NUTRITIONAL PROFILING AND DEVELOPMENT OF IRON RICH POTATO THROUGH PROTOPLAST FUSION

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Vegetable Science

By

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2026

DECLARATION

I, hereby declared that the presented work in the thesis entitled **“Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion”** in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Dr. Deven Verma, Assistant Professor, Department of Horticulture, School of Agriculture of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE-I

This is to certify that thesis entitled **"Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion"** submitted in partial fulfillment of the requirement for the award of degree of **Doctor Of Philosophy** in the discipline of **Vegetable Science**, is a Bonafide research work carried out by **Vidushi (Registration Number 12208573)** is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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CERTIFICATE-II

This is to certify that the thesis entitled **“Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion”** submitted by **Vidushi (Registration Number 12208573)** to the Lovely Professional University, Phagwara in partial fulfilment of the requirements for **Doctor Of Philosophy** in the discipline of **Vegetable Science** in the Department of Horticulture, School of Agriculture, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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ABSTRACT

Potato (*Solanum tuberosum* L.) is an eminent crop and is ranked fourth in respect of production after wheat, rice and maize in the world. The comprehensive research titled “Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion” was conducted in the field and plant tissue culture lab at Lovely Professional University, Punjab. The experiment consisted of three objectives and was arranged in CRD and FCRD with three replications. In the first objective fifteen different varieties of potato namely, Kufri Uday, Kufri Chipsona-1, Kufri Jyoti, Kufri Pukhraj, Kufri Surya, Kufri Lalima, Kufri Lima, Kufri Khyati, Kufri Mohan, Kufri Himsona, Kufri Chandramukhi, Diamond, 5758, 302, and 3797 were collected, and mineral content was analyzed using ICP-OES, whereas, moisture content, starch content, ash content, dry matter content, skin & flesh colour, total soluble solids and specific gravity were analyzed using specific methods. Whereas, in the second objective, first, the varieties were selected, one with high iron content (Kufri Uday) and one processing variety with low iron content (Kufri Chipsona-1); and the protoplasts were isolated under nine different treatments with varying incubation temperature and enzymes concentrations *i.e.,* T_1- E1 + C1 (0.25% Macerozyme + 1.5% Cellulase at 22°C); T_2- E2 + C1 (0.4% Macerozyme + 2.0% Cellulase at 22°C); T_3- E3 + C1 (0.5% Macerozyme + 1.0% Cellulase at 22°C); T_4- E1 + C2 (0.25% Macerozyme + 1.5%

Cellulase at 25°C); T_5- E2 + C2 (0.4% Macerozyme + 2.0% Cellulase at 25°C); T_6- E3 + C2 (0.5% Macerozyme + 1.0% Cellulase at 25°C); T_7- E1 + C3 (0.25% Macerozyme + 1.5% Cellulase at 27°C); T_8- E2 + C3 (0.4% Macerozyme + 2.0% Cellulase at 27°C) and T_9- E3 + C3 (0.5% Macerozyme + 1.0% Cellulase at 27°C), where E = Enzyme Combinations, C = Temperature. In this experiment, the viability, density and size of the protoplasts was observed under the fluorescence microscope by using fluorescein diacetate and hemocytometer. Afterward, the fusion of both the selected cultivar protoplasts were carried out by using the fusogen PEG 6000 and then cultured them into the culture medium. In the last objective the formation of calli, its color and growth were observed and then formed calli were subcultured in MS media with four different concentrations of plant hormone *viz.*, T1 [Zeatin (0 mg/L) + 2,4-D (0 mg/L)]; T2 [Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)]; T3 [Zeatin (1 mg/L) + 2,4-D (3 mg/L)]; T4 [Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)].

The results of the first objective revealed that Kufri Chandramukhi exhibited higher levels of Ca (1084 ± 1.0 mg/kg), Mn (26.48 ± 0.90 mg/kg), and P (586.66 ± 1.52 mg/kg); Kufri Uday had the greatest Fe (55.20 ± 0.88 mg/kg), Zn (42.12 ± 0.30 mg/kg), and Cu (34.00 ± 0.12 mg/kg) content and Kufri Lalima had the highest Mg content (1111.66 ± 1.52 mg/kg). In addition, Kufri Khyati displayed greater concentrations of K (1048 ± 2.00 mg/kg), while Kufri Jyoti had a higher S content (1494.33 ± 2.51 mg/kg) and Kufri Lima had the highest B (29.80 ± 0.56 mg/kg) than the other varieties. The skin colour of the tubers ranged from red to yellow and the flesh colour was determined between white to yellow. On the other hand, highest total soluble solids was observed in Kufri Himsona with 7.01°Brix and ash content was observed maximum in Kufri Chandramukhi (2.95%). Additionally, it was observed that Kufri Himsona, Kufri Chipsona-1, and Kufri Chandramukhi were superior for processing due to their higher level of dry matter content, specific gravity, and starch content.

The analysis of the second objective claimed that the viability, density and size of the protoplasts of the Kufri Uday and Kufri Chipsona-1 was highest with treatment E3+C3 (0.5% macerozyme + 1% cellulase at 27°C). It showed that by changing the temperature and concentrations of the enzymes variation in viability and density took

place, as they played an important role in the isolation of protoplasts that helps in to increase the viability, density as well as the size of the protoplast. That’s why the proper combination of enzymes is important and the fluctuations in the viability, density and size of the protoplasts were also taken by increasing or decreasing the incubating temperature, increase in temperature helps in isolating the protoplasts. Temperature is important for dissolving the cell wall of leaves. Both the factors are crucial for the isolation of protoplasts. Furthermore, we also observed that the high density of protoplasts increases the chances of protoplast fusion because higher density allows the cell division and cell signalling and this enzyme combination supports the density of the protoplasts when kept at 25°C or 27°C. Thus, 0.5% macerozyme and 1% cellulase at 27°C is suitable for the isolation of protoplasts of cultivars Kufri Uday and Kufri Chipsona-1 and can be used for isolation of protoplasts. A protocol has been developed for the isolation of protoplasts from the leaves of the potato plant.

Keywords: Potato, *Solanum tuberosum*, potato varieties, protoplast isolation, potato fusion, iron content, ICP-OES.

Signature of Advisor **Signature of Student**

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ABBREVIATIONS USED

Abbreviated Form		Full Form
%	:	Per cent
@	:	At the rate of
+	:	Plus
ANOVA	:	Analysis of Variance
°Brix	:	Degree Brix
CD	:	Critical Difference
CRD	:	Completely Randomized Design
CPW solution	:	Cell Protoplast Washing solution
Cm	:	Centimeter
cv.	:	Cultivar
°C	:	Degree Celsius
DW	:	Dry weight
EC	:	Electrical Conductivity
et al.	:	*Et alia* (and others)
FCRD	:	Factorial Completely Randomized Design
FDA	:	Fluorescein diacetate
Fig.	:	Figure
g	:	Gram
g/L	:	Gram per litre
g/FW	:	Gram per Fresh weight
i.e.	:	That is
Kg	:	Kilogram
M	:	Molarity
MES	:	2-Morpholinoethanesulfonic acid
mL	:	Millilitre
mg/kg	:	Milligram per kilogram
mg/g	:	Milligram per gram
mg/L	:	Milligram per litre

mM	:	Millimolar
MW	:	Molar weight
P	:	Plate
PCM	:	Protoplast Culture Medium
ppm	:	Parts per million
SE(m)	:	Standard Error of Mean
µg	:	Microgram
µL	:	Microlitre
µg/g	:	Microgram per gram
var.	:	Variety
viz.	:	Videlicet (namely)
w/v	:	Weight by volume
WS solution	:	Washing solution

Chapter-I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an eminent crop and is ranked fourth in respect of production after wheat, rice, and maize in the world **(Dutt, 2008)**. It is often referred to as the Irish or white potato. It is a dicotyledonous annual herb with underground horizontal stems that produce multiple tubers. Potato is a member of the Solanaceae family, usually referred to as the Nightshade family in the genus *Solanum*, with a chromosome number of $2n= 4x= 48$. It is a starchy, tuberous crop. It has produced tetraploid species that are heterozygous in nature. During the 17th century, the Portuguese introduced potatoes to India. The ideal conditions for potato growth are a cold environment with over 15 hours of light every day. It is a cool-season crop. If the temperature goes down by 10°C and rises upto 30°C, the tuber growth may be drastically reduced **(Chakrabarti *et al.,* 2017)**. It is a perishable crop since it contains 80% water and cannot be kept under conventional storage conditions for more than 3–4 months. As a result, potato may be kept in cold storage to lengthen their shelf life and satisfy the demands of both the public and the processing sector.

After China, India is the second-largest producer of potato worldwide. According to **Food and Agriculture Organization** estimation, world's potato production reached above 376 million metric tonnes in 2021, reflecting a growth from the production level of 333.6 million tonnes in 2010 and in India, potato covered 22,480 ha, the average yield was 24.12 t/ha, and production was 54.23 mt. Except Kerala, it is cultivated under a variety of agro-climatic conditions almost anywhere in India. The peninsular region's plateau comprises 4% of the overall potato area, 6% of the hills, and 90% of the subtropical plains **(Chadha, 2009)**. It is cultivated in plain areas during the winter months from October to March in short-day conditions and in a limited area of mountains during the summer months from April to September under long-day conditions **Rana (2008)**. The major states where potato is grown are Uttar Pradesh, Tamil Nadu, Haryana, Punjab, Madhya Pradesh, Gujarat, West Bengal, Bihar, and Andhra Pradesh.

Due to the increasing population and the processing industries, the demand for potatoes has been increasing over the past few years. It has a very special position in terms of human food. It offers adequate nutrition and energy for dietary intake, which makes it a staple food. It is one of the most affordable dietary sources of carbohydrates and provides an adequate quantity of vitamins B and C, as well as a small quantity of various minerals **(Burgos *et al.,* 2020; Lal *et al.,* 2020)**.

Iron deficiency negatively affects about 1.7 billion people worldwide. A lack of iron causes the death of about 60,000 pregnant women each year. A quarter of the world's poorest population has iron deficiency. Because of its widespread availability, the potato is a crop that may eradicate deficiencies among individuals and promote child growth. In 100 g of potato, the tubers' iron concentration ranges from 0.3 to 2.3 mg. According to **Brown (2008)**, red-skin tubers contain more iron than other cultivars. Zinc is an important mineral for the human body as it promotes growth, boost immunity, and it plays a crucial role in proteins which contains zinc. Zinc deficiency leads to the death of around 4.4% of children (aged under 5 years) in the developing countries due to acute diarrhea and pneumonia **(Sharma *et al.,* 2017)**. It also causes hair loss, poor hair development, weak eyesight and memory loss **(Dalamu *et al.*, 2017)**. Boron deficiencies have also been seen in the human body like bone deformation in older adults, hormonal imbalance in women, short-term memory loss, join stiffness, slow wound healing etc. Copper helps in functioning of humans' nervous systems and is also an antioxidant. It also helps in the formation of hemes and catalyse the process of the healing of wounds. Deficiency of copper can cause joint inflammation, infection, loss of appetite, etc. Calcium plays a crucial role in the human body like maintenance as well as strengthening the body and teeth, blood clotting, hormonal secretion, maintaining nerve functions and heartbeat, etc. Deficiencies may cause the rickets, muscle cramps, osteomalacia, etc. Manganese is a cofactor of many enzymes in the body, and it also plays a crucial role in the forming of bone cartilage, aids in the healing of wounds, etc. Its deficiency may cause weakness in the bones, joint pain, stunted growth and delay puberty in the children, increase the susceptibility to infection and diseases, etc. Magnesium is mostly stored in the bones and helps in the maintain the bone density, regulates blood pressure, and is involved in the protein synthesis, etc. Its deficiency symptoms are tingling, numbness, weakness, fatigue, depression, anxiety etc. Potassium role in the human body has seen by balancing the electrolytes and fluid in the body, maintains heart beats, functioning of muscles, regulates blood pressure. Its deficiency symptoms are, abnormal heart beats, constipation, weakness, mood swings, etc. Phosphorus is an important mineral of teeth and bones, helps in the formation of DNA, balances the acid- base in the body, involves in the transformation of the nerve impulses, etc. its deficiency may cause bone pain, weakness, tingling, numbness, weaking in the immune system etc. Sulphur helps in the detoxification of the human body, insulin production, helps in the keratin and collagen formation etc. Its deficiency causes joint pain, skin problems, weak and brittle hairs, slow healing of wounds, digestive issues etc.

The world's problems with hunger and poverty may be resolved with the help of the potato crop **(Bakhsh, 2020)**. Additionally, it also plays an essential role in the production of bioenergy (biofuels) and food processing **(Ahmed *et al.,* 2018)**. In addition, starch is used to make papers, cosmetics, textiles, and plastics as well as a stabilizing and thickening factor in food **(Craze *et al.,* 2018)**. Thus, the development of new potato cultivars is a top priority because of their high market value. To improve the quality of potatoes, a variety of breeding and molecular strategies have been used. Conventional breeding may improve potato processing, storage quality, and production **(Halterman *et al.,* 2016)**. Contrarily, traditional breeding may sometimes result in incompatibility and inbreeding depression that further hinder the assimilation of traits in polyploid crops. Abiotic factors like temperature, rainfall, drought, salt, and various post-harvest issues, as well as biotic challenges like insects, pests, bacteria, and fungus, also have an impact on conventional breeding lines. Biotechnological approaches are crucial to resolving all of these issues and improving production along with the quality of the potato **(Bekele, 2021)**.

Modern breeding methods aim to improve the nutritional concentration of potato. The mineral content of potato tubers is not entirely determined by the mineral concentration in the growing substrate **(Subramanian *et al.,* 2017)**. Furthermore, even within species, the mineral composition changes genetically. To construct an experiment to grow nutrient-rich potatoes using a variety of strategies, it is crucial to acknowledge the mechanisms that are involved in the accumulation, transit, as well as storage of minerals in tubers. Over the past ten years, several research has been conducted on this subject; however, little is yet known about the genetic variations connected to tuber mineral content.

Biotechnological techniques, including tissue culture, have been used extensively for the growth of plants and cells. The growth of the whole plant via the explants is known as totipotency, and this happens primarily under aseptic and controlled conditions, allowing adaptation to the new environment **(Alexopoulos and Petropoulos, 2021)**. Murashige and Skoog (MS) medium, which includes all the minerals, sucrose, and vitamins necessary for the healthy development of the *in vitro* grown plant, is the growth medium used in tissue culture. In terms of practical applications, it is also referred to as micropropagation, which is valuable and beneficial **(Shahzad *et al.,* 2017)**. Potatoes are cultivated *in vitro* via meristem culture, and plant material is then multiplied and acclimated in controlled environments for the production of mini-tubers, which are then given to farmers (**Delgado-Paredes *et al.,* 2021**).

There are various techniques of tissue culture technique, depending on the main plant material used, including calluses, somatic embryogenesis, protoplast culture, microspores (anthers, pollens, or ovaries), roots, node cuttings, meristem tips of shoots, and seeds **(George, 2008)**. Protoplast culture and somatic embryogenesis may prove to be the most effective of the tissue culture techniques for potato crop improvement **(Kaur *et al.,* 2018)**.

Protoplasts are described as live, naked plant cells whose cell walls are broken down by enzyme digestion. Their ability to differentiate into any kind of cell demonstrates their usefulness as an enormous biotechnological instrument in both fundamental and practical scientific investigations. Protoplasts in economic cultivars provide the opportunity for *in vitro* alteration and enhancement of crops, without the need for sexual reproduction **(Davey *et al.,* 2005)**. Protoplast culture techniques must be applicable to a diverse variety of materials in order to be used effectively in plant breeding work. The technique developed by **Shepard and Totten (1977)** has effectively been initiated to generate plants from cells of protoplasts of various cultivars of *Solanum tuberosum*.

Protoplast fusion is a special technique for plant improvement, enabling the combination of cells that are somatic in nature (complete or partial) from various genera, cultivars or species. This process results in the creation of new genetic combinations, such as symmetric hybrids, asymmetric hybrids, or cybrids. Somatic hybridization has a unique ability to simultaneously integrate nuclear and cytoplasmic genes, which distinguishes it from sexual hybridization or genetic engineering methods. This method could accelerate the process of breeding and transferring genes, circumventing issues often encountered in traditional sexual reproduction **Cocking (1960)**. **Carlson *et al.* (1972)** were the first to describe the generation of somatic hybrids in solanaceae family crop tobacco using the cell fusion approach. Currently, this technique has been expanded to encompass numerous genera, resulting in the production of somatic hybrids that possess symmetrical nuclear genomes from both parents, as well as asymmetrical hybrids that incorporate the nuclear genome from the donor parent into the recipient parent's genome. Additionally, cybrids have been developed by combining the nuclear genome of one parent with the mitochondrial genome of the other parent. This has now been expanded to many genera to generate cybrids. By avoiding problems like sexual incompatibilities, polyembryony, and female or male sterility that come with traditional sexual breeding, this method may make reproduction and gene transmission easier **(Swapnil *et al.,* 2020)**. Protoplast culture has several steps, like isolation of cells, fusion and regeneration of new plants.

Isolation of protoplasts can be done through the leaves, stems or any part of the plant and can only be possible by using enzymes like cellulose, pectinase or macerozyme. In the absence of the cell wall, the plasma membrane of more than two protoplasts may directly and closely interact. When two protoplast plasma membranes contact one another, they may adhere to one another, like the way two soap bubbles cling together, under certain circumstances. Subsequently, like two soap bubbles, when provided with the right stimulation, they will merge and create an entire sphere enclosed by a single membrane **(Withers & Cocking, 1972; Burgess & Fleming, 1974)**.

When protoplasts fuse, their nuclei also fuse and organelles, which include chloroplasts and mitochondria, combine, leading to the formation of allotetraploids. Hybrid plants usually exhibit characteristics that blend the qualities seen in their parent species. However, they may also possess unique properties not found in either parent species **(Eeckhaut *et al.,* 2013)**. Consequently, the suitability of these plants as breeding materials has been extensively recognized. Furthermore, cybrids, hybrids with fused nuclei from both parents and organelles like chloroplasts or mitochondria from each parent, have been successfully produced. However, the characteristics of these hybrid types are not known.

Since somatic hybrids have the genomes of both of their parents, it is possible for them to display desirable or undesirable characteristics from the fusion partners. This might lead to unexpected performance or phenotypes, and therefore, they could not be of direct value **(Xu *et al.,* 2007)**. Even while an incomplete or combined expression of the genomes is conceivable, as well as both, there is always the capability of inheritance of both desirable as well as undesirable characteristics simultaneously **(Grosser *et al.,* 2000)**. This means that there is always the chance of inheritance of both.

For genetic analysis and genetic improvement, the protoplast fusion method provides tremendous potential. Plant protoplasts from many different species may now be utilized to regenerate new plants. Therefore, genetic modification through *in-vitro* protoplast fusion is an alternative. This area of research is significant because it makes it possible to combine protoplasts from different sources to create unique genome combinations that aren't possible using conventional techniques. Therefore, protoplast fusion may be used to introduce unique germplasm to breeding operations. Somatic hybridization has been widely employed to create new hybrids with improved yields and disease resistance in a variety of horticulture crops.

As discussed above, the issue of unavailability of Fe rich potato varieties can be addressed by using protoplast technique.

Hence, the present study on **“Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion”** has been proposed with the following objectives.

- To identify the high iron content germplasm of potato
- To determine the isolation and fusion of protoplast
- To determine the regeneration of plantlets *in-vitro* from fusion products

Chapter II

REVIEW OF LITERATURE

This thorough chapter offers an extensive review of the existing literature related to the research problem and highlights significant findings from various scientific studies. It presents a detailed analysis of key factors, including analysis of iron content, isolation of protoplasts and fusion of protoplast cells and regeneration of plantlets from the fusion products especially in potato and other agricultural crop species.

2.1 To identify the high iron content germplasm of potato

2.1.1 Iron content in potato

2.1.2 Iron content in other agricultural crops and products

2.2 To determine the isolation and fusion of protoplast

2.2.1 Protoplast isolation and fusion

2.2.1.1 Protoplast isolation

2.2.1.1.1 Protoplast isolation in Potato

2.2.1.1.2 Protoplast isolation in Other Crops

2.2.1.2 Protoplast fusion

2.2.1.2.1 Protoplast fusion in potato

2.2.1.2.1 Protoplast fusion in other crops

2.3 To determine the regeneration of plantlets *in-vitro* from fusion products

2.3.1 Regeneration of plantlets *in-vitro* from protoplast fusion products in potato

2.3.2 Regeneration of plantlets *in-vitro* from protoplast fusion products in other crops

2.1 To identify the high iron content germplasm of potato

2.1.1 Iron content in potato

Pandey *et al.* (2023) studied mineral content in 214 distinct genotypes of tetraploid potato. The panel consisted of 62 russets, 31 chippers, 68 reds, 32 yellows, and 21 purples: including Atlantic, White LaSoda, Russet Burbank, Russet Norkotah, and a Yukon Gold strain (TXYG79), as well as improved genotypes from the Potato Breeding Program of Texas A&M. They evaluated the phenotypic diversity of tubers derived from improved potato genotypes in terms of their mineral content. Minerals such as K, Mg, P, S, Zn, Fe, Na, Ca, Cu, Mn, and B were measured with the help of Inductively coupled plasma-mass spectrometry. The analysis stated the level of Fe varied from 18.71 to 27.96 µg/g on tuber based on dry weight.

Saar-Reismaa *et al.* (2020) objective was to assess and contrast the characteristics of Blue Congo variety and its hybrids with Granola as well as with Desiree to two yellow-fleshed potato cultivars. The elements Mg, Zn, Mn, Fe, Cu, Ca, K and Se were investigated with the use of the Atomic Spectroscopy. The level of Fe ranged from 48.3 to 133 mg/kg of dry weight, while the concentrations of other elements were also detected.

Singh *et al.* (2020) conducted research to assess the mineral contents in the tubers of 37 Andigena (*Solanum tuberosum* ssp. *andigena*) accessions. Some micronutrient contents in the tubers' flesh were examined using Inductively Coupled Plasma-Mass Spectrometry. Significant fluctuations were noted in the levels of Iron, Copper, Manganese, and Zinc within the flesh of the tubers. The iron concentration in the flesh of the tubers varied from 18.03±0.12 mg/kg in JEX/A-707 to 46.43±1.09 mg/kg in JEX/A-539. Four genotypes, *viz.* JEX/A-539, JEX/A-299, JEX/A-288 and JEX/A-877 had more than 40 mg/kg Fe based on dry weight.

Sosa *et al.* (2018) stated that calibration, external validation, and independent validation processes demonstrated strong coefficients of determination, ranging from 0.93 to 0.96 iron and 0.92 to 0.97 zinc content. Additionally, the standard errors were found to be low, ranging from 1.10 to 1.44 mg/kg dry weight iron whereas 0.91 to 1.06 mg/kg dry weight zinc content. The analysis was done by using both X-ray fluorescence spectrometry and ICP-OES. The established evaluations were used to measure the contents of iron and zinc in several clones of biofortified potato from the breeding program of International Potato Centre. These results indicated that X-ray fluorescence spectrometry can accurately analyse zinc and iron levels with a high level of precision. These clones were cultivated in three separate sites in Peru. A total of twenty clones were found to had maximum iron content, more than 32 mg/kg DW. Additionally, thirteen clones were detected with maximum zinc concentration, surpassing 25 mg/kg DW. Specifically, 80 freezer-dried and crushed samples of potato tuber had Fe and Zn contents ranging from 10 to 33 mg/kg dry weight and 9 to 29 mg/kg dry weight.

Sharma *et al.* (2017) analyzed the changes in the mineral content in the potato flesh as well as in the peel of the potato and examined the loss of nutrients after peeling of 48 different varieties of the Indian potato cultivars. They analyzed the Fe, Zn, Cu, and Mn through the digestion of the samples, and the analysis was done through an Atomic Absorption Spectrophotometer. The iron content in the tubers' flesh ranged from 19.96±2.3 mg/kg in Kufri Garima to 49.51±6.7 mg/kg in Kufri Girdhari, with an average value of 28.49±6.7 mg/kg based on dry weight.

Dalamu *et al.* (2017) inquired forty-six germplasm of potato, exhibiting a range of flesh colours from white to yellow to violet, were assessed for their iron and zinc concentrations with the help of an Atomic Absorption Spectrophotometer in both raw and peeled tubers. The iron concentration in flesh varied from 14.90 to 67.13 ppm in terms of dry weight. The genotypes with the highest iron concentration, listed in decreasing order, were CP 1435 (67.13 ppm), CP 3443 (62 ppm), CP 3772 (55.73 ppm), CP 1239 (52 ppm), and CP 2067 (50.20 ppm).

Rahman *et al.* (2015) determined potassium, magnesium, phosphorus, calcium, zinc, and iron in potato, peeled potato immediately after boiling, and peeled potato before boiling using spectrophotometric techniques. The potato with its peel intact had the greatest mineral content, namely K (413.91 mg/100 g), P (60.57 mg/100 g), Zn (0.29 mg/100 g), Mg (21.6 mg/100 g), Ca (9.40 mg/100 g), and Fe (0.78 mg/100 g) compared to the other samples.

Subramanian *et al.* (2011) determined the three dimensions variation of mineral composition inside the tuber in, after the determination of potato orientation in soil. The freezer-dried tuber samples of tuber were examined through Inductively Coupled Plasma-Mass Spectrometry. The analysed minerals include iron, copper, zinc, magnesium, manganese, phosphorus, calcium, sulphur, and potassium. The iron content was elevated around the stolon attachment site and also exhibited a gradual rise from the end part of bud of potato to the end part of stem of potato. The potato skin included around 17% of the total zinc content in the tuber, 34% of the calcium content, and 55% of the iron content.

Andre *et al.* (2007) examined 74 genetically diverse potato landraces, screened out of 1000 potato germplasm available at CIP using SSR markers. The investigation aimed to ascertain the concentrations of calcium, zinc, iron, total vitamin C, total phenolic compounds, and total carotenoids in various landraces. The mineral level of the samples of potato was determined using Inductively Coupled Plasma Atomic Emission Spectroscopy. The iron concentration varied between 29.87±4.39 and 154.96±49.14 µg/g of dry weight (DW).

Burgos *et al.* (2007) analyzed the zinc and iron levels in 49 Andean potato varieties, which showed substantial variance across different genotypes. The samples were determined for zinc, iron, and aluminium using Inductively Coupled Plasma-Optical Emission Spectrophotometry. The concentrations of iron (Fe) and zinc (Zn) in raw and peeled tubers varied from 9.4 to 36.7 mg Fe/kg and 8 to 20 mg Zn/kg (dry weight). Accession 703274 had the greatest amount of Fe, whereas accession 701165 had the highest level of Zn.

Rivero *et al.* (2003) examined the concentration of K, Na, Ca, Mg, Fe, Cu, Zn, Mn, and Rb within eight different potato varieties collected from Tenerife, Spain. Atomic Emission Spectrometry was employed to ascertain the concentration and presence of elements. Azucena (11.2±1.25 mg/kg), Palmera (10.4±2.91 mg/kg), and Peluca (9.37±3.13 mg/kg) cultivars had the greatest average amounts of Fe whereas Cara (7.19±3.13 mg/kg) cultivar had the lowest quantity.

2.1.2 Iron content in other agricultural crops and products

Louppis *et al.* (2019) studied the mineral content of Asfaka, flower, fir, orange blossom honeys, and woodland flowers obtained in the larger region of Hellas by experienced beekeepers were examined by Inductively Coupled Plasma Optical Emission Spectrometry. There were twenty-five minerals discovered and measured. The analysis revealed that the honey samples included high concentrations of Fe as found in the Samos Island's flower was 7.68 mg/kg and lowest was in the Lakonia's orange blossom 0.59 mg/kg.

Ördög *et al.* (2017) aimed to ascertain and contrast the elemental composition of honey samples derived from black locust, sunflower, and oilseed rape, which were gathered from the Great Plain of Hungary of southern region. The samples of these honeys were examined with the help of Inductively Coupled Plasma-Mass Spectrometry to detect the primary minerals (Mg and K), trace elements (Zn, B, Mn, Mo, Fe, Ni, Al, Se, Cu, and Co), and hazardous elements (Cd, As, and Pb). The concentration of Fe in unifloral honey varieties ranged from 2.71 to 9.67 mg/kg.

Kumaravel & Alagusundaram (2014) assessed different Indian spices (ajwain powder, aniseed, cloves, poppy seed, fenugreek seeds) for analyzing trace elements namely phosphorus (P), mercury (Hg), zinc (Zn), lead (Pb), arsenic (As), copper (Cu), cadmium (Cd), iron (Fe), manganese (Mn), chromium (Cr), magnesium (Mg), selenium (Se), carbon (C), sodium (Na), and potassium (K) through Inductively Coupled Plasma Optical Emission Spectroscopy. The composition of iron in Cloves was 1.699±0.2 mg/kg, the Aniseed had 5.40±2.0 mg/kg, ajwain powder contained

17.87±8.0 mg/kg, fenugreek seeds had 4.094±1.0 mg/kg and Poppy seeds had 5.475±2.5 mg/kg Fe content in them on dry weight basis.

Rehman *et al.* (2014) depicted that nutrient content of widely eaten vegetables, including *Abelmoschus esculentus, Lathyrus aphaca, Amaranthus caudatus, Brassica rapa, Raphanus sativus,* and *Solanum melongena*. The nutritional properties and mineral content of these vegetable species were assessed. The nutritional study of these selected vegetable species included evaluating the carbohydrates, total proteins, lipids, moisture, and ash contents. Additionally, Na, Mg Fe, Cr, Cu, Pb, Cd, and Mn were assessed using the Atomic Absorption Spectrophotometric technique. The concentration of iron (Fe) was observed in *Amaranthus caudatus* (10.68±0.2 ppm), *Lathyrus aphaca* (2.11±0.3 ppm), *Abelmoschus esculentus* (5.01±0.1 ppm), *Solanum melongena* (10.69±0.4 ppm), *Raphanus sativus* (11.64±0.7 ppm), and *Brassica rapa* (9.12±0.2 ppm) on dry weight basis.

Hussain *et al.* (2011) examined the mineral and nutritional composition in *Cucurbita maxima, Momordica charantia, Allium sativum, Solanum melongena* var. *esculentum, Brassica oleraceae* var. *capitata* and *Capsicum fruitescens.* Minerals like Cu, Cr, Co, Zn, Cd, Fe, Ni, and Pb were analyzed through an Atomic Absorption Spectrophotometer. The findings of their investigation revealed that *M. charantia* had maximum Fe concentration of 612 ppm, followed by *B. oleraceae* var. *capitata*, *C. fruitescens*, *S. melongena* var. *esculentum*, *A. sativum*, and *C. maxima*, which had concentrations of 102 ppm, 104 ppm, 70 ppm, 164 ppm, and 66 ppm of Fe respectively.

Onwordi *et al.* (2009) analyzed the mineral composition of three vegetables, namely *Celusia argenta, Amaranthus cruentus,* and *Corchorus olitorius* leaves. The mineral content was determined using the Atomic Absorption Spectrophotometric technique. The nutritional minerals, including iron, calcium, and zinc, were analyzed in these crops. The leaves of *C. argenta*, *A. cruentus*, and *C. olitorius* contain iron with average concentrations of 0.39±0.04 mg/g, 0.6±0.00 mg/g, and 0.47±0.09 mg/g respectively.

2.2 To determine the isolation and fusion of protoplast

2.2.1 Protoplast isolation and fusion

Various studies conducted around the world regarding isolation and fusion of protoplasts in different crops has been compiled below under the following subheadings:

2.2.1.1 Protoplast isolation

2.2.1.1.1 Protoplast isolation in Potato

Moon *et al.* (2021) chose potato cultivar Desiree and employed various techniques to extract the protoplast, including chopping leaves and leaf strips. The enzymes cellulase (1%) and macerozyme (0.2% & 0.5%) were used for the extraction of protoplast. The maximum protoplast efficiency was recorded with cellulase 1% and 0.5% macerozyme which lied between 36.40×10^5 and 63.59×10^5 protoplast/g FW.

Sadia (2017) used enlarged leaves of plants grown in *in vitro* of the potato wild diploid species *S. chacoense* Bitt and cultivar Desiree. The different enzyme and their combinations were used by taking five enzymes- Xylanase, Macerozyme, Hemicellulase, Cellulase R10, Cellulase RS, and Pectolyase Y23. The yield with the combinations of cellulase (15 g/L), macerozyme (5 g/L) and hemicellulose (10 g/L) showed the lesser viability for both the varieties (28.66 ± 3.6%, 29.33 ± 2%) in comparison to other combinations. The Xylanase (15 g/L) without any enzyme combination produced the highest viability in both the species (81 ± 1.7%, 78.33 ± 3.60%).

Wang *et al.* (2017) did their research on two dihaploid lines of *Solanum tuberosum* 81–15 and 81–8 as well as two tetraploid local varieties of China named Gannongshu No. 1, Gannongshu No. 3. Following the gathering of immature flower buds, only buds with tetrad-containing anthers were chosen. After cutting each anther's basal end under water and using a scapel to squeeze out the pollen tetrads, the supernatants were filtered through a steel sieve with a pore size of 150 μm to get rid of extra anther wall debris. After centrifuging the filtrate three times for five minutes at 500 rpm, the supernatants were added to 10 milliliters of filter-sterilized enzymatic mixture that contained snailase (0.5, 0.8, 1.0, 1.2, and 1.5%), hemicellulose (0.5%), pectinase (0.5%), cellulase (1%), CH (0.01%), MES 3mM, PVP (1%). The solution of enzymes also contained 0.03M osmoticum (sorbitol, sucrose, glucose, mannitol) with the pH of 5.6. The mixture then kept in shaking incubator at 24-26°C for 2-4 hours at 60 rpm. The highest protoplast isolation was seen in tetraploid cv. Gannongshu No. 3 (74.6 ± 2.4%) out of the four donor materials and the same variety used further for the enzyme combination and the solution with 1.5% snailase had the highest protoplast isolation rate (74.0± 3.4%).

Chen *et al.* (2008) employed three wild diploid species of *Solanum* in their research, one from Argentina and one from Mexico, were cultivated *in-vitro* using true seeds from Inter Regional Potato Introduction Station in Sturgeon Bay, Wisconsin. Leaves of *in vitro* plant were used to separate the mesophyll protoplasts; by using a sharp surgical blade, 0.5 g of leaf tissue was removed from 4 to 6-week-old shoots that were being grown *in vitro*. The tissue was then prepared at room temperature for atleast one hour in the pre-plasmolysis mixture. After that, the solution was swapped out for digesting solutions made with different concentrations of Macerozyme R-10 (0.25-0.75%) and Cellulase Onozuka R-10 (0.8- 2%). The incubation process lasted 16 hours in the dark at different temperature 22, 25, and 29°C. The yield of protoplast was 1.85×10^6 at 22°C, 2.75×10^6 at 25°C, and 3.85×10^6 at 29°C and enzyme combination 0.35% macerozyme and 2% cellulase had the highest mean yield 3.50×10^6 FW.

Chang and Loescher (1991) aimed to explore the impact of Ca^{2+}, dark pretreatment, as well as glycine on the production of potato cultivar Russet Burbank protoplasts that are appropriate for the regeneration as well as for electroporation, with the goal of achieving consistently high yields. Leaf tissue weighing 1 g was cut into strips that were 0.5 millimetres thick. The strips were then washed once with a solution containing either 0.3 M sorbitol or sucrose. After that, the strips were transferred to a 10-millilitre solution of enzymes that had been sterilised by filtration. The enzyme mixture contained 0.3 M sorbitol or sucrose, bovine serum albumin (BSA),0.8% cellulase, 0.17% macerase, 0.107% 10 millimolar MES buffer, and 2 %

polyvinylpolypyrrolidone (PVPP). The pH was taken 5.6. To assess the impact of Ca^{2+} on enzyme incubation, the enzyme solution was supplemented with 0, 1, 5, or 10 mM $CaCl_2$. To examine the effects of glycine, solutions of glycine at concentrations of 0.0, 0.1, 0.2, and 0.3 M were adjusted to have the same osmotic pressure as solutions of sucrose at different concentrations, in the enzyme solution. Irrespective of other pre-treatment effects, such as employing stem cuttings, entire plants or detached leaves, the presence of both sucrose and glycine in the digesting media resulted in higher protoplast yields. Protoplast yields were consistently doubled in all situations.

Cardi *et al.* (1990) investigated the feasibility of isolating protoplasts and regenerating plants in three different accessions of *Solanum commersonii*. Protoplasts were extracted from the photosynthetic plants' leaves, as well as from the leaves and stems of plants that had lost their green coloration. The enzyme experiment was conducted in petri dishes and put these on the rotary shaker rotating at a speed of 30 revolutions per minute. The enzyme medium used consisted of 0.2% and 1% cellulase in the culture medium V-KM media of half strength with 0.20 M glucose and mannitol 0.20 M, resulting in a total osmolarity of 500 mOsm/kg and a pH of 5.6. Fluorescein diacetate (FDA) was mixed in the enzyme mixture containing bleached material at a final concentration of 1 microgram per millilitre. The protoplast yield obtained from shoots kept at a temperature of 24°C varied between 3.0 and 5.5 $\times 10^6$ g^{-1} of fresh tissue when the propagation medium included 10% sucrose. When the sucrose concentration was decreased by 3%, the protoplast yield varied from 4.4 to 8.5×10^6 g^{-1} protoplasts cells of fresh tissue.

Jones *et al.* (1989) discovered that mini tubers that were five weeks old were chosen. They were cut into discs that were 0.5 - 1.0 mm thick and left in a medium overnight. The medium included 0.5 mg/L BAP and 2.0 mg/L NAA, according to Shepard and Totten in 1977. The tuber slices were subjected to pre-plasmolysis for 20 minutes the next day. After that, the protoplasts were released by incubating the slices for 4-5 hours in an enzyme mixture. That mixture consisted of 0.2% Pectolyase, 0.5% Rhozyme, 1.0% Cellulase, and 0.5% BSA (bovine serum albumin). The incubation was carried out without shaking and at a pH of 5.6. The result showed that protoplast yield obtained from mini-tuber discs varied between 7.5×10^4 and 5×10^5/g of tissue.

Dai *et al.* (1987) claimed that five wild *Solanum* species (*S. tuberosum, S. bulbocastanum, S. jamesii, S. chacoense, S. microdontum, S. etuburosum*) were found to be as effective as leaf tissue in providing protoplasts. 1 g of leaves were added to the enzyme solution. The enzyme solution consisted of 1% (w/v) cellulase, 2% PVP, 0.2% macerozyme, 3 mM MES, 0.3 M sucrose, and a concentration reduced for the organic compounds and main and minor salts found in Shepard's R culture medium. The pH was modified to 5.6 using a NaOH solution (1 N) and maintained at 28°C temperature for 16 to 20 hours. It was observed that tetraploid domestic cultivars produced a slightly higher number of protoplasts per gramme of wet tissue weight compared to diploid wild species.

Bokelmann and Roest (1983) described a method by using Dutch cultivated tetraploid potato cultivar Bintje for regenerating plants. Potato shoots were used to harvest protoplast cells that cultivated in a controlled environment and placed in a liquid medium. The shoots were combined with 1 mL enzyme solution consisted of 0.3% macerozyme and 1.5% cellulase were dissolved in mannitol 0.6 M solution at 5.5 pH. The solution was then pierced with needles and chopped into fragments using a scalpel. The number of protoplasts, ranging from 20-50 mm in diameter, obtained from 1 g of fresh material of shoot ranged from 5×10^5 to 7×10^6. The yield did not significantly vary regardless of when shoots had been sub cultured for 2, 3, 4, or 5 weeks.

Carlberg *et al.* (1983) derived protoplasts from *in vitro* cultivated *Solanum tuberosum* L. plants. The 2 grammes of material were added to a petri dish along with 10 mL of an enzyme mixture. The enzyme combination consisted of 0.1% macerase, 0.4 M sucrose, 1% cellulysin mixed in medium K3. The dish was then incubated for 16-18 hours in the dark at 27°C. The result showed that yield varied from 1.5×10^6 to 8.0×10^6 protoplasts per gramme of tissue.

Binding *et al.* (1978) obtained protoplast from the leaves of six diploid clones of *Solanum tuberosum* L. shoot cultures. This was done by taking 1 gram of leaf slices and combining them with 0.3-1.0 grams of Macerozyme R-10 and 1.5-2.0 grams of Meicellase in 100 millilitres of an aqueous solution containing mannitol at a pH of

5.5-5.8. The incubation was placed at 25-30°C a temperature range for 4-15 hours. The findings indicated that a protoplast concentration of around 5 × 10^3 per mL was determined to be the most effective.

Shepard & Totten (1977) discovered protoplasts from mesophyll cells were extracted from potato variety of the Russet Burbank. The lower sides of the leaflets were delicately brushed with nylon brush until they displayed a light green colour. Subsequently, they were cut into squares with an approximate dimension of 2 cm. Four grammes of leaves were put in a 500-milliliter flask consisted of 200 millilitres of medium without agar or sucrose. The flask was then incubated at a temperature of 4 degrees Celsius in the dark. After a period of 16-24 hours, the medium was consisted of 100 mL of combined mixture of enzymes composed of contained 0.1 g Macerozyme, 0.3 M sucrose, 2 g PVP, 0.5 g Cellulase, mineral salts, and MES buffer 0.01 M and the pH was maintained at 5.6. Exposing plants to short periods of low light was particularly advantageous in achieving high yields (2-3 × 10^6/g tissue) of viable protoplasts.

2.2.1.1.2 Protoplast isolation in Other Crops

Stajič (2023) aimed to enhance the efficiency of the isolating protoplasts from the leaves of cabbage, using several enzyme mixtures. The optimal outcome was seen while using a mixture of 0.1% Macerozyme and 0.5% Cellulase in their research. This optimized mixture was effective in isolating protoplasts from cabbage cultivars of five distinct types, resulting in yields was ranged from 2.38 to 4.63 × 10^6 protoplasts per gram fw and viability was 93% or higher.

Figueroa-Varela *et al.* (2023) objective was to provide a technique for extracting, cultivating, and introducing genetic material into the protoplasts of Castor Bean (*Ricinus communis*) plants produced *in vitro*, using the PEG-mediated transfection method. The enzymatic content and incubation duration were assessed. The optimal condition for achieving the desired outcome was the enzymatic solution, which consisted of 0.8% Macerozyme R-10 and 1.6% Cellulase R-10 and was incubated for 16 hours. The protoplast yield was 481.16 × 10^4 protoplast cells per g of fresh weight, with a viability rate of 95%. Moreover, they discovered that a longer incubation period produced more protoplasts 8.5 × 10^5 protoplast/g FW, although their viability dropped.

Xue *et al.* (2023) aimed to develop a very effective technique for extracting mesophyll protoplasts and promoting their regeneration. The procedure of isolating mesophyll protoplasts was devised for the hybrid *Duboisia myoporoides* × *Duboisia hopwoodii* by carefully optimizing the parameters of size of leaf strip, incubation conditions, physical treatment, and enzyme concentration. The enzyme concentration was found optimal to be 1 & 2% cellulysin and 0.25 & 0.5% macerase. The enzyme concentrations had huge impact on the production of protoplasts. The protoplasts density recovered 1.5 × 10^5 and 8.9 × 10^5 on fresh weight basis.

Ab Wahab *et al.* (2022) provides a very effective method for extracting and regenerating protoplasts in *Ganoderma boninense*. A significant number of protoplasts was obtained utilising the approach that used the following parameters: the mycelia, which were 3 to 4 days old, were subjected to treatment with 0.02% Driselase and 1% lysing enzyme. They were then incubated at a temperature of 30°C by adding 0.6 M mannitol as an osmotic medium at a pH of 5.8 for a duration of 2 hours. The protoplast yield ranged between 8.95 × 10^9 and 3.12 × 10^{10} cells/mL per 5 g, with maximum yield seen within this range.

Zhu *et al.* (2022) studied protoplast isolation using *Cannabis sativus*. The following enzymes were used: 0.5% macerozyme, 2.5% cellulase, 10 mmol L^{-1} $CaCl_2$, 0.03% 2-mercaptoethanol, 20 mmol L^{-1} KCl, 0.4 mol L^{-1} D-mannitol, 0.1% BSA, and 20 mmol L^{-1} MES with pH 5.7. Following a vacuum treatment in the absence of light for 10 minutes, the enzymatic digestion had subjected to incubation with agitation at a rate of 40 revolutions per minute for 6 hours when there is no light present and the temperature of 22±2 degrees Celsius. The protoplast yield was recorded 1.15 × 10^7 cells g/FW, with a viability rate of 98.5% was obtained.

Geetha *et al.* (2000) extracted the protoplast from the mesophyll tissue of leaf samples of *in vitro* cultivated plantlets as well as from the cell suspension cultures of ginger (*Zingiber officinale*) and cardamom (*Elettaria cardamomum*). When cardamom leaf was kept in the enzyme mixture consisted 0.5% of macerozyme, mannitol 9%, and 2% cellulase for 18-20 hours at a temperature of 25°C in the dark, a protoplast yield of 3.5 × 10^5 per gram of leaf tissue was produced. The protoplast yield of cell suspension culture was obtained 1.5 × 10^5 per gram of tissue. This was achieved by incubating the culture in a solution consisted of 2% cellulase and 1% macerozyme for 24 hours at a temperature of 25°C. The incubation was done with gentle shaking at a speed of 53 rev/min in the dark. A vitality of leaf mesophyll protoplasts was determined to be 75% based on Evan's blue dye, whereas viability of the cell suspension was found to be 40%. A protoplast yielded 2.5 × 10^5 /g of ginger leaf and was achieved by digesting it in an enzyme solution consisting of 3% hemicellulase,0.5% macerozyme R-10, and 5% cellulase R-10. The digestion process included incubating the tissue at 15°C for 10 hours, and 6 hours at 30°C. The protoplasts viability was recorded 55%.

Badr-Elden *et al.* (2010) extracted, fused, and cultivated the protoplasts obtained from the leaves of sugarbeet seedlings, namely Francesca and Meghribel cultivars, seedlings grown in a laboratory. Several enzyme concetration and combinations were used *viz*., cellulase (1-2%), hemicellulase (1-2%), pectinase (1-2%). Protoplasts from Francesca and Meghribel were effectively extracted by incubating them in the combination of enzyme contained 1% hemicellulose, 2% cellulase, and 1% pectinase for 18 hours at a temperature of 25ºC in the absence of light. The extraction process resulted in a high yield of viable protoplasts. The genotype Meghribel produced the most favorable response, with protoplast yields of 4.85 × 10^5 per g fresh weight.

2.2.1.2 Protoplast fusion

2.2.1.2.1 Protoplast fusion in potato

Johnson *et al.* (2001) used Electro Cell Manipulator for electrofusion experiment. Prior to fusing, the protoplast cells were suspended at 1 × 10^6 protoplast cells per milliliter of density in fusion media (1 mM $CaCl_2$ in mannitol 8.5%, pH 5.6). Protoplasts in fusion chambers were seen using an inverted microscope. Using a sterile pipette, protoplasts were taken out of the fusion chamber five minutes of electrofusion and kept in a centrifuge tube. Following a centrifugation process at 500 revolutions per minute 5-minute, the supernatant which was remaining in the tube was extracted, and the pellet contained protoplast was again suspended in liquid culture with of 2.5×10^5 protoplast cells/mL of density. The protoplast culture was then put in the petri dish.

Debnath and Wenzel (1987) carried out protoplast fusion between dihaploids of potato with polyethylene glycol to form tetraploid potato. The combination of protoplasts from two parental clones (1:1) was placed at 23°C with 40% PEG 1500 solution containing KH_2PO_4 0.7 mM, 11.6 mM $CaCl_2$, and 0.2 M glucose (pH 5.8). Afterwards, 10-20% PEG and 0.08 M $CaCl_2$ with pH 10.0 was used to dilute the incubation mixture. Following the further incubations, the washing medium consisted of $CaCl_2$ 0.05 M and mannitol 0.6 M with 5.8 pH was used instead of the high pH or Ca^{2+} solution. The research was based on the incubation time of isolated protoplasts with PEG and $CaCl_2$ and they found that heterokaryon production was highest after 15 minutes of PEG treatment with 10 min of high pH/Ca^{2+}.

Austin *et al.* (1985) conducted protoplast fusion in a diploid potato line *Solanum tuberosum* Gp. Phureja- Stenotomum which bears tubers with a diploid wild species *Solanum brevidens* which is non tuber-bearing, so that tetraploid somatic hybrids could form. Protoplasts of leaf mesophyll have been extracted from the tip of the shoot. A number of high pH combinations of calcium chloride and polyethylene glycol (PEG) were taken as fusing agents. High viability isolated protoplast preparations were adjusted to 1×10^6 per milliliter. After adding an equivalent amount of fusion mixture (100 mM glucose, 0.7 mM KH_2PO_4, and 3.5 mM $CaCl_2$ with pH 5.8), the petri plate was gently shaken on a shaker, afterwards 25% of PEG 6000 of 13.6 mL in fusion solution was applied dropwise into the protoplast mixture by using burette. After 10 minutes of incubation, 27.2 mL of washing solution containing 0.6 M mannitol and 0.05 M $CaCl_2$ with pH 5.8 was added to the mixture. After 10 minutes of centrifuging the resultant suspension at 1300 revolutions per minute, the pellet was preserved, resuspended into the wash medium, then centrifuged again. Dual-fluorescent labelling of protoplasts before fusing revealed that this fusion approach consistently produced 2-3% of heterokaryons.

2.2.1.2.2 Protoplast fusion in other crops

Kastner *et al.* (2017) chose *Hydrangea macrophylla*, *Hydrangea quercifolia*, *Hydrangea paniculata*, *Hydrangea febrifuga,* and *Hydrangea arborescens* were among the 21 cultivars and accessions from which viable mesophyll protoplasts were extracted. The protoplasts fusion was accomplished using PEG. The 1×10^6 protoplasts (500,000 of each parent) in the mixed W5 solution were centrifuged for five minutes at 80 × g. A drop approximately 300 µL of protoplast suspension is placed in between 150 µL of PEG solution [20 g/L glucose, 400 g L^{-1} PEG 4000, 65 g L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$]. After combining the drops using a sterile pipette tip, allowed the solution to set for ten minutes. 3 mL of (65 g L^{-1}) $Ca(NO_3)_2 \cdot 4H_2O$ was added to finish the fusion process, followed by another 10 min for incubation and 3 mL of $Ca(NO_3)_2 \cdot 4H_2O$. Protoplasts were resuspended in protoplast culture medium PPM1 after that suspension medium was moved into centrifuge tubes and protoplast pellet was cleaned with W5. After 7–14 days in incubation in the absence of light at 25°C, plates were subjected to a 16–8 hour light–dark cycle. Nearly, 4000 protoplasts were fused ninety times using PEG. Fusion of two or more protoplasts could be seen under a microscope that structures with several cells that grew in liquid medium.

Tudses *et al.* (2015) carried out their research on crop *Jatropha curcas* L. and crop *Ricinus communis* L. for the isolation of protoplasts. Filtered and purified mesophyll protoplasts cells of *J. curcas* L. and calli protoplasts of *R. communis* L., with densities maintained to 1×10^5 protoplasts/mL, were combined in equal volumes in a 1:1 (v/v) ratio to compare the effect of various molecular weights of 30% PEG between MW6000 and MW8000, as well as 10 mM $CaCl_2.2H_2O$ and 4% sucrose on protoplast fusion efficiency. Protoplast suspension of 100 µL was added to each fresh microcentrifuge tube, along with the fusion solutions, and centrifuged for 25 minutes at 750 rev/min and 10°C. A high pH/Ca^{2+} solution (400 µL) was applied to each microcentrifuge tube. The heterokaryon's maximum viability (66%) was achieved after 10 minutes of 30% PEG (MW6000) aided macrofusion.

Badr-Elden *et al.* (2010) undertook extraction, fusion, and cultivation of protoplasts obtained from the leaves of seedlings of sugarbeet, namely the Francesca and Meghribel cultivars, in a laboratory setting. Various concentrations of PEG and varied treatment periods were examined. The frequency of fusion was found by quantifying the frequency of heterokaryons. Overall, the combination of 20% PEG with treatment durations of 20 and 25 minutes resulted in the greatest fusion frequency. However, the viability was achieved best with 20% PEG and a treatment time of 20 minutes. The cell division was recorded after about 4-7 days after protoplasts were cultured.

Geerts *et al.* (2008) used the donor species as female parents for the interspecific breeding of *Phaseolus vulgaris* L. (PV) with either *Phaseolus coccineus* L. (PC) or *Phaseolus polyanthus* Greenm. (PP). Once pre-germination in Petri plates was completed, the protoplasts were extracted from hypocotyl explants that were 10 days old for the PV (NI638 and NI637) as well as PP (NI1015) accessions. Protoplast fusion occurred between NI637, and all PP and PC accessions was accomplished via chemical fusion. The fusing agent utilized in this experiment was PEG 6000. The fusing agent polyethylene glycol (PEG 6000) was employed has been used to produce many heterokaryons using various genotypes and fusion processes. The development of microcalli produced from heterokaryons as well as the division of heterokaryons were noted.

Assani *et al.* (2005) isolated the protoplasts using embryogenic suspension cultures of banana cv. Gros Michel (AAA) and cv. SF265 (AA), which were obtained three to four days after the *in vitro* subculture. The two fusion partners for protoplasts, which had 5×10^5 protoplast cells per milliliter of a density, were combined equally in the fusion solution that included 0.5 mM $CaCl_2$ and 0.5 M mannitol. Drops of resultant 300 µL solution with 1.5×10^5 protoplasts were put into each 5.5 cm diameter Petri plate. Four to six drops of PEG solution (50 µL each of 50% PEG, 0.5 mM $CaCl_2$, and 0.5 M mannitol) were gradually added after the protoplasts had settled. The protoplasts attached to the Petri plate 20 to 30 minutes after being treated with PEG. Afterwards, 3 milliliters of fusion solution were used to gradually dilute the PEG solution. The diluted PEG-solution was carefully taken out and replaced with liquid culture ten minutes after dilution. Protoplast fusing with the fusogen polyethylene glycol had the highest binary fusion frequency.

Belarmino *et al.* (1996) examined protoplast cells of sweet potatoes and their relatives *Ipomoea lacunosa* L. and *Ipomoea trifida* Don. were fused asymmetrically in experiment. The three drops of protoplast mixture progressively coalesced under static circumstances after a drop of 300 g/L PEG6000 mixture was introduced diametrically opposed to the drops for 15 minutes at 22°C in the dark. The coalesced droplets were then subjected to three additions of WS solution (0.2 mL) spaced five minutes apart. The protoplasts were then suspended in liquid PC medium after being cleaned and centrifuged twice using W5 solution. Following PEG treatment, the incidence of homo-fusion and hetero-fusion was 4%.

Chen and Adachi (1998) did protoplast fusion by using *Lycopersicon chilense* cultivar PI128652, *Lycopersicon peruvianum* cultivar PI270435 and a Japanese variety *Lycopersicon esculentum* cv. Kyoryokutoko. A plastic petri dish was filled with a 0.3 mL (4×10^5 protoplasts) and ratio of 1:1 mixture of the two partners protoplasts, which was then left to settle for 15–20 minutes. After that, drops of the PEG mixture (0.15 mL) which included 0.3 M glucose, 30% PEG 6000, 50 mM $CaCl_2 \cdot 2H_2O$, and pH 9 were put on the periphery of the protoplast suspension and left to combine with the droplet contained protoplast cells. Then W5 medium (1.5 mL) was added after 5–10 minutes, and after 5 minutes, the whole fluid was carefully drained from the plate. The process of washing was carried out twice. The culture plates were sealed with parafilm and kept at 25°C in the absence of sunlight.

Kobayashi and Ohgawara (1988) used hayashi satsuma mandarin (*Citrus unshiu* Marc.), Troyer citrange, *Poncirus trifoliata,* F.N. Washington Naval Orange, Trovita orange,and Murcott tangor in different combinations. Protoplasts from all the former species in combinations of two were combined at an equal volume, acclimated to a density of 10^6 cells/mL, and fused with the help of polyethylene glycol (PEG). PEG was separated by centrifugation at 150 × g for 5 minutes after being diluted with 50 mM $CaCl_2$ and 0.6 M mannitol. Heterokaryons cells were easily identified under a microscope after the fusion procedure.

Kao and Michayluk (1974) examined protoplasts of *Glycine max* L. that obtained from suspension protoplast cultures, cultured in B5 medium consisted of 1 mg/L 2,4-D. Protoplasts cells of crop *Vicia hajastana* were extracted from colonies cultured in Eriksson's culture medium consisting of 2,4 D (0.5 mg/L). The leaves of plants cultivated in greenhouses were used to isolate protoplasts of *Pisum sativum* L. and *Hordeum vulffare* L. The protoplast culture was gradually supplemented with 450 µL of a PEG solution. The PEG solution was used to incubate the protoplasts for 40–50 days at room temperature. This combination was then gradually supplemented with 500 µL of a culture medium. After 10 minutes the protoplasts were washed and cleaned 4 - 5 times with a total of 6 to 7 milliliters of culture media. Following such treatment, soybean and *Vicia* protoplasts isolated from cultivated cells had prolonged cell division and repaired cell walls. One division was seen in several *Vicia*-pea heterokaryons. In only seven days, more over ten percent of the soybean-barley hybrids were divided. During the observation period, neither the PEG-treated nor untreated protoplasts from pea and barley leaves experienced cell division.

2.3 To determine the regeneration of plantlets *in-vitro* from fusion products

2.3.1 Regeneration of plantlets *in-vitro* from protoplast fusion products in potato

Cheng *et al.* (1995) selected the USW2225 diploid genotype of *Solanum tuberosum* and the 320287-1 genotype of *Solanum chacoense* for their study. Following the electrofusion of the genotypes' protoplasts, the pellet contained protoplasts was again suspended in culture media consisting MS culture media with pH 5.8 and 10 g/L sucrose, 30 g/L glucose, 50 g/L sorbitol, 1.25 mg/L of NAA, 1 mg/L of zeatin, 0.25 mg/L of 2,4-D, 100 mg/L glutamine, 500 mg/L of casein hydrolysate and, 100 mg/L of myo-inositol. Following a 6-week period, the formedcalli were moved to the MS culture medium that included pH adjusted to 5.8, 10g/L sucrose, 30g/L sorbitol, 100 mg/L of casein hydrolysate, 2 mg/L zeatin, 100 mg/L myo-inositol, 100 mg/L KH_2PO_4, 0.2 mg/L GA_3, and 2 g/L phytagel. The shoot regenerated medium was prepared using 100 mg/L casein hydrolysate MS media,100 mg/L myo-inositol. While p-calli obtained from the diploid *S. chacoense* could not be able to regenerate any shoots under the circumstances of shoot regeneration, however, p-calli obtained from the diploid *S. tuberosum* could regenerated the plants efficiently. From the fusion products, 50 p-calli were only selected for shoot regeneration out of which 13 shoots were produced (12 were putative somatic hybrids whereas, 1 shoot was from *Solanum tuberosum* based on visual characteristics).

Cheng and Veilleux (1991) investigated the culturability of protoplasts in *Solanum phureja* Buk and Juz., a diploid potato species. A protoplast culturable selection (NBP2) was crossed with a protoplast which are not able to culture, androgenetically formed AM3-8which is a homozygous line. Protoplast culturability tests were performed on seven randomly chosen F_1 plants. Two distinct growth media—liquid culture and embedding culture consisted of 30 g/L glucose, 50 g/L D-sorbitol, sucrose 10 g/L, 1.25 mg/L NAA, 1 mg/L Zeatin, and 0.25 mg/L of 2,4-D were used to culture the protoplasts. However, 2 mg/L of zeatin, 0.01 mg/L IAA, 0.2 mg/L of GA_3, 100 mg/L adenine sulfate, 10 g/L sucrose, 36 g/L mannitol, 100 mg/L of casein hydrolysate, glutamine 100 mg/L, 100 mg/L myo-inositol, 100 mg/L of potassium phosphate, and agar 7 g/L in MS vitamins and salts at pH 5.8 were utilized for the shoot regeneration culture medium. After seven days in culture, most of the AM3-8 protoplasts rupture. Several protoplasts of AM3-8 neither divided but instead remained alive and rebuilt their cell walls. After three days in culture, the NBP2 isolated protoplasts began to divide. Every F_1 plant could produce many protoplasts calli, or p-calli. By selecting and crossing two randomly chosen F_1 hybrids ($F_1$21 x $F_1$37), the F_2 progeny was formed. The F_2 generation underwent segregation for protoplast culturability: out of twenty erratically chosen F_2 plants, only twelve plants were culturable for protoplasts and eight were not.

Feher *et al.* (1989) successfully isolated the protoplast cells from the *Solanum tuberosum* cultivars Gulbaba, Boro, Somogygyöngye, Desiree, and Gracia and they were cultivated in V-KM medium contained 0.5 mg/L Zeatin, 1 mg/L NAA, and 2,4-D 0.2 mg/L for cell development. They later discovered that the protoplasts of the cultivar Somogygyöngye died during isolation, inhibiting the development of colonies and shoots. For shoot regeneration, they used multiple kinds of culture medium with various growth regulators. The remaining cultivars showed just 1% to 2% of isolated protoplasts forming calli. These calli showed a 50% or higher regeneration frequency when placed in medium consisted of 0.5% NAA and 0.5% Zeatin.

Tavazza *et al.* (1988) isolated the protoplast cells from the potato of cultivar Desiree. They used modified MS culture medium consisted of mannitol 0.4 M, 2 mg/L 2,4-D, BAP 0.5 mg/L, and 2 mg/L of NAA for protoplast culture. The cell wall started to regenerate within 2 days and underwent the first division within 3 days on the culture media. They recorded the first initiation of regenerated shoot after 3-4 months of the protoplast isolation. A sudden reduction in growth and inhibition of cell division were seen when the calli which was formed were moved from a culture medium with maximum auxin level (2,4-D @ 5 mg/L) to one with minimum auxin level (2,4-D @ 0.1 mg/L) as well as auxin-free media.

Binding *et al.* (1978) carried out research on potato, after being pollinated with *Solanum phureja*, tetraploid clones MPI 44.1016/10 and MPI 49.540/2 transformed into dihaploid clones of the species *Solanum tuberosum* L. using parthenogenesis. For protoplast culture, the well-regenerating clones H^2 140, H^2 258, H^2 260, H^2 345, H^2 411, and H^2 439 were selected. The ideal hormone composition 2.5 µM benzyladenine or zeatin, 5µM NAA, and 5 µM 2,4-D were used. All hormone levels were not very important in the 2.5–10.0 µM range, and NAA seemed to be helpful but not necessary for protoplast regeneration. However, cell division required 2,4-dichlorophenoxyacetic acid and a cytokinin. After 15 days on KM media, the regenerants were moved to either culture media B5, which included either the 2.5 µM benzyladenine, solely, or 20 µM zeatin, 5% cocos endosperm, and 5 µM NAA, or medium MS which contained 15 µM kinetin and 5 µM IAA. Both mediums were suitable for the development of shoots, which appeared three months after the protoplast isolation.

2.3.2 Regeneration of plantlets *in-vitro* from protoplast fusion products in other crops

Figueroa-Varela *et al.* (2023) used *Ricinus communis* L.- VERC02 for the culture of the cells of protoplasts. They used WPM liquid media without using any growth regulators in the protoplast culture, then the level of sucrose was maximized to 60 g/L, and they were incubated for seven days in an incubator at 25°C in the dark. After that, they were kept on a semi-solid medium for 45 days in the dark with supplements of 1.0 mg/L of NAA, 1.0 mg/L of BAP, and 2 mg/L of Kinetin to induce callus. The semi-solid medium was combined with the protoplast suspension for this process. The tiny calluses that were seen were subcultured into the same media. The culture medium for protoplast that was used earlier for the development of the microcolonies with consistent concentrations of BAP (1 mg/L), NAA (1 mg/L), and Kinetin (2 mg/L) were used for the development of microcalli and formed within 8 weeks after they kept into the culture.

Guan *et al.* (2010) carried out research on the regeneration of plantlets of ginger cultivars Sichuan Zhugen Jiang (SZ), Chenggu Huang Jiang (CH), and Lushan Zhangliang jiang (LZ) by chemically fusing them and to form a somatic hybrid. Their culture medium included solid MS culture media consisted of 3.0% sucrose, 0.2 mg/L Kinetin, 0.7% agar, and 1.0 mg/L of 2, 4-D. MS medium with 5.0 mg/L BA, and 0.2 mg/L 2, 4-D was used to transfer and cultivate the proliferating calli. The whole plant was regenerated when the produced shoots which contained leaves were moved to MS media that contained 0.6 mg/L of NAA and 2.0 mg/L of BA for root formation. It takes over 15 months to renew the whole plant, starting with protoplast fusion. Every fusion combination effectively formed micro-colonies and underwent redifferentiation. Only the CH and SZ fusion combination was able to produce plantlets again. In the fusion combinations LZ + CH and LZ + SZ, no plantlets were regenerated.

Venkatachalam and Jayabalan (1996) obtained protoplast cells from the plant leaves which grew *in vitro* of *Arachis hypogaea*. On modified Kao's medium consisted of 6.0% (w/v) sucrose, 1.0 mg/L of BAP and 2.0 mg/L of 2, 4-D, solidified with 0.4% agarose at pH 5.6, isolated protoplasts were cultivated at a concentration of 0.5 to 4.0×10^4 protoplasts/mL. Following the visual observation of tiny colonies (1-2 mm in size), colonies extracted from the beads were further subcultured onto medium that included MS salts, B5 organic supplements with NAA 0.5-2.5 mg/L and BAP 1.0

mg/L, 3% sucrose, 0.4% agarose, or 0.2% gelrite. Colonies multiplied into calli that were actively growing. Root development occurred in the culture medium, but none of the callus colonies produced from protoplasts developed shoots or buds.

Toriyama *et al.* (1987) studied protoplast fusion of *Moricandia arvensis* (L.) with cauliflower (cv. Nozakiwase) and red cabbage (cv. Ruby Ball). The protoplasts which were treated with fusion were cultivated in the petri plate with a liquid medium (2 mL) at a density of 2 × 10^5/mL. DBN and D2B1 were the two types of cultural media that were employed. While both were made of B5 medium consisted of 8% mannitol, the hormone level of DBN culture medium was 0.1 mg/L of BAP, 0.5 mg/L of NAA, and 0.25 mg/L of 2,4-D, while D2B1 medium had 1 mg/L BAP and 2 mg/L 2,4-D. Following two to three weeks of culture, each petridish acquired an equal volume (1 mL) of fresh medium consisted of 4% mannitol. At the fourth week, again added the fresh medium of 1 mL in the absence of mannitol. The calli which was developed were moved to MS culture medium consisted of 1 mg/L of BAP, 0.8% agar, 1 mg/L NAA, and 100 mg/L casamino acids five to six weeks of the fusion took place. The calli then transplanted to new NB media every three weeks for regeneration. Numerous calli were formed by the fusion of protoplast cells grown in B5 culture medium with high doses of 6-benzylaminopurine and 2,4-D; out of most of the calli, eight calli formed hybrid plants. Some of the plants flowered when grown in pots kept in greenhouse. The morphological characters of both parental species had been combined in all the regenerated plants.

Chapter- III

MATERIALS AND METHODS

The proposed research work entitled **“Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion”** was carried out in the Tissue Culture Lab as well as in the field at Lovely Professional University, Phagwara, Punjab during 2023-2024 and 2024-2025, which is located at 31°14’46’’N latitude and 75°42’12’’E longitude with an altitude of 245.98 m above sea level. The details of the materials used, and the techniques adopted during this experiment are described in this chapter.

3.1 Experiment I: To identify the high iron content germplasm of potato

The first objective of the current research was fulfilled in the form of this experiment. The plant material used, growing conditions, observations taken, experimental designs etc. are described below:

3.1.1 Experimental Details

Crop**-** Potato (*Solanum tuberosum* L.)

Genotypes/treatments- 15

Planting- In Grow Bags

Replications- 3

Design- Completely Randomized Design (CRD)

Analysis of Iron Content**-** Induced Couple Plasma- Optical Emission Spectroscopy

3.1.2 Description of the Plant Material

There were fifteen different varieties of potato that are grown in North-Indian plains, were procured from the commercial cold store **[Plate 3.1 (a)]**. Their characteristics are as under:

Table 3.1.1: Characteristics of Plant material

Varieties	Characteristics
Kufri Lalima	Large to medium-sized, round, red, with white flesh and medium-deep eyes. The crop takes 100–110 days to mature. Yield: 20–25 t/ha on average. Resistant to early blight to a moderate extent. Able to withstand Potato Virus Y (PVY). Unsuitable for processing.
Kufri Uday	Medium to large, 8-9 tubers per plant, Ovoid, Smooth and red skin. Early maturing variety. Average yield is 36-38 t/ha. Crop matures in 90 days. Suitable for table purpose.
Kufri Surya	White-cream, cream-fleshed and oblong with shallow eyes. It is a cultivar that matures early. The yield is 25–30 t/ha on average. Ideal for preparing French fries.
Kufri Jyoti	White skin and large, round, fleeting eyes. In hills, crops mature early; the average yield is 20 t/ha. Resistant to early and late blight to a moderate extent. Degeneration is slowly. It is appropriate for use in processing.
Diamond	They are round, smooth, have shallow eyes, and have white skin and white flesh. Variety is medium to early in maturity. Tolerant of late blight to a moderate extent.
Kufri Lima	The variety yields ovoid tubers, white-cream and cream flesh also shallow eyes. The average yield is 30-35 t/ha. Tolerance to mite burn and hopper. Suitable for table purpose.
Kufri Chandramukhi	Large, oval, white, with dull white flesh and eyes are fleeting and somewhat flattened. The yield is 25 t/ha on average. The crop takes 80–90 days to mature. Ideal for chips and flakes.
Kufri Pukhraj	Large, oval, yellow skin, white, fleeting eyes, and slightly tapered. The crop takes 70 to 90 days for growth. The yield is 40 t/ha on average. Moderately resistant to early blight and late blight. Suitable for table purpose.
Kufri Mohan	Ovoid in shape, white cream flesh, white skin and shallow eyes. This is a medium-mature variety. 35–40 t/ha is the yield of tuber.

	Resistant to late blight to a moderate extent. Suitable for table purpose.
Kufri Khyati	Ovoid, white-cream, with flesh that is white-cream and eyes that are medium-deep. This cultivar matures early. The yield is 25–30 t/ha on average. Suitable for table purpose.
Kufri Chipsona-1	Oval, white, medium to big, with dull white skin and fleeting eyes. The crop takes 90–110 days to develop. The yield is 40 t/ha on average. Frost-tolerant and resistant to late blight. Ideal for preparing French fries and chips.
Kufri Himsona	This cultivar is suitable for chip production and is cream flesh white-cream, round, and shallow eyes. It matures late and yields an average of 15-20 t/ha. It has a low phenols, low reducing sugars, and high dry matter content.
5758	Advanced breeding line
302	Advanced breeding line
3797	Advanced breeding line

3.1.3 Growing Media

These fifteen varieties potato were planted in 24 × 40 cm size grow bags at the field of LPU in the month of September in 2023. The soil, vermicompost, and cocopeat mixture was prepared in a 4:3:1 (v/v) ratio to fill the fifteen grow bags. Each bag contains around 15-18 kg of soil mixture, and each bag contains 4-6 potato tubers **[Plate 3.1 (b,c)]**.

The pH, EC, and mineral content of the soil mixture were examined. In addition to having a neutral pH of 7.0 and electrical conductivity of 0.18 mmhos/cm, the soil mix included 45 mg/kg of Nitrogen, 34 mg/kg of Phosphorus, 120 mg/kg of Potassium, 150 mg/kg of Iron, Manganese (0.79 mg/kg), Copper (5.42 mg/kg), Sulphur (14 mg/kg), Zinc (1.68 mg/kg), Calcium (254 mg/kg), Boron (0.64 mg/kg)were present in the soil.

Table 3.1.2: Average temperature and relative humidity in weather in 2023

Average Temperature and Relative Humidity during the Experiment

Date	**Max Temp (°C)**	**Min Temp (°C)**	**RH (%)**	**RH (%)**	**Windspeed (km/hr)**	**Rain (mm)**	**Evaporation (mm)**	**Sunshine (hrs)**
September 20- September27	33	23	93	63.42	4	0	3	7
September28- October 05	34	19	93	50.99	6	0	2	9
October 06- October 13	33.00	18.56	92.04	52.31	4.46	0.90	2.53	8.64
October 14- October 21	29	15.92	92.01	52.83	5.13	0.53	2.85	8.45
October 22- October 29	30.31	13.95	92.71	47.54	5.00	0.13	2.84	8.25
October 30- November 06	30	13.30	93.39	45.66	3.92	0.00	1.95	6.01
November 07- November 14	26.11	12.26	92.55	56.85	5.67	0	1.71	6.19
November 15- November 22	27	10.95	93.26	46.69	5.00	0.03	1.81	7.48
November 23- December 02	24.2	10.0	92.1	55.6	4.4	0.7	1.4	4.1

3.1.4 Location and Conditions

The experiment was conducted at the farm of School of Agriculture, Lovely Professional University, Punjab under department of horticulture, located at 31°14’46’’N latitude and 75°42’12’’E longitude with an altitude of 245.98 m above sea level, during winter season of 2023-24.

3.1.5 Package of practices

The tubers of the fifteen varieties and germplasm mentioned in the previous section were exposed to the following package of practices **(Plate 3.1, 3.2)** were as follows:

Table 3.1.3: Timeline of package of practices followed for grow bag cultivation of potato

Activities	**Date of performance**
Planting of tubers	20- September- 2023
First irrigation	05- October- 2023
N:P:K spray	15-October-2023
Weeding	20- October-2023
Earthing up	20-October-2023
Second irrigation	23- October-2023
Third irrigation	10- November-2023
De- haulming	25- October- 2023
Harvesting	04- December-2023
Surface drying in well-ventilated room	04 - 07 December- 2023

3.1.6 Details of observations recorded

The observations taken in this experiment has been explained as under:

3.1.6.1 Skin and Flesh Colour

The freshly harvested potato tubers were sliced in half, cutting them vertically and horizontally. The flesh color was determined according to **CPRI, DUS guidelines**.

3.1.6.2 Specific Gravity

The specific gravity was calculated using the water displacement method. The tubers were then weighed in the air and also weight in water. **Raj *et al.*, 2011** procedure was followed to carry out this observation.

$$Specific\ Gravity = \frac{Weight\ of\ tuber\ in\ air}{(Weight\ of\ tuber\ in\ air - Weight\ of\ tuber\ in\ water)}$$

3.1.6.3 Total Soluble Solids

To determine the tubers TSS, a digital refractometer (HANNA model number HI96801) was used. This was accomplished by using two drops of sample on the well of the refractometer. Observations were recorded at room temperature and the value was recorded in °Brix. The procedure was followed according to **Sarkar *et al.* (2018)**.

3.1.6.4 Moisture Content

The tubers were gently washed, surface dried and peeled. The tuber slices were finely cut, their wet weight was recorded, and they were subsequently oven-dried for two to five days at 70°C by using hot air oven (Narang Scientific Works PVT. LTD model number NSW-143). Then, at the end of drying period dry weight was measured. **AOAC, 2005** guidelines were followed to analyse the moisture content as per the formula mentioned below:

$$Moisture\ Content = \frac{Wet\ Weight - Dry\ Weight}{Wet\ Weight} \times 100$$

3.1.6.5 Dry Matter Content

The tubers were first thoroughly cleaned, washed, then peeled. The fresh weight of the thin slices of tubers was measured, followed by oven-drying the same slices at 70°C for 2 to 5 days. The dry matter content was calculated by measuring the fresh weight and dry weight, followed by determining the percentage of dry weight **(Singh *et al.*, 2020)** using the formula mentioned below:

$$Dry\ Matter\ Content = \frac{Dry\ Weight}{Fresh\ Weight} \times 100$$

3.1.6.6 Ash Content

The tubers were sliced and dried for 48-72 hours in a hot air oven, followed by grinding of dried samples. Subsequently, 2 g finely powdered sample was added in a crucible. To eliminate the organic carbon content, the sample was heated on a burner until white fumes ceased and then charred sample was placed in a muffle furnace at 450–470°C for 5-6 hours. After the crucible was removed from the furnace then, allowed to cool in a desiccator at room temperature. Subsequently crucible with ash content was weighed. **AOAC, 2005** guidelines were followed to analyse the ash content. The following formula was used to determine the sample's ash content:

$$Ash\ Content = \frac{(Weight\ of\ crucible + ash) - (Weight\ of\ empty\ crucible)}{(Weight\ of\ crucible + sample) - (Weight\ of\ empty\ crucible)} \times 100$$

3.1.6.7 Analysis of Iron and other Mineral Content in Potato Tubers

After the harvesting of the tubers, potatoes were washed with tap water as well as with 1 N HCl, then washed the tubers with distilled water to remove the contamination from the surface of the tubers. Before the potatoes went through the procedure, tubers were surface-dried with an absorbent paper towel, then afterward placed in a dark space for few hours. For mineral analysis, each potato tuber was sliced into small and thin slices using stainless steel vegetable peeler, and several slices were obtained from each tuber. Samples were dried in glass petri plates at 60°C in an oven to determine the mineral composition. From the dried samples, a one-gram powdered sample was extracted, and 20 milliliters of HNO_3 (65%) and $HClO_4$ (70%) in the combination 4:1 (v/v) were used to digest the crushed samples and digestion process was done on a hot plate (90°C) until white fumes ceased to emanate from the conical flask. Following digestion, in the conical flask only 5 mL samples that were left were diluted with 100 mL of double-distilled water. An Induced Coupled Plasma Optical Emission Spectroscope (ICP-OES) was used to analyse the mineral content in the acid digest. The 5 mL sample was loaded in the ICP-OES at various wavelengths for the determination of the minerals and measured in mg/kg. **Andre *et al.* (2007)** procedure was followed for the analysis of mineral concentrations.

3.1.6.8 Analysis of Starch Content in Potato Tubers

The procedure was similar to methodology adapted by **Clegg (1956)** for the starch content estimation. Firstly, crushed dehydrated potato tubers were taken and weighed 0.2 g of sample of dried tubers and transferred in 50 mL centrifuge tube. For enhancing the process of mixing of sample and water, two droplets of the 80% ethanol were added and then added water (5 mL) and thoroughly the combined mixture was agitated. Heated ethanol 25 mL of 80% concentration was combined and mixed well. The mixture was then allowed to rest for 5 minutes, then centrifuge it. Ethanol solution was separated from the mixture and performed the ethanol extraction process again by add 30 mL of hot 80% ethanol to the remaining substance. After that the two ethanol extracts were merged. Achromatization of the anthrone-sugar reaction was inhibited by ethanol. The residue aqueous solution was diluted with water until the concentration of sugars reached about 100 µg of glucose per milliliter. It was extracted from the extract mixtures that were combined by evaporating at decreased pressure in a water bath and water bath was boiled that time. Then, sugars were analyzed using anthrone reagent. The procedure of isolating the starch from remaining substance was done at room temperature. The 5 mL of water mixed with the remaining product obtained after the ethanol extraction, then pour in 6.5 mL perchloric acid (52%) (made by mixing 270 mL of 72% perchloric acid with 100 mL water), and stirring gently. The mixture was then agitated steadily for five minutes using glass rod and stir it again for the next following 15 minutes. Then, added 20 milliliters of water and centrifuged it. Transfer that supernatant into the volumetric flask with a capacity of 100 milliliters and combined the water (5 mL) with a residue left in the centrifuge and repeat extraction using perchloric acid. The mixture was continuously agitated at regular intervals for the following half an hour. Then pour the contents which was left in centrifuge tube in the flask containing the initial extract, being sure to thoroughly rinse off all the contents. Then merge the extracts and dilute them with water for the total volume make up of 100 mL. The mixture was then filtered out, discarded the first 5 mL of the filtered solution. To get a final concentration of around 100 µg glucose/mL, a part of the sample was diluted. The diluted sample was then analyzed using the anthrone reagent. A standard aqueous solution with 1 mg/mL concentration of glucose also prepared. For preparing the anthrone reagent, one gram of anthrone was dissolved in 1.0 L H_2SO_4 (containing 760 mL of concentrated then cooled to room temperature). Add 10.0 mL anthrone reagent each to two tubes of blank (2.0 mL of water), three tubes of test extract of each variety (1.0 mL of diluted perchloric acid extract and 1.0 mL of distilled water) and three tubes of glucose standard and extract of each variety (1.0 mL glucose standard solution with 1.0 mL diluted perchloric acid extract) and mix them thoroughly. The tubes were sealed with plastic caps and immersed into a boiling water bath for 12 minutes. Afterward, transfer the tubes to a separate container filled with water at room temperature to allow them to cool down. Quantify the chromatic intensity generated by the test extracts by using a spectrophotometer, with the blank reagent serving as the standard. To verify the quality of anthrone reagent, compare the readings of the blanks with distilled water. The anthrone-sugar combination exhibits peak absorption at a wavelength of 630 nanometers. The green color remains unchanged for a minimum of 3 hours.

Calculation-

The mean of the two sets of three measurements were computed and determine the corresponding amount of glucose in the extract by comparing it to the elevated absorption caused by 100 µg of glucose. The ratio of sugars and starch in the initial substance were determined by using the provided value, a dilution coefficient, and a conversion coefficient of 0.9 for the starch extract. The conversion factor is used because 0.9 grams of starch produces about 1.0 grams of glucose upon hydrolysis.

Statistical analysis

The data collected was thoroughly analysed using Analysis of Variance (ANOVA) as the primary test of significance, instigated with SPSS version 25.0 statistical software package with a fixed significance level of 5% ($p \leq 0.05$). To compare the different treatment means in detail, Duncan's Multiple Range Test was used as a post-hoc analysis to pinpoint specific differences between groups. The findings are shown as the mean ± SD (Standard deviation). To facilitate clear understanding and effective communication of the findings, the analyzed results were systematically organized and visually represented through a combination of detailed tables and informative graphs, enabling thorough interpretation of the experimental outcomes.

3.2 Experiment II: To determine the isolation and fusion of protoplast

3.2.1 Plant Materials

The variety rich in iron content and a commercially important cultivar Kufri Uday was used for this research and another suitable cultivar with least iron content Kufri Chipsona-1 was taken for the isolation of protoplast followed by fusion.

3.2.2 Chemicals Used

The chemicals that were used in this experiment were bought from Himedia except for two chemicals Cellulase *Aspergillus* species and 2-Morpholinoethanesulfonic acid (MES) that were bought from Sigma-Aldrich company.

3.2.3 Glasswares, Equipments and Instruments

All the glasswares used during the experiments were laboratory grade from Borosil. Analytical and scientific equipments like autoclave was bought from Narang Scientific Works PVT. LTD (model number NSW-227), incubator from Narang Scientific Works PVT. LTD (model number NSW-256). Laminar air flow was bought from Narang Scientific Works PVT. LTD (model number NSW-201), centrifuge from REMI R-8C BL, water bath from Narang Scientific Works PVT. LTD (model number NSW-125), hemocytometer from Today Tech, digital fluorescence microscope from Labomed company (model number LX-400 eFL 2).

3.2.4 Location

This experiment was carried out at Tissue Culture Lab at School of Agriculture, Lovely Professional University, Punjab during winter season of 2024-2025.

3.2.5 Experimental Details

Plant Part Used- Surface sterilized leaves from *in-vitro* grown plantlets (4-6 weeks old)

Isolation Method- One step method

Design- Factorial Completely Randomized Design (FCRD)

Number of Treatments- 2 × 3 × 3 = 18

Replication- 3

3.2.6 Methodology

The detailed methods of the present investigation are described under the following headings:

3.2.6.1 Treatment Details

The whole experiment was followed as mentioned in the **Giri and Giri (2007)** with slight modifications. To isolate protoplasts, leaves from *in vitro* grown plants from both the above-mentioned varieties in section 3.2.1 were used to eliminate the microbial contamination threat in the cultures. The leaves were sliced into 2-3 mm long thin strips using a sterilized scalpel blade and lightly crushed with mortar and pestle in a sterile environment inside laminar air flow **[Plate 3.3 (a,b)]**.

Table 3.2.1: Treatment Details

Treatments		**Treatment Combination**
T1	E1 + C1	0.25% Macerozyme + 1.5% Cellulase at 22°C
T2	E2 + C1	0.4% Macerozyme + 2.0% Cellulase at 22°C
T3	E3 + C1	0.5% Macerozyme + 1.0% Cellulase at 22°C
T4	E1 + C2	0.25% Macerozyme + 1.5% Cellulase at 25°C
T5	E2 + C2	0.4% Macerozyme + 2.0% Cellulase at 25°C
T6	E3 + C2	0.5% Macerozyme + 1.0% Cellulase at 25°C
T7	E1 + C3	0.25% Macerozyme + 1.5% Cellulase at 27°C
T8	E2 + C3	0.4% Macerozyme + 2.0% Cellulase at 27°C
T9	E3 + C3	0.5% Macerozyme + 1.0% Cellulase at 27°C

*E = Enzyme Combinations, C = Temperature

3.2.6.2 Protoplast Isolation

The digestion combination used in isolation of protoplast was Cell Protoplast Washing (CPW) **(Table 3.2.2)** that included mannitol and maintained pH 5.8 with MES buffer. Various concentrations of cellulase were derived from *Aspergillus* species (ranging from 1.00 to 2.00 per cent) and Macerozyme R-10 derived from *Rhizopus* species (ranging from 0.25 to 0.50 per cent) were used in the CPW solution to make a digestion mixture for the isolation of protoplasts.

The crushed one gram of macerated leaves was pre-plasmolysed in the CPW solution for 30 minutes afterwards enzyme solution (as per the treatments) was combined with the CPW solution to achieve efficient protoplast isolation **[Plate 3.4 (a)]**. The combination was incubated for 16 hours, with a shaking speed of 50 revolutions per minute, at a temperature of 22, 25, 27°C without light.

Table 3.2.2: Preparation of CPW solution

Components	Concentration (mg/L)
KNO_3	101
$CaCl_2.2H_2O$	1480
$MgSO_4.7H_2O$	246
KH_2PO_4	27.2
KI	0.16
$CuSO_4.5H_2O$	0.025
Mannitol	192000

3.2.6.3 Protoplast Purification

Following the incubation period of 16 hours (as mentioned in 3.2.6.2), the digested tissue was filtered through an 80-micron nylon mesh to recover protoplasts and eliminate debris **[Plate 3.4 (b)]**. The filtrate was then collected in screw-capped 15 mL centrifuge tubes and centrifuged at 1000 revolutions per minute for 10 minutes to further isolate the protoplasts from the residual fine debris. The pellet of protoplast cells was resuspended in the 1 mL of CPW media to eliminate all existing enzymes, and this process was repeated three times. The pellet was resuspended in 1 mL of CPW solution and thereafter placed over 9 mL of floatation medium containing sucrose in 15 mL screw-capped centrifuge tubes. The components of floatation media are listed in **Table 3.2.3.** Subsequently, it was centrifuged again for five minutes at 1000 rpm. Transparent protoplast band was produced at the site of interphase between the flotation medium and the CPW. The interphase was meticulously extracted using a Pasteur pipette and then mixed with an equal volume of fresh CPW medium **[Plate 3.5 (a,b)]**.

Table 3.2.3: Preparation of Floatation Medium

Components	**Concentration (mg/L)**
KNO_3	101
$CaCl_2.2H_2O$	1480
$MgSO_4.7H_2O$	246
KH_2PO_4	27.2
KI	0.16
$CuSO_4.5H_2O$	0.025
Sucrose	150000

3.2.6.4 Protoplast Viability and Density

Both the coverslips and the hemocytometer were cleaned with 70% ethanol. The protoplast viability was assessed using fluorescein diacetate (FDA) staining. A stock solution of fluorescein diacetate (FDA) was made by dissolving 1 mg FDA in a little acetone to dissolve the dye and 10 mL distilled water was added. The protoplast solution and FDA dye solution was mixed in the equal ratio in centrifuge tube (10 µL each). The density and cell viability of the protoplast were evaluated using a hemocytometer under a digital fluorescence microscope at 40x. The FDA stained the viable cells, resulting in fluorescence ranging from yellow to green, but the cells that were dead remained uncolored. Assessing the concentration of the FDA solution is essential to assure the protoplast viability. An increase in concentration increases the probability of protoplast rupture.

3.2.6.5 Protoplast Fusion

A protoplast suspension was combined with 0.5 milliliters from each fusion partner in a centrifuge tube. The protoplast mixture was centrifuged at a velocity of 50 × g for five minutes. Most of the supernatant was removed, retaining 0.5 mL of protoplast solution. Then tapping motion was executed with a finger to agitate the centrifuge tube. The protoplast suspension was transferred to a sterile petri plate with a diameter of 90 millimeters, dispensing droplets of 0.1 milliliters (4-5 drops per dish). Afterwards, the dish was gently agitated to concentrate the protoplasts in the centre of the drops and let the protoplast mixture to settle for five minutes. After five minutes, 0.2 milliliters of a high pH Ca^{++} solution containing 30% PEG 6000 was added around the drops containing protoplasts. The mixture was incubated for five to ten minutes at room temperature inside a laminar airflow cabinet **[Plate 3.6 (a)]**. Gradually the stabilizer solution (0.74% $CaCl_2.2H_2O$) was added at a rate of 0.5 mL per drop and thereafter eluted the same combination together containing PEG. Calcium ions (Ca^{++}) exhibit a high pH level that's why stabilizer was added again for two to three times. This whole procedure took fifteen to twenty minutes. After washing the protoplast 2-3 times with stabilizer, the protoplasts were washed 4-5 times with either CPW solution or PCM **(Table 3.2.4)**. The composition of CPW is mentioned in Table and composition of PCM 1 & 2 is mentioned in Table. The procedure was repeated to remove any traces of fusogen and stabilizer. The PCM was added for the culture of the protoplasts. The petri dishes and culture vessels were carefully sealed with parafilm and incubated in a humid environment at 22-27°Celsius in darkness to promote protoplast culture growth. The PCM 2 media was added when the white micro colonies were started to grow **[Plate 3.6 (b)]**.

Table 3.2.4: Composition of PCM (Protoplast Culture Medium)

Components	**Concentration (for 100 mL) PCM 1**	**Concentration (for 100 mL) PCM 2**
Ready-made MS media	3.41 g	3.41 g
Sucrose	3 g	0.5 g
Mannitol	9 g	7 g
NAA	0.05 mg	0.20 mg
BAP	0.05 mg	0.05 mg
2,4-D	0.10 mg	-
Coconut water	5 mL	5 mL
pH	5.8	5.8

3.2.7 Details of Observations

The observations taken in this experiment has been explained as under:

3.2.7.1 Protoplast Viability

The viability was calculated by including the number of intact protoplasts and the total number of protoplasts (dead + alive) under the digital fluorescence microscope with the help of the haemocytometer. It is expressed in percentage by using the following formula:

$$Protoplast\ Viability = \frac{Number\ of\ intact\ protoplast}{Total\ number\ of\ protoplasts} \times 100$$

3.2.7.2 Protoplast Density

The density of protoplast was estimated by counting the average number of cells per square under the digital fluorescence microscope with the help of the haemocytometer and multiplied it with the dilution factor and 10^4. It is expressed in cells/mL.

Dilution factor is calculated by the final volume of the solution divided by the volume of cells.

$$\text{Protoplast Density} = \text{Average number of cells/squares} \times \text{Dilution Factor} \times 10^4$$

3.2.7.3 Size of the cells

The size of the cells was measured with the help of software (micaps 3.7 for Digital Camera) in part of digital fluorescent microscope and expressed in µm.

Statistical analysis

The data collected was thoroughly analysed using Analysis of Variance (ANOVA) as the primary test of significance, instigated with SPSS version 25.0 statistical software package with a fixed significance level of 5% ($p \leq 0.05$). To compare the different treatment means in detail, Duncan's Multiple Range Test was used as a post-hoc analysis to pinpoint specific differences between groups. To facilitate clear understanding and effective communication of the findings, the analyzed results were systematically organized and visually represented through a combination of detailed tables and informative graphs, enabling thorough interpretation of the experimental outcomes.

3.3 Experiment III: To determine the regeneration of plantlets *in vitro* from fusion products

3.3.1 Materials

The fragile callus was obtained after the fusion of the two selected cultivars' protoplasts and used for the further experiment.

3.3.2 Chemicals Used

All the chemicals used in this experiment were bought from Himedia company.

3.3.3 Location

This experiment was carried out at Tissue Culture Lab at School of Agriculture, Lovely Professional University, Punjab during winter season of 2024-2025.

3.3.4 Experimental Design

Plant Growth Hormones- Zeatin, 2,4-D

Media- Modified MS nutrient media

Design- Completely Randomized Design (CRD)

Number of Treatments- 4

Replication- 3

3.3.5 Methodology

The detailed methods of the present investigation are described under the following headings:

3.3.5.1 Treatment details

The whole experiment was followed as mentioned in the **Giri and Giri (2007)** with slight modifications. All the work was done under the sterile laminar air flow to avoid the contamination. The fragile callus was collected with the help of filter paper and then washed three to four times with distilled water to remove the culture media and then kept it in the petri plates. The callus was then distributed equally for the sub-culturing on solidified MS media with several concentrations of zeatin and 2,4-D.

Table 3.3.1: Treatment Details

Treatments	**Treatment Combination**
T1	Zeatin (0 mg/L) + 2,4-D (0 mg/L)
T2	Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)
T3	Zeatin (1 mg/L) + 2,4-D (3 mg/L)
T4	Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)

3.3.6 Details of Observations

The observations taken in this experiment has been explained as under:

3.3.6.1 Formation of calli

The calli was observed in culture vessels per treatment. The fragile callus was collected on the filter paper from each culture vessel.

3.3.6.2 Growth of calli

The calli growth was recorded by measuring it on the digital weighing balance and expressed in grams.

3.3.6.3 Colour of calli

The colour of calli in all the treatments was observed and recorded visually.

3.3.6.4 Number of regenerated calli

The number of regenerated calli from the sub-culturing of the mother calli on MS media with different growth regulators was to be observed.

3.3.6.5 Number of plantlets

The number of plantlets formed per calli was to be calculated in each and every treatment.

3.3.6.6 Fusion product identification

The fusion product was to be identified using leaves of the regenerated plantlets with the help of Flow cytometry.

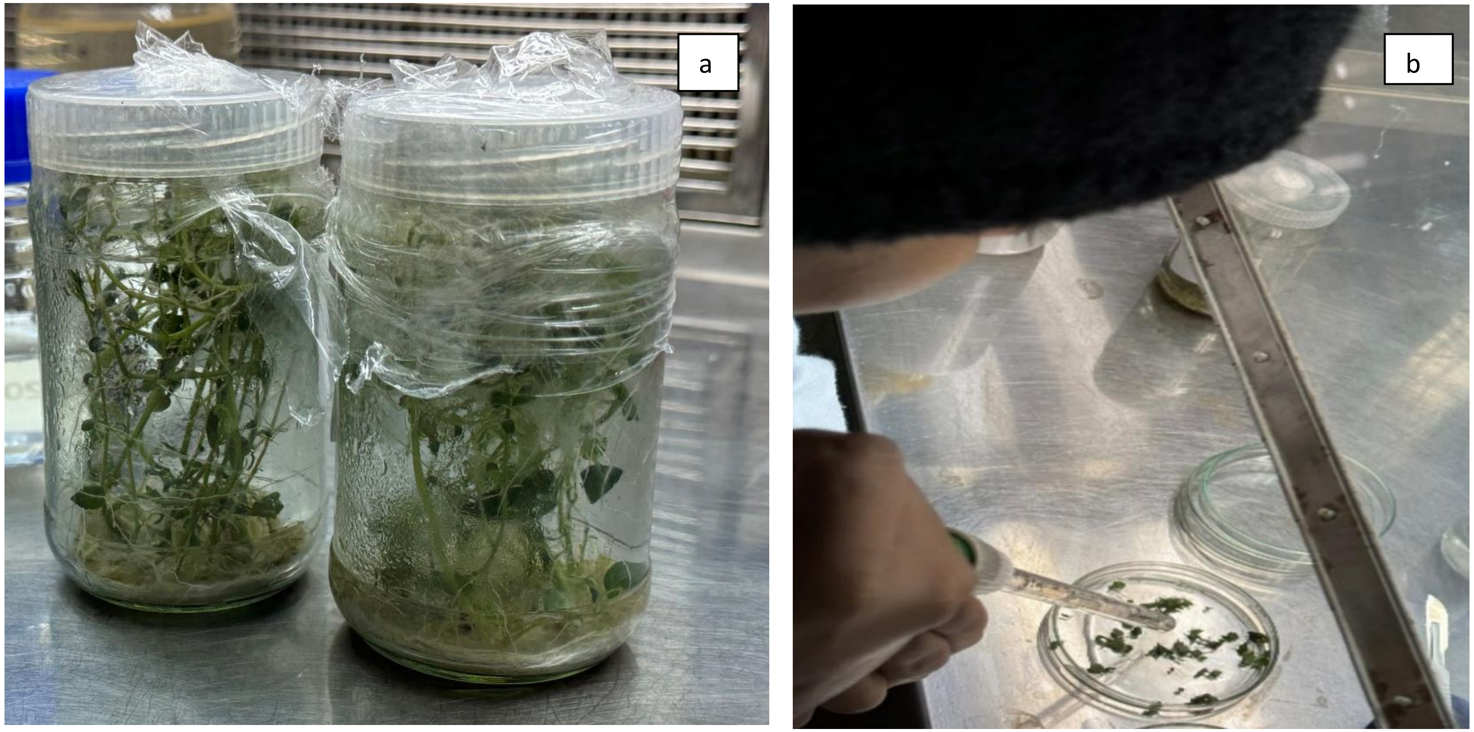
Statistical analysis

This experiment was analyzed through coding and thematic analysis, the primary test of significance, instigated with SPSS version 25.0 statistical software. To facilitate clear understanding and effective communication of the findings, the analyzed results were systematically organized and visually represented through the detailed tables enabling thorough interpretation of the experimental outcomes.

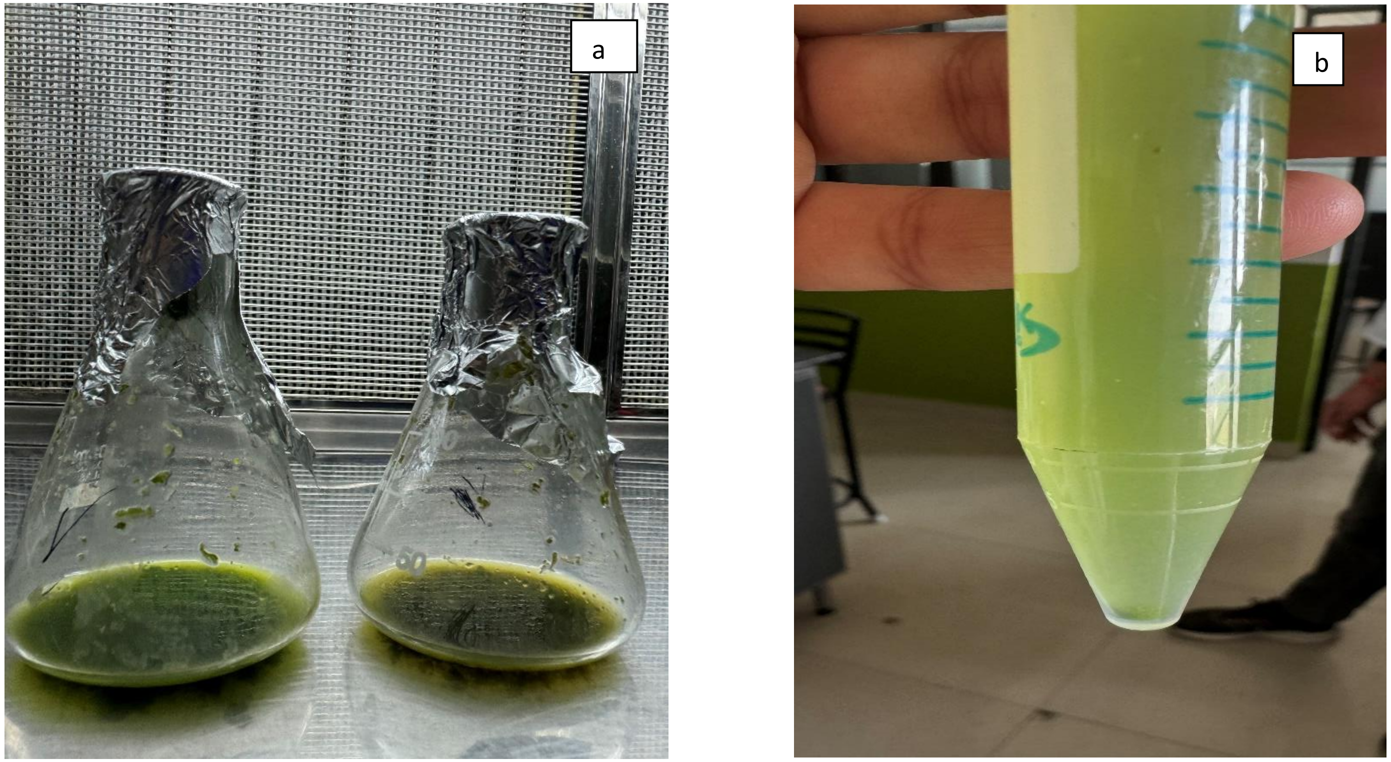
P 3.1: (a) Collection of 15 different varieties of potato; (b) Filing of soil mixture into grow bags; (c) Planting of potato tubers

P 3.2: (a) Growth of potato plants in grow bags; (b) Harvesting of tubers

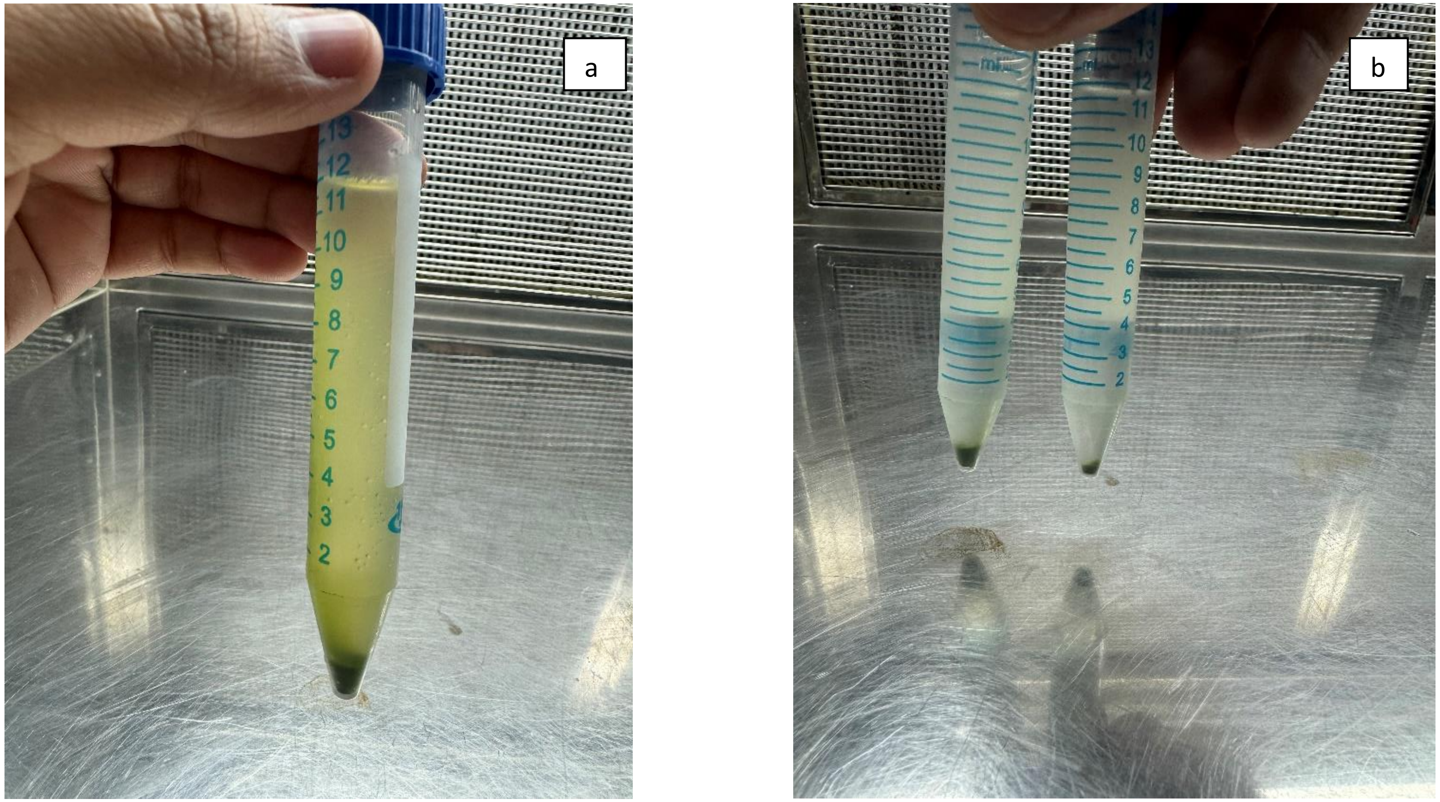
P 3.3: (a) *In-vitro* plants of Kufri Uday and Kufri Chipsona-1; (b) Adding CPWM solution for preplasmolysis



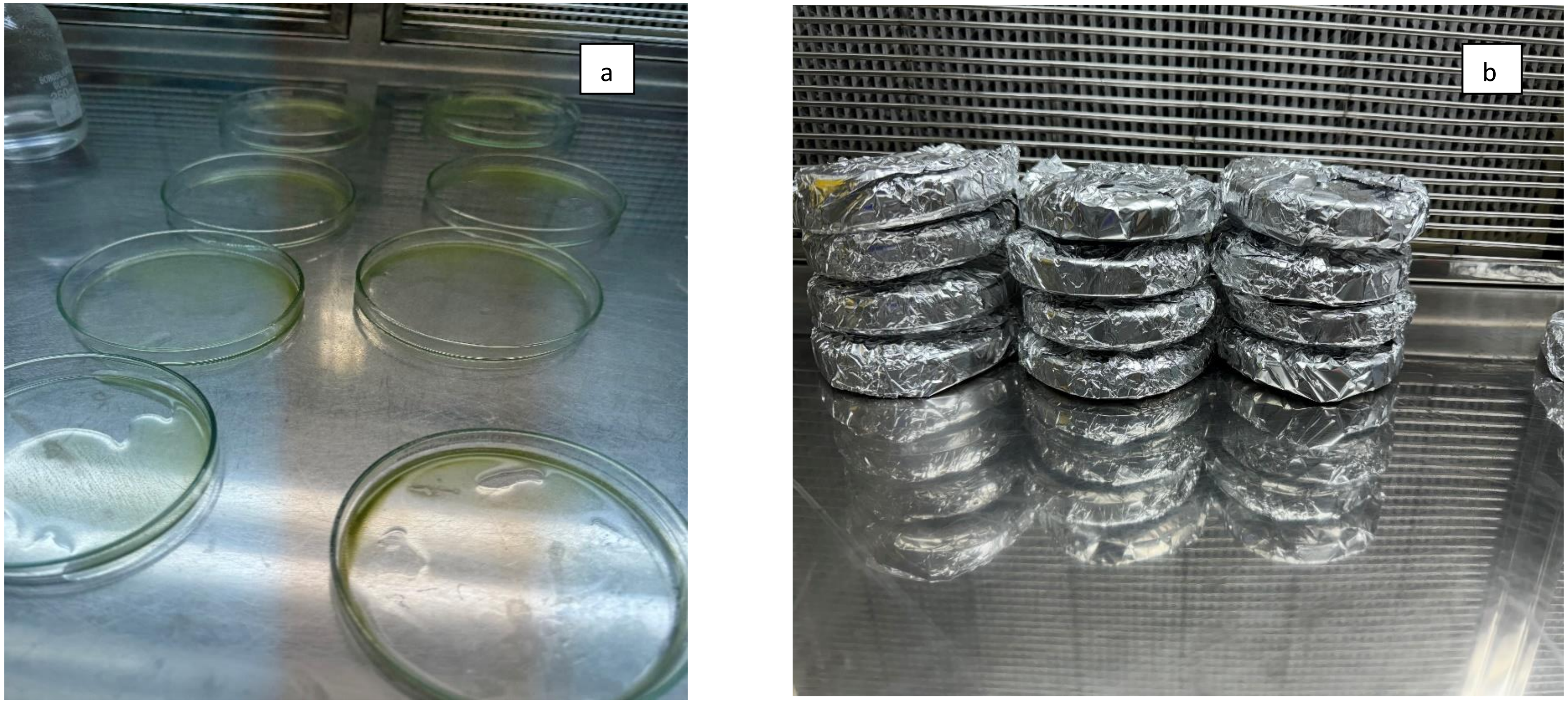
P 3.4: (a) Addition of Enzyme solution into the preplasmolysed solution; (b) Protoplasts suspension after filter with nylon mesh



P 3.5: (a) Solution containing protoplasts after few washing; (b) Protoplast in floating media



P 3.6: (a) Protoplast fusion with PEG under laminar air flow; (b) Cultured protoplast in PCM media



Chapter- IV

RESULTS AND DISCUSSIONS

The proposed research work entitled **"Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion"** was carried out in the Tissue Culture Lab as well as in the field at Lovely Professional University, Phagwara, Punjab. The comprehensive experimental study was conducted over an extensive period of 2023-2024 and 2024-2025, enabling careful data collection and observation. The results of this research are meticulously presented and thoroughly analysed in this chapter. The findings are fully illustrated through comprehensive tables and informative graphs, which are then deliberately discussed and elucidated in the following subsections to provide a complete understanding of the results.

4.1 To identify the high iron content germplasm of potato

4.1.1 Iron Content

It was shown that iron content had most of the variations between 21.73 mg/kg and 55.20 mg/kg. The highest iron content among the potato varieties was found in Kufri Uday (55.20 ± 0.88 mg/kg) which was significantly higher than other varieties, followed by Kufri Lalima (52.36 ± 1.06 mg/kg) and the least in Kufri Chipsona-1 (21.73 mg/kg). Iron concentration over 40 mg/kg were detected in the following potato varieties: Kufri Surya, Kufri Mohan, Kufri Jyoti and Diamond which were significantly similar. The concentration of iron in tuber flesh ranged between 30-40 mg/kg in Kufri Lima, Kufri Chandramukhi, Kufri Pukhraj, Kufri Khyati, Kufri Himsona, and 5758. The concentration ranged below 30 mg/kg were observed in 3797, 302, and Kufri Chipsona-1 **(Table 4.1.1, Fig. 4.1.1 and Plate 4.2 (c))**.

In the findings of **Trehan and Sharma (1996)** the iron content in Indian potato cultivars was ranged between 21-53 ppm which was almost similar to our findings. **Singh *et al.* (2020)** also analysed the iron content in different potato genotypes and found that the iron concentration ranged between 18.03 and 46.43

mg/kg and in the research of **Dalamu *et al.* (2017)** the iron concentration in potato flesh was ranged from 14.90 to 67.13 ppm. Additionally, **Brown *et al.* (2010)** showed that the iron concentration of the tubers varied more. Recommended dose of iron according to National Institute of Nutrition, Hyderabad for man is 17 mg/day, woman 21-35mg/day, infants 46 µg/kg, children 09-16 mg/day, teenagers 21-32 mg/day. Research conducted by **Burgos *et al.* (2007)**, claimed that consuming a potato variety with a higher Fe content as compared to one with a lower Fe content might greatly enhance the Fe intake of women and children. According to **Saar-Reismaa *et al.* (2020)** iron present in potato belongs to the non heme iron group and is an essential source for human diet. Iron deficiency might cause to serious health issues, such as less work ability and stunted adolescent development, while it also has advantages against anaemia since they also include vitamin C, which improves the absorption of iron from potato.

Table 4.1.1: Iron content of potato cultivars in mg/kg on dry weight basis

Varieties	**Iron Content**
Kufri Lalima	52.36^{b}±1.02
Kufri Uday	55.20^{a}±0.88
Kufri Surya	43.46^{c}±0.55
Kufri Jyoti	44.22^{c}±0.31
Diamond	44.02^{c}±0.45
Kufri Lima	38.75^{d}±0.26
Kufri Chandramukhi	32.46^{f}±0.54
Kufri Pukhraj	33.81^{e}±0.24
Kufri Mohan	43.62^{c}±0.26
Kufri Khyati	34.26^{e}±0.92
Kufri Chipsona-1	21.73^{h}±0.24
Kufri Himsona	31.87^{f}±0.67
5758	32.50^{f}±0.19
302	22.33^{h}±0.45
3797	29.25^{g}±0.50
C.D. ($p \leq 0.05$)	0.98
SE(m)±	0.33

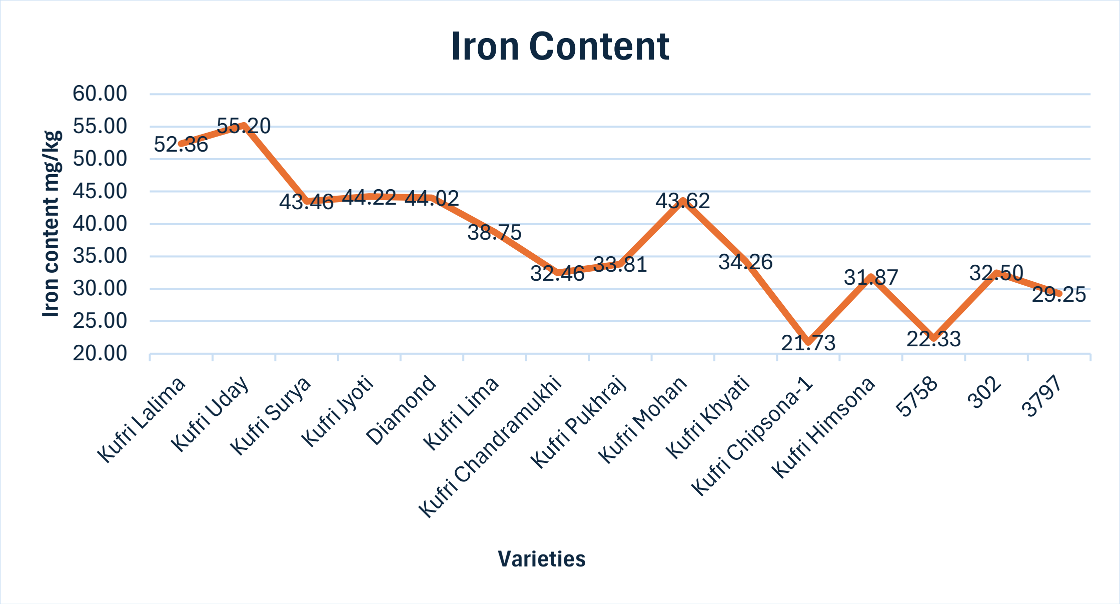


Fig. 4.1.1: Iron content of potato cultivars in mg/kg on dry weight basis

4.1.2 Zinc content

The zinc content in the peeled potatoes was highest in Kufri Uday (42.12 ± 0.30), followed by Kufri Jyoti (39.08 ± 0.23 mg/kg) which was at par with Kufri Surya (38.25 ± 0.63), Diamond (38.15 ± 0.63) and Kufri Lalima (38.05 ± 0.18), and least was observed in 302 (21.38 ± 0.51) **(Table 4.1.2 and Fig. 4.1.2)**. The substantial level of zinc *i.e.*, above 30 mg/kg, in peeled tubers of potato varieties was found in: Kufri Jyoti, Kufri Uday, Kufri Lalima, Kufri Surya, Diamond, Kufri Lima, Kufri Pukhraj, and 5758. The cultivars had zinc content over 20 mg/kg were: Kufri Khyati, Kufri Himsona, Kufri Mohan, Kufri Chandramukhi, Kufri Chipsona-1, 302, and 3797.

Saar-Reismaa *et al.* (2020) determined the zinc content in potato which ranged from 9.8 to 26.0 mg/kg. **Sharma *et al.* (2017)** analyzed the zinc in flesh of tubers of different varieties with the range of 12.59 mg/kg and 22.85 mg/kg. Numerous studies worldwide have shown genetic differences in the zinc content of different potato types, and it was found that, when evaluated on a dry weight basis, the zinc concentration in potato tubers ranged from 3 to 37 mg/kg (**Brown *et al.*, 2011**; **Rivero *et al.*, 2003** and **Burgos *et al.*, 2007**;**)**. According to the recommendation of National Institute of Nutrition, Hyderabad zinc daily dietary dose for man is 12 mg/day while for women is 10-12 mg/day, for children 5-8 mg/day, and for teenagers 9-12 mg/day. It aids in strengthening the immune system to fight against diseases. It also enhances logical skills among children, that's why its deficiency directly affects brain development. Zinc has three biological roles mainly structural, catalyst, and regulatory ions. It also helps to maintain homeostasis, the immune system, and apoptosis and alleviates the aging process. Zinc deficiency leads to the death of around 4.4% children (age under 5 years) in the developing countries due to acute diarrhoea and pneumonia **(Sharma *et al.,* 2017)**. It also causes hair loss, poor hair development, weak eyesight and memory loss **(Dalamu *et al.*, 2017)**. The development of numerous chronic diseases, including neurological disorders, inflammation, autoimmune diseases, gastrointestinal infection, loss of appetite, and aging-related disorders, is due to zinc deficiency in the human body.

Table 4.1.2: Zinc content of potato cultivars in mg/kg on dry weight basis

Varieties	**Zinc Content**
Kufri Lalima	$38.05^{b}\pm0.18$
Kufri Uday	$42.12^{a}\pm0.30$
Kufri Surya	$38.25^{b}\pm0.63$
Kufri Jyoti	$39.08^{b}\pm0.23$
Diamond	$38.15^{b}\pm0.63$
Kufri Lima	$34.04^{c}\pm0.56$
Kufri Chandramukhi	$21.63^{h}\pm0.25$
Kufri Pukhraj	$30.65^{d}\pm1.19$
Kufri Mohan	$25.50^{f}\pm0.33$
Kufri Khyati	$25.82^{f}\pm0.98$
Kufri Chipsona-1	$23.81^{g}\pm0.25$
Kufri Himsona	$26.88^{e}\pm0.16$
5758	$30.58^{d}\pm0.84$
302	$21.38^{h}\pm0.51$
3797	$23.76^{g}\pm1.02$
C.D. ($p \leq 0.05$)	1.061
SE(m)±	0.366

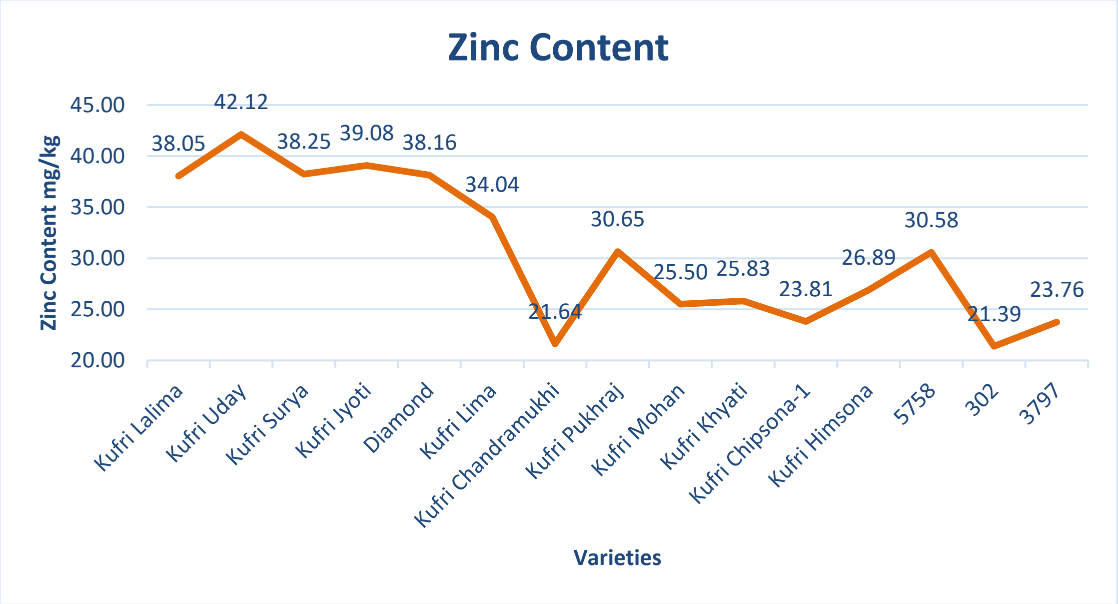


Fig. 4.1.2: Zinc content of potato cultivars in mg/kg on dry weight basis

4.1.3 Boron content

The boron content in potato varieties lied between 17.06 and 29.80 mg/kg. The boron content was found maximum in Kufri Lima (29.80 ± 0.56), followed by Kufri Khyati (25.56 ± 0.98) which was at par with Kufri Chipsona-1 (25.48 ± 0.96) and Kufri Pukhraj (24.85 ± 0.80), and minimum in Kufri Uday (17.06 ± 0.79) **(Table 4.1.3 and Fig. 4.1.3)**. High boron concentration (≥ 25 mg/kg) were observed in Kufri Lima, Kufri Khyati, Kufri Chipsona-1 and Kufri Pukhraj. Potato varieties with medium boron concentration *i.e.*, 20-25 mg/kg were Kufri Surya, Kufri Mohan, 5759, 302, Kufri Jyoti and Diamond. Low concentration (< 20 mg/kg) of boron was observed in varieties: Kufri Uday, Kufri Chandramukhi, Kufri Himsona, and 3797. The potato tubers exhibited lower boron levels in comparison to other minerals.

Pandey *et al.* (2023) also identified the boron concentration in different potato genotypes which was generally found between 2.83 and 3.74 µg/g. According to the National Health and Nutrition, the recommended daily allowance of diet for adults is 0.87 to 1.35 mg/day, 1.05 to 1.08 for pregnant women, 0.55 mg/day for infants, 0.54 mg/day for toddlers, 0.75 to 0.96 for pre-school children. Boron deficiencies have also been seen in the human body like bone deformation in older adults, hormonal imbalance in women, short-term memory loss, join stiffness, slow wound healing etc.

Table 4.1.3: Boron content of potato cultivars in mg/kg on dry weight basis

Varieties	**Boron Content**
Kufri Lalima	20.28^{d}±0.19
Kufri Uday	17.06^{f}±0.79
Kufri Surya	22.04^{c}±0.63
Kufri Jyoti	21.10^{c,d}±0.79
Diamond	20.21^{d}±0.51
Kufri Lima	29.80^{a}±0.43
Kufri Chandramukhi	17.74^{e,f}±1.19
Kufri Pukhraj	24.85^{b}±0.80
Kufri Mohan	21.81^{c}±0.80
Kufri Khyati	25.56^{b}±0.36
Kufri Chipsona-1	25.48^{b}±0.96
Kufri Himsona	18.53^{e}±0.60
5758	21.18^{c,d}±0.95
302	21.33^{c,d}±0.26
3797	18.30^{e,f}±0.82
C.D. ($p \leq 0.05$)	5.217
SE(m)±	1.715

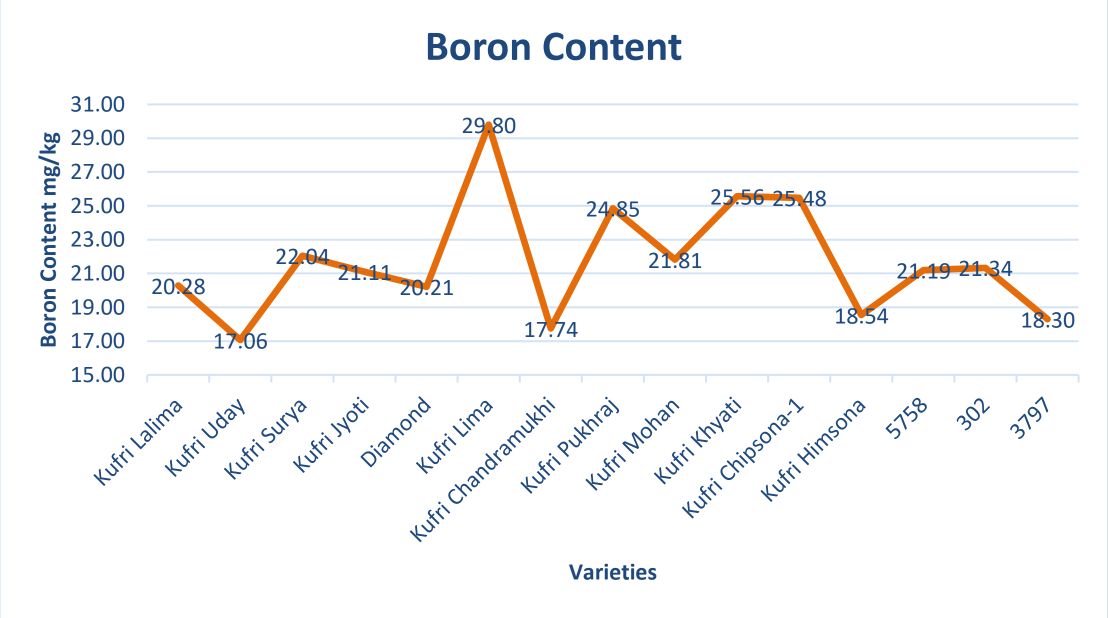


Fig. 4.1.3: Boron content of potato cultivars in mg/kg on dry weight basis

4.1.4 Copper content

As per the statistical analysis, Kufri Uday showed the maximum copper concentration at 34.00 ± 0.12 mg/kg in peeled tubers which was even significantly higher over the other varieties, followed by 302 (31.66 ± 0.01), and minimum copper content was observed in Kufri Surya 3.71 ± 0.02 mg/kg **(Table 4.1.4 and Fig. 4.1.4)**.Varieties with the concentration lied in 10.01-25.00 mg/kg were Kufri Chandramukhi, Kufri Pukhraj, Kufri Mohan, Kufri Khyati, Kufri Chipsona-1, Kufri Himsona, and 5758. The remaining varieties like Kufri Lalima, Kufri Jyoti, Diamond, 3797, Kufri Lima, and Kufri Surya had concentrations below 10 mg/kg. Copper is usually less abundant as compared to the other minerals.

The differences in the range of copper might be due to the genetic variations among the varieties of potato. **Haynes *et al.* (2012)** discovered that the highest copper content in tubers was 12 mg/kg on a dry weight basis, among all potato genotypes studied and the research of **Pandey *et al.* (2023)** copper content in different potato varieties was ranged from 3.63 µg/g to 4.01 µg/g. According to the Food and Nutrition Board, daily dietary allowance for infants is 200- 220 µg/day, children 340- 440 µg/day, males 700- 900 µg/day, females 700- 1000 µg/day. Copper helped in functioning of central nervous system and also considered as an antioxidant, development and maintenance of brain system and improve the immune system by helping the white blood cells. It contributes to the synthesis of melanin, which gives skin, hair, and eyes their color. It helps in the absorption and transportation of blood. Copper helps in to prevent anaemia. It also helps in the formation of hemes and catalyse the process of the healing of wounds. Deficiency of copper can cause joint inflammation, infection, loss of appetite, fatigue.

Table 4.1.4: Copper content of potato cultivars in mg/kg on dry weight basis

Varieties	Copper Content
Kufri Lalima	6.09^{k}±0.17
Kufri Uday	34.00^{a}±0.12
Kufri Surya	3.71^{m}±0.02
Kufri Jyoti	5.76^{l}±0.01

Diamond	$4.80^{m}\pm0.03$
Kufri Lima	$6.07^{k}\pm0.03$
Kufri Chandramukhi	$13.85^{g}\pm0.02$
Kufri Pukhraj	$15.52^{f}\pm0.02$
Kufri Mohan	$22.57^{d}\pm0.01$
Kufri Khyati	$12.16^{h}\pm0.01$
Kufri Chipsona-1	$11.84^{i}\pm0.01$
Kufri Himsona	$17.68^{e}\pm0.04$
5758	$23.46^{c}\pm0.01$
302	$31.66^{b}\pm0.01$
3797	$6.22^{j}\pm0.01$
C.D. ($p \leq 0.05$)	0.099
SE(m)±	0.034

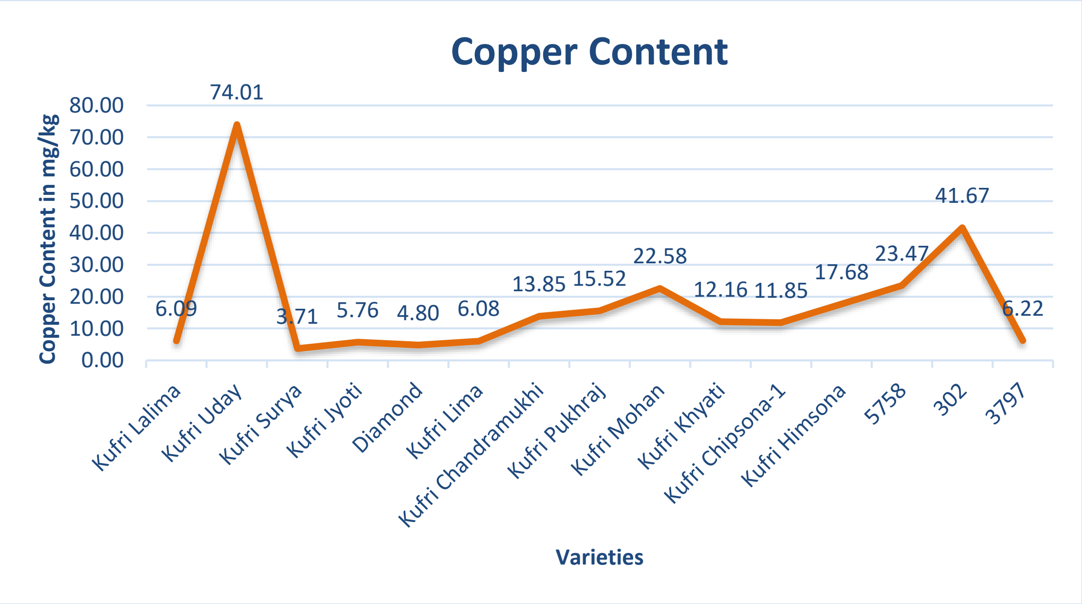


Fig. 4.1.4: Copper content of potato cultivars in mg/kg on dry weight basis

4.1.5 Calcium content

The range of calcium content in potato varieties had been found between 1084 mg/kg and 393 mg/kg. The calcium content was significantly higher in the Kufri Chandramukhi, with a concentration of 1084 ± 1.0 mg/kg. It was followed by the Kufri Mohan (1075 ± 2.0), whereas Kufri Surya had the minimum (393 ± 2.0) calcium content, and the significant differences were observed among all the varieties **(Table 4.1.5 and Fig. 4.1.5)**. The varieties which had calcium content above 800 mg/kg were Kufri Chandramukhi, Kufri Mohan, Kufri Pukhraj, Kufri Chipsona-1, and Kufri Himsona, while the other varieties had lower calcium content (less than 800 mg/kg) were Kufri Lima, 5758, Kufri Khyati, Diamond, Kufri Uday, Kufri Lalima, Kufri Jyoti, and 3797.

Pandey *et al.* (2023 studied on the calcium content of potato tubers, with findings falling between 0.19 and 0.79 mg/g. **Rivero *et al.* (2003)** also investigated the calcium content in peeled tubers where they found the range of calcium between 47.6 mg/kg and 87.3 mg/kg, however, our finding showed higher than findings of above researchers. According to National Institute of Nutrition, Hyderabad, the recommended dose for man is 600 mg/day, woman 600- 1200 mg/day, infants 500 mg/day, for teenagers 600- 800 mg/day. Calcium plays a crucial role in the human body like maintenance and strengthening the body and teeth, blood clotting, hormonal secretion, maintaining nerve functions and heartbeat, etc. Muscle contractions, reflexes, and other reactions are made possible by its facilitation of seamless communication between the brain and the body. Deficiencies may cause the rickets, muscle cramps, osteomalacia, brittle nails, tooth decay, tingling in feet, toes and hands, etc.

Table 4.1.5: Calcium content of potato cultivars in mg/kg on dry weight basis

Varieties	**Calcium Content**
Kufri Lalima	481.33^{k}±1.15
Kufri Uday	505.66^{j}±5.50
Kufri Surya	393.00^{n}±2.00
Kufri Jyoti	453.00^{l}±2.00
Diamond	597.00^{i}±1.00
Kufri Lima	725.33^{f}±4.50
Kufri Chandramukhi	1084.00^{a}±1.00
Kufri Pukhraj	837.66^{e}±2.51
Kufri Mohan	1075.00^{b}±2.00
Kufri Khyati	664.33^{h}±2.08

Kufri Chipsona-1	937.66^{d}±3.51
Kufri Himsona	985.33^{c}±3.05
5758	687.33^{g}±8.02
302	567.33^{h}±2.51
3797	426.00^{m}±2.00
C.D. (p ≤ 0.05)	5.690
SE(m)±	1.961

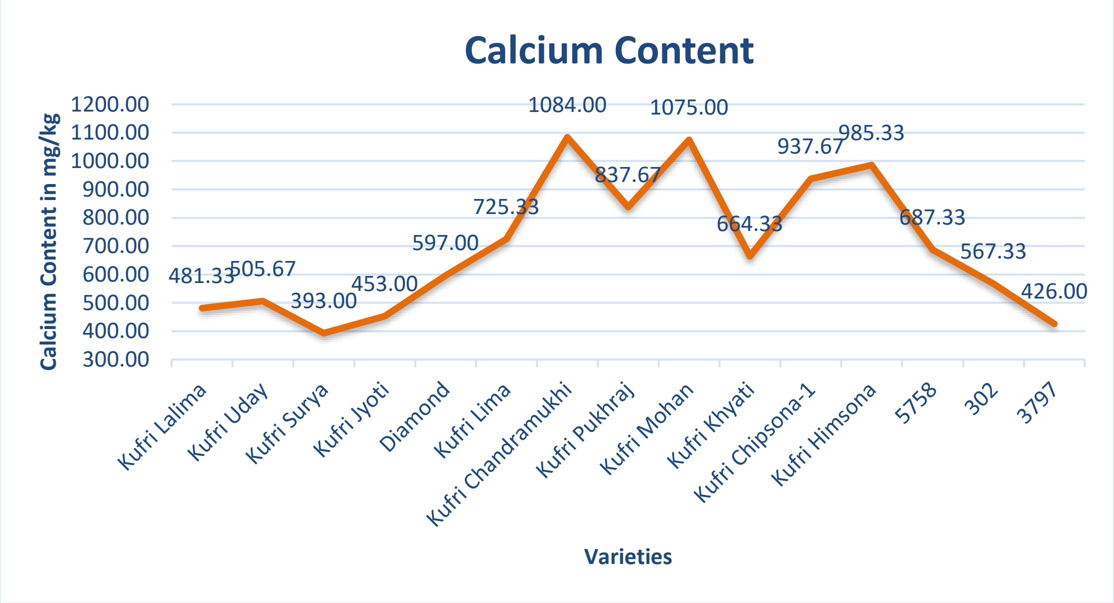


Fig. 4.1.5: Calcium content of potato cultivars in mg/kg on dry weight basis

4.1.6 Manganese content

The manganese content in variety Kufri Chandramukhi (26.48 ± 0.90) was observed highest which was significantly higher among the varieties and was followed by Kufri Jyoti (24.62 ± 0.89) and the least content was in variety Kufri Lima (6.67 ± 0.33) **(Table 4.1.6 and Fig. 4.1.6)**. Compared to iron and zinc, manganese is less common in potatoes. Most of the varieties lied ≥ 10 mg/kg like Kufri Chandramukhi, Kufri Jyoti, Kufri Lalima, Kufri Uday, Kufri Surya, Kufri Pukhraj, Kufri Khyati, Kufri Chipsona-1, Kufri Himsona, 3797, and 302 while rest of the varieties 5758, Kufri Mohan, Diamond, Kufri Lima < 10 mg/kg.

The manganese in peeled Indian potato tuber was ranged from 17.34 mg/kg-30.52 mg/kg in the research of **Sharma *et al.* (2017)** which was almost similar to our research. According to **Haynes *et al.* (2012)**, the Mn content of the peeled potato tubers ranged from 8 to 14 mg/kg on a dry weight basis. **Saar-Reismaa *et al.* (2020)** also determined the Mn content in the potato flesh and found out that 5.3 to 12.0 mg/kg was present in the tuber varieties, the differences in the content were due to the genetic variability of potato varieties. Recommended dose of manganese suggested by the Food and Nutrition Board for infants is 0.003-0.6 mg/day, children 1.2-1.5 mg/day, males 1.9-2.3 mg/day, females 1.6- 2.0 mg/day. Manganese is a cofactor of many enzymes in the body. Collagen and other bone matrix proteins are produced using manganese. It also plays a role in the formation of bone cartilage, helps in wound healing, etc. Its deficiency may cause weakness in the bones, fatigue and weakness, increase the risk of fractures, joint pain, skin problems, memory problems, blood sugar level imbalance, mood swings, stunted growth and delay puberty in the children, increase the susceptibility to infection and diseases, etc.

Table 4.1.6: Manganese content of potato cultivars in mg/kg on dry weight basis

Varieties	**Manganese Content**
Kufri Lalima	$18.16^{d}\pm0.51$
Kufri Uday	$13.48^{g}\pm0.65$
Kufri Surya	$11.34^{h}\pm0.67$
Kufri Jyoti	$24.62^{b}\pm0.89$
Diamond	$6.94^{j}\pm0.34$
Kufri Lima	$6.67^{j}\pm0.33$
Kufri Chandramukhi	$26.48^{a}\pm0.90$
Kufri Pukhraj	$12.03^{h}\pm0.54$
Kufri Mohan	$7.28^{j}\pm0.38$
Kufri Khyati	$18.51^{c,d}\pm0.87$
Kufri Chipsona-1	$19.56^{c}\pm0.34$
Kufri Himsona	$15.51^{e,f}\pm0.46$

5758	9.50^{i}±0.97
302	16.42^{e}±1.71
3797	14.70^{f,g}±0.24
C.D. (p ≤ 0.05)	1.261
SE(m)±	0.435

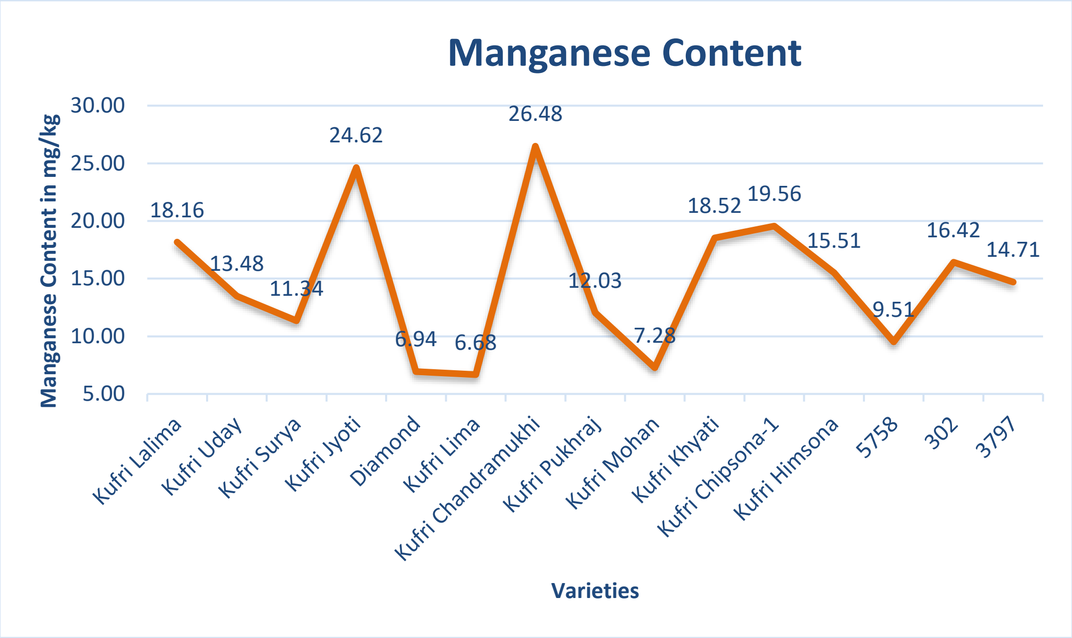


Fig. 4.1.6: Manganese content of potato cultivars in mg/kg on dry weight basis

4.1.7 Magnesium content

The range of magnesium levels was 557–1112 mg/kg. The potato variety Kufri Lalima had the highest magnesium concentration (1111.66 ± 1.52), followed by Kufri Uday (1010.66 ± 14.22), which was significantly greater than the other varieties. The Diamond variety had the lowest magnesium level (557.33 ± 4.16) **(Table 4.1.7 and Fig. 4.1.7)**. The magnesium content was between 700- 1112 mg/kg observed in Kufri Lalima, Kufri Uday, Kufri Surya, 3797, 5758, Kufri Mohan, Kufri Chipsona-1, Kufri Jyoti, while rest of the varieties came under 500- 700 mg/kg Kufri Khyati, Kufri Lima, 302, Kufri Chandramukhi, Diamond, Kufri Pukhraj, and Kufri Himsona.

On the basis of dry weight, **Brown *et al.* (2012)** also measured the magnesium content and found that the range of concentrations in the potato tubers was 700–1200 mg/kg. **Pandey *et al.* (2023)** investigated the Mg content between 0.81 mg/g and 1.44 mg/g in the flesh of the tubers. These variations in the different varieties of potato were due to the genetic adaptability of the tuber. The magnesium dose per day for man is 340 mg/day, woman 310 mg/day, infants 30-45 mg/day, children 50-100 mg/day and for teenagers 160-235 mg/day suggested by National Institute of Nutrition, Hyderabad. Magnesium is mostly stored in the bones and helps in the maintain the bone density, regulates blood pressure, involves in the protein synthesis, etc. It is the cofactor for many enzymes which are involved in the various roles of the human body such as nucleic acid synthesis, protein, carbohydrates, and energy metabolism. This element is used to treat acute myocardial infarction (heart attack) and to treat atherosclerosis. Its deficiency symptoms are tingling, numbness, weakness, fatigue, depression, anxiety, abnormal heart beats, spasms, etc.

Table 4.1.7: Magnesium content of potato cultivars in mg/kg on dry weight basis

Varieties	**Magnesium Content**
Kufri Lalima	1111.66^{a}±1.52
Kufri Uday	1010.66^{b}±14.22
Kufri Surya	962.00^{c}±4.58
Kufri Jyoti	790.33^{e}±2.51
Diamond	557.33^{k}±4.16
Kufri Lima	610.65^{i}±2.51
Kufri Chandramukhi	576.00^{k}±1.00
Kufri Pukhraj	684.66^{g}±2.51
Kufri Mohan	756.33^{f}±1.52
Kufri Khyati	648.00^{h}±1.00
Kufri Chipsona-1	753.33^{f}±1.52
Kufri Himsona	655.00^{h}±2.00
5758	784.66^{e}±1.52
302	584.00^{j}±1.00
3797	874.33^{d}±1.52
SE(m)±	2.487

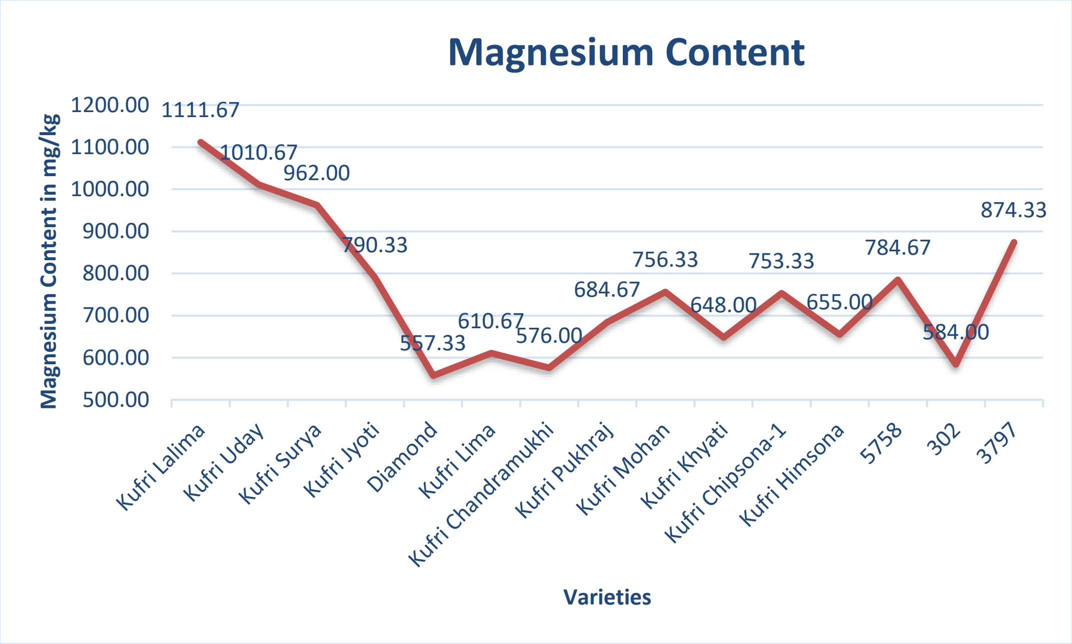


Fig. 4.1.7: Magnesium content of potato cultivars in mg/kg on dry weight basis

4.1.8 Potassium content

Potassium was found highest in Kufri Khyati (1048 ± 2.00) significantly similar with Kufri Chandramukhi (1015 ± 100) followed by Kufri Mohan (952.33 ± 6.50) was at par with Kufri Chipsona-1 (943.33 ± 1.52), Kufri Jyoti (940 ± 51.09) and Kufri Pukhraj (912.66 ± 0.57) and lowest was observed in Kufri Lima (512.33 ± 2.08) **(Table 4.1.8 and Fig. 4.1.8)**. It is also observed that the remaining varieties were ranged between 500- 900 mg/kg of potassium content Kufri Himsona, 3797, Kufri Lalima, Kufri Surya, Diamond, 302, Kufri Uday, Kufri Lima.

Pandey *et al.* (2023) measured the potassium content in a variety of potato genotypes, ranging from 20.39 mg/g to 32.46 mg/g, in proportion to the weight of the dried potato. **Subramanian *et al.* (2011)** also analysed the potassium in tubers which came out 22.4 mg/g in the flesh. According to the Food and Nutrition Board, the dietary allowance for infants is 400- 860 mg/day, children 2000-2300 mg/day, males 2500- 3400 mg/day, 2300-2900 mg/day. Its role in the human body has seen by balancing the electrolytes and fluid in the body, maintains heart beats, functioning of muscles, regulates blood pressure. Its deficiency symptoms are, abnormal heart beats, constipation, weakness, mood swings, muscle contraction, tingling in hands, etc.

Table 4.1.8: Potassium content of potato cultivars in mg/kg on dry weight basis

Varieties	Potassium Content
Kufri Lalima	837.66^{d,e}±41.18
Kufri Uday	616.00^{b}±3.00
Kufri Surya	711.66^{f}±2.08
Kufri Jyoti	940.00^{b}±51.09
Diamond	783.66^{e}±3.05
Kufri Lima	512.33^{g}±2.08
Kufri Chandramukhi	1015.00^{a}±100.00
Kufri Pukhraj	912.66^{b,c}±0.57
Kufri Mohan	952.33^{b}±6.50
Kufri Khyati	1048.00^{a}±2.00
Kufri Chipsona-1	943.33^{b}±1.52
Kufri Himsona	864.33^{c,d}±1.52
5758	825.33^{d,e}±3.05
302	678.00^{f}±13.07
3797	842.66^{d}±1.52
C.D. ($p \leq 0.05$)	52.218
SE(m)±	17.993

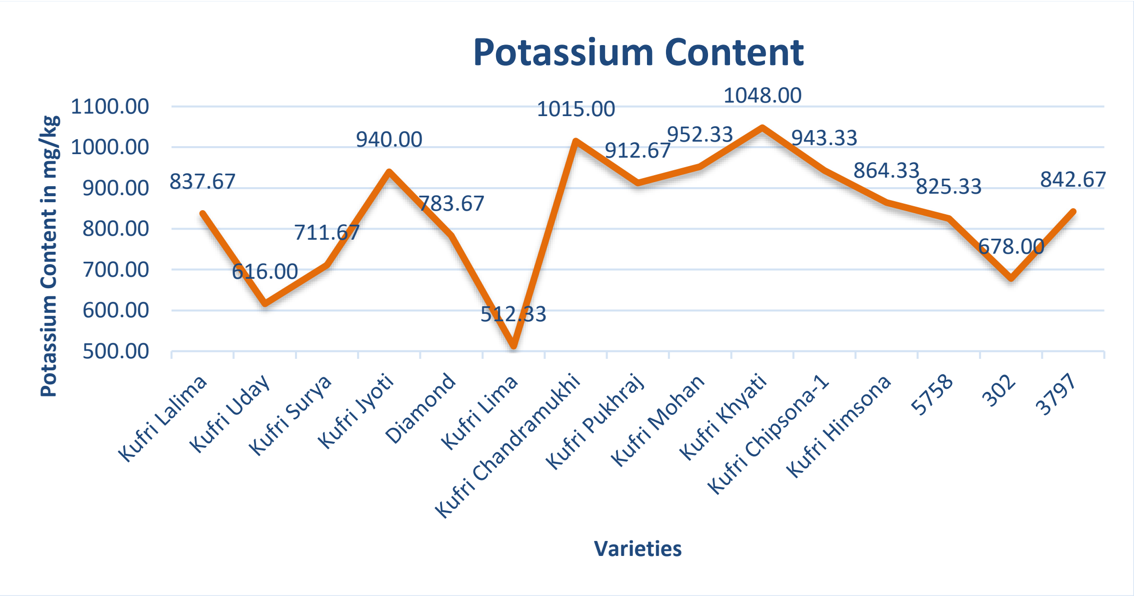


Fig. 4.1.8: Potassium content of potato cultivars in mg/kg on dry weight basis

4.1.9 Phosphorus content

Phosphorus was analysed in peeled tubers and found that Kufri Chandramukhi (586.66 ± 1.52) was significantly higher over other varieties followed by Diamond (547.33 ± 2.08) had the greatest levels of phosphorus, while 5758 (169 ± 3.00) had the lowest levels of phosphorus **(Table 4.1.9 and Fig. 4.1.9)**. In few potato varieties phosphorus concentration was over 400 mg/kg. Specifically, Kufri Chandramukhi, Diamond, Kufri Mohan, Kufri Uday. Other varieties with phosphorus content above 200 mg/kg were Kufri Himsona, 5797, Kufri Chipsona-1, Kufri Lalima, Kufri Surya, Kufri Jyoti, Kufri Lima, Kufri Khyati, 302, while varieties with phosphorus less than 200 mg/kg were Kufri Pukhraj, 5758.

Pandey *et al.* (2023) observed the variation in phosphorus content in several potato genotypes by 2.96 mg/g-4.79 mg/g. **Subramanian *et al.* (2011)** analysed phosphorus and found out 2.8 mg/g in the tuber variety flesh. These variations in varieties occur due to the difference in the variety genetic makeup and the type of soil in which they had been planted. According to the Food and Nutrition Board, the dietary allowance for infants is 100- 275 mg/day, children 460- 500 mg/day, males 700- 1250 mg/day, females 700- 1250 mg/day. It is a major component of teeth and bones, helps in the formation of DNA, balances the acid- base in the body, involves in the transformation of the nerve impulses, etc. its deficiency may cause bone pain, weakness, tingling, numbness, weaking in the immune system etc.

Table 4.1.9: Phosphorus content of potato cultivars in mg/kg on dry weight basis

Varieties	Phosphorus Content
Kufri Lalima	340.33^{f}±2.51
Kufri Uday	424.00^{d}±3.00
Kufri Surya	321.33^{g}±3.51
Kufri Jyoti	287.66^{h}±2.51
Diamond	547.33^{b}±2.08
Kufri Lima	266.33^{i}±2.51
Kufri Chandramukhi	586.66^{a}±1.52
Kufri Pukhraj	190.00^{k}±7.00

Kufri Mohan	485.66^{c}±3.05
Kufri Khyati	247.00^{d}±2.00
Kufri Chipsona-1	338.33^{f}±2.51
Kufri Himsona	380.00^{e}±7.00
5758	169.00^{l}±3.00
302	238.33^{j}±5.03
3797	375.33^{e}±2.51
C.D. ($p \leq 0.05$)	6.199
SE(m)±	2.136

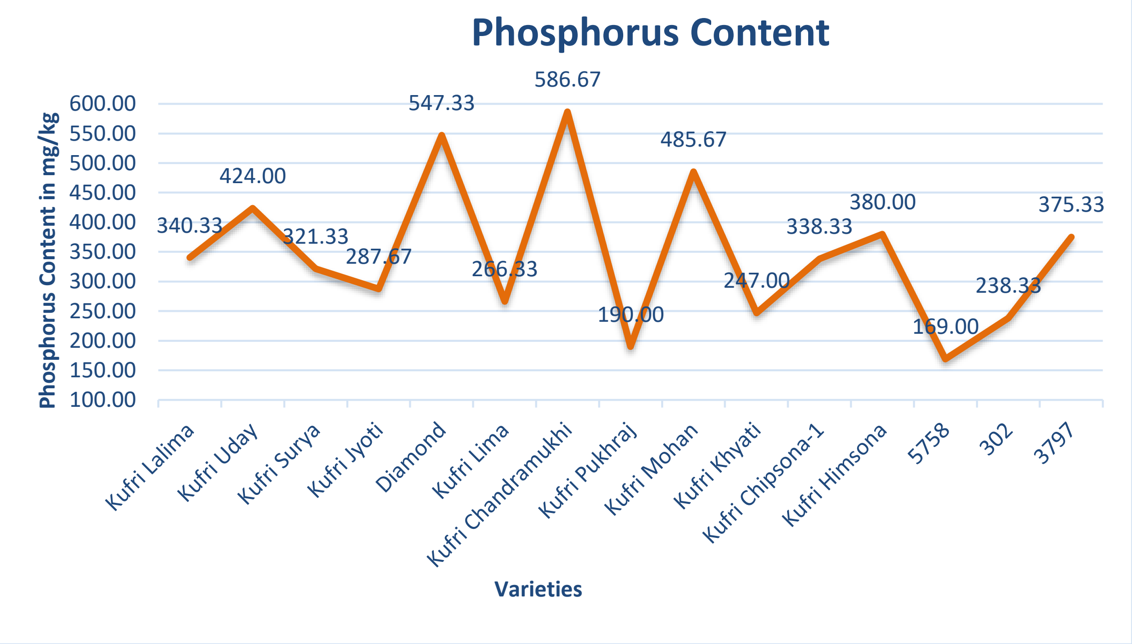


Fig. 4.1.9: Phosphorus content of potato cultivars in mg/kg on dry weight basis

4.1.10 Sulphur content

Sulphur is an essential element for human health. The concentration of this element was greatest in Kufri Jyoti (1494.33 ± 2.51) significantly higher over the other varieties, followed by Kufri Himsona (1373.00 ± 2.00) and as significantly similar with Kufri Mohan (1369 ± 1.00), and minimum in Kufri Lima (1074.66 ± 3.05), as shown in **Table 4.1.10 and Fig. 4.1.10**. The higher concentration above 1200 mg/kg were observed in Kufri Jyoti, Kufri Himsona, Kufri Mohan, Kufri Uday, 302, Kufri Khyati, Kufri Chandramukhi and 3797. Varieties with less than 1200 mg/kg was found in 5758, Kufri Pukhraj, Kufri Chipsona-1, Diamond, Kufri Lalima, Kufri Surya and Kufri Lima.

A study done by **Brown *et al.* (2012)**, determined that the levels of sulphur in potato tubers varied from 1169 to 1408 µg/g on dry weight basis. **Pandey *et al.* (2023)** also observed the sulphur in the potato tubers which lied in the range of 1.07 mg/g-1.86 mg/g. The significant variations were observed due to the difference in the varieties. According to the Sulphur Institute, there is no recommended dietary allowance of sulphur for our diet. Sulphur helps in the detoxification of the human body, insulin production, helps in the keratin and collagen formation etc. Its deficiency causes joint pain, skin problems, weak and brittle hairs, slow healing of wounds, digestive issues etc.

Table 4.1.10: Sulphur content of potato cultivars in mg/kg on dry weight basis

Varieties	**Sulphur Content**
Kufri Lalima	1154.66^{j}±1.52
Kufri Uday	1344.33^{c}±1.52
Kufri Surya	1109.33^{l}±4.50
Kufri Jyoti	1494.33^{a}±2.51
Diamond	1177.33^{h}±3.51
Kufri Lima	1074.66^{m}±3.05
Kufri Chandramukhi	1255.33^{e}±2.08
Kufri Pukhraj	1157.33^{j}±4.04
Kufri Mohan	1369.00^{b}±1.00
Kufri Khyati	1242.33^{f}±2.51
Kufri Chipsona-1	1147.33^{k}±3.05
Kufri Himsona	1373.00^{b}±2.00
5758	1167.33^{i}±2.08
302	1327.00^{d}±2.00
3797	1237.33^{g}±1.52
C.D. ($p \leq 0.05$)	4.433
SE(m)±	1.528

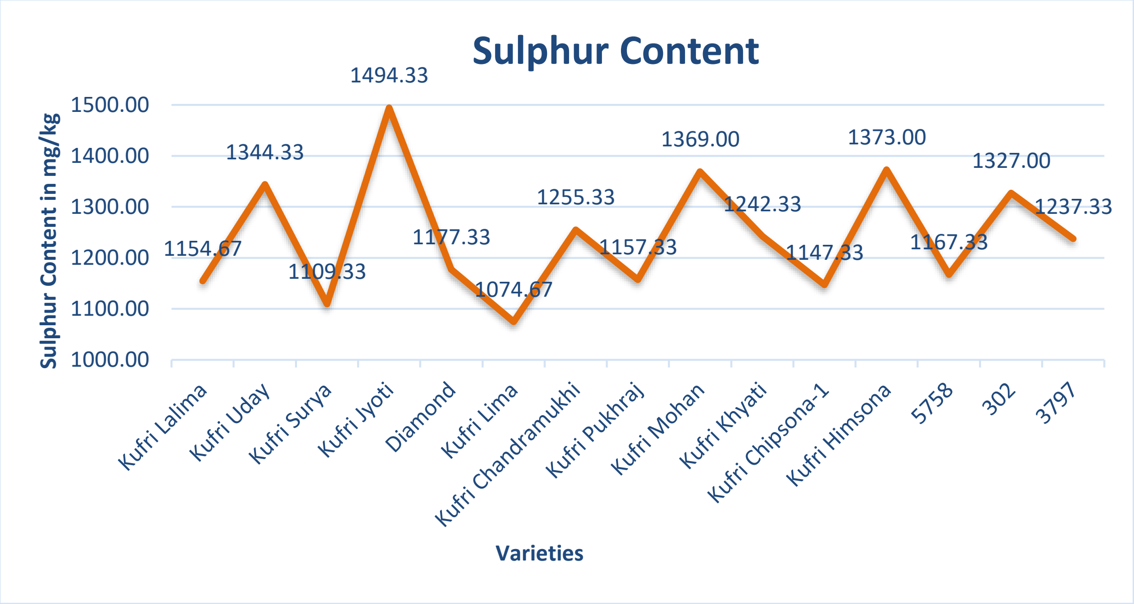


Fig. 4.1.10: Sulphur content of potato cultivars in mg/kg on dry weight basis

4.1.11 Starch content

The starch content in various potato varieties found in the range between 19.71 and 10.98% where significant variations were observed. It was found that Kufri Himsona (19.71%) had the highest starch in peeled tubers followed by Kufri Chipsona-1 (18.81%) and Kufri Lalima (10.98%) had lowest starch content. The significant differences in starch content among the potato varieties indicate variability in their starch characteristics, which could influence their appropriateness for various uses, such as industrial or culinary purposes **(Table 4.1.2, Fig. 4.1.11 and Plate 4.3 (b))**. Varieties with high starch content (17-19%) were Kufri Himsona, Kufri Chipsona-1, Kufri Chandramukhi, varieties with medium starch content (13-16%) were 302, 5758, Kufri Jyoti, Diamond, 3797 and the remaining contained the least starch content (10-13%) Kufri Pukhraj, Kufri Surya, Kufri Mohan, Kufri Uday, Kufri Khyati, Kufri Lima, Kufri Lalima.

According to **Salunkhe *et al.* (1991)**, the primary component of potato is comprised of starch, which accounts for 65–80% of its dry matter. The variance in starch content might be ascribed to the fact that various cultivars of potatoes have variable amounts of dry matter. **Donnelly and Kubow (2011)** claimed that starch content in potato varieties ranged from 10 to 30%. High starch content is desirable for high yield and texture because it increased the gelatinization at the time of processing **(Bandana *et al.*, 2016)**. If the starch is more than 19%, those potato varieties is considered to be the best for mashing. If the range lies between 16-19% than such potato may be used for roasting purposes, the varieties ranged between 13-15.9% are basically used for cooking as well as for roasting and varieties with starch content less than 12% are suitable for boiling purposes **(Ekin, 2011)**.

Potato varieties are also divided according to their starch content: those with 15% or above may be used to make starch, while those with 16–20% can be used to make chips, 15–18% for making the french fries, and 15–19% for dried products **(Lisiěska *et al.*, 2009)**.

Table 4.1.11: Starch content of potato cultivars in per cent

Varieties	**Starch Content (%)**
Kufri Lalima	10.98^{i}±0.18
Kufri Uday	11.43^{h}±0.18
Kufri Surya	12.21^{g}±0.13
Kufri Jyoti	13.80^{e}±0.13
Diamond	13.11^{f}±0.13
Kufri Lima	11.31^{h}±0.13
Kufri Chandramukhi	17.76^{c}±0.28
Kufri Pukhraj	12.27^{g}±0.22
Kufri Mohan	12.00^{g}±0.22
Kufri Khyati	11.94^{g}±0.13
Kufri Chipsona-1	18.81^{b}±0.27
Kufri Himsona	19.71^{a}±0.18
5758	13.98^{e}±0.13
302	15.72^{d}±0.13
3797	13.08^{f}±0.22
C.D. ($p \leq 0.05$)	0.318
SE(m)±	0.11

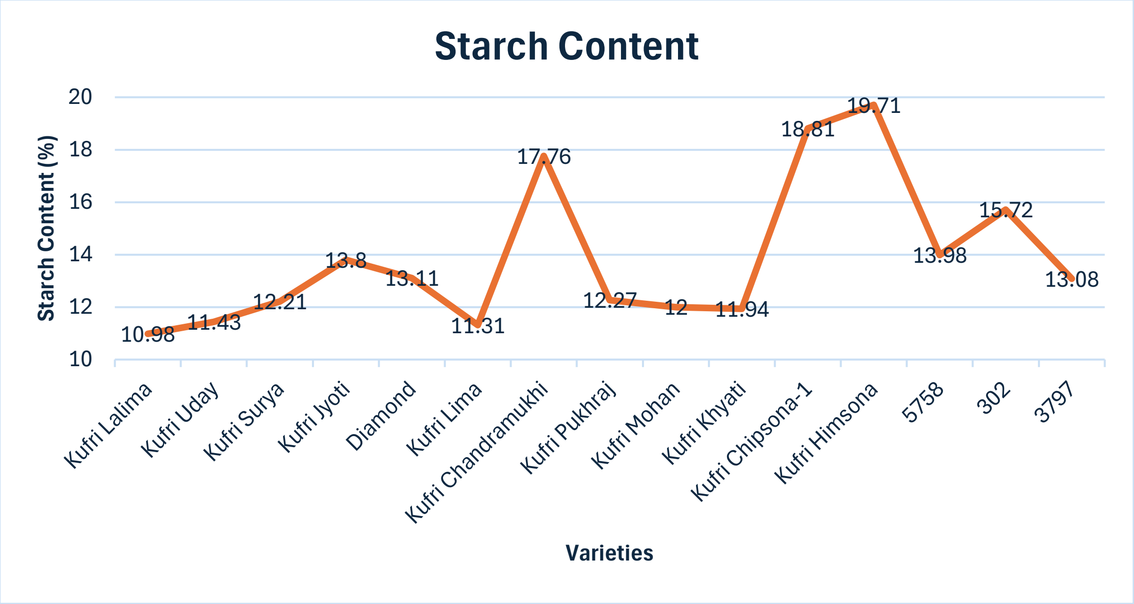


Fig. 4.1.11: Starch content of potato cultivars in percent

4.1.12 Skin and Flesh colour

The colour of the skin of the tuber varieties were found from red to yellow. The red colour varieties were Kufri Lalima, Kufri Uday; white colour varieties were Kufri Jyoti, Diamond, Kufri Chandramukhi, Kufri Mohan, Kufri Chipsona-1, while white-cream coloured varieties were Kufri Lima, Kufri Surya, Kufri Khyati, Kufri Himsona, 302, 3797, and the yellow colour of skin was observed in Kufri Pukhraj and 5758 **(Table 4.1.12, Fig. 4.1.12 and Plate 4.1 (c))**.

It is observed that the red colour of the skin of potato might be due to the presence of the anthocyanin pigments while the yellow colour of the potato skin might be due to the presence of carotenoids. It can also be possible that the white-cream or cream colour of the skin can also be occurred due to the less amount of carotenoids and white skin by the absence amount of carotenoids.

Similarly, the flesh colour of various potato varieties ranged from white to yellow. It is found that the Kufri Lalima, Kufri Mohan and 302 have white flesh colour while white-cream coloured were Kufri Khyati and Kufri Chipsona-1; varieties with cream flesh were Kufri Surya, Kufri Jyoti, Kufri Himsona, 3797 and yellow flesh-coloured varieties were Kufri Uday, Kufri Pukhraj and 5758.

Earlier studies have also demonstrated comparable findings regarding flesh colour **(Gaur *et al.,* 1999)**. The colour of the flesh of a potato significantly impacts its economic value, it is a main characteristic in the processing sector. Prior research has shown that the pigmentation of potato tubers is influenced by the concentration of carotenoids and anthocyanins **(Nesterenko and Sink, 2003)**. The tubers with minimum amount of carotenoids is indicated white-coloured flesh, whereas the high carotenoid content results in yellow colour **(Zhang *et al.,* 2009)**.

Table 4.1.12: Skin and flesh colour of potato varieties

Varieties	Skin Color	Flesh Color
Kufri Lalima	Red	White
Kufri Uday	Red	Yellow
Kufri Surya	White- cream	Cream
Kufri Jyoti	White	Cream
Diamond	White	White
Kufri Lima	White- cream	Cream
Kufri Chandramukhi	White	White
Kufri Pukhraj	Yellow	Yellow
Kufri Mohan	White	White
Kufri Khyati	White- cream	White- cream
Kufri Chipsona-1	White	White- cream
Kufri Himsona	White- cream	Cream
5758	Yellow	Yellow
302	White- cream	White
3797	White- cream	Cream

4.1.13 Dry matter content

The dry matter content of fifteen varieties was evaluated and concise in **Table 4.1.13, Fig. 4.1.13 and Plate 4.2 (b)**. The dry matter was highest in Kufri Chandramukhi (26%) which was significantly at par with Kufri Chipsona-1 (25.66%) and Kufri Himsona (25.33%), whereas variety 3797 (11.58%) was found to have the lowest dry matter. Significant difference among the varieties showed that varieties with high dry matter (20-24%) are suitable for industrial purpose *i.e.* Kufri Chandramukhi, Kufri Chipsona-1, Kufri Himsona, Diamond; and with medium dry matter (17-20%) can be used for both culinary and industrial purposes *viz.*5758, Kufri Pukhraj, Kufri Surya, and lowest dry matter (11-17%) varieties are thus only be used for table purpose *i.e.* Kufri Khyati, Kufri Lima, 302, Kufri Uday, Kufri Mohan, Kufri Lalima and 3797.

In the study of **Ndungutse *et al.* (2019)**, the dry matter content was estimated in varieties and clone of potato where it ranged from 20.94 to 25.93% and **Kaur and Aggarwal (2014)** also analysed the dry matter content in the several Indian potato cultivars and found the same between 14.06 to 24. 31% which was close to our results. The dry matter content of potatoes is a crucial factor in potato processing. A greater dry matter content results in reduced absorption of oil, a desired texture, and improved yields in the final products **(Marwaha, 1997)**. There is a significant degree of variation across cultivars when it comes to the quantity of dry matter that is present in tubers, as stated by **Abbas *et al.* (2011)**. The ideal range of dry matter for French fries is 20 to 24% **(Kabira and Lemaga, 2003)**. According to **Marwaha *et al.* (2010)** dry matter should be more than 20% for fries and crisps, while for canning and dehydrated products it should be less than 18%.

Table 4.1.13: Dry matter content of potato varieties in per cent

Varieties	Dry Matter (%)
Kufri Lalima	$14.19^{e,f}\pm1.29$
Kufri Uday	$15.22^{d,e}\pm1.34$
Kufri Surya	$17.33^{c,d,e}\pm2.08$
Kufri Jyoti	$16.66^{c,d,e}\pm3.05$
Diamond	$22.33^{b}\pm1.52$
Kufri Lima	$15.66^{d,e}\pm1.52$
Kufri Chandramukhi	$26.00^{a}\pm1.00$
Kufri Pukhraj	$18.00^{c,d}\pm1.00$
Kufri Mohan	$14.33^{e,f}\pm2.08$
Kufri Khyati	$16.12^{c,d,e}\pm1.19$
Kufri Chipsona-1	$25.66^{a}\pm2.51$
Kufri Himsona	$25.33^{a}\pm1.52$

5758	$19.25^{c}\pm1.40$
302	$15.18^{d,e}\pm1.73$
3797	$11.58^{f}\pm1.01$
C.D. ($p \leq 0.05$)	2.878
SE(m)±	0.992

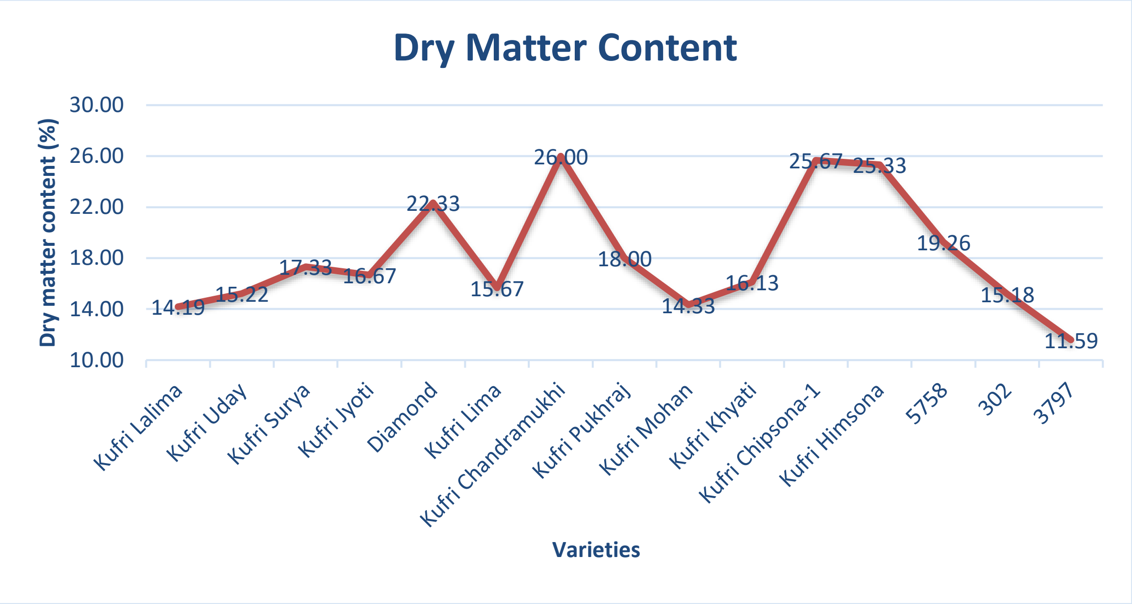


Fig. 4.1.13: Dry matter content of potato varieties in percent

4.1.14 Moisture content

The moisture content was found minimum was in Kufri Chandramukhi (74%) which was significantly at par with Kufri Chipsona-1 (74.33%), and Kufri Himsona (74.66%) also significantly different from other varieties, whereas maximum moisture content was observed in 3797 (88.41%) as shown in **Table 4.1.14 Fig. 4.1.14 and Plate 4.2 (a)**. The variations in moisture content may be ascribed to the distinct genetic makeup, or varietal traits or due to the adaptability in growing environment.

In the findings of **Dev Raj *et al.* (2011), Rai and Verma (1989)** and **Raj *et al.* (2007)**, the moisture content in several potato varieties lied between 77.40% and 83.03% which were nearly to our results. The tubers with high moisture content are considered to be an undesirable trait because it favours the microorganisms to grow thus reduce the shelf life of the potato. In addition, moisture content causes blistering while frying the potato, that's why variety with less moisture content is mainly used for fries and chips **(Nawaz *et al.*, 2020)**. Tubers with high moisture content is only used for mashing and boiling.

Table 4.1.14: Moisture content of potato varieties in per cent

Varieties	Moisture Content (%)
Kufri Lalima	85.81^{e,f}±1.29
Kufri Uday	84.78^{d,e}±1.34
Kufri Surya	82.66^{c,d,e}±2.08
Kufri Jyoti	83.33^{c,d,e}±3.05
Diamond	77.66^{b}±1.52
Kufri Lima	84.33^{d,e}±1.52
Kufri Chandramukhi	74.00^{a}±1.00
Kufri Pukhraj	82.00^{c,d}±1.00
Kufri Mohan	85.66^{e,f}±2.08
Kufri Khyati	83.87^{c,d,e}±1.19
Kufri Chipsona-1	74.33^{a}±2.51
Kufri Himsona	74.66^{a}±1.52
5758	80.74^{c}±1.40
302	84.82^{d,e}±1.73
3797	88.41^{f}±1.01
C.D. ($p \leq 0.05$)	2.878
SE(m)±	0.992

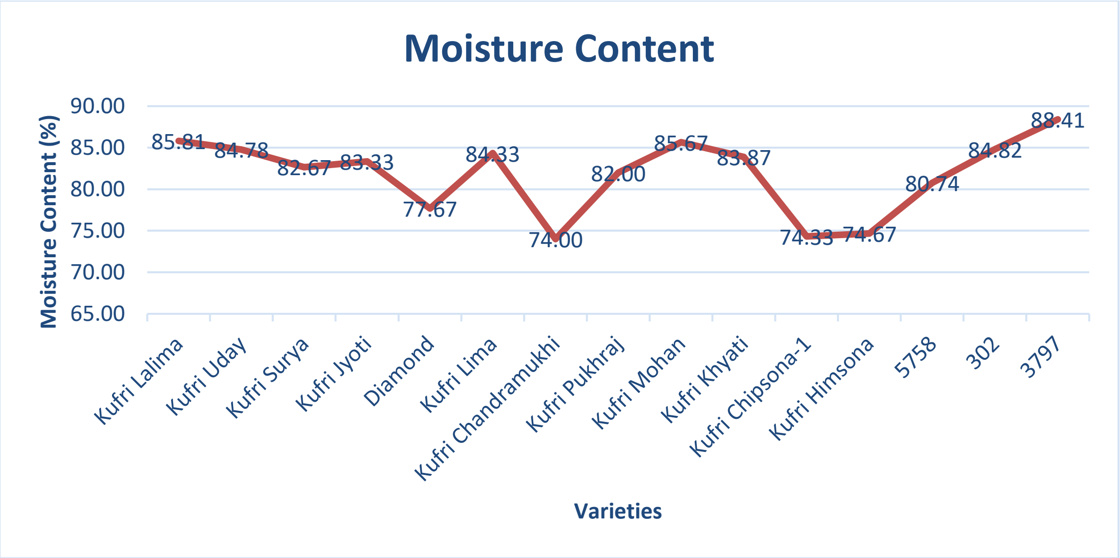


Fig. 4.1.14 Moisture content of potato varieties in percent

4.1.15 Specific Gravity

Specific gravity was found maximum in Kufri Chipsona-1 (1.097) significantly at par with Diamond (1.077) and Kufri Chandramukhi (1.079) whereas, minimum in Kufri Mohan (1.025) **(Table 4.1.15, Fig. 4.1.15 and Plate 4.1 (a))**. These variances may be attributed to genetic variability among various cultivars. The specific gravity with high specific gravity ranged between 1.070 and 1.097 included varieties Kufri Chipsona-1, Kufri Chandramukhi, Kufri Himsona, Diamond, Kufri Surya. Varieties with medium specific gravity (1.040- 1.060) were Kufri Lima, Kufri Uday, Kufri Pukhraj, Kufri Khyati, Kufri Jyoti, 302. Minimum specific gravity (1.025- 1.040) was observed in 5758, 3797, Kufri Lalima, and Kufri Mohan.

Marwah *et al.* (2010) claimed that the potato with specific gravity 1.080 or above was used for the french fries, chips and specific gravity lower than 1.070 was used for the canning purposes. **Kaur and Aggarwal (2014)** analysed the specific gravity which was ranged between 1.040 and 1.096. **Sandhu and Parhawk (2002)** investigated the specific gravity in various potato tuber varieties and found that the differences in the specific gravity were due to the genetic variations. It has been observed that cultivars that have a high specific gravity have a higher percentage of dry matter content. In contrast, cultivars with a low specific gravity had a lower dry matter content **(Abbas *et al.,* 2011)**.

Table 4.1.15: Specific gravity of potato varieties

Varieties	Specific Gravity
Kufri Lalima	$1.027^{h}\pm0.001$
Kufri Uday	$1.056^{c,d,e,f}\pm0.002$
Kufri Surya	$1.075^{b,c,d}\pm0.011$
Kufri Jyoti	$1.044^{e,f,g,h}\pm0.028$
Diamond	$1.077^{a,b}\pm0.021$
Kufri Lima	$1.060^{b,c,d,e}\pm0.004$
Kufri Chandramukhi	$1.079^{a,b}\pm0.007$
Kufri Pukhraj	$1.054^{d,e,f,g}\pm0.010$
Kufri Mohan	$1.025^{h}\pm0.001$
Kufri Khyati	$1.052^{e,f,g}\pm0.003$
Kufri Chipsona-1	$1.097^{a}\pm0.002$

Kufri Himsona	$1.073^{b,c,d}\pm0.016$
5758	$1.036^{f,g,h}\pm0.003$
302	$1.045^{e,f,g,h}\pm0.003$
3797	$1.034^{g,h}\pm0.003$
C.D. ($p \leq 0.05$)	0.019
SE(m)±	0.007

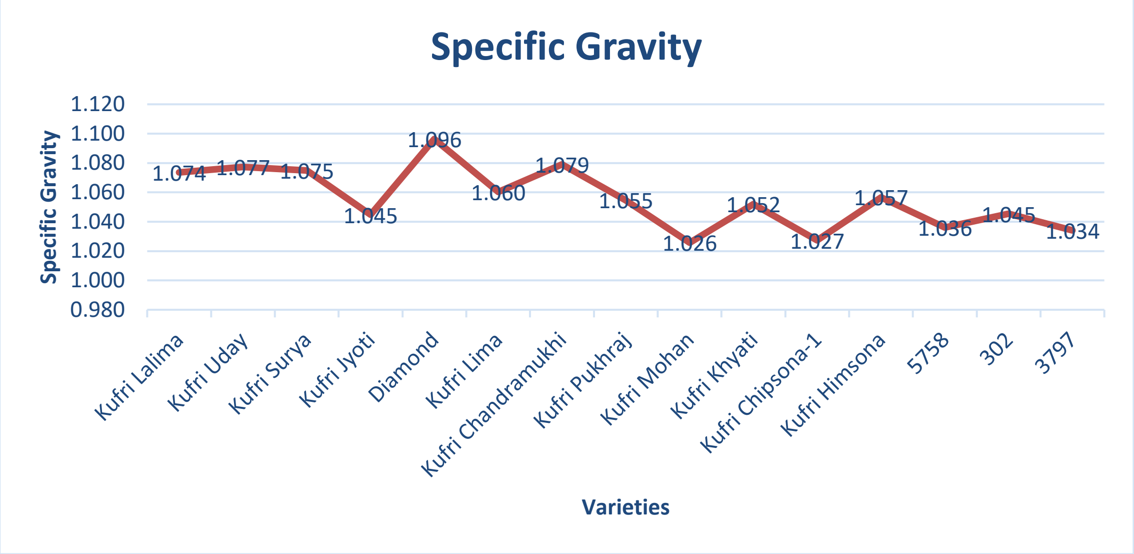


Fig. 4.1.15: Specific gravity of potato varieties

4.1.16 Ash content

The ash content in this study ranged between 0.66% and 2.95%. The highest ash content was found in Kufri Chandramukhi (2.95%) which was significantly higher than the other varieties followed by Kufri Uday (1.86%) and the lowest was recorded in Kufri Lalima (0.66%) **(Table 4.1.16, Fig. 4.1.16 and Plate 4.3 (a))**. The varieties with ash content ranged between 1.00% and 2.00% were Kufri Uday, Kufri Khyati, Kufri Mohan, Kufri Himsona, Kufri Chipsona-1, 302, 5758, 3797, Kufri Pukhraj, Kufri Jyoti and the ash content less than 1% was observed in Kufri Surya, Diamond, Kufri Lima, and Kufri Lalima.

In the previous studies of **Kaur and Aggarwal (2014)** the ash content was analysed which was ranged between 0.70% and 2.09% found in synchrony with current findings. Similar findings were also observed by **Nesterenko and Sink (2003)** for different potato varieties and predicted that the genotype is responsible for the variations in ash content. Higher ash content indicates the higher essential minerals present in the potato tuber.

Table 4.1.16: Ash content of potato varieties in per cent

Varieties	Ash Content (%)
Kufri Lalima	$0.66^{m}\pm0.01$
Kufri Uday	$1.86^{b}\pm0.03$
Kufri Surya	$0.91^{k}\pm0.01$
Kufri Jyoti	$1.03^{j}\pm0.01$
Diamond	$0.87^{k}\pm0.01$
Kufri Lima	$0.74^{l}\pm0.01$
Kufri Chandramukhi	$2.95^{a}\pm0.02$
Kufri Pukhraj	$1.16^{i}\pm0.03$
Kufri Mohan	$1.53^{d}\pm0.03$
Kufri Khyati	$1.66^{c}\pm0.02$
Kufri Chipsona-1	$1.37^{f}\pm0.02$
Kufri Himsona	$1.46^{e}\pm0.02$
5758	$1.28^{h}\pm0.01$
302	$1.32^{g}\pm0.01$
3797	$1.12^{i}\pm0.02$
C.D. ($p \leq 0.05$)	0.038
SE(m)±	0.013

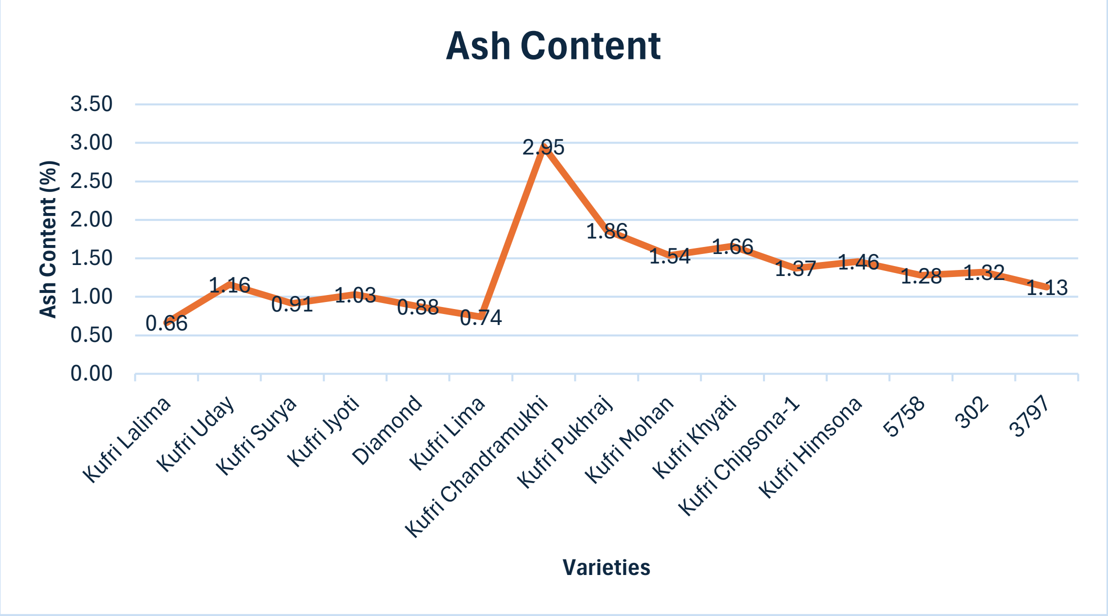


Fig. 4.1.16 Ash content of potato varieties in percent

4.1.17 Total soluble solids

The total soluble solids of different varieties of potato were varied between 5.32 and 7.01°Brix. The highest TSS observed in Kufri Himsona which was 7.01°Brix followed by Kufri Chandramukhi with 6.85°Brix and the lowest was observed in Diamond with 5.32°Brix **(Table 4.1.17, Fig. 4.1.17 and Plate 4.1 (b))**.

The values of total soluble solids that were found in cultivars were somewhat higher than those that were reported in earlier investigations **Sandhu and Parhawk (2002);** and **Sandhu *et al.* (2002)**. The increase might be ascribed to a greater concentration of soluble proteins and vitamins. According to **Ndungutse *et al.* (2019)** the sugar content is basically dependent on the genotypes of the variety and the pre harvest components (temperature, irrigation, mineral nutrition, crop maturity) and post-harvest components (storage and mechanical stress).

Table 4.1.17: Total soluble solids of potato varieties in °Brix

Varieties	Total Soluble Solids (°Brix)
Kufri Lalima	5.90^{i}±0.02
Kufri Uday	6.01^{h}±0.00
Kufri Surya	6.47^{d}±0.02
Kufri Jyoti	5.67^{l}±0.02
Diamond	5.32^{n}±0.01
Kufri Lima	5.86^{i}±0.00
Kufri Chandramukhi	6.85^{b}±0.03
Kufri Pukhraj	5.41^{m}±0.01
Kufri Mohan	5.81^{j}±0.01
Kufri Khyati	6.25^{f}±0.02
Kufri Chipsona-1	6.58^{c}±0.07
Kufri Himsona	7.01^{a}±0.00
5758	6.32^{e}±0.00
302	6.07^{g}±0.02
3797	5.73^{k}±0.01
C.D. ($p \leq 0.05$)	0.043
SE(m)±	0.015

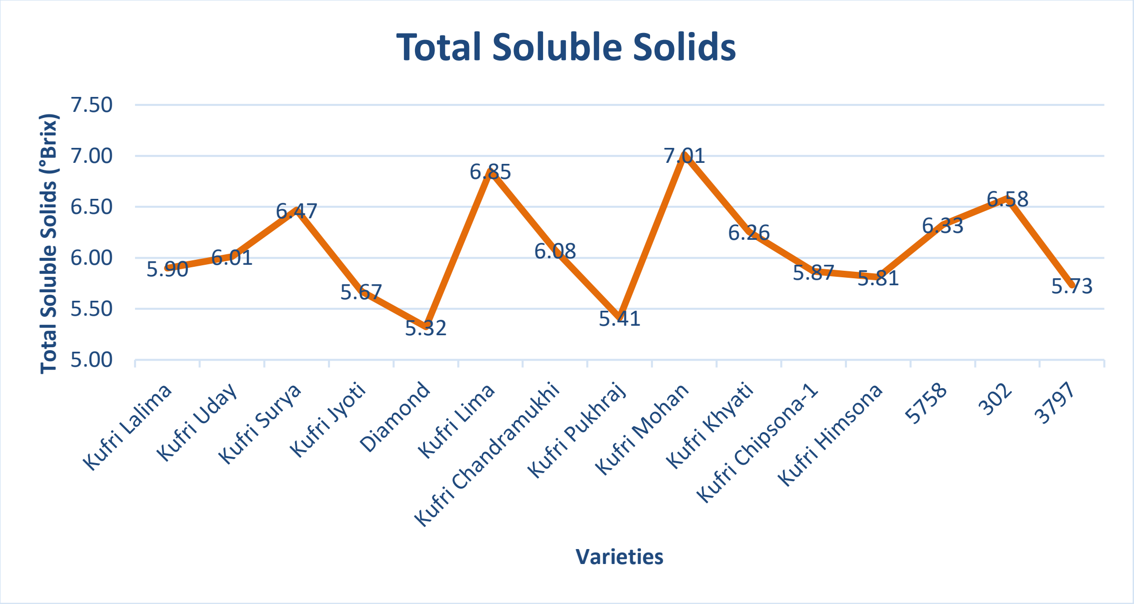


Fig. 4.1.17 Total soluble solids of potato varieties in °Brix

4.2 To determine the isolation and fusion of protoplast

4.2.1.1 Protoplast Viability of variety Kufri Uday

The protoplast viability of the variety Kufri Uday was significantly affected by the different temperatures. The different temperatures were used during the isolation of the protoplasts from the *in-vitro* leaves of the potato plant. The mean viability of the protoplast was higher when the enzymatic solution containing *in-vitro* leaves were kept at 27°C in treatment C3 (81.70%) which was significantly higher than other treatments followed by C2 (72.30%) and lowest was observed at 22°C in treatment C1 (34.45%) **(Table 4.2.1.1 and Fig. 4.2.1.1)**.

The finding suggested that the Kufri Uday performed well at high temperature and produced a high percentage of viable protoplasts, however the viability decreased under C1, showing that the lower temperature reduced the efficiency of protoplasts isolation which may have reduced the viability of the protoplasts. The temperature plays a vital role in the isolation of protoplasts. According to **Reed and Bargmann (2021)**, temperature affects the viability of protoplasts, too high or too low temperature can reduce the viability of the protoplasts. That's why it is important to choose the temperature that promotes the viability of the protoplasts. Temperature enhances the degradation of cell walls of the leaves.

Similarly, enzyme combinations had significant effect on the protoplast viability of Kufri Uday. The mean viability of Kufri Uday was highest in E3 (73.30%) where the enzyme combinations were 0.5% macerozyme + 1 % cellulase followed by E2 i.e., 0.4% macerozyme + 2% cellulase (66.98%) and lowest was observed in E1 [0.25% macerozyme + 1.5% cellulase (48.18%)] with significant difference observed among the treatments **(Table 4.2.1.1 and Fig. 4.2.1.1)**.

According to the previous findings, the enzyme combinations played a vital role in enhancing the protoplast viability by isolating the protoplasts without rupturing it. If the concentration of the enzymes increased, then the chances of rupturing the protoplasts increases which simultaneously reduce the viability of the protoplasts. **Moon *et al.* (2021)** used the same enzymes and described their roles of macerozyme which targets the pectic substances, while cellulase focuses on breaking down the cellulose present in the leaves of the plant. They also observed that using the different enzyme concentrations can affect the viability of the protoplasts. When both the enzymes were combined, they would perform their own functions, which would result in a more effective isolation of protoplasts **(Xue *et al.*, 2023)**. **Stajič (2023)** used different enzymes at different concentrations to analyze the viability of protoplasts of *Brassica oleracea* var. *capitata* L. leaves and found that the variations in the viability occurred while changing enzymes concentrations, but no effect was shown while changing the enzymes. However, the best results were seen while using 0.5% cellulase and 0.1% macerozyme.

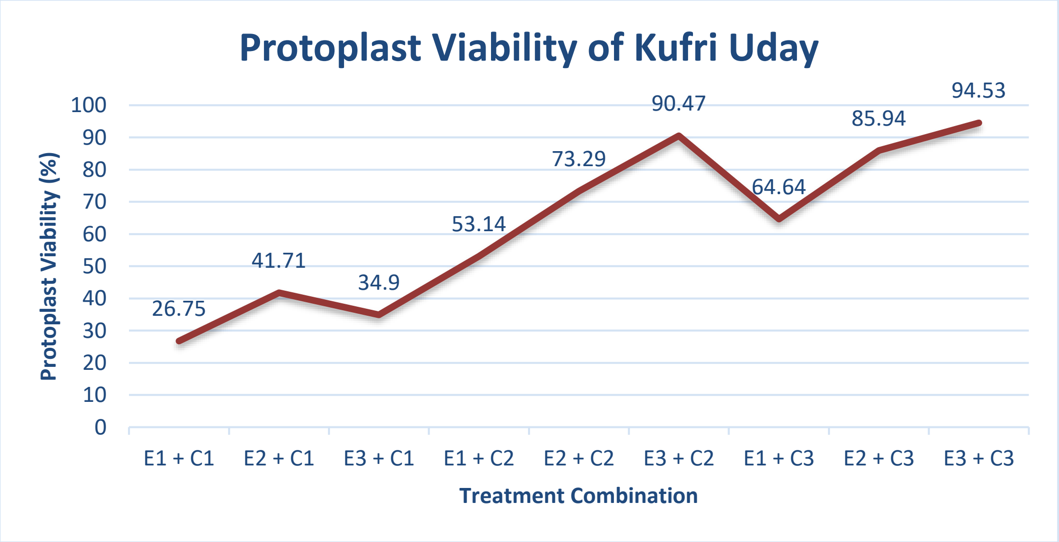
The interaction between the enzyme combinations and temperature showed maximum viability in E3+ C3 (0.5% macerozyme + 1% cellulase at 27°C) with 94.53% which was significantly higher among all the treatments followed by E3+C2 (0.5% macerozyme + 1% cellulase at 25°C) with 90.47% and least viability was observed in E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 26.75% **(Table 4.2.1.1 and Fig. 4.2.1.1)**.

The results showed that both enzyme combinations and temperature work simultaneously for increasing the viability of the protoplasts. **Reed and Bargmann (2021)** claimed that the temperature promotes enzymatic activity. Enzymes degrade the cells wall. According to **Tee *et al.* (2010)**, the enzyme concentration and combinations differs from variety to variety and crop to crop. The enzyme concentration is also worked properly with the suitable temperature for the protoplasts isolation that promotes the protoplasts viability. High and low concentrations of the enzymes effect the viability of the protoplasts and same as high and low temperature also affects the viability of the protoplasts.

Table 4.2.1.1 The effect of temperature and enzyme combination interaction on the protoplast viability of Kufri Uday in percentage

Treatment	E1	E2	E3	Mean C
C1	26.75	41.71	34.90	34.45^{c}
C2	53.14	73.29	90.47	72.30^{b}
C3	64.64	85.94	94.53	81.70^{a}
Mean E	48.18^{c}	66.98^{b}	73.30^{a}	
	C	**E**	**C × E**	
C.D. ($p \leq 0.05$)	1.855	1.855	3.212	
SE(m)±	0.619	0.619	1.073	

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C



Fig, 4.2.1.1: The effect of temperature and enzyme combination interaction on the protoplast viability of Kufri Uday in percentage

4.2.1.2 Protoplast Viability of variety Kufri Chipsona-1

The viability of the Kufri Chipsona-1 was significantly affected by the temperature. The drastic variations had been observed in the percentage of viability by changing the temperature. The maximum viability was observed in C3 (83.73%) at 27°C followed by C2 (71.54%) at 25°C and minimum was observed in C1 (37.99%) at 22°C **(Table 4.2.1.2 and Fig. 4.2.1.2)**.

According to the findings observed in Kufri Chipsona-1, temperature plays a great role in increasing or decreasing the viability with low temperature had less viability of protoplasts and with high temperature had the highest viability. High temperature promotes isolation and thus promotes viability **(Reed and Bargmann, 2021)**. The very high temperature can rupture the protoplasts by damaging the cell wall. Temperature for isolation also varies from plant to plant and variety to variety. **Chen *et al.* (2008)** also observed that the temperature is very important for the isolation of the protoplast. They also carried out experiments on several temperatures in potato species and found that at high temperature *i.e.* 29°C isolation was done successfully.

As due to the enzyme combinations, the fluctuations in the viability of Kufri Chipsona-1 had been observed. It showed that the E3 (0.5% macerozyme + 1 % cellulase) had the highest viability 78.74% which was found highest over the other enzyme combinations and second highest was observed in E2 (0.4% macerozyme + 2% cellulase) with 59.35% viability, however the lowest viability was found in enzyme combination E1 (0.25% macerozyme + 1.5% cellulase) with 55.17% of viability **(Table 4.2.1.2 and Fig. 4.2.1.2)**.

The findings state that the enzyme combinations are crucial for increasing the viability of the protoplasts of the potato *in-vitro* leaves. Increasing or decreasing the concentration of enzymes could affect viability. It has been suggested that the macerozyme works to break the cell wall by rupturing the pectic substance, while cellulase mainly targets the cellulose so that the protoplasts can easily be isolated **(Moon *et al.*, 2021)**. According to the research of **Wang *et al.* (2017)** the highest viability was observed when they use the snailase with macerozyme, cellulase and hemicellulase and showed that the specific amount of enzymes are beneficial for the extraction of protoplasts from the leaves of the dihaploid potato lines.

The interaction between the temperature and enzyme combinations showed that the highest viability was observed in E3+C3 (0.5% macerozyme + 1% cellulase at 27°C) with 94.96% which was significantly higher than other treatments followed by E3+C2 (0.5% macerozyme + 1% cellulase at 25°C) with 89.45% and least viability was observed in E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 24.88% **(Table 4.2.1.2 and Fig. 4.2.1.2)**.

In the previous research of **Reed and Bargmann (2021)**, observed that the enzymatic activity was fastens at high temperature. Moderate amount of enzyme concentration and high temperature successfully isolated the protoplast and increased the viability of potato. These results suggested that both enzymes' combinations and temperature are dependent on each other, and both play a great role in increasing the viability of protoplasts by isolating the protoplasts from the leaves. Both enzymes could be toxic if their concentration exceeds. Similarly, increasing or decreasing the temperature could also degrade the protoplast quality, thus the viability of the protoplasts decreases.

Table 4.2.1.2: The effect of temperature and enzyme combination interaction on the protoplast viability of Kufri Chipsona-1 in percentage

Treatment	E1	E2	E3	Mean C
C1	24.88	37.27	51.83	37.99^{c}
C2	67.70	57.48	89.45	71.54^{b}
C3	72.94	83.30	94.96	83.73^{a}
Mean E	55.17^{c}	59.35^{b}	78.74^{a}	
	C	**E**	**C × E**	
C.D. ($p \leq 0.05$)	1.914	1.914	3.315	
SE(m)±	0.639	0.639	1.107	

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C

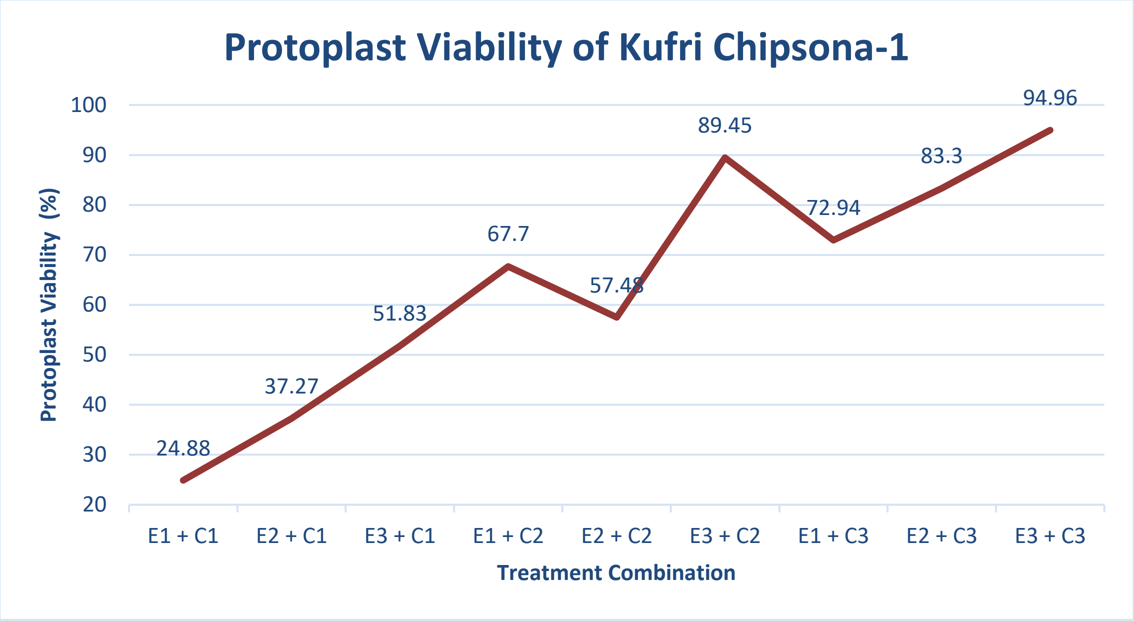


Fig. 4.2.1.2: The effect of temperature and enzyme combination interaction on the protoplast viability of Kufri Chipsona-1 in percentage

4.2.2.1 Protoplast Density of Kufri Uday

The density of Kufri Uday was greatly influenced by increasing or decreasing the temperature. The highest protoplast density was observed in C3 (27°C) with 2.03 $\times 10^5$cells/mL which was observed to be the highest amongst the other treatments and it was followed by the C2 (25°C) with 1.58 $\times 10^5$cells/mL and the lowest protoplast density was found in the treatment C1 (22°C) with 0.48 $\times 10^5$ cells/mL **(Table 4.2.2.1 and Fig. 4.2.2.1)**.

According to the findings, as the density of the variety Kufri Uday had been drastically changed by increasing or decreasing the temperature. As the temperature the density of the protoplast increased and by decreasing the temperature the density was decreasing. Temperature helps the protoplasts to be released from the leaves. **Dai *et al.* (1987)** also maintained the temperature at 28°C for the isolation of protoplasts from the leaves of different species in potato. **Chen *et al.* (2008)** did their research on the different species of potato, to standardize the temperature (29°C) for isolation of protoplasts, the density of the protoplasts was significantly higher compared to other temperatures. If the isolation is successfully done, then it ultimately increases the viability percentage of the protoplasts which had emerged out from the *in-vitro* leaves of the plant.

Similarly, the enzyme combinations also play a role in protoplast density. The density was observed highest with treatment E3 (0.5% macerozyme + 1% cellulase) with 1.77 × 10^5 cells/mL followed by E2 (0.4% macerozyme + 2% cellulase) with 1.43 × 10^5 cells/mL and the treatment with low density was observed in E1 (0.25% macerozyme + 1.5% cellulase) with 0.89 × 10^5cells/mL and significant difference was observed amongst the treatments **(Table 4.2.2.1 and Fig. 4.2.2.1)**.

The result showed that protoplasts density was greatly impacted by enzyme concentrations. Enzyme concentration is very important for increasing the density of protoplasts. Firstly, the macerozyme dissolves the layer called middle lamella of the leaves and then the cellulase breaks down the cell wall made up of hemicellulose and cellulose so that the protoplasts can easily be isolated. That's why the proper combination of enzyme is important **(Capitana and McCann, 2000)**. **Bokelmann and Roest (1983)** used the same combination of enzymes, but different concentrations; they observed the highest yield in commercial tetraploid potato cv. Bintje. **Cardi *et al.* (1990)** isolated protoplasts from three different accessions of *Solanum commersonii* by using macerozyme and cellulase and used different concentration of macerozyme from our research, but the cellulase concentration was same and their protoplast yield was from 4.4 to 8.5 ×10^6 g^{-1} protoplasts of fresh tissue. **Moon *et al.* (2021)** used two different concentrations of macerozyme, however, the cellulase concentration was constant for the isolation of protoplasts from two different varieties of potato and they analyzed the protoplast efficiency higher with cellulase 1% and 0.5% macerozyme. In our research we used the same concentration, and our density was also highest by using this same concentration.

The interaction between the enzyme combination and temperature affected the density of the protoplasts of variety Kufri Uday. According to the findings, the highest density of protoplasts was observed in treatment E3+C3 (0.5% macerozyme + 1% cellulase at 27°C) with 2.53 ×10^5 cells/mL followed by E3+C2 (0.5% macerozyme + 1% cellulase at 25°C) with 2.28 ×10^5 cells/mL and lowest protoplast density was found in treatment E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 0.34 $\times 10^5$ cells/mL **(Table 4.2.2.1 and Fig. 4.2.2.1)**.

The result clearly shows that higher concentration of enzyme along with higher temperature resulted in maximum recovery of protoplasts from the explants of Kufri Uday. The results also indicate that lower temperature resulted in least recovery of protoplasts. This interaction clearly shows the impact of temperature on enzymatic activities which have been proven in many enzymes related studies. For instance, **Daniel *et al.* (2008)** studied the effect of temperature on enzymes activity. Enzymes activity catalyzes with the increase in temperature and activity inactive with the decrease in temperature, which clearly showed that for the cell division both the temperature and enzymes are required, solely they won't be able to break the bond present in the layers of the leaves. The enzymes macerozyme and cellulase could be stable at high temperatures. **Den (2024)** also discussed the effect of temperature on enzymatic activity.

Table 4.2.2.1: The effect of temperature and enzyme combination interaction on the protoplast density of Kufri Uday ($\times 10^5$cells/mL)

Treatment	**E1**	**E2**	**E3**	**Mean C**
C1	0.34	0.60	0.50	0.48^c
C2	0.89	1.57	2.28	1.58^b
C3	1.44	2.12	2.53	2.03^a
Mean E	0.89^c	1.43^b	1.77^a	
	C	**E**	**C × E**	
C.D. ($p \leq 0.05$)	0.05	0.05	0.08	
SE(m)±	0.01	0.01	0.02	

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C

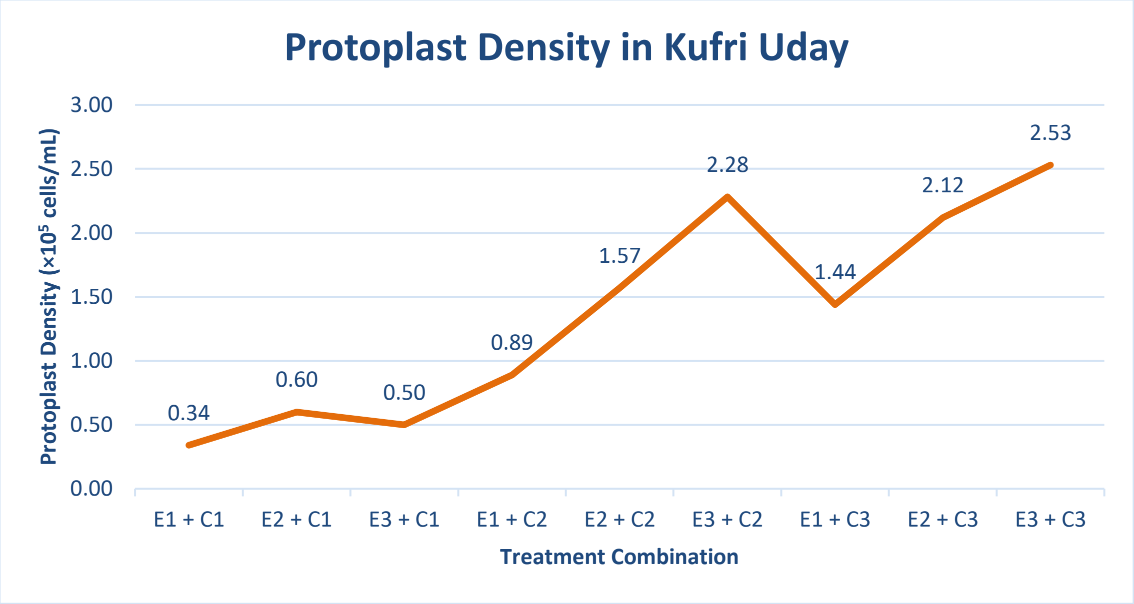


Fig. 4.2.2.1: The effect of temperature and enzyme combination interaction on the protoplast density of Kufri Uday (×10^5cells/mL)

4.2.2.2 Protoplast Density of Kufri Chipsona-1

The density of the protoplast of Kufri Chipsona-1 was highly affected by the temperature as shown in results that the maximum density was observed in treatment C3 (2.07 × 10^5cells/mL) at 27°C which was maximum amongst all the treatments and the second highest was observed in treatment C2 with 1.59 ×10^5cells/mL at 25°C and the lowest was observed in C1 0.54 ×10^5cells/mL at 22°C and significant difference was observed among the treatments **(Table 4.2.2.2 and Fig. 4.2.2.2)**.

The results that are shown in **Table 4.2.2.2 and Fig. 4.2.2.2** claimed that the density of protoplasts of Kufri Chipsona-1 was increasing by increasing the incubating temperature, which helps in isolating the protoplasts. Temperature is important for dissolving the cell wall of leaves. **Dai *et al.* (1987)** chose the high temperature 28°C for their research for analyzing the protoplast density in five different species of potato. **Carlberg *et al.* (1983)** successfully isolated the protoplasts by choosing the temperature 27°C for their research which helped them to get the highest yields from potato leaves. **Chen *et al.* (2008)** also analysed the isolation of protoplasts at various temperatures (22°C, 25°C, 29°C) to standardize the protocol for their research and found that isolation was highest at 29°C so they chose higher temperature for their further research work.

Furthermore, it has also been noticed that the density of potato variety Kufri Chipsona-1 was also affected by changing the concentration of enzymes. That's why the highest density was recorded in E3 (0.5% macerozyme + 1% cellulase) with 1.84 $\times 10^5$cells/mL followed by E2 (0.4% macerozyme + 2% cellulase) with 1.21 $\times 10^5$cells/mL which was significantly at par with E1 (0.25% macerozyme + 1.5% cellulase) with 1.15 $\times 10^5$cells/mL **(Table 4.2.2.2 and Fig. 4.2.2.2)**.

The results showed that the little change in concentration of enzymes affects the density of protoplasts of Kufri Chipsona-1. Enzymes and their concentrations play a major role in the isolation of protoplasts. Excessive concentration of enzymes can cause toxicity in the cells, and this toxicity leads to the death of the protoplasts as well as reduce the viability which ultimately reduce the density of the protoplast. Both the enzymes did their work separately even when they mixed together like cellulase break the cell wall made up from cellulose and hemicellulose, which macerozyme mainly targets the pectic substances present in the cell wall of the leaves. **Shepard & Totten (1977)** used macerozyme and cellulase of different concentrations for their research and found that they had isolated the protoplasts successfully from the leaves of the potato variety *i.e.* Russet Burbank. **Moon *et al.* (2021)** also observed the protoplast density by using the same enzyme and concentrations in the potato leaves of Desiree variety. **Figueroa-Varela *et al.* (2023)** also used the same enzymes of different concentrations in castor beans, and they also isolated protoplasts and found the substantial yield form the isolated protoplasts.

The interaction of enzymes and temperature showed that the treatment E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C) showed the highest density of 2.50 $\times 10^5$cells/mL followed by E2 + C3 (0.4% macerozyme + 2% cellulase at 27°C) 1.21 $\times 10^5$cells/mL and lowest density was observed in the treatment E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 0.32 $\times 10^5$cells/mL and significant difference was observed in all treatments **(Table 4.2.2.2 and Fig. 4.2.2.2)**.

The results suggested that with the change in the concentration of the enzymes significant changes in the density were observed. Similarly, by changing the temperature the fluctuations had been observed in the density of protoplasts of Kufri

Chipsona-1. **Daniel *et al.* (2008)** reviewed the synergistic effect of temperature and enzymes on the division of the cell wall. They claimed that with the rise in temperature enzymatic activity increases and eases the cell division.

Table 4.2.2.2: The effect of temperature and enzyme combination interaction on the protoplast density of Kufri Chipsona-1 (×10^5 cells/mL)

Treatment	**E1**	**E2**	**E3**	**Mean C**
C1	0.32	0.54	0.76	0.54^c
C2	1.48	1.02	2.26	1.59^b
C3	1.65	2.06	2.50	2.07^a
Mean E	1.15^b	1.21^b	1.84^a	
	C	**E**	**C × E**	
C.D. ($p \leq 0.05$)	0.07	0.07	0.01	
SE(m)±	0.02	0.02	0.04	

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C

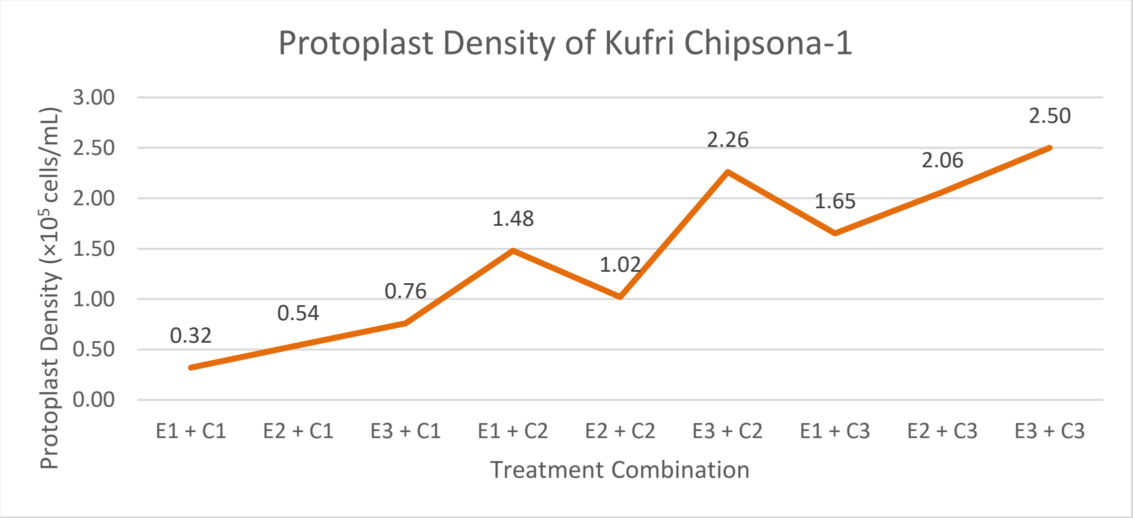


Fig. 4.2.2.2: The effect of temperature and enzyme combination interaction on the protoplast density of Kufri Chipsona-1 (×10^5cells/mL)

4.2.3 Size of the Protoplasts

4.2.3.1 Size of the protoplasts of Kufri Uday

The mean size of the protoplasts of Kufri Uday influenced by temperature was observed bigger in treatment C3 (27°C) with 20.47 µm followed by C2 (25°C) with

13.11 µm and smaller was observed in treatment C1 (22°C) with 7.11 µm and significant differences were observed with other treatments **(Table 4.2.3.1, Fig. 4.2.3.1 and Plate 4.4 & 4.5)**.

The temperature had impacted on the size of the protoplasts of the potato variety. The moderate temperature catalyses the digestion of cell wall which results in the successful isolation of intact protoplasts without giving stress to the protoplasts cells which might help in maintaining the size of the protoplasts they are usually larger in size. However, lower temperatures might lead to unsuccessful isolation and usually give stress to the protoplasts which could make them small and usually find shrinking of cells. It has also been noted that extreme high temperatures also rupture the cells of the protoplasts. **Chen *et al.* (2008)** in their research have found that there is variation in size of the protoplasts in *Solanum* species.

Similarly, the effect of different enzymes has also been observed when the maximum size of protoplasts was observed in treatment E3 (0.5% macerozyme + 1% cellulase) 16.13 µm followed by E2 (0.4% macerozyme + 2% cellulase) 13.15 µm and minimum size was found in E1 (0.25% macerozyme + 1.5% cellulase) 11.41 µm and there was no statistical difference was observed among treatments **(Table 4.2.3.1 and Fig. 4.2.3.1)**.

The variations in the size of the protoplasts of Kufri Uday due to the change in the concentration of enzymes. High and low concentrations affect the size of the protoplasts. Sometimes they may die or break, which directly affects the size of the protoplasts.

The synergistic effects of temperature and enzymes on size of protoplasts of Kufri Uday showed that the larger size was observed in E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C) 23.33 µm followed by E2 + C3 (0.4% macerozyme + 2% cellulase at 27°C) 19.73 µm with and lowest size was observed in the treatment E1 + C1(0.25% macerozyme + 1.5% cellulase at 22°C) with 5.20 µm and significant difference was observed among the treatments **(Table 4.2.3.1 and Fig. 4.2.3.1)**.

The results showed that at high temperatures promote the enzymes to isolation of the protoplasts without any rupturing or damaging them while the lower temperature with suitable enzyme concentration cannot promote the isolation as they stop working. Enzymes alone cannot work properly if they won't get suitable environment to isolate, thus the size of the protoplast decreases, or they break down or start shrinking while the isolation process.

Table 4.2.3.1: The effect of temperature and enzyme combination interaction on the protoplast size of Kufri Uday (µm)

Treatment	**E1**	**E2**	**E3**	**Mean C**
C1	5.20	6.63	9.50	7.11^{c}
C2	10.66	13.10	15.56	13.11^{b}
C3	18.36	19.73	23.33	20.47^{a}
Mean E	11.41^{c}	13.15^{b}	16.13^{a}	

	C	**E**	**C × E**
C.D. ($p \le 0.05$)	0.349	0.349	0.604
SE(m)±	0.117	0.117	0.202

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C

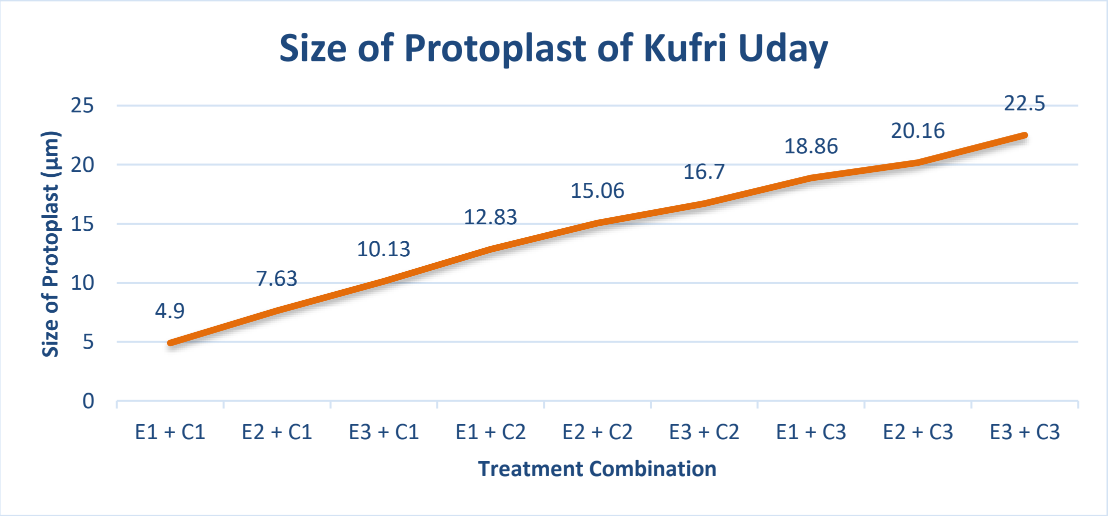


Fig. 4.2.3.1: The effect of temperature and enzyme combination interaction on the protoplast size of Kufri Uday (µm)

4.2.3.2 Size of the protoplasts of Kufri Chipsona-1

The effect of temperature had also been observed on the size of the protoplasts of Kufri Chipsona-1. The results showed that the maximum size was observed in treatment C3 (27°C) with 20.51 µm followed by C2 (25°C) 14.86 µm and minimum size was observed in C1 (22°C) 7.55 µm and significant difference was observed in all treatments **(Table 4.2.3.2, Fig. 4.2.3.2 and Plate 4.6 & 4.7)**.

The temperature is essential for getting the intact protoplast through the isolation. According to our research, the optimum temperature for the size of protoplasts is 25°C and 27°C. At these temperatures the size is larger while lowering the temperature *i.e.* 22°C the size of protoplasts was smaller. This might occur, at high temperatures the digestion rate increases which isolate the protoplasts faster and come out from the middle lamella of the leaves in proper size, while at lower temperature sometimes protoplasts went in shock due to this reason their size becomes smaller and start to shrink.

The size had also been affected by different enzyme concentrations, and it was clearly shown in **Table 4.2.3.2 and Fig. 4.2.3.2**. The larger size protoplasts were observed under the treatment E3 (0.5% macerozyme + 1% cellulase) with 16.44 µm followed by E2 (0.4% macerozyme + 2% cellulase) with 14.28 µm and smaller size protoplasts were observed in E1 (0.25% macerozyme + 1.5% cellulase) with 12.20 µm and statistical variations were observed in these treatments.

According to the results observed, the size of the protoplasts was affected by the different enzymes concentrations. Suitable concentrations of enzymes enhance the digestion without damaging the cells which often resulting the larger size protoplasts. However, the high concentration of the enzymes and unsuitable enzymes can damage the cells results small or broken cells will observe.

The interaction between the temperature and the enzyme was also showed the variations as maximum size was observed in treatment E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C) 22.50 µm followed by E2 + C3 (0.4% macerozyme + 2% cellulase at 27°C) 20.16 µm with and lowest density was observed in the treatment E1

+ C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 4.90 µm and significant difference was observed among the treatments **(Table 4.2.3.1 and Fig. 4.2.3.1)**.

The result showed that the both temperature and enzyme concentration affects the size of the protoplasts of Kufri Chipsona-1. As at high temperature and 0.5% macerozyme+1% cellulase concentration the large sizes were observed. The enzymatic activity only occurs if the enzymes get the suitable temperature for the isolation of protoplasts and because of this the size of the protoplasts was maintained as they released properly from the middle lamella of leaves of the Kufri Chipsona-1.

Table 4.2.3.2: The effect of temperature and enzyme combination interaction on the protoplast size of Kufri Chipsona-1 (µm)

Treatment	**E1**	**E2**	**E3**	**Mean C**
C1	4.90	7.63	10.13	7.55^{c}
C2	12.83	15.06	16.70	14.86^{b}
C3	18.86	20.16	22.50	20.51^{a}
Mean E	12.20^{c}	14.28^{b}	16.44^{a}	
	C	**E**	**C × E**	
C.D. ($p \leq 0.05$)	0.41	0.41	0.71	
SE(m)±	0.137	0.137	0.237	

*E- Enzyme Combination; C- Temperature; E1- 0.25% macerozyme + 1.5% cellulase; E2- 0.4% macerozyme + 2% cellulase; E3- 0.5% macerozyme + 1% cellulase; C1- 22°C; C2- 25°C; C3- 27°C

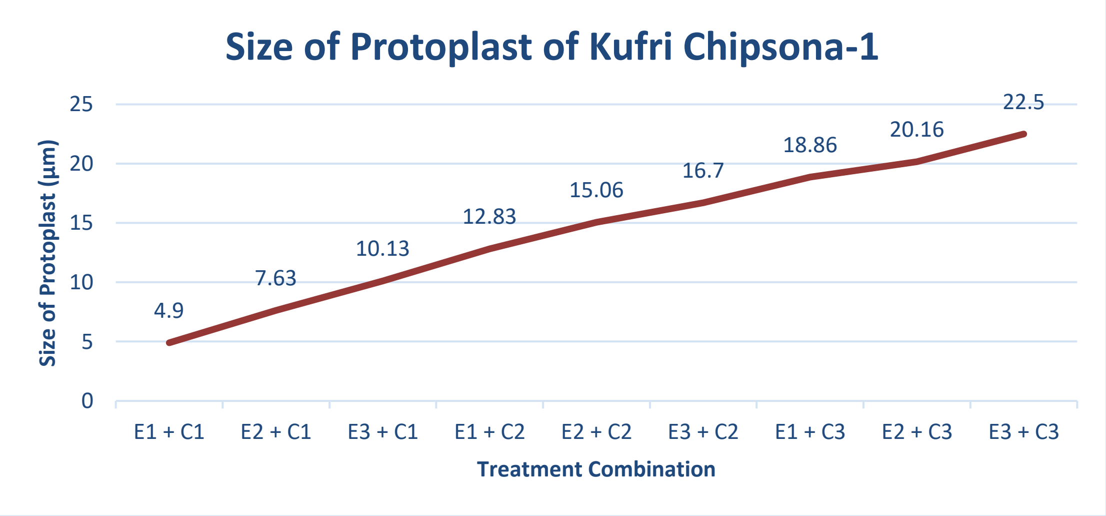


Fig. 4.2.3.2: The effect of temperature and enzyme combination interaction on the protoplast size of Kufri Chipsona-1 (µm)

4.3 To determine the regeneration of plantlets *in-vitro* from fusion products

4.3.1 Formation of calli

The calli was observed in the treatment T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) and T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) after twelve weeks of culturing in the PCM media, whereas the microcolonies were started to form after the 6 weeks of the culturing in T5 (0.4% Macerozyme + 2.0% Cellulase at 25°C), T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C), T7 (0.25% Macerozyme + 1.5% Cellulase at 27°C), T8 (0.4% Macerozyme + 2.0% Cellulase at 27°C) and T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) **(Table 4.3.1 and Plate 4.8 (a,b))**.

The formed calli was fragile in nature and exhibited the lower formation rate. The callus was fragile might be due to weak cell wall formation or incomplete formation which might sometimes lead to the soft and friable calli. The protoplasts have capability to regenerate the cell wall after the fusion and it also depends on the density of the protoplasts. It was observed that the density of the protoplasts of both the varieties was highest in T_6 and T_9 treatments in which the calli was formed. As the density increased the chances of forming the calli increased because higher density allows the cell division and cell signalling which is not possible with low density of protoplasts. The protoplasts cells were active in the PCM media and formed micro colonies in most of the treatments, but they might be unable to form the calli. Microcolony formation indicated that the cell division took place however unable to form the mass of callus. Microcolonies formation also indicates that the mitotic division occurred, however for the callus formation cells need the dedifferentiation and ability to form the undifferentiated mass *i.e.,* callus. **Nakano *et al.* (2003)** observed that if the density is low then the growth of the colony is also low which later impact on the formation of calli. **Cheng and Veilleux (1991)** also observed that some cells were viable in the media and formed the cell wall but did not divide. **Bajaj and Dionne (1967)** also observed the cell division in the form of milky white appearance on the surface of the culture media which later divide and formed the friable calli. **Feher *et al.* (1989)** observed no colony formation after the protoplasts' fusion.

Table 4.3.1: Formation of calli in different treatment combinations

Treatments	Treatment Combination	Formation of Microcolony	Formation of calli
T1	0.25% Macerozyme + 1.5% Cellulase at 22°C	-	-
T2	0.4% Macerozyme + 2.0% Cellulase at 22°C	-	-
T3	0.5% Macerozyme + 1.0% Cellulase at 22°C	-	-
T4	0.25% Macerozyme + 1.5% Cellulase at 25°C	-	-
T5	0.4% Macerozyme + 2.0% Cellulase at 25°C	+	-
T6	0.5% Macerozyme + 1.0% Cellulase at 25°C	+	+
T7	0.25% Macerozyme + 1.5% Cellulase at 27°C	+	-
T8	0.4% Macerozyme + 2.0% Cellulase at 27°C	+	-
T9	0.5% Macerozyme + 1.0% Cellulase at 27°C	+	+

* + = Calli form, - = calli not form

4.3.2 Growth of calli

The calli was suspended in the liquid medium was fragile and collected on the filter paper then weighed them and found T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) was 0.89 g and T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) was 0.77g **(Table 4.3.2)**.

The calli grow after the division of the cells. The calli growth was highest in treatment T9 represents that more viable protoplasts fused in this treatment and formed undifferentiated fragile masses. The nutrients present in the PCM media as well as the plant growth hormones helped the calli to grow. Although the culture media was same in all treatments, but the growth was observed in two treatments and differences in growth was also observed this might be due to the fused cell capability to uptake the nutrients and the internal environment of the culture vessels. **Nakano *et al.* (2003)** also observed the growth of callus after the protoplast fusion of ornamental plant *Agapanthus praecox* in millimeters. **Chen *et al.* (2008)** also analysed the diameter of the protoplast fused callus of the potato species.

Table 4.3.2: Growth of calli in different treatments

Treatments	Treatment Combination	Growth of calli (g)
T1	0.25% Macerozyme + 1.5% Cellulase at 22°C	-
T2	0.4% Macerozyme + 2.0% Cellulase at 22°C	-
T3	0.5% Macerozyme + 1.0% Cellulase at 22°C	-
T4	0.25% Macerozyme + 1.5% Cellulase at 25°C	-
T5	0.4% Macerozyme + 2.0% Cellulase at 25°C	-
T6	0.5% Macerozyme + 1.0% Cellulase at 25°C	0.77
T7	0.25% Macerozyme + 1.5% Cellulase at 27°C	-
T8	0.4% Macerozyme + 2.0% Cellulase at 27°C	-
T9	0.5% Macerozyme + 1.0% Cellulase at 27°C	0.89

4.3.3 Colour of calli

The colour of the calli was observed in the treatments and found that in treatment T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) and T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) both had the light brown coloured calli **(Table 4.3.3)**.

The light brown colour of the calli might be due to capability of the cells to absorb sugars present in the culture media. Colour of the calli may also be varied from species to species of the plant. It could be due to the oxidation of the phenolic compounds. **Aisyah *et al.* (2022)** observed the brown colored callus due to the sucrose concentration available in the protoplasts culture media. **Nakano *et al.* (2003)** observed the creamy-white colored callus of *Agapanthus praecox*.

Table 4.3.3: Colour of calli in different treatments

Treatments	**Treatment Combination**	**Colour of calli**
T1	0.25% Macerozyme + 1.5% Cellulase at 22°C	-
T2	0.4% Macerozyme + 2.0% Cellulase at 22°C	-
T3	0.5% Macerozyme + 1.0% Cellulase at 22°C	-
T4	0.25% Macerozyme + 1.5% Cellulase at 25°C	-
T5	0.4% Macerozyme + 2.0% Cellulase at 25°C	-
T6	0.5% Macerozyme + 1.0% Cellulase at 25°C	Light brown
T7	0.25% Macerozyme + 1.5% Cellulase at 27°C	-
T8	0.4% Macerozyme + 2.0% Cellulase at 27°C	-
T9	0.5% Macerozyme + 1.0% Cellulase at 27°C	Light brown

4.3.4 Number of regenerated calli

The sub-cultured calli did not grow in any of the treatments. In treatment T_1 [Zeatin (0 mg/L) + 2,4-D (0 mg/L)], the calli became dark brown in color by day 20 after sub-culturing. The calli, in treatment T_2 [Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)], were initially healthy but later deteriorated by the 31st day. Treatment T_3 [Zeatin (1 mg/L) + 2,4-D (3 mg/L)] also indicated signs of browning with time, and the health of the calli kept on deteriorating despite best efforts to sustain it. In treatment T_4 [Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)], the calli lost their vitality, with increasing browning and ultimate death after 38 days of sub-culturing. **(Table 4.3.4 and Plate 4.8 (a,b))**.

Many studies have reported inability to regenerate calli which may be due to the combinations of growth hormones that do not affect the regeneration of calli or it might be due the genetic incompatibility or instability **(Feher *et al.,* 1989)**. It could also be possible that sometimes when the calli are transferred from liquid to solid media, it might go to shock and start to brown. The fungal infection had occurred due to contamination. **Chen *et al.* (2008)** reported the growth rate of regeneration of calli was very low in potato species.

Table 4.3.4: Regeneration of calli after the formation of callus

Treatments	Treatment Combination	Regenerated calli
T1	Zeatin (0 mg/L) + 2,4-D (0 mg/L)	0
T2	Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)	0
T3	Zeatin (1 mg/L) + 2,4-D (3 mg/L)	0
T4	Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)	0

4.3.5 Number of plantlets

The plantlets were not formed because calli was not further grew after sub culturing on the solid media containing different growth hormone concentrations **(Table 4.3.5)**.

Guan *et al.* (2010) reported no shoot formation in some treatments by NAA and BA as growth hormones in ginger. **Radke and Grun (1986)** observed no shoot formation in some *Solanum tuberosum* varieties. **Chen *et al.* (2008)** also stated that the shoot formation was none or less when the callus of potato species was kept on the solid media.

Table 4.3.5: Observation of number of plantlets from the regenerated calli

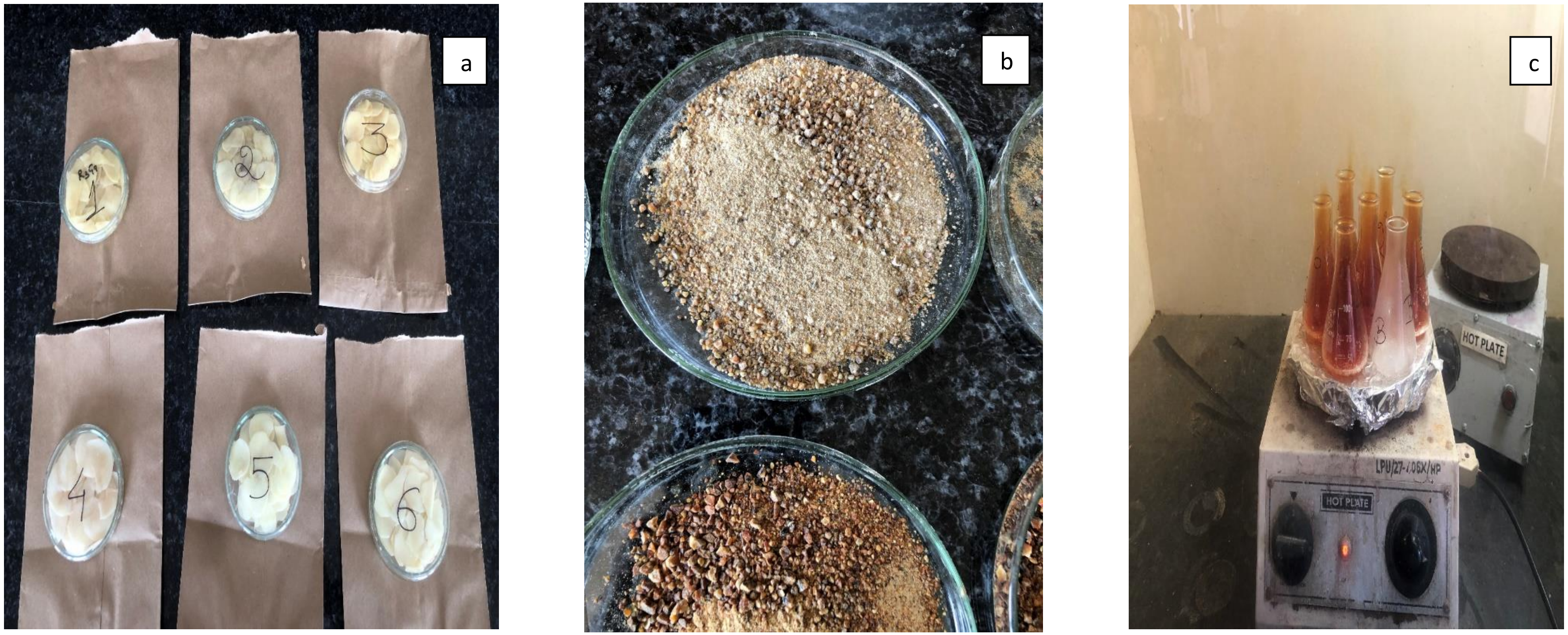
Treatments	Treatment Combination	Number of plantlets
T1	Zeatin (0 mg/L) + 2,4-D (0 mg/L)	0
T2	Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)	0
T3	Zeatin (1 mg/L) + 2,4-D (3 mg/L)	0
T4	Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)	0

4.3.5 Fusion product identification

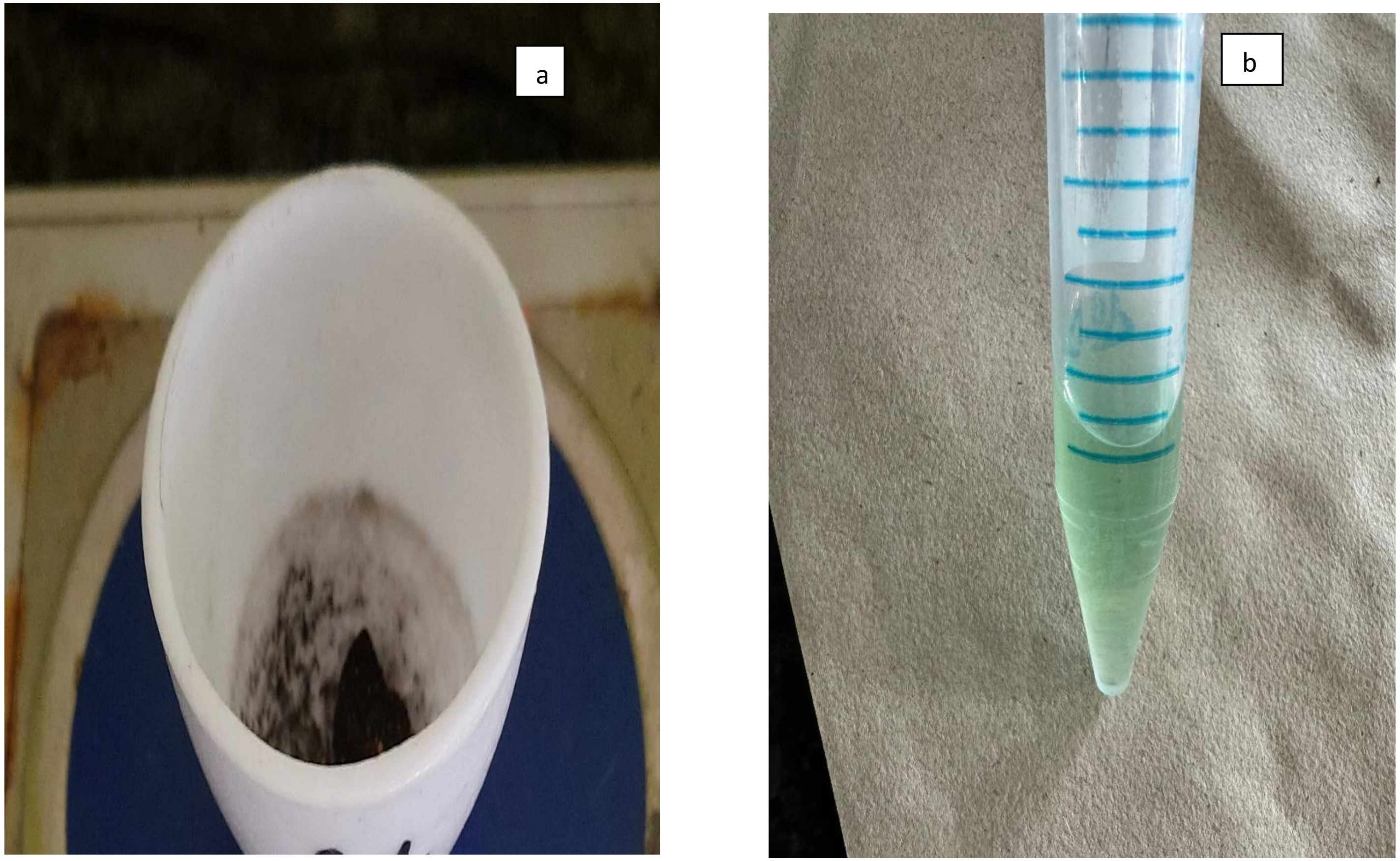
As the research did not reach up to the stage of development of plantlets, which is why the fusion product did not identify. The fusion product is only identified with the help of the leaves of the plantlets and examined through the flow cytometry technique.

P 4.1: (a) Specific gravity of potato; (b) Total soluble solids of potato; (c) Skin and flesh of tuber

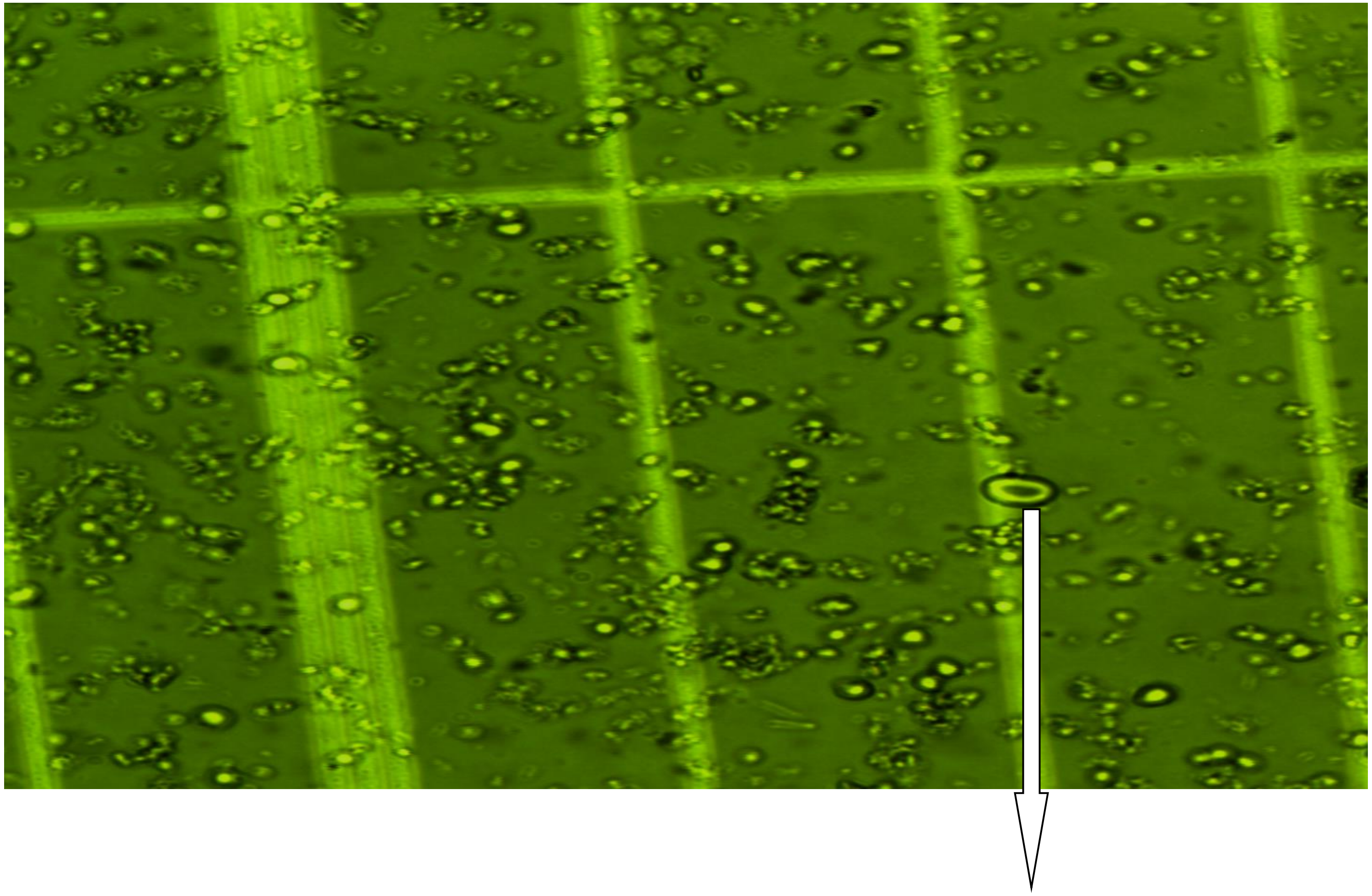
P 4.2: (a) Moisture Content of tubers; (b) Dry matter content of tubers; (c) Mineral content of tubers



P 4.3: (a) Ash content of tubers; (b) Anthrone reagent method used for the analysis of starch content of tubers

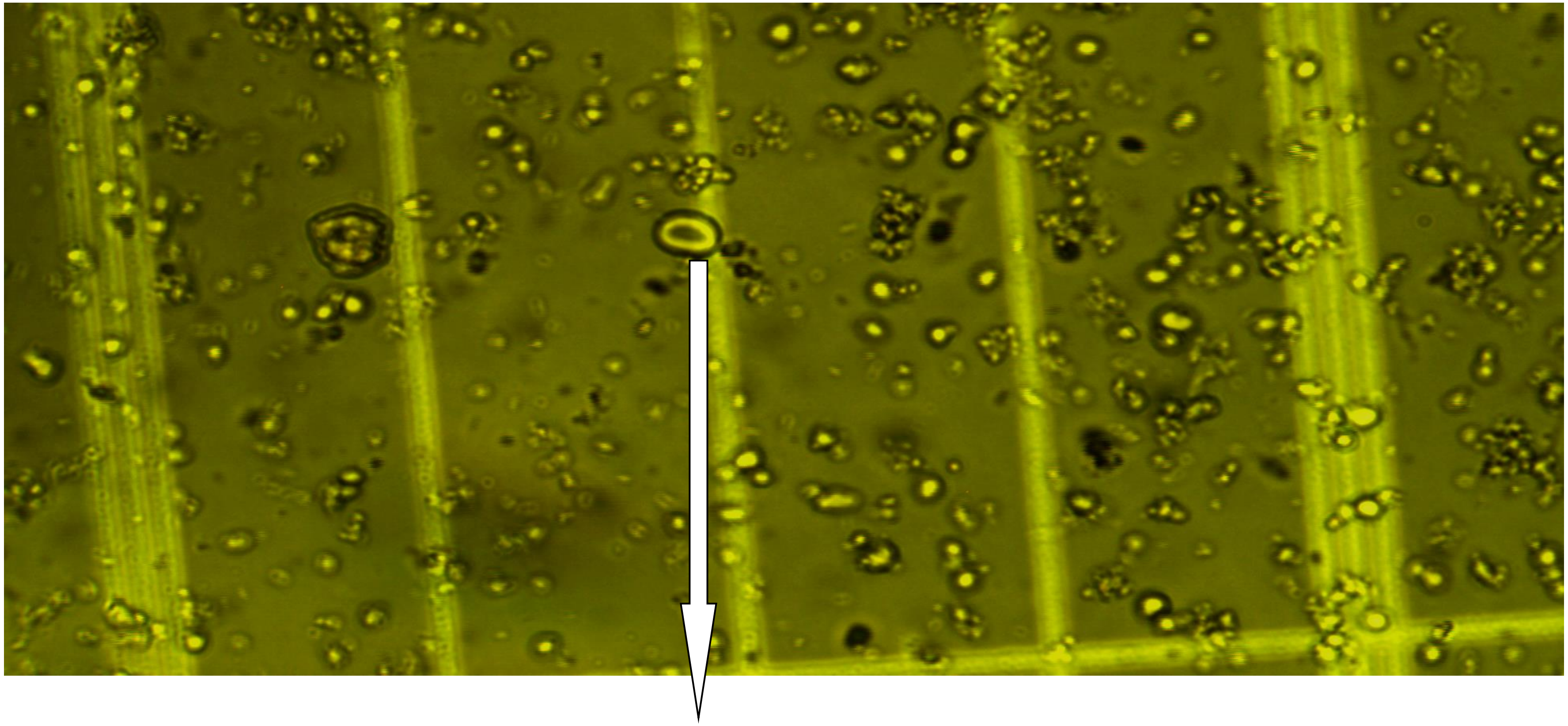


P 4.4: Size of protoplast of Kufri Uday in Treatment T1[E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C)]



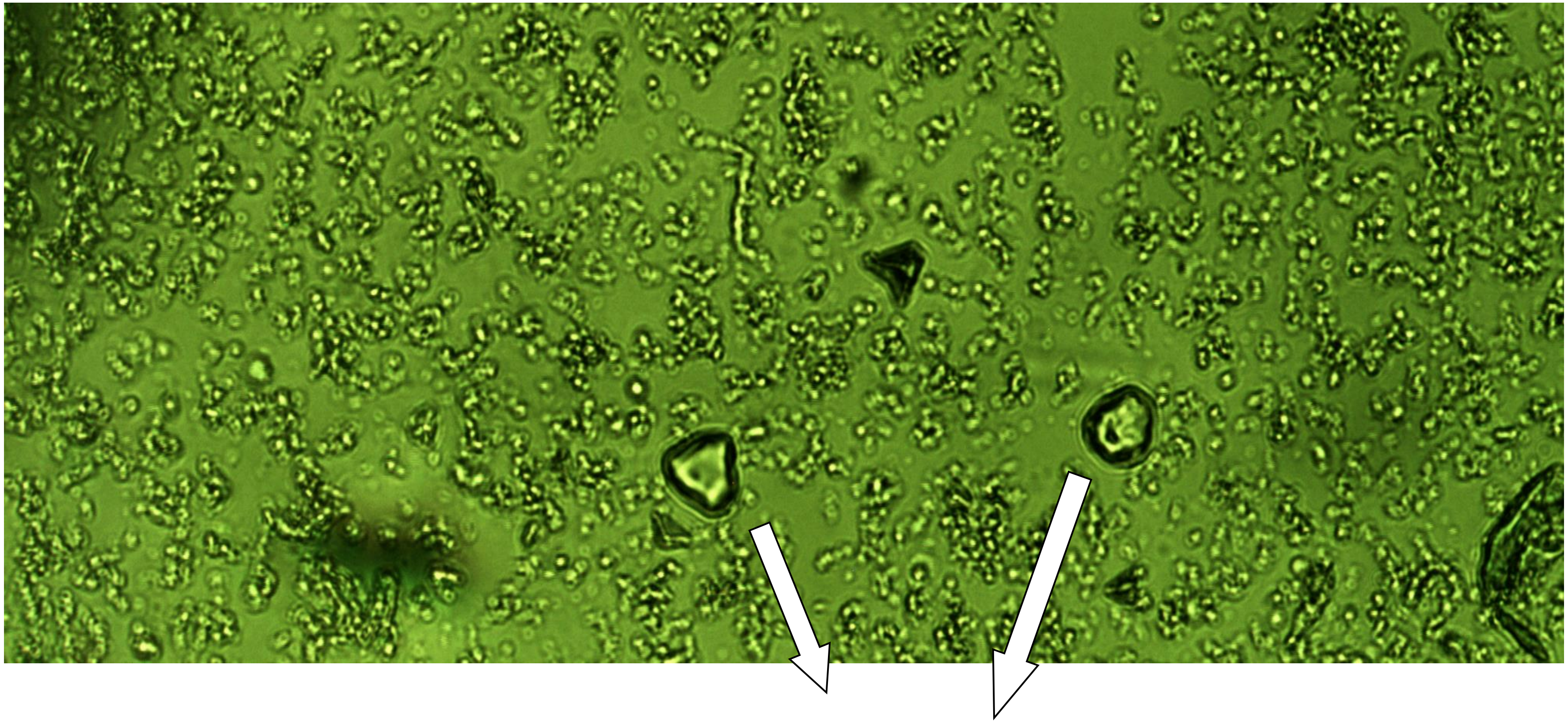
P 4.5: Size of protoplast of Kufri Uday in Treatment T9 [E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C)]

P 4.6: Size of Kufri Chipsona-1 in Treatment T1 [E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C)]



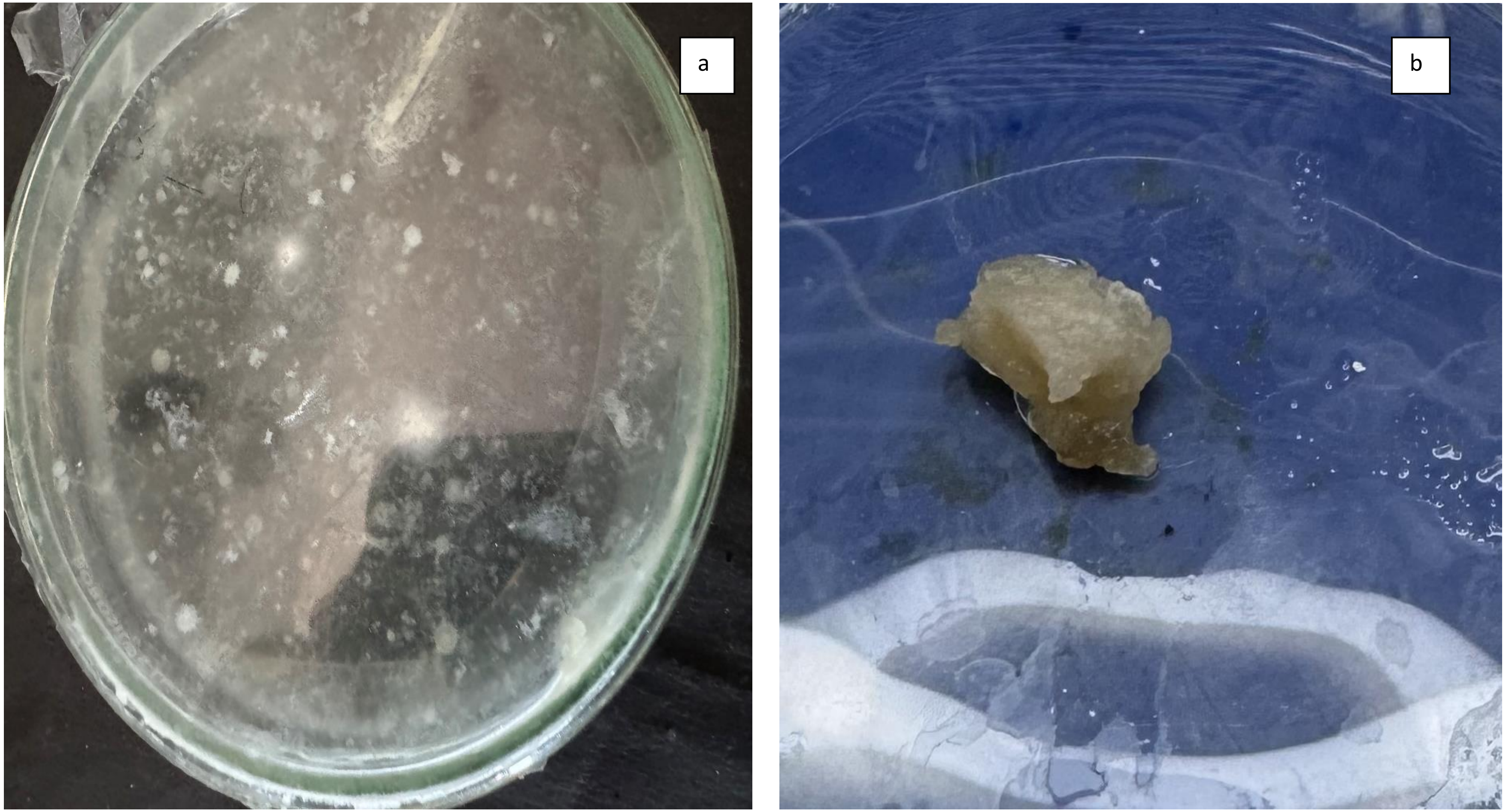
Protoplast Cell

P 4.7: Size of Kufri Chipsona-1 in Treatment T9 [E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C)]

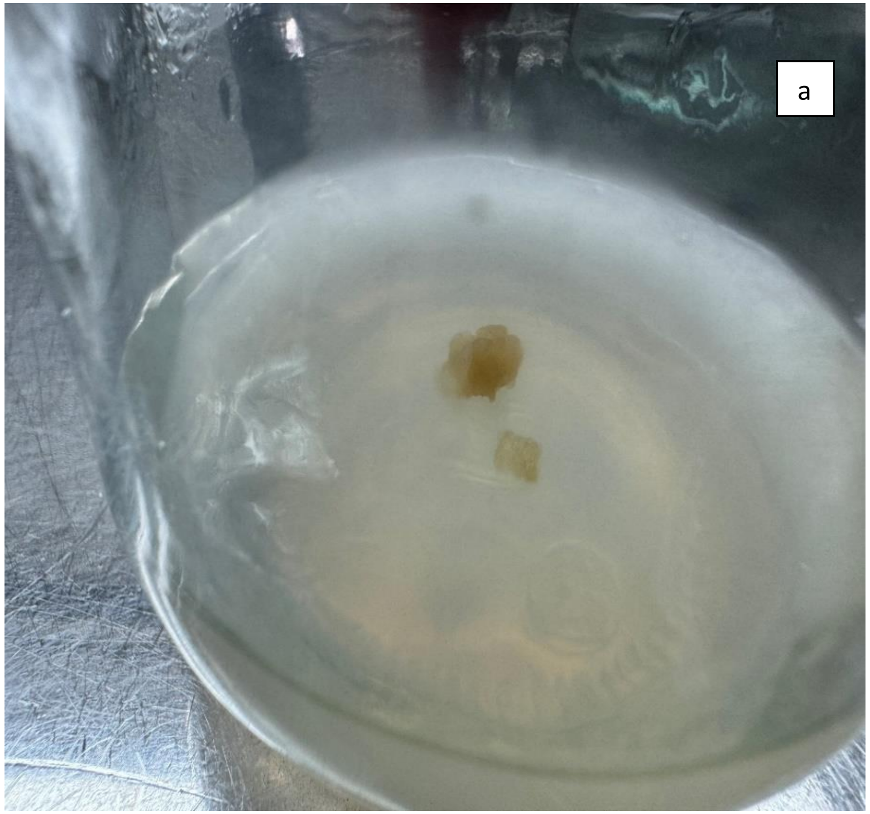


Protoplast Cells

P 4.8: (a) Micro calli formation after fusion; (b) Calli formed



P 4.9: (a) Browning of callus



Chapter-V

SUMMARY AND CONCLUSION

The proposed research work entitled **"Nutritional Profiling and Development of Iron Rich Potato through Protoplast Fusion"** was carried out in the Tissue Culture Lab as well as in the field at Lovely Professional University, Phagwara, Punjab in 2023-2024 and 2024-2025.

The summary of findings of the current study are outlined as follows:

5.1 To identify the high iron content germplasm of potato

- The highest iron content among the potato varieties was discovered in Kufri Uday (55.20 ± 0.88 mg/kg) and the lowest in the variety Kufri Chipsona-1 (21.73 mg/kg).
- The zinc content was greatest in variety Kufri Uday (42.12 ± 0.30 mg/kg) and lowest zinc was found in line 302 (21.38 ± 0.51 mg/kg).
- The boron content was found to be greatest in Kufri Lima (29.80 ± 0.56 mg/kg) and Kufri Pukhraj (24.85 ± 0.80 mg/kg) whereas, the lowest in Kufri Uday (17.06 ± 0.79 mg/kg).
- According to the statistical analysis, Kufri Uday exhibited the highest copper concentration at 34.00 ± 0.12 mg/kg, and lowest copper content was observed in Kufri Surya 3.71 ± 0.02 mg/kg.
- The calcium content was significantly higher in the Kufri Chandramukhi, with a concentration of 1084 ± 1.0 mg/kg, whereas Kufri Surya variety had the lowest calcium content (393 ± 2.0 mg/kg).
- The manganese content was observed maximum in variety Kufri Chandramukhi (26.48 ± 0.90 mg/kg) and the minimum content was in variety Kufri Lima (6.67 ± 0.33 mg/kg).
- The magnesium content was found maximum in the potato variety Kufri Lalima (1111.66 ± 1.52 mg/kg) and the minimum was found in variety Diamond (557.33 ± 4.16 mg/kg).

- Potassium is recorded highest in Kufri Khyati (1048 ± 2.00 mg/kg) and lowest was observed in Kufri Lima (512.33 ± 2.08 mg/kg).
- Phosphorus content was highest in Kufri Chandramukhi (586.66 ± 1.52 mg/kg) while 5758 (169 ± 3.00 mg/kg) had the lowest levels of phosphorus.
- The concentration of sulphur was greatest in Kufri Jyoti (1494.33 ± 2.51 mg/kg) and minimum in Kufri Lima (1074.66 ± 3.05 mg/kg).
- The starch content was found in variety Kufri Himsona (19.71%) had the highest in peeled tubers and Kufri Lalima (10.98%) had lowest starch content.
- The colour of the skin of the tuber varieties were found from red to yellow. The red colour varieties were Kufri Lalima, Kufri Uday; white colour varieties were Kufri Jyoti, Diamond, Kufri Chandramukhi, Kufri Mohan, Kufri Chipsona-1, while white- cream coloured varieties were Kufri Lima, Kufri Surya, Kufri Khyati, Kufri Himsona, 302, 3797, and the yellow colour of skin was observed in Kufri Pukhraj and 5758. Similarly, the flesh colour of various potato varieties ranged from white to yellow. It is found that the Kufri Lalima, Kufri Mohan and 302 have white flesh colour while white-cream coloured was observed in Kufri Khyati and Kufri Chipsona-1; varieties with cream flesh were Kufri Surya, Kufri Jyoti, Kufri Himsona, 3797 and yellow flesh-coloured varieties were Kufri Uday, Kufri Pukhraj and 5758.
- The dry matter was highest in Kufri Chandramukhi (26%), whereas line 3797 (11.58%) was found to have the lowest dry matter.
- The moisture content was found minimum was in Kufri Chandramukhi (74%), whereas maximum moisture content was observed in 3797 (88.41%).
- Specific gravity was found maximum in Kufri Chipsona-1 (1.097), whereas minimum in Kufri Mohan (1.025).
- The highest ash content was found in Kufri Chandramukhi (2.95%) and the lowest was recorded in Kufri Lalima (0.66%).
- The highest TSS observed in Kufri Himsona which was 7.01°Brix and the lowest was observed in Diamond with 5.32°Brix.

5.2 To determine the isolation and fusion of protoplast

- The mean viability of the protoplast was higher when the enzymatic solution containing *in-vitro* leaves kept at 27°C in treatment C3 (81.70%) and lowest was observed at 22°C in treatment C1 (34.45%).
- Similarly, enzyme combinations had significant effect on the protoplast viability of Kufri Uday. The mean viability of Kufri Uday was highest in E3 (73.30%) where the enzyme combinations were 0.5% macerozyme + 1% cellulase and lowest was observed in E1 [0.25% macerozyme + 1.5% cellulase (48.18%)].
- The interaction between the enzyme combinations and temperature showed maximum viability in E3+C3 (0.5% macerozyme + 1% cellulase at 27°C) with 94.53% and least viability was observed in E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 26.75%.
- The viability of the Kufri Chipsona-1 was significantly affected by the temperature. The maximum viability was observed in C3 (83.73%) at 27°C and minimum was observed in C1 (37.99%) at 22°C.
- As due to the enzyme combinations, the fluctuations in the viability of Kufri Chipsona-1 had been observed. It showed that the E3 (0.5% macerozyme + 1% cellulase) had the highest viability 78.74%, which was found highest over the other enzyme combinations. However, the lowest viability was found in enzyme combination E1 (0.25% macerozyme + 1.5% cellulase) with 55.17% of viability.
- The interaction between the temperature and enzyme combinations showed that the highest viability was observed in E3+C3 (0.5% macerozyme + 1% cellulase at 27°C) with 94.96%. The least viability was observed in E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 24.88%.
- The density of Kufri Uday was greatly influenced by increasing or decreasing the temperature. The highest protoplast density was observed in C3 (27°C) with 2.03×10^5 cells/mL which was, and the lowest protoplast density was found in the treatment C1 (22°C) with 0.48×10^5 cells/mL.

- Similarly, the enzyme combinations also play a role in protoplast density. The density was observed highest with treatment E3 (0.5% macerozyme + 1% cellulase) with 1.77 × 10^5 cells/mL and the treatment with low density was observed in E1 (0.25% macerozyme + 1.5% cellulase) with 0.89 × 10^5 cells/mL.
- The interaction between the enzyme combination and temperature affected the density of the protoplasts of variety Kufri Uday. According to the findings, the highest density of protoplasts was observed in treatment E3+C3 (0.5 % macerozyme + 1% cellulase at 27°C) with 2.53 ×10^5 cells/mL and lowest protoplast density was found in treatment E1+C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 0.34 ×10^5 cells/mL.
- The density of the protoplast of Kufri Chipsona-1 was highly affected by the temperature as shown in results that the maximum density was observed in treatment C3 (2.07 ×10^5cells/mL) at 27°C which was maximum amongst all the treatments and the lowest was observed in C1 0.54 ×10^5 cells/mL at 22°C.
- Furthermore, it has also been noticed that the density of potato variety Kufri Chipsona-1 was also affected by changing the concentration of enzymes. That's why the highest density was recorded in E3 (0.5 % macerozyme + 1% cellulase) with 1.84 ×10^5cells/mL and lowest in E1 (0.25% macerozyme + 1.5% cellulase) with 1.15 ×10^5 cells/mL.
- The interaction of enzymes and temperature showed that the treatment E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C) showed the highest density of 2.50 ×10^5cells/mL and lowest density was observed in the treatment E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 0.32 ×10^5 cells/mL.
- The mean size of the protoplasts of Kufri Uday influenced by temperature was observed bigger in treatment C3 (27°C) with 20.47 µm and smaller was observed in treatment C1 (22°C) with 7.11 µm.
- Similarly, the effect of different enzymes has also been observed when the maximum size of protoplasts was observed in treatment E3 (0.5 % macerozyme + 1 % cellulase) 16.13 µm and minimum size was found in E1 (0.25% macerozyme + 1.5% cellulase) 11.41 µm.

- The synergistic effects of temperature and enzymes on size of protoplasts of Kufri Uday showed that the larger size was observed in E3 + C3 (0.5 % macerozyme + 1% cellulase at 27°C) 23.33 µm lowest size was observed in the treatment E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 5.20 µm.
- The effect of temperature had also been observed on the size of the protoplasts of Kufri Chipsona-1. The results showed that the maximum size was observed in treatment C3 (27°C) with 20.51 µm and minimum size was observed in C1 (22°C) 7.55 µm and no significant difference was observed in all treatments.
- The larger size protoplasts were observed under the treatment E3 (0.5 % macerozyme + 1 % cellulase) with 16.44 µm and smaller size protoplasts were observed in E1 (0.25% macerozyme + 1.5% cellulase) with 12.20 µm.
- The interaction between the temperature and the enzyme was also showed the variations as maximum size was observed in treatment E3 + C3 (0.5 % macerozyme + 1% cellulase at 27°C) 22.50 µm and lowest density was observed in the treatment E1 + C1 (0.25% macerozyme + 1.5% cellulase at 22°C) with 4.90 µm.

5.3 To determine the regeneration of plantlets *in-vitro* from fusion products

- The calli was observed in the treatment T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) and T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) after twelve weeks of culturing in the PCM media, whereas the microcolonies started to form after the 6 weeks of the culturing.
- The calli was suspended on the liquid medium was fragile and collected on the filter paper then weighed them and found T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) was 0.89 g and T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) was 0.77g.
- The colour of the calli was observed in the treatments and found that in treatment T6 (0.5% Macerozyme + 1.0% Cellulase at 25°C) and T9 (0.5% Macerozyme + 1.0% Cellulase at 27°C) both had the light brown coloured calli.

- The sub-cultured calli did not grow in any of the treatments. In treatment T_1 [Zeatin (0 mg/L) + 2,4-D (0 mg/L)], the calli became dark brown in color by day 20 after sub-culturing. The calli, in treatment T_2 [Zeatin (0.5 mg/L) + 2,4-D (2 mg/L)], were initially healthy but later deteriorated by the 31st day. Treatment T_3 [Zeatin (1 mg/L) + 2,4-D (3 mg/L)] also indicated signs of browning with time, and the health of the calli kept on deteriorating despite best efforts to sustain it. In treatment T_4 [Zeatin (1.5 mg/L) + 2,4-D (4 mg/L)], the calli lost their vitality, with increasing browning and ultimate death after 38 days of sub-culturing. The plantlets were not formed because calli was not further grew after sub culturing on the solid media containing different growth hormone concentrations.
- As the research did not reach up to the stage of development of plantlets, which is why the fusion product was not identified.

Conclusion

From the first experiment we can conclude that Kufri Uday had the highest levels of iron, zinc, and copper and a significant amount of magnesium, potassium and sulphur. On the other hand, Kufri Chandramukhi had higher levels of calcium, manganese, potassium and phosphorus. But Kufri Jyoti revealed a greater sulphur content compared to the other varieties. Conversely, Kufri Chipsona-1, Kufri Himsona, and Kufri Chandramukhi were the top processing varieties because they had the greatest levels of dry matter content, starch content, and specific gravity.

As the Kufri Uday had the highest iron content and Kufri Chipsona-1 had the lowest that's why they were selected for the second experiment. In the experiment, highest protoplast viability, density and size of protoplasts was observed in treatment E3 + C3 (0.5% macerozyme + 1% cellulase at 27°C). The chemical fusion was done using PEG 6000 in all the treatments and observed that the calli was formed in the treatments T_6 (0.5% macerozyme + 1% cellulase at 25°C) and T_9 (0.5% macerozyme + 1% cellulase at 27°C) and were light brown in colour. However, after the subculturing of the calli on the solid media, they could not able to survive as browning and the fungal infection took place. For that reason, the plantlets were not able to form.

From this investigation, we can conclude that by changing the temperature and concentrations of the enzymes the variation in the viability and density took place, as they played an important role in the isolation of protoplasts that helps to increase the viability, density as well as the size of the protoplast. That's why the proper combination of enzymes is important and the fluctuations in the viability, density and size of the protoplasts were also taken by increasing or decreasing the incubating temperature, increase in temperature helps in isolating the protoplasts. Temperature is important for dissolving the cell wall of leaves. Both factors are crucial for the isolation of protoplasts. Furthermore, we also observed that the high density of protoplasts increases the chances of protoplast fusion because higher density allows the cell division and cell signalling and this enzyme combination supports the density of the protoplasts when kept at 25°C or 27°C.

Potato offers adequate nutrition and energy for dietary intake, which makes it a staple food. It is one of the most affordable dietary sources of carbohydrates and provides an adequate quantity of vitamins as well as a small quantity of various minerals. This crop is consumed in every part of the world in different forms. To elevate the iron in the potato was the main initiative of this research. Traditional breeding methods take time to improve the cultivars of the potato. That's why biotechnological technique was used. Protoplast fusion increases the chances of improvement if the required material is available in bulk. Though the concentration of the enzymes and incubation temperature may vary from plant to plant and species to species. A potent biotechnology method for crop enhancement in potatoes is protoplast fusion, which combines favourable features from sexually incompatible species or cultivars. This method makes it possible to create new hybrid lines with better yield and quality attributes. Thus, 0.5 % macerozyme and 1% cellulase at 27°C is suitable for the isolation of protoplasts of potato cultivars Kufri Uday and Kufri Chipsona-1 and can be used isolation of protoplasts. A protocol has been developed for the isolation of protoplasts from the leaves of the potato plant.

Instead of that, there are future prospects for this research, efficient screening of potato germplasms for the analysis of mineral content should be focused on advanced technologies. Such technologies could accelerate the identification of mineral rich genotypes. Additionally, for the protoplast fusion, the required chemicals and materials are quite expensive, so it is important to standardize the protocols of isolation and fusion of the potato (Indian) cultivars by using different enzymes at variable concentrations. Also, different incubation temperatures, durations, light intensity, photoperiod, etc. could also be assessed. Furthermore, for the fusion and regeneration of plants, different fusogens, various culture media with/without growth hormones need to be evaluated.

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APPENDICES

Annexure- I

Weekly average temperature and relative humidity in weather in 2023

Average Temperature and Relative Humidity during the Experiment

Date	Max Temp (°C)	Min Temp (°C)	RH (%)	RH (%)	Windspeed (km/hr)	Rain (mm)	Evaporation (mm)	Sunshine (hrs)
September 20- September27	33	23	93	63.42	4	0	3	7
September28- October 05	34	19	93	50.99	6	0	2	9
October 06- October 13	33.00	18.56	92.04	52.31	4.46	0.90	2.53	8.64
October 14- October 21	29	15.92	92.01	52.83	5.13	0.53	2.85	8.45
October 22- October 29	30.31	13.95	92.71	47.54	5.00	0.13	2.84	8.25
October 30- November 06	30	13.30	93.39	45.66	3.92	0.00	1.95	6.01
November 07- November 14	26.11	12.26	92.55	56.85	5.67	0	1.71	6.19
November 15- November 22	27	10.95	93.26	46.69	5.00	0.03	1.81	7.48
November 23- December 02	24.2	10.0	92.1	55.6	4.4	0.7	1.4	4.1

Annexure- II

Annexure- II (a) ANOVA for Iron content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	4,047.307	289.093	898.882	< 0.001
Error	30	9.648	0.322		
Total	44	4,056.956			

Annexure- II (b) ANOVA for Zinc content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	4	35.537	8.884	44.400	< 0.001
Error	10	2.001	0.200		
Total	14	37.538			

Annexure- II (c) ANOVA for Boron content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	505.730	36.124	68.127	< 0.001
Error	30	15.907	0.530		
Total	44	521.637			

Annexure- II (d) ANOVA for Calcium content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	2,372,116.000	169,436.857	14,691.057	< 0.001
Error	30	346.000	11.533		
Total	44	2,372,462.000			

Annexure- II (e) ANOVA for Manganese content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	1,552.686	110.906	195.807	0.000
Error	30	16.992	0.566		
Total	44	1,569.679			

Annexure- II (f) ANOVA for Magnesium content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	1,197,912.133	85,565.152	4,611.296	< 0.001
Error	30	556.667	18.556		
Total	44	1,198,468.800			

Annexure- II (g) ANOVA for Potassium content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	947,404.533	67,671.752	69.677	< 0.001
Error	30	29,136.667	971.222		
Total	44	976,541.200			

Annexure- II (h) ANOVA for Phosphorus content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	640,698.578	45,764.184	3,343.163	< 0.001
Error	30	410.667	13.689		
Total	44	641,109.244			

Annexure- II (i) ANOVA for Sulphur content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	581,921.911	41,565.851	5,937.979	< 0.001
Error	30	210.000	7.000		
Total	44	582,131.911			

Annexure- II (j) ANOVA for Copper content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Treatment	14	14,377.118	1,026.937	292,431.046	0.001
Error	30	0.105	0.004		
Total	44	14,377.224			

Annexure- II (k) ANOVA for Starch matter

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	335.036	23.931	664.666	< 0.001
Error	30	1.080	0.036		
Total	44	336.116			

Annexure- II (l) ANOVA for Dry matter

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	876.908	62.636	21.227	< 0.001
Error	30	88.525	2.951		
Total	44	965.433			

Annexure- II (m) ANOVA for Specific gravity

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	0.019	0.001	10.375	< 0.001
Error	30	0.004	0.000		
Total	44	0.022			

Annexure- II (n) ANOVA for Ash content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	13.263	0.947	1,853.855	< 0.001
Error	30	0.015	0.001		
Total	44	13.278			

Annexure- II (o) ANOVA for Moisture content

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	876.893	62.635	21.225	< 0.001
Error	30	88.529	2.951		
Total	44	965.421			

Annexure- II (p) ANOVA for Total soluble solids

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Treatment	14	10.270	0.734	1,098.925	< 0.001
Error	30	0.020	0.001		
Total	44	10.290			

Annexure- III

Annexure- III (a.a) ANOVA for Viability of Kufri Uday

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	11,260.649	5,630.325	1,630.539	< 0.001
Factor B	2	3,073.874	1,536.937	445.096	< 0.001
Interaction A X B	4	778.480	194.620	56.362	< 0.001
Error	18	62.155	3.453		
Total	26	15,175.158			

Annexure- III (a.b) ANOVA for Viability of Kufri Chipsona-1

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	10,099.548	5,049.774	1,373.370	
Factor B	2	2,847.920	1,423.960	387.270	< 0.001
Interaction A X B	4	571.902	142.975	38.885	< 0.001
Error	18	66.185	3.677		
Total	26	13,585.554			

Annexure- III (b.a) ANOVA for Density of Protoplast Kufri Uday

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	113,963,851,851.852	56,981,925,925.926	2,185.386	< 0.001
Factor B	2	35,421,629,629.630	17,710,814,814.815	679.250	
interaction A X B	4	12,695,703,703.704	3,173,925,925.926	121.727	< 0.001
Error	18	469,333,333.333	26,074,074.074		
Total	26	162,550,518,518.518			

Annexure- III (b.b) ANOVA for Density of Protoplast Kufri Chipsona-1

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	110,578,962,962.963	55,289,481,481.481	1,036.678	< 0.001
Factor B	2	26,486,518,518.519	13,243,259,259.259	248.311	< 0.001
Interaction A X B	4	10,964,148,148.148	2,741,037,037.037	51.394	< 0.001
Error	18	960,000,000.000	53,333,333.333		
Total	26	148,989,629,629.630			

Annexure- III (c.a) ANOVA for Size of Kufri Uday

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	806.805	403.403	3,300.517	
Factor B	2	102.627	51.314	419.833	< 0.001
Interaction A X B	4	1.646	0.411	3.367	0.032
Error	18	2.200	0.122		
Total	26	913.279			

Annexure- III (c.b)ANOVA for Size of Kufri Chipsona-1

Source of Variation	DF	Sum of Squares	Mean Squares	F-Value	Significance
Factor A	2	759.476	379.738	2,248.473	
Factor B	2	81.076	40.538	240.030	< 0.001
Interaction A X B	4	2.975	0.744	4.404	0.012
Error	18	3.040	0.169		
Total	26	846.567			