Borowska, 1996) and temperature effect on germination (Munro *et al*, 2004).

Substantial information about the temperature at which seeds will germinate has now been accumulated (Roberts, 1972; Khan, 1978; Bewley and Black, 1982, 1985, 1994; Mayer and Poljakoff-Mayber,

1989; Copland and McDonald, 1995). According to Munro *et al*, (2004) *Pisum sativum* seeds are chilling resistant because they germinate in chilling temperature with no difference in the rate of carbon loss from cotyledon, carbon gain in the embryonic axis or total carbon loss of whole seedling measured by dry weight between control and chilled plants.

Effect of higher temperature on seed germination behaviour of *Pisum sativum* can be interpreted based on field emergence and germination percentage. Egli and Tekrony (1995) have drawn a correlation between germination percentage and field emergence in soyabean seeds and stated that if the field emergence index (FEI) is 100 or above an ideal condition for germination is indicated. In the present study control seeds and seeds treated at 35°C showed FEI=100 and in the treatments of 40°C, 45°C and 50°C FEI was reduced to 97.5, 99.4 and 96.1 respectively indicating an adverse effect of temperature on field emergence as reported in soybean seeds (Egli and Tekrony, 1995). Similar observations were made by TeKrony *et al* (1987) and Torres *et al* (2004) according to whom, under ideal sowing conditions, seedling field emergence percentage would be almost the same obtained in the standard germination test, and FEI would be equal to or higher than 100.

Eventhough cent percent germination was shown by control and experimental seeds the imbibition pattern showed significant difference. The imbibition rate of seeds treated at 35°C and 40°C became constant at about 6-8 hours irrespective of the treatment and control. The enhanced rate of imbibition in the case of seeds treated at 45°C and 50°C is due to wide gradient in water potential. The water potential of control seeds is much lower than that of the imbibition medium and in the case of overdried (at 45°C and 50°C) seeds also

very low water potential exists. But the observation of more or less similar pattern of imbibition curve in all treatments and control (Table 5) is found to be related to the biochemistry of metabolites of *Pisum sativum* seeds as suggested by Vertrucci and Leopold (1987) according to whom the integrated hygroscopic properties of cellular constituent especially the moisture absorbing capacity of legume seeds may be related to carbohydrate and lipid content of cotyledons. Temperature dependent changes observed in the histochemical localization of metabolites (Plate 7 to 18) and the anatomy of embryonic axis (Plate 5 & 6) are getting probably changed/resumed during hydration.

Data on moisture content distribution of *Pisum sativum* seeds treated at high temperature reveal strong influence of temperature on seed 'bound water' removal without losing viability upto 50°C when the moisture content was only 6.5% (Table 2). This observation is in partial agreement with the view of Vertrucci and Leopold (1987). According to Vertrucci and Leopold (1987) as desiccation of legume seeds proceeds during storage as well as prolonged drying there is no evidence of large structural changes in their membranes. It has been suggested that the lack of major conformational changes in membranes upon drying is an essential characteristic of desiccation tolerant orthodox seeds in general and the legume seeds in particular (Seewaldt *et al,* 1981; Leopold and Vertrucci, 1986; Crowe and Crowe, 1992).

One of the important characteristic features of seeds during germination is imbibitional injury and resultant solute leakage (Simon *et al,* 1976; Powell and Matthews, 1978, 1981; Prusinski and Borowska, 1996). Studies of imbibitional injury on *Pisum sativum* (Powell and

Matthews, 1978) showed that the sensitivity to imbibitional injury is caused by rapid water uptake by seeds. Seeds under dry storage as well as aged seeds exhibited increased leakage of solutes due to more imbibitional injury. The present author observed that seeds with reduced moisture content as a result of drying for 7 days at 45°C and 50°C showed abundant leakage of solutes (Table 6a & 6b) and were more sensitive to imbibitional injury compared to the control seeds. Studies of temperature effect on *Pisum sativum* seed (Prusinski and Borowska, 1996) revealed that overdried seed containing 6-8% moisture content was readily germinable irrespective of the mechanical damage shown by highest electrolytic conductivity of the exudates during germination.

Imbibitional injury of seeds is dependent on several factors such as seed maturation, age, moisture content and temperature (Tully *et al,* 1981; Powell, 1989; Taylor and Prusinski 1990). Rate of solute leakage is considered as a parameter of imbibitional injury (Bruggink *et al*, 1991; Doijode, 1988). According to Woodstock (1988) high leakage of organic and inorganic substances occurs during imbibition of seeds. In *Pisum sativum* (Prusinski and Borowska, 1996) high rate of solute leakage occurred during first 2 minutes of imbibition when dry embryo (decoated seed) was imbibed and the rate of leakage declined rapidly to a low constant leakage value after about 5 minutes. A comparison between the above observations and the present study is not feasible because of difference in structure of seeds due to temperature treatment and the mode of germination. In the present study conductivity measurement showed highest leachate conductivity in seeds treated at 45°C and 50°C when cumulative (Table 6a, Fig. 4a) and non-cumulative (Table 6b, Fig. 4b) methods were applied for the analysis of leachate and this finding is in conformity with the views of

Prusinski and Borowska (1996) according to whom *Pisum sativum* seeds with 6-8% moisture content and lowest germinability were more sensitive to imbibitional damage as shown by highest electric-conductivity of their exudates.

Role of testa as a barrier for imbibition and the difference in compounds from imbibing seeds have been studied in many seeds, inclusive of *Pisum sativum* (Simon 1974; Powell and Matthews, 1977). According to these authors in decoated embryo and seeds with damaged testa, more water was imbibed and leakage of solute increased about 117% compared to intact seeds. Greater leakage of electrolytes from the overdried and mechanically damaged seeds suggested that initial water content of the embryo (Taylor and Prusinski, 1990) and testa (Powell and Matthews, 1979) determine the result of conductivity test as well as plant establishment. During initial period, the conductivity of leachate of seeds treated at higher temperature (45°C and 50°C) only showed enhanced values compared to the control and treatments at 35°C and 40°C plausibly due to the cracks on the testa (Plate 19), which enhanced leakage of electrolytes or metabolites. Incidentally, leachate analysis showed maximum sugar content (Table 7) in these samples. Powell and Matthews (1978) suggested that in *Pisum sativum* seeds imbibitional damage was more due to more leakage through damaged testas. Taylor *et al* (1993) showed that in *Brassica* seeds leakage of the "sinapine" an alkaloid compound was increased by seed coat cracks. Similarly, positive correlation was drawn between the number of seed coat crack and leakage of solute in onion and leek seeds (Beresniewicz *et al,* 1995) and these authors suggested that seed coat cracks enhanced leakage of solute compared to seeds with intact testa. Several cracks are observed in *Pisum sativum* seeds in the outermost layer of the testa

and irregular distribution of osteosclerieds and the parenchymatous layer in the inner region of testa in seeds treated at 50°C (Plate 19, Fig. b). Ultra structural studies by scanning electron microscopy on *Cassia* sp. seeds (Bhattacharya and Saha, 1990) showed that these seeds exhibit seed coat imposed dormancy and testa breaking by H_2SO_4 treatment resulted in germination and the strophiole and the micropyle play critical role in imbibition. On the contrary, *Pisum sativum* seeds are readily germinable and imbibition takes place through the entire testa and the damage occurring on the testa during temperature treatment may result in more absorption of water, while intact testa imposes some limit to the leakage of solutes. Leakages of solute through testa have been used as a rapid method to assess seed quality (Simon and Mathavan, 1986; Abdul-Baki and Anderson, 1970). Nevertheless, leakage tests may be confounded by seed coat cracks that influence leakage rate.

Eventhough the loss of solutes from imbibing seeds is an important factor in determining the seedling vigour the conductivity measurements have been shown to be an unreliable indicator of pea seed viability particularly when applied to individual seeds (Daczmal and Ratajczak, 1993). A reliable explanation of the influence of imbibitional damage resulting in leakage of minerals and metabolites has been given by Powell (1986); Spaeth (1987), Bruggink *et al* (1991). Crowe and Crowe (1986) stated that membranes become disorganised in dry seeds the cause of which is not fully known. Cortes and Spaeth (1993) investigated the origin and characterisation of electrolytes loss as a result of ageing of seeds by compartmental analysis of potassium efflux in submerged *Pisum sativum* embryos during imbibition and suggested that aged seeds showed increased amount of potassium

since the potassium ions were available for efflux from both compartments.

Temperature treatment or heat shock has been proposed to assess the vigour of seeds exhibiting high germination despite some deteriorated qualities like ageing and/or poor seedling emergence and vigour (Dell'Aquila, 1999). According to Dell'Aquila (1999) in lentil seeds, the heat shock induces ageing of seeds and a measurement of leachate conductivity varies in relation to temperature treatment. However, the leachate conductivity test is expressed to be a poor indicator of seed vigour level in legumes (Fernandez and Johnston, 1995).

Studies on the effect of higher temperature treatment on seeds viability/germination are very scanty, however in *Zizyphus mauritiana* seeds higher temperature treatment (30°C) compared to room temperature (20°C-25°C) resulted in germination reduction (Murthy and Reddy, 1989). Seeds treated at various temperatures and subjected to standard laboratory germination tests showed that germination percentage reached 93% and 78% in *Pisum sativum* seeds treated at 45°C and 50°C respectively (Table 1). Ellis and Roberts (1982) found that *Pisum sativum* seeds soaking injury resulted in a reduction of only 7% germination when the seeds were desiccated to reduce the moisture content from 16.8% to 7.2%. Another investigation on the germination behaviour of pea seeds (Ellis *et al*, 1990) showed that the proportion of seeds germinated normally in standard germination tests declined progressively from 94% to 50% when the seeds were dried from 14.8% to 3.7% moisture content at ambient temperature.

Occurrence of seed hardedness (at 50°C only) was an important observation in the present study (Table 1). Irreversible seed hardedness induced by desiccation has been already reported in

Pisum sativum seeds at moisture content of 7.9% (Ellis *et al*, 1990). However, in the present study seed hardedness was observed when the moisture content was reduced to 6.5% by treating at 50°C for 7 days. On scarification it was found that the seeds were not at all viable. According to Sundstrom (1990) in tabasco pepper seeds temperature treatment resulted in reduced germination percentage due to induction of temporary dormancy at low moisture content. But seed vigour retained unchanged even at lower level of moisture content. These observations are interpreted in such a way that low moisture content as a result of overdrying in tabasco pepper seeds and the resultant moisture content reduction to 5.8% might be due to removal of water content only from sorption zones II and III (Vertrucci and Leopold, 1984; and 1987) which did not affect any significant desiccation injury in the seeds. Slow germination rate was another character shown by seeds treated at 45°C and 50°C (Fig. 2) compared to control seeds. In a similar study Mai-Hong *et al,* (2003) observed that the seeds of *Peltophorum pterocarpum*, a tree legume, the hardseededness was induced when seeds were dried to about 15% moisture content. The normal seedling growth or the germination percentage was affected by over drying. It increased the time for germination and caused abnormalities in seedlings. In *Raphanus sativus* L. var, *longipinnatus* Bailey cv. Xin Li Mei seeds on treatment with dry-heat (76°C) for 12 hour to 72 hour, at 3.2% seed moisture content, germination and vigour were maintained well after 48hour drying, but vigour was reduced after 60 hour and germination after 72hour. At 9.4% seed moisture content, germination was reduced significantly after 36 hour and vigour was reduced after 12 hour. These reductions of seed quality were increased as initial seed moisture content was increased (Meng *et al,* 2003).

Critical moisture content in *Pisum sativum* to obtain 100% germination was found to be 9.2%. Afterwards moisture content and viability are getting proportionally reduced. So desiccation injury is imposed on *Pisum sativum* seeds below moisture content 9.2% despite viability retained to reasonable extent (Table 2). It was observed that cent percent germination was obtained in seeds treated at 40°C when the moisture content was only 9.2% even though the parameters like imbibition pattern, SVI (Table 3) etc were significantly changed compared to the control. A significant reduction in the moisture content of seeds did not show any adverse effect on germination of pea seeds. This observation is in accordance with earlier reports in *Pisum sativum* seeds (Ellis and Roberts, 1982; Ellis *et al*, 1990).

Contradictory to the earlier reports, adverse effect of high temperature on seed germination in *Catharanthus roseus* seeds showed that pre-treatment at 30°C made the seed more permeable and the leachates were significantly higher at 30°C due to more permeability caused by high temperature treatment compared to the ambient temperature around 25°C (Choudhury and Gupta, 1995). More or less similar results have been reported in *Festuca* sp. (Brar and Palazzo, 1997) and *Abelmoschus esculentus* (okra) seeds (Demir, 2001). *Pinus sylvestris* and *Pinus halepensis* seeds treated at 70°C showed increase germination percentage (Núñez and Calvo, 2000). In wheat (Dell' Aquila and Di Turi. 1996) and in barley (Dell' Aquila *et al*, 1998) high germination potential was observed in terms of leachate electrical conductivity and protein synthesis by heat treatments of seeds.

When temperature treated seeds were germinated in refrigerator (3±1°C) the germination performance was entirely different from that of room temperature. A chronological comparison of

germination at room temperature and in refrigerator can not be made because it took more or less 10 days (Plate 2) for the radicle emergence in refrigerator while at room temperature this period was only 1 day (Plate 1). However, germination pattern was almost similar to that of room temperature even though more period was required in refrigerator. Morphologically, seedlings showed more vigorous growth especially proliferation of root system in the cold condition compared to that of room temperature and there was no significant difference between control and experimentals (Plate 3).

Deteriorative effect of desiccation due to higher temperature treatments was more when germination was conducted at room temperature (28±4°C) compared to germination performance in the cold condition (3±1°C) in terms of germination percentage and seedling vigour. Nevertheless, in refrigerator there was no difference in biomass between control and treatments (Table 4). The hardseededness was not observed when seeds treated at 50°C were germinated under cold condition. Zheng *et al* (1979) reported that seeds subjected to priming by Polyethylene Glycol (PEG) treatment responded to chilling stress at about 10°C during 1st hour of imbibition. But if the chilling stress was imposed following some hours of warm soaking it resulted in little or no distinct reduction in percentage germination or seedling vigour (Zheng *et al*, 1980). According to Zheng (1991) soybean seeds treated with PEG, resulted in a decreasing gradient of water potential between outside and inside of seeds and therefore the rate of water uptake was varied. Germination decrease occurred when the rate of water influx was enhanced resulting in more imbibitional injury. Similarly in *Pisum sativum* seeds temperature treatment resulting in reduction of seed moisture content is also a positive factor causing imbibitional injury due to wide water potental gradient.

Chilling injury is a physical disorder that occurs at temperature below 12°C. One of the symptoms of chilling injury is delayed germination and stunted radicle growth during early phase of germination in *Cucumis sativus* (Saltviet and Morris, 1990). . In the present study seeds germinated in refrigerator exhibited chilling injury expressed as stunted radicle growth during early phase of germination only (Plate 2) but the normal growth was regained after 10 days and profuse root growth was established. The heat shock effect seems to be nullified during germination in the cold condition, whereas seedlings raised at room temperature showed retarded growth in seeds treated at 45°C and 50°C (Plate 1).

The anatomy of radicle tip of temperature treated seeds germinated at room temperature showed considerable damage on the cells of root cap region as well as in the epidermal cell of the entire root tip in the seeds treated at 45^0C and 50^0C (Plate 5). But, this damage was not seen when the seeds were germinated in refrigerator (Plate 6). More or less a similar observation was made by Collins *et al* (1995) who stated that in mung bean hypocotyls, acquisition of chilling tolerance was established when subjected to heat shock.

According to Markowski (1998) chilling injury can be prevented if seeds are exposed to chilling in a more hydrated state, moisture content being more than 10% or after a first imbibition period at warm temperature. The present study in *P. sativum* seeds shows imbibitional injury is prevented or ameliorated during germination in refrigerator (Table 2 and 8) even when the moisture content is less than 10% (about 6.5%).

Berry and Raison (1982) hypothesised that imbibition of seeds is necessarily controlled by the structure of cell membrane lipids. At a

given temperature if the membrane lipids are in gel phase, formation of continuous bilayer may be functionally imperfect. The semipermeability of membrane lipid is lost and membrane structure is disrupted by inrush of water during imbibition since a wide water potential gradient exists between the seed and medium. In the present study imbibitional injury is highly reduced under cold condition compared to room temperature presumably due to very slow or moderate rate of water diffusion occurs in the cold condition instead of rapid inrush occurring at room temperature. It is well established that temperature is one of the important factors influencing water diffusion. Moreover magnitude of imbibition as well as germination rate is very slow under cold condition. It takes roughly 10-times increase in the period of imbibition and germination. Hence in *Pisum sativum* seeds chilling injury during imbibition is almost absent and this observation is inconsistent with the view of Stewart and Bewley (1981) who suggested no relationship between fatty acid composition of membrane and chilling sensitivity in soybean embryonic axis.

In *Pisum sativum* seeds as in the case of germination behaviour in refrigerator, leachate conductivity also did not show any difference between the control and all treatments (Table 10a & b). Contradictory to the germination behaviour of seeds at room temperature, seeds germinated in the refrigerator showed no direct relationship with water content (expressed in mg/g fresh tissue) and treatment temperature since all tissues showed almost similar values (Table 9). According to Bochicchio *et al* (1991) maize seeds absorbed water very slowly and hence imbibitional injury was independent of chilling temperature and seeds experienced the lowest inrush of water into, showed highest radicle growth.

When the leachate conductivity and rate of water uptake (Table 9) were correlated they did not show their highest values when seeds with low moisture content were germinated under chilled condition. In the cold the leachate conductivity was more compared to that of room temperature even though the speed of germination was delayed. Nevertheless, changes were not significant between control and experimentals germinated in refrigerator. The pronounced accumulation of leachate in the cold condition is due to reduced cellular membrane repair at low temperature (Douglass *et al*, 1993). However, it is concluded that imbibitional injury and low moisture content are not at all directly related to chilling temperature at which loss of semipermeability of membrane lipid is believed to occur according to the hypothesis of Berry and Raison (1982).

Many hypotheses have been offered to explain the cellular events of chilling injury and a phase transition of membrane is still thought to be the initial step in a chain of events that results in chilling injury (Nishida and Murata, 1996). In chilling sensitive plants, radicle growth is an indication of chilling stress and the chilling sensitivity is shown by the incremental decrease in subsequent elongation of cucumber *(Cucumis sativus)* radicle (Rab and Saltviet, 1996). Eventhough the slow growth rate of radicle was shown by *Pisum sativum* seeds during early stages of germination, profuse root development occurred as seedling growth advanced and hence the inhibitory effect of high temperature (45^0C and 50^0C) was not expressed (Plate 2 & 3). Mangrich and Saltviet (2000) reported that cucumber radicle exposed to chilling temperature for 6-7 days resulted in severe injury and the radicle failed to elongate. These observations are similar to the finding of Jennings and Saltviet (1994) who reported that irreversible chilling injury occurs in cucumber seedling after 4 days

at 25°C. According to Mangrich and Saltviet (2000) cucumber seeds exposed to heat shock of 40°C for 4-12 minutes increased chilling tolerance such that 4 days of chilling caused only 30% decrease in radicle growth compared to 66% for seedling which are not heat shocked. The ability of heat shock to ameliorate the effect of chilling on subsequent radicle elongation was affected by the severity of chilling. In *Pisum sativum* seeds the heat shock given at 45°C and 50°C resulted in seedling growth retardation at room temperature (Plate 1) but the heat shock was ameliorated by chilling treatment since in all experimentals (treatments at 35°C, 40°C, 45°C and 50°C) seedlings exhibited uniform proliferated growth in cold condition (Plate 2 & 3). This quality of *Pisum sativum* seeds seems to be an ecological adaptation to grow at a wide range of temperature regime as suggested by Davies *et al* (1985). Hence *P. sativum* seeds are chilling tolerant since chilling sensitivity restricts wide geographical distribution of plants.

Recently Munro *et al* (2004) suggested that *Pisum sativum* is a chilling tolerant plant with respect to respiration rate, lipid peroxidation and ubiquinone content distribution. Pea seedlings under chilled condition showed no difference in the rate of carbon loss from cotyledon, carbon gain in the embryonic axis or total carbon loss of the whole seedlings measured by dry weight between control and chilled plants. During low temperature treatment lipid peroxidation was measured by malondialdehyde (MDA) accumulation and was not increased in comparison with control seedlings and total ubiquinone content was not decreased. According to these authors, antioxidant system might have protected both total ubiquinone and membrane lipids in pea seedlings owing to their chilling tolerance.

Data of biomass productivity expressed as seedling vigour showed no difference between control and treatments when temperature treated seeds were germinated under chilled condition (Table 8). At the same time inhibitory effect of temperature treatment was evident when the germination/seedling growth was done at room temperature either in Petri dishes or under field conditions. So it is confirmed that *Pisum sativum* seeds are chilling tolerant and heat shock effect is ameliorated in the cold condition. *Pisum sativum* seeds are characterised by desiccation tolerance also owing to their viability retention (100% germination) when treated upto 50°C and more than 50% germination in seeds treated at 60°C and 70°C (Table 8).

Pisum sativum is included in the non-endospermic group of legumes in which endosperm is absorbed during seed development and cotyledons then become the major storage organ. Fewer studies have been conducted on the mobilisation of carbohydrate in legumes compared to the cereal (Bewley and Black, 1994). Nevertheless, a general pattern of events in legume seed reserve mobilisation during germination has emerged (Davis, 1979; Sumathi *et al,* 1995; Nkang, 2002).

Amylase activity of *P. sativum* seeds during germination in the dark at room temperature was very slow upto 3 days and maximum activity was shown on 9^{th} and 10^{th} days by control as well as treatments of 35°C and 40°C. But in high temperature treated experimentals (seeds treated at 45°C and 50°C) amylase activity was comparatively lower than the control and other experimentals (Table 14a). Earlier studies on pea cotyledons (Swain and Dekker, 1969; Juliano and Varner, 1969; Yomo and Varner, 1973) showed dramatic

increase of amylase activity between days 7-10 when germination occurs in the dark.

Oishi and Bewley (1990), after studies on α-amylase activity in maize kernels during germination, suggested that endosperm abscisic acid (ABA) plays a role in inhibiting/preventing germination by suppressing α-amylase synthesis. Temperature stress is known to induce increased production of ABA (Buchanan *et al,* 2000) and hence in *P. sativum* seeds treated at 45°C and 50°C, temperature stress induced synthesis of ABA and resultant inhibition of α-amylase synthesis cannot be ruled out. Similarly water stress tolerance also is controlled by abscisic acid (Zhu, 2002). In the present study seeds treated at 45°C and 50°C suffer both temperature and water stress (over desiccation) both process leading to enhanced synthesis of ABA, (Buchanan *et al,* 2000 ; Hopkins, 1999; Taiz and Zeiger, 2002) which in turn inhibits amylase synthesis.

According to Davis (1979) in pea seeds the changes in α-amylase activity during germination are due to changes in enzyme synthesis and the increase was independent on seedling development. In the present study the above view of increased enzyme synthesis is evident by the enhanced amylase activity and concomitant reduction in starch (Table 11a) observed during 7th day onwards in control and treatments of 35°C and 40°C. Histochemical localisation of starch by PAS reaction also confirmed the reduction of starch in the cotyledon of these seedlings (Plate 9, Fig. b &c). But in the case of seeds treated at 45°C and 50°C amylase activity and starch distribution proportionality are not agreeable with the above concept because significant decrease in starch as well as significantly reduced amylase activity was observed (Table 14a & 11b). So it is presumed that non-enzymatic degradation

of starch might have occurred at high temperature (45°C and 50°C). Shephard *et al* (1995) has correlated α-amylase activity with sun drying and seed vigour depression in rice seeds. Contradictory to the present study α-amylase showed an increased activity followed by desiccation in rice seeds.

A reason for reduced starch content of seeds treated at 45°C and 50°C after 4 days of germination at room temperature may be the efflux of granular starch along with other leachate constituents as already reported in pea seeds during imbibition (Speath, 1987) The cracks appeared on the testa could be another reason associated with the efflux of starch grain to the germination medium (Plate 19). This conclusion is confirmed by the observation that very few number of starch grains are present in the cotyledon of seeds treated at 45°C and 50°C shown by histochemical localisation after 5-10 days of germination compared to the control seeds (Plate 8 & 9). Starch grain was scattered all over the cells showing broken cell walls. The testa of seeds treated at 45°C and 50°C showed number of cracks facilitating easy efflux of starch grains from the damaged cells of the cotyledon (Plate 19, Fig. d).

Distribution of soluble carbohydrates particularly sugars are related to desiccation tolerance in maturing seeds (Hoekstra *et al,* 1994; Popp and Smirnoff, 1995; Nkang, 2002). The role of sucrose and raffinose has been interpreted as agents of stabilisation of membrane lipid during desiccation (Koster, 1991; Koster *et al,* 1994; Wolkers *et al,* 1998; Buitink *et al,* 2000) and sucrose vitrification around fluid membrane phosopholipid stabilise the membrane during desiccation (Koster *et al,* 1994). According to Buchanan *et al* (2000) increase in the soluble non-reducing sugars occurs during desiccation.

The accumulation of raffinose is associated with desiccation tolerance and it is important for the longevity of mature dry seeds (Bewley and Black, 1994).

In *P. sativum* total sugars of control and seeds treated at higher temperature did not show much significant qualitative/quantitative changes. So it is inferred that *Pisum sativum* seeds are highly tolerant to desiccation since sucrose and raffinose family oligosaccharides are present at significant levels in the control seeds and further changes were not occurring during further higher temperature treatment. Desiccation tolerance and accumulation of sugars in general, raffinose family oligosaccharides in particular are related to each other (Blackman, 1992, 1995; Sun *et al,* 1994). The sugar content of seeds treated at 45°C and 50°C was comparatively less than that of control and other treatments and this reduction was noticed only during 4^{th} day of germination onwards probably indicating maximum leakage of soluble sugars through the broken cell wall and damaged testa as mentioned earlier (Table 12 a, b, c, d & e).

In the seeds of maize (Brenac *et al,* 1997) and soyabean (Blackman *et al,* 1992, 1995) desiccation tolerance and concomitant accumulation of raffinose family oligosaccharides and/or sugars have been reported. According to Chen and Burris (1990) and Brenac *et al,* (1997) raffinose accumulation has been associated with tolerance to high temperature drying of mature maize seeds. In *P. sativum* seeds the ratio of raffinose/sucrose is around 1:10 in control and experimentals (Table 12 a, b, c, d & e) and this observation indicates desiccation tolerance of pea seeds and this finding is in agreement with the view of Bernal-Lugo and Leopold (1992 and 1995), according to

whom the desiccation tolerant seeds must have raffinose to sucrose mass ratio 1:10.

The decrease of all raffinose family oligosaccharides during the 24 hour of imbibition in *P. sativum* seeds is similar to the pattern that has been found in many seeds in which these oligosaccharides are present (Kuo *et al,* 1988) and the current theory regarding the role of raffinose family oligosaccharide in the seed is that they are stable storage forms of sugar and positively correlated with longivity (Bernal-Lugo and Leopold, 1992; Lin and Huang, 1994).

According to Koster and Leopold, (1988) sucrose and oligosaccharides play a role in promotion of vitrification of water and facilitate the stabilization of lipid and protein in the cell membranes by binding to phospholipids forming hydrogen bonding. Similarly sucrose, raffinose and stachyose are abundant in desiccation tolerant seeds (Bewley and Black, 1994). In soybean seeds it has been reported that there is a conversion of previously existing monosaccharide to sucrose and/or oligosaccharide and these sugars facilitate stabilization of protein and lipids in the cell membranes during desiccation period (Blackman *et al,* 1992). Lahuta *et al* (1998) reported relationship between desiccation tolerance and soluble sugars from pea seed germination. The loss of desiccation tolerance in the seedlings was accompanied by rapid degradation of raffinose family oligosaccharides and accumulation of fructose and galactose. In *Lupinus luteus* highest rate of oligosaccharide accumulation appeared during seed desiccation and was correlated with the acquisition of the ability to germinate (Gorecki *et al,* 1997).

Another correlation between desiccation tolerance and sugar distribution was interpreted by Brenac *et al,* (1997) and Corbineau *et*

al, (2000) and they suggested that the ratio of sucrose/raffinose + stachyose is 20:1 to acquire desiccation tolerance in seeds. In *Pisum a sativum seed* the ratio of sucrose/raffinose + stachyose is 10:2 (Table 12 a, b, c, d & e) in control seeds and during further desiccation due to temperature treatment this ratio remains unchanged and the viability also is retained in all the experimental samples.

Sucrose is the most abundant sugar of control and temperature treated seeds and their concentration showed absolutely no changes during drying upto 50°C. This observation once again confirms that the desiccation tolerance of *Pisum sativum* seed acquired during seeds maturation drying and further drying at higher temperature does not affect this quality.

During germination of *Pisum sativum* seeds in the control and treatments at 35°C and 40°C reducing sugars (fructose and glucose) were getting reduced and raffinose disappeared from second day onwards (Table 12 a, b & c). Almost similar observations were made in maize seeds (Koster and Leopold, 1988) and it was interpreted that loss of raffinose and increased level of reducing sugars are associated with desiccation tolerance. However, in *P. sativum* seeds treated at 45°C and 50°C reducing sugars are significantly reduced during germination and raffinose content was not changed compared to the control (Table 12 a, b, c, d & e). This reduction of total sugar as well as reducing sugars during germination is presumably associated with leakage through damaged cotyledonary cells and testa as shown in leachate analysis (Plate 7).

The significant reduction in the distribution of soluble sugars in the seeds treated at 45°C and 50°C during earlier period of germination is correlated with enhanced desiccation or ageing. Ageing is believed

to occur by different stress such as temperature stresses (Buchnan *et al,* 2000). In soybean seeds soluble sugar content reduction is correlated with desiccation and ageing (Robert and Yaklichi, 1985).

Despite the abundance of raffinose family oligosaccharides in dry seeds of control and experimentals of *P. sativum* their complete disappearance after imbibition indicates the cleavage/degradation of raffinose family oligosaccharides but a concomitant reduction in the sucrose also is evident. These findings lead to the speculation that the sucrose is metabolised/ transported to the developing seedling. This is a common metabolic scenario in the seeds of many angiosperms (Bewley and Black, 1994; Kuo *et al,* 1988. Downie and Bewley, 2000). The role of raffinose family oligosaccharides as an energy source for events early in the seed germination has been a well established concept (Kuo *et al,* 1988; Nichols *et al,* 1993; Buckeridge and Dietrich, 1996; Dirk *et al,* 1999; Downie and Bewley, 2000).

Sugars are suspected to have cryoprotective effect as; they stabilize proteins and lipids of membranes during dehydration induced by low temperature. In winter wheat, the greater the sucrose concentration, the greater the freezing tolerance. Sucrose predominates among soluble sugars associated with freezing tolerance, but in some species raffinose, fructans, sorbitol or mannitol serve the same function (Taiz and Zeiger, 2002).

The increased content of total sugars in control and treated seeds during the early phase of germination in the cold is apparent though statistically not significant. This observation is inconsistent with the findings of Kao and Rowan (1970) in *Pinus radiata* where glucose and fructose were increased during germination under cold condition.

Contradictory to the distribution of sugars during germination at room temperature, germinating seeds in the cold showed the occurrence of oligosaccharides raffinose, rhamnose and stachyose (Table 13 a, b, c, d & e). A similar observation was made in *Picea glauca* by Downie and Bewley (2000) who suggested that in cold condition raffinose family oligosaccharides are synthesised after about 6 days of imbibition and this synthesis is continued until the germination is completed at 4°C. According to Wiemken and Ineichen (1993) the enzymes responsible for raffinose family oligosaccharides synthesis are activated at low temperature in conifers. Sprenger and Keller (2000) and Pennycooke *et al* (2003) also reported cryoprotective nature of raffinose family oligosaccharides. The raffinose family oligosaccharide content which remained unchanged during treatment at high temperature declined and completely disappeared during germination at room temperature (Table 12 a, b, c, d & e) whereas in refrigerator (Table 13 a, b, c, d & e) these raffinose family oligosaccharide except verbascose was found to be present up to 50 day of germination even though some slight fluctuations were observed during earlier phase of germination. The physiological significance of this finding is not clear. However, according to Hurry *et al,* (1995) exposure of tolerant plant to low temperature corresponds with an accumulation of soluble carbohydrate predominantly sucrose and hexose phosphates in plants.

It is now generally accepted that sucrose synthesis occurs in germinating seeds and it appears that predominantly sucrose phosphate synthase (SPS) is the enzyme (along with sucrose synthase) responsible for sucrose synthesis (Winter and Huber, 2000). Contradictory to this statement, in *P. sativum* seeds germinated in the cold sucrose was getting gradually decreased despite a transient

increase on 10th day. According to the view of Rosa *et al,* (2004) in *Chenopodium quinoa* seeds at low temperature induce sucrose phosphatesynthase, and sucrose synthase enzymes. The significant decrease in sucrose is presumably due to an enhanced rate of translocation from cotyledon to profusely growing seedling which is a very powerful metabolic sink. In otherwards in the cold sucrose synthesis is presumed to occur via SPS pathway followed by sucrose phosphate phosphatase activity which is energetically more feasible (Buchanan *et* al., 2000) then the sucrose is immediately getting translocated. An indirect correlation between SPS activity and germination/seedling growth in the cold is in conformity with the view of Dell' Aquila and Di Turi (1996) and Dell' Aquila *et al* (1998) in wheat and Barley respectively that high potential of germination is induced by heat shock treatment of seeds followed by germination under cold conditions.

Protein turnover during germination of seeds is regulated by synthesis and degradation. There is a general trend of decline in total protein distribution during germination of control and experimentals in *P. sativum* seeds (Table 16a). But there is no significant difference between control and experimentals in each interval. Electrophoretic protein profile showed no difference in protein profile during germination of control and experimental seeds at room temperature. Contradictory to this observation soybean seeds treated at 40°C (Key *et al,* 1981) and maize seeds treated at 41°C (Riley, 1981) showed protein reduction during germination. In wheat, ribosomal inactivation and resultant inhibition of protein synthesis occurred at 45°C (Fehling and Weidner, 1986).

It is commonly found that with exposure to high temperature there is decline in normal protein synthesis together with selective synthesis of characteristic set of heat shock proteins having molecular masses in the range of 15-40KDa (Vierling, 1991). Studies on synthesis and breakdown of heat shock proteins revealed that abundance of heat shock proteins occur at high temperature during earlier hours of temperature treatment and get decreased or disappeared thereafter (Iba, 2002).

Late-Embryogenesis-Abundant (LEA) protein, which accumulates during seed development and desiccation, contributes to the total protein in *P. sativum* since it is desiccation tolerant. This total protein is not directly related to seed viability during storage or ageing (Grelet *et al,* 2005). In the present study despite the temperature treatment at 45°C and 50°C total protein is not significantly reduced (Table 16a). It seems that *P. sativum* seeds are well adapted to high fluctuation in temperature, that is, temperature up to or more than 50°C is not stressful to protein metabolism as well as viability of *Pisum sativum* seeds.

During germination at room temperature both control and experimental seeds exhibited a fast reduction in protein content retaining only less than one fourth on 12^{th} day as a result of catabolism and mobilisation to the growing seedling (Fig. 11a). Histochemical localisation studies also showed gradual depletion of protein content during germination up to 12 days (Plate 13 to 15). It seems that protein metabolism/mobilisation is very significant in *P. sativum* seeds during later days of germination/seedling growth since starch content mobilisation is very fast retaining only less than 3-4% on 12^{th} day. Juliano and Varner (1969) reported a fast reduction in extractable

protein content along with the increasing activity of α-amylase during 4-9 days of germination at dark in *P. sativum*. Almost similar observations are obtained in the present study also.

SDS-PAGE of protein profile in control seeds and treatments did not show any significant changes during germination at room temperature in *P. sativum* seeds (Plate 4). So correlation cannot be drawn between temperature stress given to dry seeds and synthesis of heat shock proteins. However, Gumilevskaya *et al,* (1996) reported the synthesis of high molecular weight and low molecular weight HSPs at high temperature that is from 28°C to 38-40°C for 2-4 hours in pea seedlings but not in seeds. Notwithstanding, a number of authors have made reports on germination parameters and synthesis of heat shock proteins in seeds such as *Festuca* sp. seeds (Brar and Palazzo, 1997), wheat (Dell' Aquila and Di turi, 1996) and Barley (Dell' Aquila *et al,* 1998). Involvement of HSP and their chaperon activity is interpreted in plant tissues like seedlings (Gumilevskaya *et al*, 1996), cotyledon (Lafuente *et al*, 1991), embryonic axis (Mansfield and Key, 1987) and aleurone layer (Brodl and Ho, 1991) subjected to high temperature treatments. In the present study it seems that *P. sativum* seeds are highly tolerant to heat stress and so involvement of HSPs is not essential.

When germination of control and heat-treated seeds were conducted in refrigerator, additional protein polypeptides were observed in comparison with the germination at room temperature (Plate 4c) but these bands can not be considered as proteins produced to withstand chilling stress because according to Guy (1990) a number of enzymes and proteins are produced/activated during cold acclimation and all of them are not HSPs. A number of enzymes show

shift in isozymic composition upon exposure to low temperature, and numerous electrophoretic studies have shown both qualitative and quantitative differences in the protein content between non acclimated and cold acclimated tissues. This apparent increase in protein synthetic capacity of *P. sativum* seeds during germination under cold supports the idea that the synthesis of housekeeping proteins are continued to be synthesised during low temperature acclimation (Laroche and Hopkins, 1987).

Chilling tolerant plants such as potato, pea, soybean, cucumber, egg plant, rice etc. show increase in respiration particularly via the cyanide insensitive alternative respiratory pathway as a response to chilling (Moynihan, *et al,* 1995). Because the electron transport chain of the alternative respiration has no energy conservation site between ubiquinone and oxygen it dissipates almost all the chemical energy of respiratory substrate as heat. It has been suggested that heat generated by the increased electron flux through the alternative pathway can counteract the deleterious effect of chilling on the fluidity of mitochondrial membrane as well as on the activity of respiratory enzyme (Buchanan *et al*, 2000). Significant reduction of biomass and insoluble metabolites like starch (Table 8) in seedlings germinated in refrigerator in comparison with that of room temperature (irrespective of temperature treatment of seeds) (Table 11b) can be correlated to the enhanced utilisation of respiratory substrate via alternative cyanide insensitive pathway in *Pisum sativum* when germination is performed in the cold condition.

Legume seeds in general and *P. sativum* seeds in particular show aspargine as the transporting form of amino acid (Mayer and Poljakoff-Mayber, 1989). The metabolism of amino acids in *P. sativum*

cotyledon is unusual in that a major transported form of amino acid and the one that accumulates in the cotyledon is homoserine (Bewley and Black, 1994). Glutamine is another transporting form of amino acid.

In the present study instead of homoserine, threonine is the abundant free amino acid (Table 17 a, b, c, d & e). The threonine is synthesised from homoserine via phosphorylation and threonine synthase activity. The deviation in this pathway of threonine synthesis is not clear. Nevertheless, threonine along with lysine and methionine is very essential for plant growth and development and they are needed in vastly different amount at each stage of development (Anderson and Beardall, 1991; Buchanan *et al,* 2000). During germination of *P. sativum* seeds at room temperature as well as in the refrigerator the rate of combined occurrence of threonine, lysine and methionine are clear inspite of the fluctuations in methionine, since seedling growth is an important developmental phase of growth and life cycle.

The predominant occurrence of aspartic acid and glutamic acid is a characteristic feature of legume seeds (Bewley and Black, 1994) since major transporting form of amino acid from cotyledon to growing seedling is their respective amides that are aspargine and glutamine.

Distribution of free amino acids of control and treatments at 35°C and 40°C was similar. But γ-aminobutyric acid (GABA) was the only non-protein amino acid present in pea cotyledons. Quantitatively maximum GABA was present in seeds treated at 45°C and 50°C during germination (Table 17 d & e). The synthesis and accumulation of GABA is induced by temperature stress (Taiz and Zeiger, 2002) and according to them during heat stress GABA accumulates the level to more than 6-fold higher than non-stressed seeds. *Pisum sativum* seeds

treated at 45°C and 50°C showed reduced amount of glutamic acid, which presumably is utilised for the synthesis of GABA via glutamate decarboxylase pathway, which is getting controlled and accumulated by calmodulin (Snedden *et al*, 1995). According to Satyanarayanan and Nair (1990); Bown and Shelp (1997) GABA synthesis increased rapidly in response to variety of environmental signals, including hypoxia, acidiosis, mechanical stress or cold stress.

Generally, during final stages of germination in both room temperature and in refrigerator (Table 18 a, b, c, d & e) a significant reduction of total amino acid is observed in samples treated at 45°C and 50°C compared to control and other treatments. This is presumably due to the leaching of liberated amino acid through broken cell membrane/cell wall/cracked testa in the samples germinated at room temperature. But in the cold this reduction may be due to enhanced translocation/transport of amino acid from cotyledons to the growing embryonic axis.

EFFECT OF TEMPERATURE ON PHYSIOLOGY OF RESERVE MOBILISATION IN *PISUM SATIVUM* L. SEEDS DURING GERMINATION

Dissertation submitted to the Faculty of Science
University of Calicut
In part fulfillment of the requirements for the degree of
Doctor of Philosophy
In Botany

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Conclusions

Pisum sativum seeds are highly viable and rapidly germinable. Temperature treatment at higher temperature regimes that is 35°C and 40°C show 100% germination when moisture content is reduced from 12.3% to 9.2% and at 50°C germination percentage is above 70% inspite of reduced moisture content, hence *P. sativum* seeds are highly desiccation tolerant.

Under chilled condition (refrigerator) germination percentage and seedling growth are improved compared to that of room temperature, but time taken for germination and seedling growth is enhanced about 10 fold in the refrigerator showing chilling tolerant character of *P. sativum* seeds. Field emergence index data reveals that treatment of seeds up to 40°C does not cause any adverse effect on seedling emergence

Over drying of seeds exhibit enhanced efflux of leachate during imbibition due to mechanical damage of testa and rapid uptake of water resulting in reduced germination rate at temperature above 40°C.

Anatomical studies of radical tip show slight cellular damage of seeds treated at 45°C and 50°C but these damages are getting ameliorated during germination in the cold conditions.

During germination, amylase activity and starch depletion are characteristics of *P. sativum* seeds but high temperature treatments result in inhibited amylase activity *vis-à-vis* reduced starch content owing to non-enzymatic degradation of starch as well as efflux of starch grains form the cotyledonary cells through the cracked seed coat as observed in sections stained by histochemical techniques.

Soluble carbohydrates such as sucrose and raffinose family oligosaccharides are present in considerable quantities and do not get reduced due to higher temperature treatment, inferring the desiccation tolerance of *P. sativum* seeds. Occurrence of raffinose family oligosaccharides in seeds treated at higher temperature when germinated in the cold is another observation which confirms the tolerance of *P. sativum* seeds towards chilling temperature.

Abundance of protein content which does not change during temperature treatment also is characteristic feature of temperature tolerant seeds. Additional polypeptides shown by SDS-PAGE in treated seeds germinated under cold, support the view of enhanced synthesis of housekeeping proteins during cold acclimation.

The distribution pattern of free amino acids shows the abundant occurrence of γ-aminobutyric acid and a concomitant reduction in glutamic acid only in seeds treated at 45°C and 50°C as a response of seeds towards temperature stress and/or cold stress.

Pisum sativum seeds are tolerant to prolonged temperature treatments up to 40°C and the treatments at 45°C and 50°C are not much stressful as shown by the germination behaviour anatomical/histochemical characters and also by the distribution

pattern of metabolic changes. Similarly tolerance towards chilling also is shown by the above parameters.

Mobilisation pattern of seed reserves such as starch, protein, sugar and amino acids during germination is almost uniform in control and temperature treated seeds when germinated at room temperature as well as in the cold condition. Nevertheless, significant reduction of metabolites in general and starch in particular was observed during germination in samples treated at 45°C and 50°C and this is mainly due to leaching of metabolites through damaged/broken cotyledonary tissue.

The origin and distribution of *P. sativum* seeds throughout the tropical, subtropical and temperate regions (to some extend) are positively correlated to the tolerance of the seeds towards high and low temperature regime.

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