Ascorbic acid, a major non-enzymatic antioxidant in plants: Estimation in different potato (*Solanum tuberosum* L.) tissues

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Dissertation
Submitted in the partial fulfillment of the requirement for the award of degree of
Master of Science
In
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CANDIDATE'S DECLARATION

I, hereby declare that the work which being presented in the thesis entitled, "**Ascorbic acid, a major non-enzymatic antioxidant in plants: Estimation in different potato (*Solanum tuberosum* L.) tissues**" in the partial fulfillment of the requirement for the award of degree of Master science in Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is an original record of my own research work carried out under the guidance and supervision of **Dr. N. Das**, Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, India. The content in the dissertation has not been submitted to any other university or institute for award of any other degree.

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CERTIFICATE

This is to certify that the dissertation entitled "**Ascorbic acid, a major non-enzymatic antioxidant in plants: Estimation in different potato (*Solanum tuberosum* L.) tissues**" submitted by **Zeeninder Kaur** (Regd. No 301601025) in the partial fulfillment of the requirement for the award of the degree of Master in Science in Biotechnology, to Thapar Institute of Engineering & Technology is a record of student's own work carried out by her under my guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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LIST OF ABBEREVATIONS

Abbreviation	Name
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
CAT	Catalase
APX	Ascorbate peroxidase
MDHAR	Monodehydroascorbate reductase
DHAR	Dehydroascorbate reductase
DOS	Days of storage
CS-1	Kufri Chipsona- 1
CS-2	Kufri Chipsona -2
KJ	Kufri Jyoti
KCM	Kufri Chandramukhi
PR	Kufri Pukhraj
DE	Desiree
$O_2^{\cdot -}$	Superoxide radical
·OH	Hydroxide radical
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen
H_2O	Water
E C	Enzyme commission number
NADPH	Nicotinamide adenine dinucleotide phosphate
(HPO3)	Metaphosphoric acid
DNPH	Dintrophenylhydrazine
$SC(NH2)_2$	Thiourea
µg	Microgram
µl	Microlitre
FW	Fresh weight
mL	Mililitre
g/mol	Grams/mol

ABSTRACT

Reactive oxygen species (ROS), by-products of aerobic metabolism, were initially recognized as toxic to living cells but now their role in plant growth, signalling and especially response to various stresses is gaining considerable importance. Plants have evolved enzymatic and non-enzymatic antioxidants to overcome the effect of these ROS. Potato is the fourth major cultivated crop and also forms a significant proportion in the Indian diet because of the presence of proteins, nutrients, starch, carbohydrates, vitamins, minerals along with fibres beneficial for human health. In mature tubers L-Ascorbic acid (AA) also known as Vitamin C, is present in relatively high amount. Ascorbic acid is a powerful antioxidant which helps in modulating cellular H_2O_2 level to a permissible range. The extent of ascorbate synthesis varies in the different potato cultivars depending on the genotype and other factors. This study focused on the estimation and comparison of ascorbate level in various tissues namely tuber, leaf and stem of different potato cultivars at different time intervals during growth under field condition and also storage conditions. The highest ascorbate content was noticed in the leaf tissue of all cultivars. The experimental data as presented in this report suggest that mature tubers contain more ascorbic acid in comparison to the growing tubers. Vitamin C content was found to vary in the tissues under different storage conditions. The apparent fluctuations of ascorbate level as observed in different tissues under storage conditions need to be understood carefully.

Keywords:

Potato (*Solanum tuberosum* L.); Reactive oxygen species (ROS); Non-enzymatic antioxidants; Vitamin C (Ascorbate); Spectrophotometric & Titrimetric method

Chapter 1: Introduction

1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are the molecules or ions, which are unstable in nature. Reactive oxygen species are also termed as free radicals possessing the ability to cause various deleterious effects to cell leading cell injury and even cell death. Moreover several studies prove that reactive molecules are also important in cell signalling pathways etc. Hence these molecular species are dual in nature having both beneficial and harmful effects on cell life. They are beneficial at low concentrations by acting as secondary messangers in cell signalling pathways, whereas they cause damage to biomolecules like DNA, lipids and proteins (Levitt, 1980). Reactive oxygen species (ROS) are produced through aerobic metabolism and are toxic in nature (Das and Roychoudhury, 2014). These molecules are generated by various cell mechanisms like electron transport chain in mitochondria and as necessary intermediates of metal catalysed oxidation reactions. They are produced widespread in various cell compartment sites such as mitochondria, chloroplast, peroxisomes, apoplast, endoplasmic reticulum.

Various environmental factors lead to ROS generation in plants viz. drought, salinity, metal toxicity, UV radiation etc. ROS when generated at lower levels, are not harmful because they are continuously being removed by antioxidant machinery of cells. Under unfavourable conditions when the balance between their generation and elimination gets disturbed, ROS causes cellular damages in the form of degradation of major molecules like DNA, lipids and proteins etc. As a result of normal aerobic respiration ,the various types of ROS are Singlet oxygen ($^1O_2$), Hydrogen peroxide (H_2O_2), Superoxide radical ($O_2^{\cdot -}$) , Hydroxyl radical (OH **.**). These oxygen species on accumulation , can cause damage to cells by disrupting their metabolic function (Elstner,1987).

1.2 Overproduction of ROS under stressful conditions

ROS are produced at basal levels in plants, under normal conditions. As a consequence of various environmental stresses, ROS are produced above the critical value. These environmental stresses include abiotic factors like drought, metal toxicity, salinity, UV –B radiation and biotic factors like microbial and pathogen attack etc. Hence a number of factors such as extremity and period of stress conditions, ability of adaptation of plant etc. decide the survival of plant in stress conditions.

1.3 Types of ROS

Superoxide radical ($O_2^{\cdot -}$): The superoxide radical is generated mainly inside the chloroplast and mitochondria. In chloroplasts, it is generated in photo system 1 during non – cyclic electron transport chain. It gets scavenged by enzymatic antioxidant ie superoxide dismutase. This radical is moderately reactive in nature and generally reacts with compounds containing double bonds i.e. proteins and also possess low reactivity with DNA.

Hydroxyl radical (OH**.**): The hydroxyl radical is highly reactive ROS among others. It is highly toxic in nature. It can cause protein damage, lipid peroxidation and even cellular death due to non availability of any enzymatic scavenging system (Pinto et al., 2003). It is generated inside mitochondria, chloroplast and membranes.

Hydrogen peroxide (H_2O_2): This radical is produced under normal as well as under stress conditions. It is generated in mitochondria, chloroplasts, membranes and peroxisomes. It is moderately reactive in nature. It causes cellular damages by oxidizing proteins. Catalases and various peroxidase are the enzymatic scavengers of hydrogen peroxide.

Singlet oxygen ($^1O_2$)**:** singlet oxygen is produced by reaction of chlorophyll (chl) and oxygen. It is produced in membranes, chloroplasts and mitochondria. It causes damage by oxidising proteins, polyunsaturated fatty acids (PUFAs) and DNA. Singlet oxygen is scavenged by non enzymatic antioxidants i.e. carotenoids and α- Tocopherol.

1.4 Sites of ROS production

The major sites of ROS production in plant cell are mitochondria, chloroplast, plasma membrane, endoplasmic reticulum, peroxisomes and cell wall. The major sources of ROS production in presence of light are Chloroplasts and peroxisomes whereas under dark conditions, mitochondrian is the leading producer of ROS (Choudhary et al., 2013). ROS are generally termed as consequence of electron transport activities of mitochondria, plasma membrane and chloroplast. Also they are generated by various metabolic pathways that take place inside the cellular compartments.

1.5 Targets of ROS

ROS target the genetic material i.e., DNA and other major biomolecules like proteins and lipids. ROS may break the DNA strand and cause depurination, depyrimidination , mutation of bases and crosslinking of DNA and proteins. Such kind of crosslinks are not repairable in nature and are destructive to cell. They target lipids by increasing membrane fluidity and permeability and by lipid peroxidation which is highly damaging for the cells under the stress conditions. The lipid peroxidation makes the plasma membrane leaky which allows the entry of substances into the cell and causes damage to various membrane proteins receptors and enzymes (Das and Roychodhury; 2014*)*. ROS causes oxidation of proteins, modification of amino acids, breakage of peptide bonds and by inactivation of enzymes (Fig. 1).

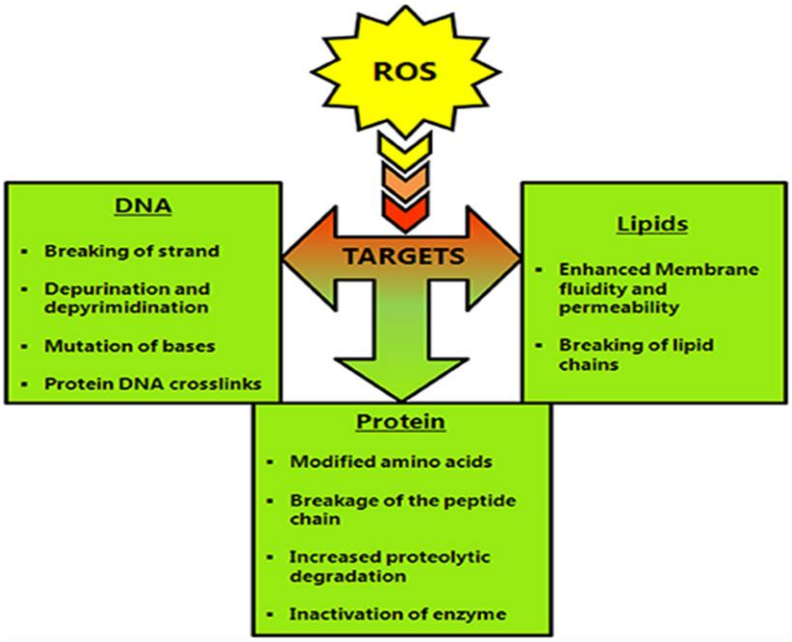


Fig 1 :- Various targets of ROS (Das and Roychoudhury,2014)

1.6 Antioxidative defense mechanism

Reactive oxygen species (ROS) are constantly being generated as a result of aerobic metabolism. Drought ,salinity, metal toxicity, chilling are the various environmental stresses that lead to increased production of ROS due to disturbance of cellular homeostasis. (Sharma et al., 2012). Plants possess active oxygen scavenging system termed as ROS defense system which helps in maintaining oxygen below critical levels. When this delicate equilibrium between ROS generation and scavenging gets perturbed, oxidative damage to lipids, proteins and nucleic acids take place. This balance between ROS production and removal is disturbed by various environmental factors which results in increased levels of ROS inside the cells. In order to avoid oxidative stress, the production and removal of ROS must be strictly controlled. Plants overcome this imbalance by raising their antioxidant defense mechanism. The antioxidant defense mechanism consists of an antioxidant machinery which scavenge ROS and mitigate the damages caused by them. Antioxidants are the substances that delay or inhibit oxidation of other molecules.

Apart from their vital roles in defense mechanism, these antioxidants are also crucial in plant growth and development processes. The antioxidants in plants are becoming important due to their roles in maintaining human health by extending shelf life of foods (Ali Ghasemzadeh et al., 2012).The activation of antioxidant defense machinery overcomes the balance of ROS production in plants. The antioxidant machinery comprises of two main elements i.e. Enzymatic antioxidants and Non enzymatic antioxidants.

1.7 Enzymatic antioxidants

Enzymatic scavangers are Superoxide dismutase (SOD), Ascorbate peroxidise (APX), Catalase (CAT), Monodehydroascorbate reductase (MDHAR), Dehydroascorbate reductase (DHAR). These enzymes are present inside the various subcellular compartments and scavenge the free radicals in efficient ways. Superoxide dismutase (SOD) reacts with superoxide radicals ($O_2^{\cdot-}$**)** and detoxifies them into oxygen and hydrogen peroxide (H_2O_2). CAT converts Hydrogen peroxide into water.

Superoxide dismutase (SOD)

SOD (E.C.1.15.1.1) is one of the important enzyme in defense against oxidative stress. It belongs to the family of metalloenzymes and is present in all aerobic oraganisms. SOD catalyses the dismutation of superoxide radical into oxygen and hydrogen peroxide (Duman et al .,2013). It forms the first line of defense against ROS attack under stress conditions (Das and Roychoudhury, 2014*)*. SOD is present in all cell organelles which produces activated oxygen.

Catalase (CAT)

Catalase (E.C.1.11.1.6) is an important antioxidant enzyme. It is a tetrameric enzyme containing heme group. It catalyzes the dismutation of H_2O_2 to form water and oxygen. It has stronger affinity for H_2O_2 and shows lesser activity against other organic peroxides. Though the turn over number of catalse is very high but APX (Ascorbate peroxidase) has high affinity for H_2O_2 than catalase.

Ascorbate peroxidase (APX)

Ascorbate peroxidase (E.C.1.1.11.1) is another antioxidant enzyme of defense mechanism. It scavanges H_2O_2 in cytosol and chloroplast. H_2O_2 is reduced to DHA (dehydroascorbate) and water through APX with the help of ascorbic acid as a reducing agent. APX is extensively found in cell organelles . It is considered as effective scavenger of H_2O_2 than other enzymes.

Monodehydroascorbate reducatse (MDHAR)

MDHAR (E.C.1.6.5.4) is an important enzyme for restoring the cellular ascorbic acid pool. It reforms the ascorbic acid from MDHA with the help of NADPH as a reducing agent. It is present in peroxisomes and mitochondria alongwith APX. It plays vital role in regenerating ascorbic acid.

Dehydroascorbate reductase (DHAR)

DHAR (M.C.1.8.5.1) is another enzymatic component responsible for regenerating ascorbic acid pool. With the help of Reduced Glutathione (GSH) as a electron donor, DHAR forms ascorbic acid by reducing dehydroascorbate (DHA). It functions as MDHAR by regenerating cellular ascorbic acid pool.

1.8 Non-enzymatic antioxidants

Non-enzymatic antioxidants include ascorbic acid, reduced glutathione, tocopherol, carotenoids, flavanoids and proline. Apart from protection against cell damage, these antioxidants also play crucial role in various plant growth and development processes like cell elongation, cell death, mitosis and senescence (de Pinto and De Gara, *2004*). Likewise enzymatic antioxidants, the non enzymatic scavangers also diminish the damaging effects of ROS.

L- Ascorbic acid

Vitamin C (L- Ascorbic acid) is considered as a major component of human nutrition. Being an electron donor ascorbic acid is an important water soluble antioxidant in humans (Upadayatty et al., 2009). Ascorbic acid is a six carbon compound. It is used as a reducing agent and as a coenzyme in various metabolic pathways. It plays an important role in maintaining connective tissues and bones and is also important for maintaining normal functioning of immune system. Biosynthesis of ascorbic acid occurs through D-mannose/ L-galactose which further gets oxidised to form precursor of ascorbic acid i.e. L- galactono-γ-lactone (Wheeler et al., 1988). Further galacturonic acid is also primary component in Ascorbic acid synthesis (Agius et al., 2003).

1.9 About potato plant

The potato (*Solanum tuberosum* L.*)* is an important staple crop of *Solanaceae* family that is widely used as food for human consumption and is at fourth position after rice, wheat and maize. Potato is an annual, herbaceous, dicotyledonous and vegetatively propagated plant *(*Percy et al.,1984). The cultivated potatoes have originated from the Andes of South America and have widely distributed to 160 countries around the world. Potato was introduced to Europe in 16th century through Spanish conquerors from where it got distributed to other countries through colonial expansion. In India, potato is cultivated in 1.2 million hectares of land producing about 23.5 million tonnes. The ease of cultivation and nutritive value made potato a valuable food crop. It is considered as the most essential basic vegetable worldwide. It is the fourth largest food crop in the world after rice, wheat and maize. *(*Akyol et al., 2016*)* Potatoes are widely used as vegetables, seeds, feed for animals and as major raw material for food products, starch and alcohol. The potato plant grows to 0.4 to 1.4 m tall having compound leaves which may vary from medium to dark green in color. It is a nightshade plant with fibrous root system. The stems may or may not be hairy and are medium to dark green in color as leaves. Also, the potato plant possesses white, pink, blue, or purple flowers with yellow stamens. Tubers are the storage organs that originate from swollen underground stems.

The potato plays major role in human consumption as it is a rich source of amino acids, minerals and carbohydrates. Various abiotic stresses such as heat, drought and salinity affect the production of potatoes worldwide (Gangadhar et. al., 2016*).* Potatoes are rich in various organic nutrients among which vitamin C (ascorbic acid) is most rich and strong antioxidant.

Several studies suggested that potato tubers contain high amounts antioxidant effects of potatoes. The antioxidant activity of potato is found to be comparable with synthetic antioxidants (Kanatt et al., 2005*).* A number of factors affect the antioxidant activity and vitamin C content in plants which include environmental conditions, growing and cultivation methods, processing conditions etc.

Chapter 2: Review of Literature

2.1 Brief introduction of ascorbic acid

Vitamin C (L- Ascorbic acid) is the major constituent of human nutrition. It is water soluble vitamin which is extensively used as food additive. It is an important metabolite and plays a crucial role against stress in plants. Lack of vitamin c cause a pathological disease known as scurvy. In living organisms ascorbic acid is involved in various physiological functions. It occurs in both reduced form (ascorbic acid) and oxidized form (dehydroascorbic acid) (Kapur et al., 2012) also it is a vital antioxidant in detoxification of various reactive oxygen species. Hence it acts as a good scavenger of free radicals and other oxygen derived species by oxidising them. Being a powerful antioxidant, ascorbic acid not only scavenges free radicals but also regenerates vitamin E from its oxidized form and prevents lipid peroxidation. Due to lack of gene essential for ascorbic acid biosynthesis, humans need to get this vitamin from their diet source only i.e. mainly from plants. Various citrus fruits like kiwi, mango and vegetables like tomatoes, potatoes and broccoli are rich sources of vitamin C. It is important for normal functioning of the immune system and for the collagen synthesis (Upadhaya. et al., 2010). Ascorbic acid stimulates the immune system by enhancing the protection of organism from various stresses. It is a white, crystalline solid and consists of two asymmetric carbon atoms, C-4 and C-5. Apart from cytosol, ascorbic acid is also concentrated in apoplast hence acting as a first line of defense against ROS attack (Das and Roychoudhury; 2014*)*.

2.2 Ascorbic acid biosynthesis and mode of action

Biosynthesis of Ascorbic acid in plants takes place through D- mannose / L- galactose pathway i.e. majorly known as Smirnoff- Wheeler pathway (Wheeler et al., 1998 *;* Agius et al., 2003*)*. It involves conversion of D- mannose first into GDP- D-mannose and then into GDP- L-galactose (Fig.2). Further oxidation of L- galactose results in the formation of precursor to ascorbic acid that is L – galactono-γ – lactone *(*Wheeler et al., 1998). Apart from this pathway, some of the ascorbic acid biosynthesis takes place with the help of galacturonic acid Moreover with the help of metabolic engineering, various genes responsible for ascorbate biosynthesis have been introduced to increase the levels of ascorbic acid. Oxidation of ascorbic acid takes place via two consecutive steps. First of all, it gets oxidised to form monodehydroascorbate (MDHA) which further gets coverted either immediately into ascorbate or breaks into Ascorbic acid and dehydroascorbate (DHA). In other words, we can say that ascorbic acid on losing one electron, forms monodehydroascorbate (MDHA) which is highly unstable in nature. This MDHA before further getting reduced, breaks down into Ascorbic acid and dehydroascorbate (DHA). Also it has been studied that with the help of several reductases, MDHA and DHA can be reduced back to Ascorbic acid. Ascorbic acid ultimately reacts with various free radicals like hydrogen peroxide, hydroxyl radical and superoxide radical to prevent the oxidative damage.

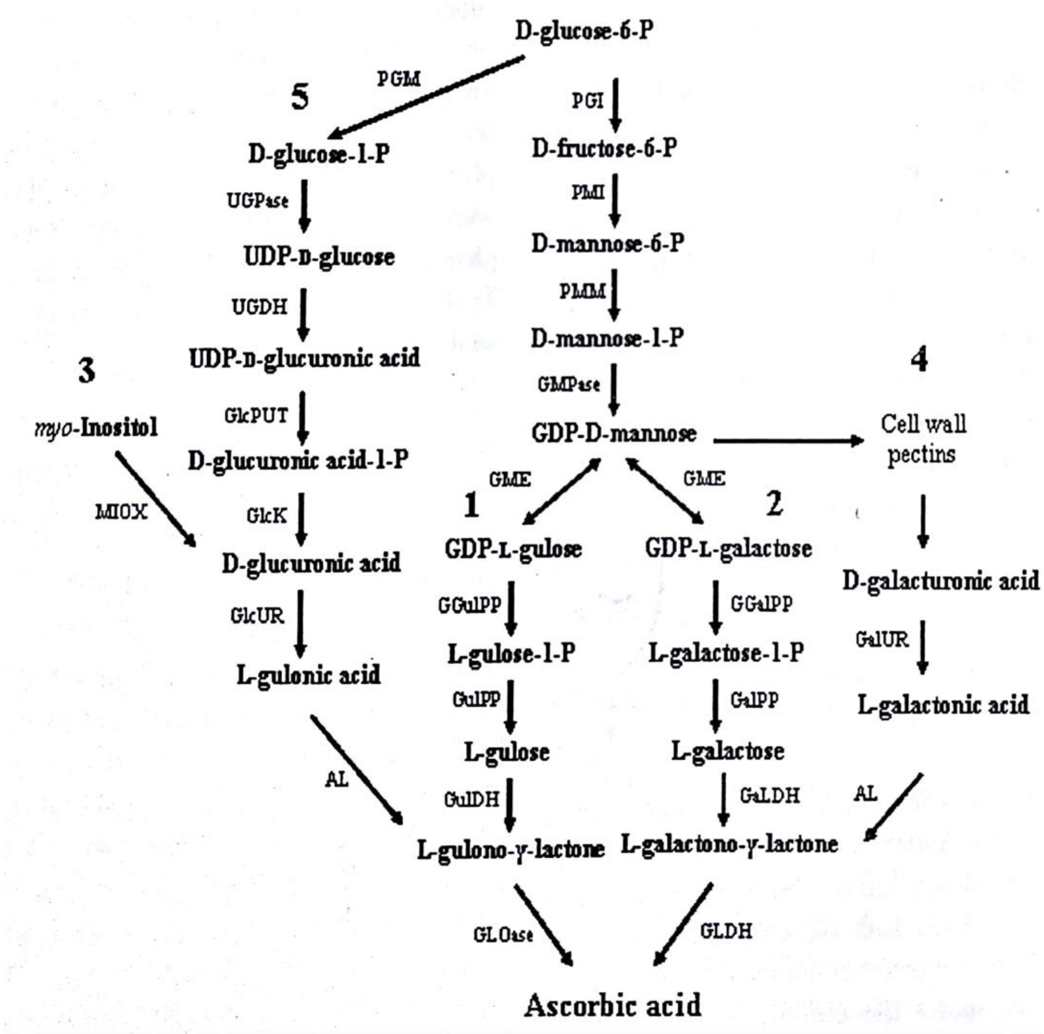


Fig 2:- Biosynthesis of ascorbate in plants (Hemavathi *et al ,2009)*

2.3 Ascorbic acid as an antioxidant

Ascorbic acid has major role in protecting plants against the oxidative damage. It acts as an important antioxidant in plants. The oxidative stress is caused by various free radicals like superoxide, hydrogen peroxide and hydroxyl radical. These radicals are generated either during photosynthesis with the help of singlet oxygen or as a consequence of abiotic factors like drought, salinity, metal toxicity etc. Several ROS like singlet oxygen, superoxide and hydroxyl radical can be eliminated directly by ascorbic acid (Padh et al., 1990*)*. Ascorbic acid also plays vital role in maintaining an antioxidant α- tocopherol in reduced state (Packer et al., 1979*)*. Various studies have shown the consequence of an oxidative stress on Ascorbic acid in plants. During stress conditions, increased oxidation and insufficient regeneration makes the Ascorbic acid pool highly oxidised.

2.4 Studies on ascorbic acid till date

Galani *et. al.,* 2016 studied the effect of storage temperature on vitamin C, total phenolics, UPLC phenolic acid profile and antioxidant capacity of eleven potato varieties. Hemavathi et al., 2009, investigated that enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. Agius et al., 2003, found that engineering increased vitamin C levels in plants by overexpression of D galacturonic acid reductase. Smirnoff N 1996 studied the function and metabolism of ascorbic acid in plants and its biosynthesis was also studied in(2001). Khan et al., 2006 proposed a simple UV- spectrophotometric method for determination of vitamin C in various fruits and vegetables at Sylhet area in Bangladesh. Chen Z, et al.,2003, through enhanced recycling of ascorbate, studied the increasing vitamin C content in plants. The metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants was studied by Jain and Nessler, 2000. The biosynthesis pathway of vitamin C in higher plants was proposed by Wheeler et al., 1998.

Abong, et al., 2011 studied the losses in ascorbic acid during storage of fresh tubers, frying, packaging and storage of potato crisps from four Kenyan potato cultivars. Also the effect of cold storage on total phenolics content, antioxidant activity and vitamin C level of selected potato clones. The effect of conditions of storage on ascorbic acid content and sprouting of tubers of some Indian potato varieties was investigated by .Mishra et al., 1985. Kulen et al.,2013 observed the effect of cold storage on total phenolics content, antioxidant activity and vitamin C level of selected potato clones . Rosenthal and Jansky, 2008 studied the effect of production and storage on antioxidant levels in potato tubers. Also the free radical scavenging ability of ascorbic acid was studied on various reactive oxygen species. For example, Nakano and Asada (1981) studied the scavenging effects of ascorbate specific peroxidases on hydrogen peroxide in spinach chloroplasts. Moreover, the effects of storage conditions and are extensively studied on ascorbic acid. Zee et al.*(*1991) studied the effect of storage conditions on the stability of vitamin C in various fruits and vegetables produced and consumed .Also the effects of storage and processing on the nutritive value of potatoes was observed by (Woolfe , 1987).

2.5 Origin of the problem

In potatoes, vitamin C is the most abundant vitamin which is considered as one of the major component of human nutrition. Ascorbic acid (vitamin C) is also termed as an effective antioxidant against oxidative stress (Navas and Gomezdiaz et al., 1995). Despite knowing the fact that during harvest, a considerable amount of vitamin C is present in the potatoes, very less is known about decrease in ascorbic acid content during storage at low temperatures (Kumar et al., 2013*)*. Consequently there is very little known regarding their behaviour during period of storage. Also it has been found that antioxidant capacity of potato tubers is affected by low storage temperature which needs to be fully explained. Moreover concerning the changes in antioxidant levels during different stages of growth and development, the focus was laid on ascorbate estimation.

2.6 Objectives

L-Ascorbate (vitamin C) is a vital nutrient with wide range of functional roles. It is a water soluble vitamin. Most importantly it is a powerful antioxidant and plays crucial role in scavenging ROS, particularly H_2O_2 in plants. Potato tuber is a rich source of vitamin C in human diet. This study focused on different tissues of potato including growing tubers. Keeping in view with the biological and nutritional importance of ascorbate, the following objectives were framed:

- To grow and harvest different tissues of potato cultivars under field condition
- Estimation of ascorbate in the potato tissues at different growing stages
- Effect of storage at various temperatures on ascorbate content in the mature tubers

Chapter 3: Materials and Methods

3.1 Establishment of plants and other materials

For the estimation of ascorbate, six major Indian potato cultivars such as Kufri Chipsona 1 (CS-1) Kufri Chipsona 2 (CS-2), Kufri Jyoti (KJ), Kufri Chandramukhi (KCM), Desiree (DE), Kufri Pukhraj (PR) were selected. All these cultivars were maintained in the growth room of laboratory no. 4 of Thapar institute of Engineering & technology (T.I.E.T), Patiala. The plants were transferred into the fields in November starting after the process of acclimatization in the green house at Centre of Relevance and Excellence (CORE), T.I.ET, Patiala. Since potato is a cold weather crop, November is best suited for cold temperature and short length day light conditions. Optimum conditions were taken care of while growth of these cultivars. Further these cultivars were harvested at different time period i.e. in the month of February and March at different stages of growth and development. After harvest, healthy tubers were obtained along with other tissues of importance like roots, stems, leaves. For the purpose of convenience, short name of each cultivar is being used in the parenthesis.

Other materials: Variety of chemicals and biochemical items were obtained from different sources. Chemicals were purchased from various laboratories like Sigma-aldrich India Pvt. Ltd., and Hi Media Laboratories Mumbai. Glassware's and Plastic wares were purchased from Borosil and Tarsons Products Pvt. Ltd.

3.2 Maintenance of potato germplasm

The potato cultivars CS-1, CS-2, KJ, KCM, PR, DE are selected because of their different genetic makeup and many other factors like time of maturation and their growth in various agro climatic zones of Indian subcontinent. Among all KCM is early maturing while others are medium and late maturing cultivars. All these cultivars, along with Desiree, are micropropagated under controlled conditions of temperature, light and humidity inside the laboratory.

3.3 Reagents used

- ***Metaphosphoric acid:*** Metaphosphoric acid (HPO3), with molecular weight 79.98 kDa, is hygroscopic in nature. It usually decomposes in water and is soluble in alcohol. Its melting point is 21ºC and boiling point is 158º C. It is a stable compound usually incompatible with the strong bases and few metals. It is a transparent or glassy substance which forms pieces. It is harmful if swallowed and can also cause serious eye damage and skin burns. To carry out ascorbate estimation, 15 g of solid metaphosphoric acid was dissolved in mixture of glacial acetic acid and distilled water. This mixture was prepared by adding 40 mL glacial acetic acid and making the volume with 450 distilled water in a 500 mL volumetric flask. This solution of metaphoshoric acid and acetic acid was filtered before use.

- *2,4-dintrophenylhydrazine*: 2,4 – dintrophenylhydrazine is a chemical compound with molecular weight 198.14 Da and is a hydrazine derivative and is potential mutagenic agent. 2,4-DNPH is widely used as reagent to detect the presence of aldehydes and ketones in proteins. This compound is also used as a colorimetric reagent in various quantitative analyses as in ascorbate estimation.

- *Thiourea*; Thiourea is an organic compound containg carbon, hydrogen,sulphur and nitrogen . Its molecular formula is $SC(NH2)_2$ and molecular weight is 76.117 g/mol. Thiourea is quite similar to urea in case of structure only but the properties are entirely different. Thiourea is a strong denaturant which is generally used to increase the solubility and recovery of proteins. In the estimation of ascorbate, 2 g of thiourea wasdissolved in 10 mL ethanol and 10 mL water.

- *Bromine water*: Bromine water is a dilute solution which is prepared by adding diatomic bromine in water. It is a dark yellow mixture which is highly oxidizing in nature. To carry out the experiment, it was prepared by adding the fumes of liquid bromine directly with water using a fume hood and proper protective clothing, mask and gloves. For estimation of vitamin C, 3% bromine water was prepared in the lab taking the protective measures and was kept inside the dark bottle.

- *85% sulphuric acid*: Sulphuric acid is a colourless oily liquid having molecular formula H_2SO_4. It releases heat on solubilising with water. Its molecular weight is 98.072 g/mol and melting point is 10ºC. It is miscible with water and alcohol with the generation of heat. Sulphuric acid is stable under recommended storage conditions. It also emits toxic fumes when heated to decomposition. For vitamin C estimation. 85% sulphuric acid was prepared in lab and was kept in refrigerator to obtain chilled sulphuric acid.
- *Iodine solution*: Iodine solution is a solution of potassium iodide with iodine in water. It is used here in titrimetric method for estimation of ascorbic acid. This solution is source of

free elemental iodine, which is result of equilibrium between elemental iodine and triiodide ions. In our experiment, 100 mL of 0.05 M iodine solution was prepared using potassium iodide in minimum amount of water.

- *Starch indicator*: 1% starch solution was used as an indicator in redox titration in determination of vitamin C in potatoes. For the purpose of titration, 1 gram of starch was dissolved in 5 mL of water. The mixture was stirred thoroughly and the volume was made about 100 mL by adding appropriate amount of water. The solution formed was boiled for few minutes, cooled and then filtered before use.

3.4 Sample preparation

The process of harvesting took place in the month of February and March to obtain samples at various stages of growth i.e. young and mature. Various tissues of six different varieties were simultaneously collected at different time intervals. The collected tissues include tubers of various sizes i.e. small, medium and large. Additionally young and mature stems, roots and leaves of the selected six potato cultivars were collected. All these collected tissues were washed with abundant tap water in order to remove dirt and soil residues. Afterwards they were rinsed with distilled water and dried with tissue paper. 2.5 grams of each type of tissue sample of respective cultivar was weighed. All the tubers of different six cultivars at different growing stages i.e. small, medium and large were packed in foil paper each having weight of 2.5 grams. Similarly the rest of the tissues like young and mature stems, roots, young and mature leaves were packed separately in the foil paper. With the help of liquid nitrogen all these packets, each containing 2.5 grams of samples were snap frozen. Finally these weighed samples after the process of snap freezing, were stored in - 80º C for further experimental usage. Since estimation of ascorbic acid in potatoes is to be checked under different storage conditions, the tubers of all six cultivars ie CS-1, CS-2, PR, DE, KCM, KJ were stored at three different temperatures i.e. at room temperature (25ºC -37ºC), in cold storage (4ºC) and in refrigerator (-20ºC). Simultaneously samples were collected for estimation at 15, 30, 45 and 60 DOS.

3.5 Estimation of vitamin C

3.5.1 Spectrophotometric analysis

Principle: The determination of total ascorbic acid (ascorbic acid and dehydroascorbic acid) can be done by spectrophotometric method. This method involves the formation of dehydroascorbic acid through oxidation of ascorbic acid with the help of bromine water in the presence of acetic acid. After the addition of 2,4- dinitrophenylhydrazine, a red coloured complex is produced. The absorbance of the coloured substance is measured with the help of spectrophotometer at wavelength 521 nm. A linear concentration range for standard solutions of ascorbic acid can be obtained using this method. Spectrophotometric analysis of ascorbic acid using 2, 4 –DNPH is the most effective and accurate method.

Sample preparation: First of all the sample preparation was done according to Kapur et al., *2016* using metaphosphoric acid. For this, 2.5 g of each of the stored samples for tubers, leaves, stems, roots at different growth conditions were taken one by one. Each 2.5 g of sample was crushed in liquid nitrogen to obtain a thick paste. The samples were crushed using mortar and pestle and were homogenised uniformly under the same set of conditions. 25 mL of metaphosphoric acid was added to each sample to obtain a homogenised mixture. This is applied to each and every stored sample of six different potato cultivars. The homogenate was then centrifuged at 4000 rpm for 15 min at 4 ºC. After centrifugation, supernatant was filtered and collected in vials. The supernatant was used further in spectrophotometric determination of ascorbate content.

Methodology: 2 mL of sample extract of each tissue was taken in test tube and 115 µl of 3% bromine water was added into it .Bromine water oxidises ascorbic acid to dehydroascorbic acid . Now after the process of oxidation by bromine water, 65 µl of thiourea was added in to each test tube in order to remove extra bromine from solution. Also 500 µl of 2, 4 – dinitrophenylhydrazine was added to form osazone. All the test tubes of each cultivar alongwith blank solution were icubated for three hours incubation at 37ºC inside the BOD incubator. After three hours, all the test tubes were cooled in ice for 30 min. After ice treatment, 2.5 mL of chilled 85% H_2SO_4 was added to each of the tubes and mixed thoroughly. Absorbance of the orange coloured solution were taken using spectrophotometer -Shimadzu at 521 nm. By obtaining absorbance of each sample extract, a calibration curve was prepared. The amount of ascorbic acid in each sample was estimated using the plot obtained.

Reactions involved – Initially with the addition of bromine water, oxidation of ascorbic acid took place. Ascorbic acid oxidised into dehydroascorbic acid. By adding 2, 4 –DNPH further L-dehydroascorbic acid produced an osazone. Finally on treatment with 85% H_2SO_4 an orange- red coloured solution was produced. The absorbance of coloured solution was measured at 521 nm and a calibration curve was plotted to estimate the amount of ascorbic acid in each of samples.

3.5.2 Titration method

Principle: Estimation of vitamin C can be done by redox (oxidation- reduction) titration. The samples to be titrated are allowed to react with iodine solution and starch solution is used as an indicator in such titration. Initially iodine on reaction with iodide, forms triodide which further oxidises vitamin C. Vitamin C on oxidation by triodide gets converted into dehydroascorbic acid. The endpoint of this titration is indicated by blue black coloured product. Triodide converts all the vitamin c present in sample to iodide ion, hence no coloured product is produced. But the excess of triodide left after the oxidation of vitamin C, reacts with starch and produces the blue black coloured product. Hence the endpoint is detected by the formation of blue black coloured product.

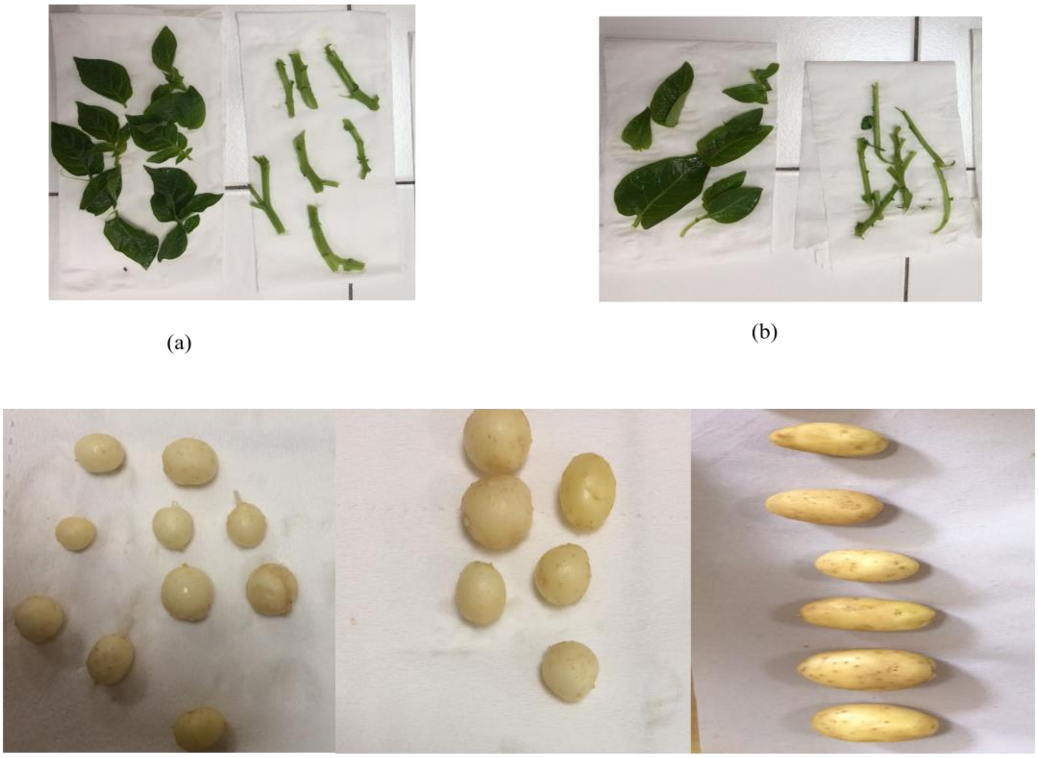
Methodology: In order to determine the amount of ascorbic acid in selected samples, a standard curve for ascorbic acid was made first. A standard solution of ascorbic acid (2 mL) having different concentrations i.e. 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL up to 100 µg/mL from the stock solution of 1 mg/mL was prepared. This solution was titrated with the help of potassium iodide taken inside the burette. Appearance of first dark blue coloured complex detected the endpoint of the titration. Now from each of the different concentrations of standard solution of ascorbic acid, 2 mL solution was taken in conical flask. Further 15 mL of distilled water and 100 µl of 1% starch indicator was added in the conical flask. Each of this mixture was titrated against potassium iodide solution in the burette. Detection of endpoint was done by appearance of first permanent dark blue black colour due to formation of starch –iodine complex. Titration was repeated to get concordant readings. The amount of ascorbic acid present in each test sample was detected with the help of standard curve. Concentration of ascorbic acid was detected by plotting the concentration against the concentration from the standard graph of ascorbic acid.

Reactions involved: The estimation of vitamin C was done using redox titration method. The redox titration was carried out with the help of iodine solution .Starch solution was used as an indicator in this titration. During titration, iodine was added in burette. Ascorbic acid was oxidized to dehydroascorbic acid by the addition of iodine solution. On the other hand, iodine was reduced to iodide ions. As long as ascorbic acid was present, the iodine was immediately reduced to iodide ions. But when all ascorbic acid was oxidized completely, the excess iodine left behind was free to react. This free iodine was responsible for the formation of blue- black coloured complex. This complex was result of reaction between the free iodine and the starch solution. The formation of this coloured complex completed the titration as the endpoint was detected by the first permanent appearance of this blue black coloured complex.

Chapter 4: Results and discussion

4.1 Plant growth and harvesting of tissues

Fig. 3 Steps of potato plants grown in lab to field conditions (a) culture maintained in MS medium in lab. (b) Acclimatization and hardening of plantlets. (c) field transfer of plantlets. (d) collection of samples



(a) (b)

(c) (d) (e)

Fig. 4 Collection of (a) young and (b) mature tissues (c) develop[iong tubers (d) growing tubers (e) mature tubers

4.2 Estimation of ascorbate by spectrophotometric method

Ascorbic acid estimation was done using various tissues of six Indian potato cultivars namely, CS-1, CS-2, KCM, KJ, PR and DE. The selected tissues like tubers, stems and leaves were harvested from the field grown cultivars (Fig.3). The respective tissues were crushed in liquid nitrogen and homogenized in the extracting solution (metaphosphoric acid) and centrifuged to obtain sample extract of different tissues of each cultivar (refer chapter 3 of materials and methods).

The standard curve for ascorbic acid estimation was done using different concentrations (10-100 µg/ standard ascorbate from SRL and the absorbance was measured at 521 nm as shown in Table 1 and Fig.6.

Test tubes : 1 2 3 4 5 6 7 8 9

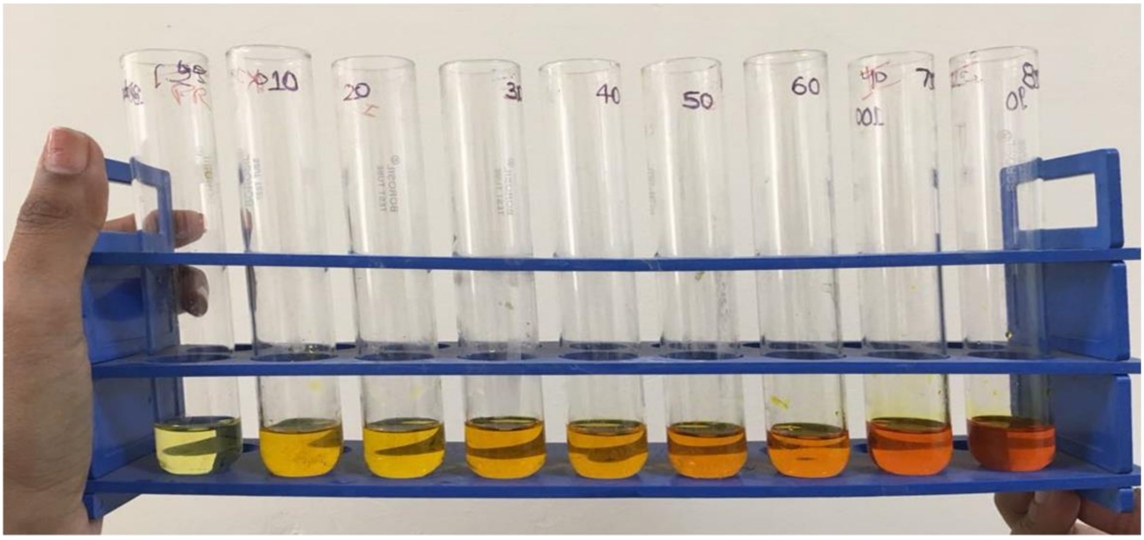


Fig. 5– colour gradient developed by different concentrations of ascorbic acid

Table1: Measurement of absorbance at 521 nm

Ascorbic acid (µg)	A_{521} Value
Blank	0
10	0.11
20	0.22
30	0.31
40	0.42
50	0.511
60	0.621
70	0.702
80	0.809
90	0.9
100	1.111
110	1.21

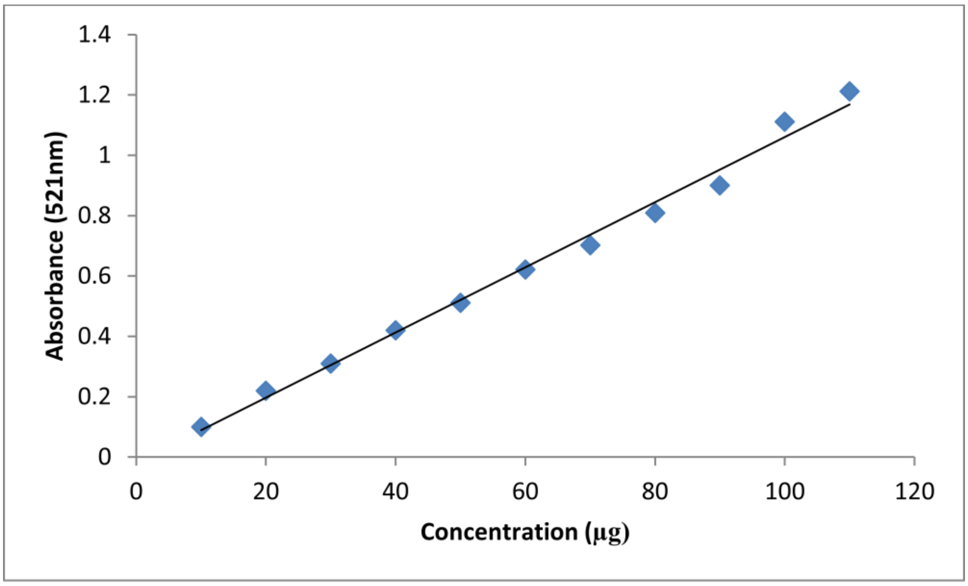


Fig 6: Ascorbic acid standard curve

4.2.1 . Estimation of ascorbate in different tissues of the potato cultivars

Absorbance was measured at 521 nm using UV-Visible spectrophotometer. The amount of ascorbic acid present in each sample was expressed in µg/gram of FW of the tissue.

*Tubers***:** The inter-varietal differences in ascorbate content in tubers at various growth stages ie developing, growing and mature were observed. A bell shaped pattern was observed i.e.

low in early growing (small), increase in growing (medium) and then decrease in mature (large) stage except in cultivar CS -1. The amount of ascorbate was found to be highest in growing stage of cultivar KCM (676.6 µg/ g FW) and lowest in mature cultivar of KCM (122.5 µg/ g FW) as shown in Table 2.

Table 2: Estimation of ascorbate (µg/g F.W.) in different stages of tuber development.

Cultivar	Early growing	Growing	Mature
CS -1	224.5	315.7	508.0
CS -2	238.7	456.4	307.3
PR	247.8	497.7	154.5
DE	261.1	512.4	238.0
KJ	192.0	652.4	127.2
KCM	245.0	**676.6**	**122.5**

Stem: The differences in ascorbate content was estimated in stems of potato at early and mature stage. To carry out this, stem samples were collected at early and late stages of growth and development. From the calculations, it was observed that the mature cultivar of KCM has highest amount of ascorbate acid i.e 746.9µg/ g FW. On the other hand, the ascorbic acid content was found to be lowest i.e 148.4 µg/ g FW in young cultivar of CS-1 as shown in Table 3. It was seen that the amount of ascorbate goes on increasing with growth of potato plant as all the mature cultivars were found to contain more amount of ascorbate as compared to the young stems of early stages except cultivar CS-2 where it was almost constant.

Table 3: Estimation of ascorbate (µg/g F.W.) in young and mature stem samples

Cultivar	Young stems	Mature stems
CS -1	**148.4**	413.7
CS -2	369.6	396.2
PR	324.8	541.8
DE	236.6	540.4
KJ	236.6	392.7
KCM	288.4	**746.9**

*Leaves***:** Highest ascorbate content was estimated in the leaf tissue indicating their high antioxidative potential. From the study as shown in Table 4, it was observed that the ascorbate content increased approx.1.5 times from young to mature stage, indicating higher antioxidative capacity towards the stage of maturity.The highest amount of ascorbate was observed in mature cultivar of CS-1 (3245.2 µg/ g FW)whereas lowest amount was observed in the young cultivar of DE (945 µg/ g FW). However, some fluctuations in the amount were also observed.

Table 4: Estimation of ascorbic acid (µg/g F.W.) in young and mature leaf samples

Cultivar	Young leaves	Mature leaves
CS -1	2030	**3245.2**
CS -2	1902.6	2937.2
PR	1587.6	1845.2
DE	**945**	1451.8
KJ	1965.6	2745.4
KCM	1325.8	1887.2

4.2.2 Estimation of ascorbate in storage tubers

Tubers stored for 15 days: As shown in Table 5, after 15 DOS no significant change was observed except in the case of cultivar CS-2 and KCM where there was a significant change at 4 ºC and at -20 ºC. The difference was not significant for the storage conditions. In the cultivars KCM and DE it increased to almost double at room temperature and at 4 ºC but at -20 ºC it almost increased four times. Interestingly in the cultivar PR it increased almost two times at all the storage temperatures. This fluctuations reflects the different antioxidant potential of the cultivars that differ in their genetic makeup

Table 5: Estimation of ascorbate (µg/g F.W.) in tuber samples stored at different temperature for 15 days

Cultivar	Room temp.	4ºC	-20ºC
CS -1	506.5	517.0	520.1
CS -2	380.0	540.3	460.0
PR	220.4	290.1	280.3
DE	205.0	310.0	370.0
KJ	276.4	390.2	320.2
KCM	258.9	260.0	490.0

*Tubers stored for 30 days***:** As shown in Table 6, we have observed inter varietal differences in ascorbate content in storage tubers after 30 days. In KCM cultivar stored at -20 ºC, the amount of ascorbate was found to be highest whereas the DE cultivar stored at room temperature was found lowest in ascorbate content. At room temperature, the ascorbic acid was found high in CS-1 cultivar. On the other hand, at 4 ºC the amount of ascorbic acid was high in CS-2 cultivar and lowest in PR cultivar. At -20ºC, the ascorbic acid content was high in KCM and lowest in PR.

Table 6 Estimation of ascorbate (µg/g F.W.) in tuber samples stored at different temperature for 30 days

Cultivar	Room temp.	4ºC	-20ºC
CS -1	504.0	528.5	564.9
CS -2	401.8	679.7	546.0
PR	241.5	331.1	334.6
DE	175.0	361.9	450.8
KJ	399.0	458.5	562.1
KCM	315.0	347.9	780.5

*Tubers stored for 45 days***:** As shown in Table 7, the inter-varietal differences were found in tubers stored at three different temperatures for a period of 45 days. The ascorbate content was found highest in KJ cultivar stored at -20ºC whereas it was found least in KJ cultivar stored at room temperature. At room temperature, the CS -1 cultivar was found to contain highest amount of ascorbate as compared to the other cultivars stored at room temperature for 45 days and the ascorbate content was least found in KJ cultivar. At 4ºC, the amount was high in CS-2 cultivar and low in PR. On the other hand, at -20º C the ascorbate content was found high in KJ and it was least in PR cultivar.

Table 7: Estimation of ascorbate (µg/g F.W.) in tuber samples stored at different temperatures for 45 days

Cultivar	Room temp.	4ºC	-20ºC
CS -1	380.8	296.1	505.4
CS -2	280.7	446.6	474.6
PR	256.9	261.8	424.9
DE	236.6	285.6	536.9
KJ	225.4	368.2	620.2
KCM	354.9	303.1	433.3

*Tubers stored for 60 days***:** As shown in Table 8, we have observed inter-varietel differences in ascorbate content in tubers stored at three different temperatures i.e. room temperature , 4ºC and -20º C. It was found that in DE cultivar stored at -20ºC, the amount of ascorbate was highest whereas it was least in CS-2 cultivar stored at room temperature.

Table 8 Estimation of ascorbate (µg/g F.W.) in tuber samples stored at different temperatures for 60 days

Cultivar	Room temp.	4ºC	-20ºC
CS -1	157.5	287.0	307.3
CS -2	113.4	228.2	271.6
PR	152.6	137.9	241.5
DE	215.6	193.9	318.5
KJ	207.9	177.1	244.3
KCM	226.1	284.9	308.7

4.2.3 Bar graph of different cultivars at different storage temperatures

- Storage at room temperature

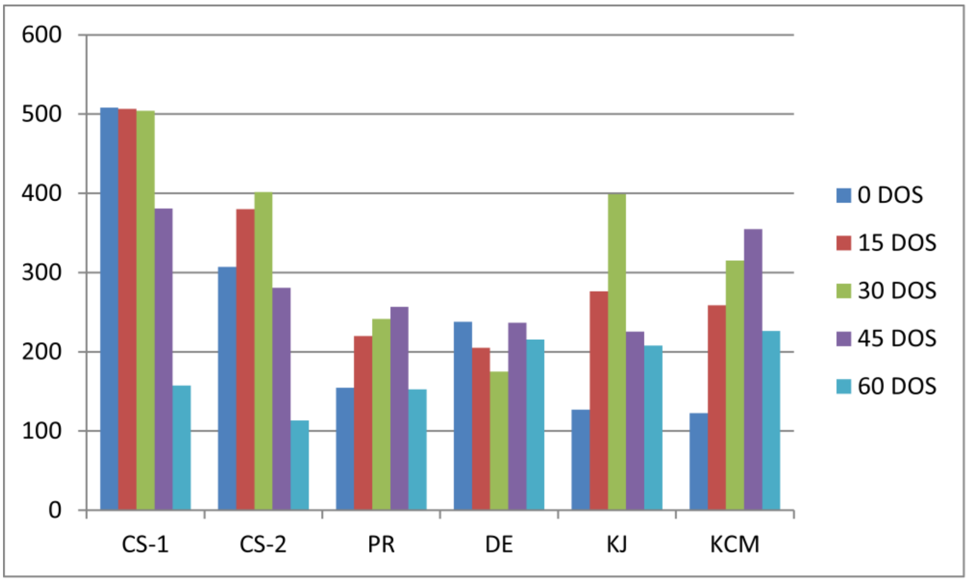


Fig.7- Bar graph for ascorbate content of different cultivars at room temperature

- <u>Storage at 4º C</u>

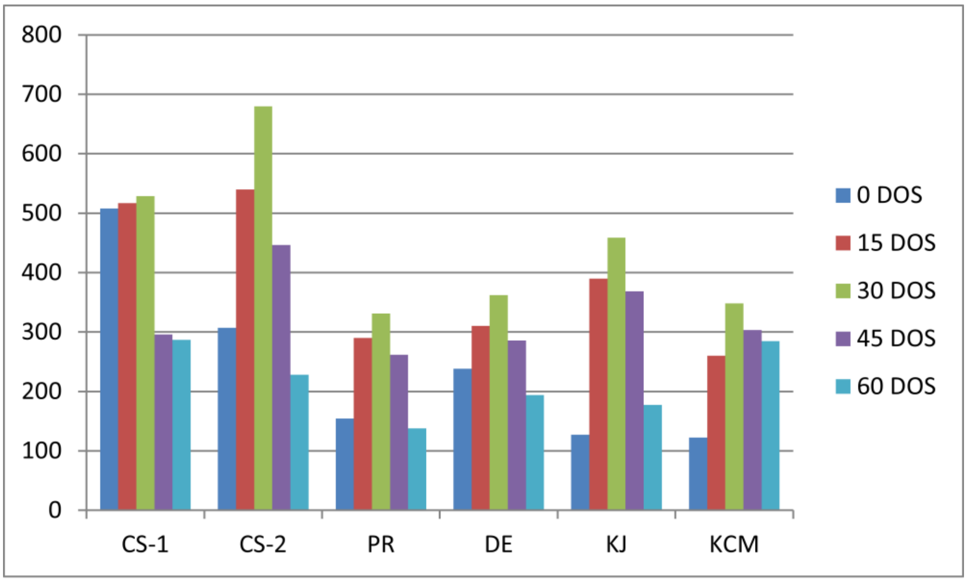


Fig.8- Bar graph for ascorbate content of different cultivars at 4ºC

- <u>Storage at -20º C</u>

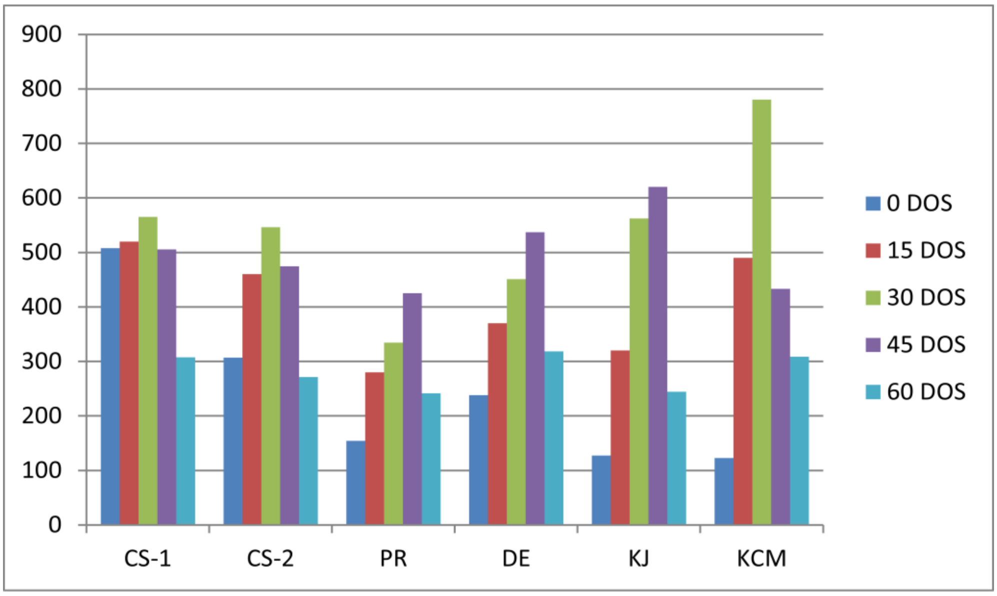


Fig. 9 – Bar graph for ascorbate content of different cultivars at – 20º C

***Ascorbate content during storage**:* The ascorbate content on 0 DOS i.e.at initial day of observation was in the range, lowest (122.5 µg/ g FW) in cultivar KCM and highest (508.0 µg/ g FW) in the cultivar CS-1. Initially the ascorbate content increased upto 30 DOS at all the storage temperatures and then decreased after it till the 60th DOS. At different temperatures, the oxidative potential of the cultivars varied. It was observed that for storage conditions at room temperature, ascorbate content was highest in cultivar CS-1.

At 4°C the cultivar CS-2 showed the highest ascorbate level after 15 DOS and at - 20° C highest level of ascorbate cultivar KCM after 30 DOS. The trend was marked with some fluctuations at - 20° C. From the 0 DOS the cultivar KJ and KCM the ascorbate content increased twice and in the case of cultivar CS-1 it was decreased three times. This might be due to the the extent of stress tolerated by the the different cultivars varying in their genetic make up. This study is in concordance with Galani et al., 2016 who studied the effect of storage temperature on vitamin C, Antioxidant capacity, UPLC phenolic acid profile and total phenolics of eleven potato varities. The study is also in agreement with Mishra et al., *(*1985) and Kulen et al.,(2013) who also studied effects of condition of storage on ascorbic acid content in potato tubers.

4.3 Estimation of ascorbate in mature tubers by titration method

The amount of ascorbate present in each test sample of large tubers was detected with the help of standard curve plotted using a standard solution of ascorbic acid (2mL) having different concentrations ie 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL up to 100 µg/mL from the stock solution of 1mg/mL. Concentration of ascorbate was detected by plotting the concentration against the concentration from the standard graph of ascorbate.

Table 9 Ascorbate at different concentration (10-100 µg/mL)

Ascorbic acid (µg)	Iodine (mL)
Blank	0
10	0.30
20	0.50
30	0.80
40	1.20
50	1.60
60	2.30
70	2.40
80	2.80
90	3.20
100	3.70

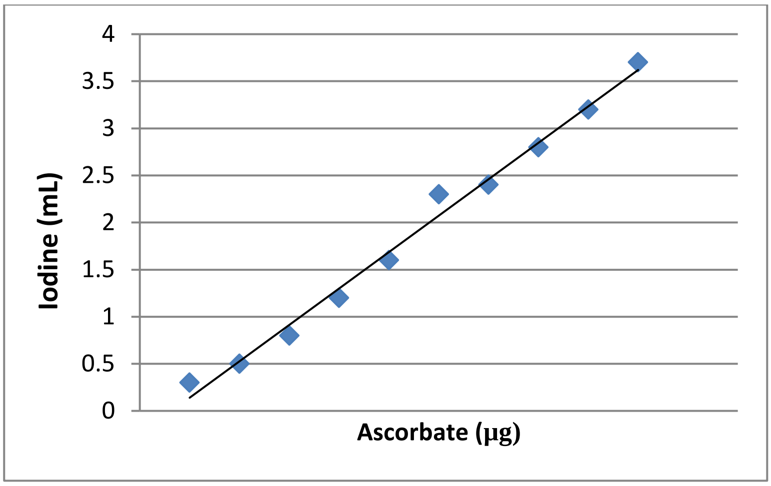


Fig 10: Ascorate standard curve

Table 10: Estimation of ascorbate (µg/g) F.W in large tuber samples

Cultivar	Amount of ascorbate
CS -1	321.842
CS -2	269.210
PR	229.700
DE	203.421
KJ	242.89
KCM	295.5

From the values obtained by spectrophotometric (as presented in the Tables 1-9) and titrimetric estimations (Table 10), it could be concluded that spectrophotometric method is more sensitive, accurate and reliable. But in the case of titrimetric method, reagents used are simple and its time saving (Fig.10). In nutshell for the precision, spectrometric estimation should be undertaken. This is a simple study which needs to be further explored.

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