GENERATION OF FROST TOLERANT POTATO (*Solanum tuberosum* L.) CLONES THROUGH INTERVARIETAL HYBRIDIZATION AND EXPRESSION ANALYSIS OF PUTATIVE FROST TOLERANCE GENES

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
HORTICULTURE (VEGETABLE SCIENCE)
(Minor Subject: Plant Breeding and Genetics)

By

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CERTIFICATE – I

This is to certify that the dissertation entitled "**Generation of frost tolerant potato (*Solanum tuberosum* L.) clones through intervarietal hybridization and expression analysis of putative frost tolerance genes**" submitted for the degree of Ph.D. of Science in the subject of **Horticulture (Vegetable Science)** (Minor subject: **Plant Breeding and Genetics)** of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Dechen Angmo (L-2016-A-43-D)** under my supervision and that no part of this dissertation has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that the dissertation entitled, **"Generation of frost tolerant potato (*Solanum tuberosum* L.) clones through intervarietal hybridization and expression analysis of putative frost tolerance genes"** submitted by **Dechen Angmo (L-2016-A-43-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of **Ph.D.** in the subject of **Horticulture (Vegetable Science) (**Minor subject: **Plant Breeding and Genetics)** has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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Date…… ***(Dechen Angmo)***

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ABSTRACT

The present study was carried out with an overall aim to generate frost tolerant potato clones and optimization of phenotyping techniques in order to complement the natural field selection criteria. The physiological responses to low temperature stress were explored using four potato genotypes *viz.* Kufri Anand, J-2/19, MS/7-645 and Kufri Pukhraj under *in vitro* controlled conditions. Significant variation was observed amongst genotypes for parameters associated with frost tolerance in potato such as cell membrane stability, activity of PSII, photosynthetic efficiency and leaf morpho-anatomical traits. The genotypes J/2-19 and Kufri Anand exhibited higher P_N, g_s and $\Delta F/Fm'$values and lower electrolyte leakage as compared to the sensitive genotype Kufri Pukhraj upon cold acclimation and during recovery days after freezing exposure. Similarly, J-2/19 genotype also recorded higher adaxial trichome density than the sensitive genotype MS/7-645. In order to identify the fold changes in the expression of three candidate genes involved in cold stress responses in potato *viz. dehydrin*, *hsp70* and *SAD* a qRT-PCR study was also performed. Overall an increase in gene expression was observed in cold acclimated plants of all the genotypes compared to non-acclimated plants. However, the increase was more pronounced in tolerant genotype (J-2/19) as compared with susceptible genotypes (MS/7-645 and Kufri Pukhraj). In the summer 2018, the segregating progenies were developed through hybridization among contrasting parents *viz.* J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) at Keylong, HP. In F_1C_1 and F_1C_2 generations, the characterization of the generated potato clones was accomplished on the basis of leaf morpho-anatomical traits and electrolyte leakage assay. To determine the association between the key traits and potential frost tolerant potato clones, various statistical approaches, such as cluster analysis, principal component analysis and genotype by trait biplot analysis were performed. Total 11, 10 and 8 clones from PAU3, PAU7 and PAU9 crosses, respectively were identified exhibiting potential frost tolerant characteristics with desirable horticultural traits for their further evaluation. Thus, the findings of this study concluded that in addition to electrolyte leakage the chlorophyll fluorescence and leaf gas exchange parameters can be effectively utilized for screening frost tolerant potato genotypes, but with certain limitations. Furthermore, the leaf morpho-anatomical traits have been confirmed to be associated with frost tolerance through decrease in foliage damage and electrolyte leakage. Therefore, these traits can potentially be used for screening of large segregating populations for freezing tolerance in the early clonal generations in potato.

Keywords: Chlorophyll fluorescence, cold responsive genes, electrolyte leakage, leaf morpho anatomical traits, low temperature stress,

Signature of Major Advisor **Signature of the Student**

ਖੋਜ ਪੱਤਰ ਦਾ ਸਿਰਲੇਖ	:	ਠੰਡ ਸਹਿਣਸ਼ੀਲ ਆਲੂ (ਸੈਲੋਨਮ ਟਿਊਬਰੋਜ਼ਮ ਐਲ) ਦੇ ਕਲੋਨਾਂ ਦੀ ਪੈਦਾਵਾਰ ਇੰਟਰਵੈਰੀਟਲ ਹਾਈਬ੍ਰਿਡਾਈਜੇਸ਼ਨ ਅਤੇ ਪੁਟੇਟਿਵ ਠੰਡ ਸਹਿਣਸ਼ੀਲਤਾ ਜੀਨਾਂ ਦੇ ਪ੍ਰਗਟਾਵੇ ਵਿਸ਼ਲੇਸ਼ਣ ਦੁਆਰਾ।
ਵਿਦਿਆਰਥੀ ਦਾ ਨਾਮ ਅਤੇ ਦਾਖਲਾ ਕ੍ਰਮਾਂਕ	:	ਡੇਚਨ ਆਂਗਮੋ ਐਲ-2016-ਏ-43-ਡੀ
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ਨਿਮਨ ਵਿਸ਼ਾ	:	ਪਲਾਂਟ ਬ੍ਰੀਡਿੰਗ ਅਤੇ ਜੈਨੇਟਿਕਸ
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ਮੁੱਖ ਸ਼ਬਦ : ਕਲੋਰੋਫਿਲ ਫਲੈਰਸੈਂਸ, ਠੰਡੇ ਪ੍ਰਤੀਕਿਆਸ਼ੀਲ ਜੀਨ, ਇਲੈਕਟ੍ਰੋਲਾਈਟ ਲਿਕੇਜ਼, ਪੱਤਾ ਮੈਰਫੋ ਸ੍ਰਰਿਕ ਗੁਣ, ਘੱਟ ਤਾਪਮਾਨ ਤਣਾਅ।

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an autotetraploid (2n=4x=48), belongs to family Solanaceae. It is considered to be originated in tropical South America, particularly in high mountains of Peru and Bolivia (Shekhawat 2015). In 1570, it was introduced in Europe, whence it spread to other parts of the world. Now it has emerged as the third most important food crop in the world after rice and wheat (Contaldi 2015). Potato is known for its high yield potential and nutritional qualities. It is a rich source of starch, high quality of proteins, vitamins and minerals. Potato is a versatile food which can be cooked in several ways, processed into number of products and fitted in any meal (Marwaha and Sandhu 2002). Thus, with its high tuber yield, digestible proteins and adequate content of vitamins B and C, it can effectively contribute to ensure the food nutritional security and improve people's livelihood in developing countries including India (Pushkarnath 1976, Sonnewald *et al* 2015).

Among the major potato growing countries of the world, India ranks second after China with a total annual production of 51.3 million tonnes (Anonymous 2020). Uttar Pradesh, West Bengal and Bihar are the major potato producing states in the country, which account for nearly 75% of the area and 80% of the total national potato production. Punjab ranks sixth in potato production with 5.6% share in the national production. In Punjab, area under potato cultivation is 107.0 thousand ha with 29.5 lac tonnes of annual production and with 27.5 t ha^{-1} of productivity (Anonymous 2021). On the basis of area potato is 5^{th} most important crop in Punjab, cultivated on 2.1% of geographical area. In Punjab, potato crop is grown during winter season. Potato is sensitive to an array of abiotic stresses such as heat, cold, salinity and drought (Diaz-Valencia *et al* 2021, Gangadhar *et al* 2013, Palta 2014). Thus, during December to January potato is exposed to severely low temperatures which affect its growth and occasionally reduce its yield up to 40-60% (Kang *et al* 2007, Luthra *et al* 2008). However, the extents of damage and yield losses vary from year to year depending on the intensity and duration of freezing stress (Stone *et al* 1993, Kang *et al* 2007, Kumar and Minhas 2013). In the years of occurrence of frost incidences and temperatures falls below 0°C which results in plant foliage damage and tubers may remain under-developed. Thereby, the yield is drastically reduced causing a huge loss to the state farm economy (Kang *et al* 2007, Luthra *et al* 2008). However, a considerable variability in frost tolerance and recovery from frost injury under field conditions had been reported in potato (Luthra *et al* 2008) and oat (Rizza *et al* 2001). Thus, the identification of genotypes possessing low temperatures tolerance is a viable option to sustain the production of potatoes in these frost prone regions, such as Punjab.

Cold stress is a major environmental constraint that limits the potato productivity in many regions around the world. The cold stress is classified in two types, chilling and

freezing. Low temperature damage without inducing freezing in the plant tissue is called chilling stress (Anjum 1994). Potato plants does not have the ability to avoid chilling injury, only tolerance mechanisms enable these to endure the effect of chilling temperature. Under low temperature conditions plants substantially alter the integrity and fluidity of membranes, which interrupts ion transport, denatures proteins and causes imbalance in normal cell metabolism (Orzechowski *et al* 2021, Wu *et al* 2019). Moreover, it also specifically affects the ultrastructure of chloroplasts altering the light harvesting chlorophyll antenna complexes and modifying thylakoid structures, thus reducing the photosynthetic ability and osmotic adjustments of crop plants (Wu *et al* 2019). Furthermore, photosynthesis has been reported to be much more sensitive than respiration under cold stress (Steffen *et al* 1989).

Frost tolerance is described as the ability of leaf tissue to endure ice formation when exposed to sub-zero temperatures. Extent of frost tolerance in crop plants depends on their ability to tolerate freeze-induced dehydration and the resultant mechanical stresses, and to avoid intracellular freezing. In potato, the term frost tolerance is used to depict their ability to endure -4°C or lower temperature without chilling injury (Li and Fennell 1985). Although, frost tolerance is a complex trait and two major components of frost tolerance involved in crop plants are freezing tolerance in non-acclimated and cold acclimated state (Orzechowski *et al* 2021, Stone *et al* 1993). Hardening, the exposure to low and non-freezing temperatures is also known as cold acclimation. Cold acclimation induces many changes in plants at physiological level such as, alterations in membrane composition, accumulation of soluble sugars, osmolytes and enhanced levels of ABA (Janská *et al* 2010). The inherent freezing tolerance before cold acclimation and the potential to acquire freezing tolerance during cold acclimation varies with plant species, even among genotypes with in a species (Liu *et al* 2017, Xi and Browse 2000). Some of these physiological changes may significantly be associated with increasing frost tolerance and can potentially be used to screen frost tolerant genotypes of potato.

Though various genetic resources for abiotic stress resistance, especially for frost resistance are available, but potato breeding programs in India had been focused on enhanced productivity, quality and disease resistance. Thus, most of the modern cultivars have very limited tolerance against abiotic stresses (Shin *et al* 2011). Furthermore, to combat serious yield losses due to frost stress, the foremost step is to identify traits associated with frost tolerance characters among the genetically diverse landraces. And some wild relative species, such as *S. commersonii*, *S. acaule* and *S. megistacrolobum* have been reported to possess a great potential in conferring freezing tolerance even up to -4 to -6 ˚C (Muthoni *et al* 2015, Palta and Li 1979,). But due to various complexities of genetic control of this trait and cross incompatibility with commonly cultivated potato (*S. tuberosum* L.) breeding efforts generally had limited success (Muthoni *et al* 2015). However, the variability in potato germplasm for

frost tolerance had been reported in India (Kang *et al* 2007, Luthra *et al* 2008, Tiwari *et al* 1986) Thus, the characterization of parental lines or the generated clones through hybridization will be useful for their utilization in breeding for frost tolerance.

In order to complement the selection under natural field conditions there is also a lack of precise controlled environment freezing tests which can be used to complement the natural field's selection criteria. Thus, there is an urgent need to optimize the precise and rapid controlled environment screening techniques in order to characterize and differentiate the available frost tolerant and susceptible potato germplasm (Stone *et al* 1993). The various freezing tests, such as electrolyte leakage assay, chlorophyll fluorescence and leaf gas exchange can be carried out to assess the cell membrane injury, activity of PSII photochemistry and photosynthetic efficiency, respectively (Maxwell and Johnson 2000, Rizza *et al* 2001, Sharma *et al* 2019).

With the advancement in crop stress physiology, the measurement of chlorophyll fluorescence (CF) and leaf gas exchange (LGE) have become well established and non–invasive techniques commonly used to assess the photosynthetic performance of plants by physiologists and eco-physiologists (Maxwell and Johnson 2000, Sharma *et al* 2015). This technique has been used to detect and quantify the damage in photosystem II (PS II) in several crops including potato (Sundbom *et al* 1982), wheat (Sharma *et al* 2015, Wu and Bao 2011), rice (Gu *et al* 2017), muskmelon (Sharma *et al* 2019) and oats (Rizza *et al* 2001) under various abiotic stress conditions. Thus, allowing a rapid and early phenotyping of genotypes to identify existing genetic variation in physiological responses associated with cold stress tolerance. However, till date there have been very limited systematic and profound studies on the frost tolerance physiology and regulation in potato under freezing stress. Therefore, in addition to the electrolyte leakage assay, chlorophyll fluorescence and leaf gas exchange based screening methods can be potentially utilized in evaluation of plants capacity for freezing tolerance in the potato breeding programme.

Other than these physiological techniques of phenotyping, the leaf morpho-anatomical characterization (LMAC) is a rapid, simple, cost-effective and accurate as compare with others physiological and molecular techniques. Moreover, LMAC can be utilized to identify genetic variations at early stages of crop growth and can also be used as screening markers for selection in the segregation populations (Kleinhenz *et al* 1995). A range of LMA traits, such as stomatal and trichome density on both adaxial and abaxial leaf surfaces, dimensions of palisade parenchyma cells are believed to get altered in response to diverse environmental stresses (Palta 2014). The stomata and palisade parenchyma cells exhibit enormous morphological and mechanical diversity which directly affect the leaf gas exchange properties of the genotype (Bisognin *et al* 2006). The variations in LMA characteristics have been studied extensively at various inter and intra specific levels in many

plants (Belhadj *et al* 2011, Campitelli *et al* 2013, Pereira *et al* 2013). In potato, the frost tolerant genotypes exhibit rosette growth habit, modified leaf forms, enhanced pigmentation, higher stomatal density on the adaxial surface and multiple, and larger leaf palisade layers are generally found in freezing tolerant members of tubers bearing *Solanum* species (Palta and Li 1979). Thus, this information will help in understanding and exploring the various factors responsible for imparting or increasing freezing tolerance in potato genotypes. This understanding will be useful in transferring the traits in to frost susceptible high yielding cultivars through inter-varietal hybridization.

The activation and regulation of some specific stress related genes are the basis of molecular mechanisms responsible for imparting tolerance against these stresses. These genes are directly involved in signaling, transcriptional control, protection of membranes and proteins, scavenging of free-radicals toxic compounds under stress conditions (Wang *et al* 2003). Cold responsive genes, such as dehydrin (dhn1), heat shock protein (hsp70), and stearoyl-acyl carrier protein desaturase (SAD) are involved in conferring freezing tolerance in potato and other plant species, such as tomato and *Arabidopsis thaliana* (Kosova *et al* 2007, Li *et al* 2015, Seppanen and Coleman 2003). A combination of expression profiling and bioinformatics analysis can be used to quantitatively analyze the expression level of these genes and their function under different sets of stress/freezing conditions with application of various PCR based techniques using specific sets of primers (Gangadhar *et al* 2013, Pérez-Torres *et al* 2009). This information would definitely be helpful in enhancing the efficiency of identification of frost tolerant potato clones.

Thus, the current study was carried out with the overall aim to generate information on understanding of physiological and molecular mechanisms involved in frost tolerance in potato, optimization screening technique and generation of frost tolerant potato clones through hybridization.

Objectives

- Optimization of controlled environment screening protocols for rapid identification of the frost tolerant clones
- Characterization of morpho- anatomical and physiological traits associated with frost tolerance in available potato genotypes
- Transcript profiling of dehydrin (*dhn1*), heat shock protein/cognate (*hsp*70), and Stearoyl-acyl carrier protein desaturase (*SAD*) genes in selected tolerant and susceptible potato clones
- Generation and identification of frost tolerant potato clones through inter-varietal hybridization

CHAPTER II

REVIEW OF LITERATURE

Potato (*Solanum tuberosum* L.), a solanaceous vegetable crop, is ranked 3rd among the major staple food crops after wheat and rice. However, the relative importance of environmental stresses affecting its productivity is poorly understood. The genetic resources for abiotic stress resistance, especially against frost are available, but in India national potato breeding programs had been focused on enhanced productivity, quality and disease resistance. Therefore, most of the modern cultivars have very limited tolerance against frost. In India, 80% of potato is grown in Indo–Gangetic Plains during winter season (*Rabi*). In these areas of cultivation the radiant frost incidences are frequent during the months of December to January when plants are in their tuber formation stage. Exposure to frost can cause foliage damage and tuber may remain underdeveloped thus, leading to heavy yield losses. The literature pertaining to frost incidences and injury, traits associated with frost tolerance in potato, screening methodologies, cold responsive gene(s) and availability of genetic resources for frost tolerance has been reviewed under the following heads.

2.1 Frost occurrence and injury

In potato, the frost damage to plant canopy under field conditions occurs due to low temperature and its associated ice nucleation in the hydrated aerial tissues. The low temperature during night hours induces freezing of the leaf tissues, thereby decreasing the energy consumption in photosynthetic carbon fixation. Freezing injury triggers an array of ABA-/ calcium dependent genes which in turn affects the overall physiology and biochemistry of the injured plants. These altered pathways lead to serious yield losses. The degree of freezing injury depends on the frequency, severity, and duration of the frost event, freezing rate, minimum temperature reached, time for exposure to low temperature, thawing rate and the post-thaw conditions (Seppanen 2000).

The frost injury is morphologically identified as occurrence of flaccid and water-soaked leaf canopy. The freezing usually starts at low temperatures *i.e.* -2 to -3ºC (Jouyban *et al* 2013). It is manifested as formation of ice crystals first in the xylem vessels and intercellular spaces. The ice crystals typically grow in the apoplastic regions *i.e.* intercellular water is crystallized. Due to physical obstruction imposed by the cell membrane and wall, the ice crystallization does not propagate in the symplast region or within the cytoplasm. However, the water potential gradient is established within the injured tissue due to ice crystallization in apoplast. The lowered chemical potential of ice compared to liquid water causes progressive loss of water from the cell cytoplasm into the intercellular space leading to growth of extracellular ice crystals (Levitt 1980). The plant cell experiences a physiological dehydration stress due to these ice crystals. This makes the canopy to appear wilted and water-soaked. Depending on the genetic potential of the candidate cultivar, potato genotypes

can either escape or endure the freezing temperature stress. However, the exact mechanisms for these characteristics are not completely deciphered (Seppanen 2000).

2.2 Phenotyping techniques for frost tolerance in potato

Many decades of research have failed to identify genotypes with frost tolerant characters to develop commercial cultivars that could offer economically significant benefit to the potato growers. However, lack of standard protocols and effective screening methods may lead to wrong identification of the partially tolerant clones due to spatial temperature differences. Thus, effective identification and elimination of the false-positives can be ensured by improving the screening methodologies under controlled conditions. Although, the field survival/ evaluation were the main criteria of selection during early frost tolerance experiments, it is not foolproof due to several inherent problems (Stone *et al* 1993) primarily because of large G × E interactions. Moreover, the field evaluation of frost tolerance is rather tedious and erroneous due to unprecedented variability in severity and timing of the frost events (Palta and Li 1979). The evaluation in frost chambers exhibiting control over timing and intensity of frost treatment by monitoring temperature, moisture and light conditions throughout the period of experiment allows out-of-season screening besides the elimination of the false-positives. The field selection criteria can be better supplemented by various laboratory freezing tests/ protocols, such as leaf gas exchange, chlorophyll fluorescence, electrolyte leakage (electrical conductivity) and microscopy observations have been evaluated in various crops, such as potato, wheat and oats to identify frost tolerance. Therefore, the lack of effective selection criteria is one of the main factors of failure to achieve greater success in the past studies. To plan a better breeding strategies and develop frost tolerant potato clones. There is necessary to have precise and rapid phenotyping techniques in order to identify the existing genetic variation in bio-physiological, genetic and leaf morpho-anatomical traits associated with cold stress tolerance.

2.2.1 Physiological traits

Under low temperature stress, a large number of plant species display physiological or cellular perturbations (Sharma *et al* 2020, Wu *et al* 2019). Thus, for survival in such adverse environments, plants need to maintain cell behavior and activity and, in particular the stability of the cell membrane and activity of photosystem II (Mishra *et al* 2011). Among the bio-physiological traits associated with frost, this study focused on the PSII and cell membrane stability and activity of plants photosynthetic system.

2.2.1.1 Leaf gas exchange (LGE)

The photosynthetic efficiency of the PSII is affected by frost episodes and has been observed to vary among the frost-hardy and susceptible plants. Steffen *et al* (1989) observed that photosynthesis was much more sensitive to freezing stress as compared to respiration.

Photosynthesis showed a slight to moderate inhibition when only 5 to 10% of the total electrolytes had leaked from the tissue (reversible injury) in contrast to respiration which showed no impairment until temperatures at which about 50% ion leakage (irreversible injury) had occurred. Similarly, in melons it was observed that the maintenance of leaf gas-exchange capacity and crop photosynthesis are more related to adaptation responses to water-deficit conditions (Sharma *et al* 2019). Thus, early maturing and short duration melon cultivars that have the capacity to maintain leaf area development under water deficit conditions, can better sustain productivity in drought prone semiarid growing regions. Recently, Meena *et al* (2021) concluded that the collective improvements in CO_2 assimilation, stomatal conductance, PSII quantum efficiency and chlorophyll contents contributes to better heterosis performance in F_1 hybrid (DHM 117) compared to parents in maize crop grown in field condition.

In addition to cell membrane stability and activity of PSII, the cell cytoplasmic and cell sap osmolyte concentration are also most important physiological traits directly linked to frost-tolerance. Sugars/ salts or similar compounds have been observed to accumulate thereby increasing the concentration of the cell sap in the leaf tissues during cold acclimation of the frost-tolerant potato plants (Levitt 1980). The sugar and starch content in the leaves of frost-tolerant *S. commersonii* was observed to be higher than in the non-acclimating species (*S. tuberosum*) (Chen and Li 1980). Similarly, the amino acid contents of the frost-tolerant plants were correlated with the non-hardy plants though inconclusive results were documented (Levitt 1980). However, proline amino acid has been reported to accumulate in response to cold acclimation in frost-hardy plants (Swaaij 1986). Thus, free proline can be a potential biochemical marker for frost tolerance in potato. Both sugars/starch and amino acids (particularly proline) are responsible for inducing frost tolerance by reduction of freeze induced dehydration (Li *et al* 1979) and not simple frost avoidance.

Wu *et al* (2019) concluded that cold acclimation enhanced cold resistance in W3 by strengthening the original cold resistance traits of the plant and understand the cold resistance mechanism of plants, to identify morphological and physiological indicators for cold-resistant germplasm resources, and to provide a theoretical basis for cold resistance breeding in potato.

2.2.1.2 Chlorophyll fluorescence

Chlorophyll fluorescence (CF) represents a potentially valuable new screening approach to study/monitor the photochemical efficiency and physiological status of plants under various abiotic stress conditions in different crops (Kalaji *et al* 2016), durum wheat (Gautum *et al* 2014) and bitter gourd (Yang *et al* 2009). Moreover, the approach may provide unique benchmarks to improve global agricultural productivity models thus improving the reliability of crop yield projections under climate change scenarios.

Sharma *et al* (2015) concluded that the three-tiered phenotyping based on Fv/Fm to

identify genetic variation among the wheat cultivars under moderate to severe heat stress was successful in identifying wheat cultivars differing in photosynthesis under moderate and agronomical more relevant heat stress. Further, it was found more relevant in terms of total carbon gain in the plant. The identified cultivars may serve as a valuable resource for further studies to understand the physiological mechanisms underlying the genetic variability in heat sensitivity of photosynthesis. Thus, such approach can be utilized to identify cultivar differences for other physiological traits in other crops against other abiotic stresses.

Mechanism, non-photochemical quenching has been evolved in plants to reduce damages caused by the production of reactive oxygen species (ROS) by dissipating excessively absorbed solar energy as heat. However, it sometimes represents as a significant loss in solar energy and photosynthetic efficiency and consequently lowers the yield potential of the crops. In crop like rice it was reported that photosynthesis is temperature dependent process and therefore causing depression in net photosynthetic rate during midday (Gu *et al* 2017). Sundbom *et al* (1982) studied two different screening methods controlled freezing of plants in a climatic chamber and temperature induced fluorescence. It has been reported to be more rapid, non-invasive and precise method in oats (Rizza *et al* 2001) as, this method highly correlated with the field evaluated frost damage.

2.2.1.3 Membrane stability

Electrolyte leakage is the method of choice to screen frost-tolerant and susceptible plants. It has been reported to be more reliable and suits well to correlate with the visual assessed freezing injury in case of *Salix eriocephala* (Tsarouhas *et al* 2000). The conductivity of electrolyte does not depend on number of segments of leaf but rather on the size of leaf discs and changed during the leakage time studied in rape and wheat crop (Prasil and Zamechik 1998). Arvin and Donnelly (2008) have evaluated ten *S. tuberosum* cultivars and 11 wild *Solanum* species on basis of electrolyte leakage from the detached leaves of *in vitro* plantlets and observed significant differences among the wild and cultivated potato species. Similarly, some changes at the physiological level such as chlorophyll content, relative conductivity, malondialdehyde (MDA) and activity of antioxidant enzymes also helps in enhancing its cold tolerance characteristics with cold acclimation (Wu *et al* 2019).

2.2.2 Leaf morpho-anatomical traits

Certain plant phonological parameters can be utilized to differentiate among the frost-hardy and frost-susceptible plants such as the shoot height, inter nodal length and the length of leaves which exhibit an inverse relationship with frost-hardiness though it is a non-consistent criterion and works well if measured during hardening period. The percent foliage damage is another useful indicator of the frost tolerance under field conditions (Luthra *et al* 2008, Kang *et al* 2007). Similarly, in adaptations to winter conditions, the two species of Rhododendron have evolved for the distinct leaf morphological and anatomical changes. *R.*

catawbiense exhibited thermonasty and is more winter-hardy whereas, *R. ponticum L.* is less hardy and non-thermonastic behavior. Thus, the thermonasty served as a desiccation avoidance strategy in *R. ponticum* (Wang *et al* 2008). Moreover, in *R. ponticum* species additional layer of upper epidermis and higher stomatal density was observed whereas, in *R. catawbiense* species thicker cuticle, extra palisade layer and higher stomatal pore was observed. Such differences represent structural adaptations for reducing light injury in leaves and could serve a photo protective function during winter.

Trichomes are epidermal protuberances and can play a buffering role in the interaction between plants and biotic or abiotic stresses. The physical properties of trichomes such as density and their types are involved in reducing desiccation and thus, increasing the resistance of plants to cold damage and drought (Dai *et al* 2010, Zhang *et al* 2020). It also acts as a physical barrier against external invasion thus, protecting plant tissues from insects and ultraviolet light (Zhang *et al* 2020). The ratio of trichomes to stomata (T/S) was positively correlated with water use efficiency (WUE), indicating an important role for both trichomes and stomata in drought tolerance in tomato, and offering a promising way to select these traits for improved water use efficiency of major crop (Galdon-Armero *et al* 2018).

The key anatomical features observed to be linked to frost-hardy plants include thick palisade generally two layered palisade parenchyma, and low stomatal density (Anjum 1994). The palisade thickness is genetically transmittable character (Estrada, 1982).Moreover, the number of stomata per unit area of the upper leaf surface or stomatal index have also been distinctly related to frost hardiness. Palta and Li (1979) have observed three times higher leaf upper surface stomatal index in frost tolerant lines compared to the non-frost hardy plants. Kleinhenz *et al* (1995) reported stomatal index as a useful marker to screen backcross populations of two wild potato species and segregating clones for freezing tolerance.

Furthermore, Tiwari *et al* (1986) had performed association studies between different frost associated leaf anatomical characters with frost injury in various accessions and species of potato. A very strong and positive correlation between leaf thickness, palisade thickness and palisade proportion were observed. However, highly significant and negative correlation was observed between frost injury and palisade thickness and its proportion. Further, it was reported that the palisade thickness to be the highest direct contributor to frost injury followed by leaf thickness in potato.

2.3 Low temperature-responsive genes (LTR)

On freezing temperature exposure, a cascade of altered expression of certain genes occurs. The products of these genes render tolerance to elevated freezing temperatures in tolerant or resistant plants. Expression profiling followed by sequencing and identification of the product of the reverse transcriptase polymerase chain reaction (RT-PCR) have been instrumental in pin-pointing certain genes which have been classified in two categories. The

protein products of the 'primary genes' have a direct role in imparting protection against freezing temperatures. However, the cis-or trans-acting 'regulatory genes' regulate the expression of the primary genes during adaptation response (Shinozaki and Yamaguchi-Shinozaki 1997). Alternatively, the genes involved in freezing tolerance can be grouped as those genes whose products mediate interactions for biochemical and physiological processes altering the growth and development at low temperature and those which have a direct role in freezing tolerance. Importantly, the major function of primary gene products is to avoid or curb freezing dehydration and toxic concentrations of solutes (Seppanen 2000).

There are several LTR genes in diverse plant genera which have a direct role in freezing tolerance though the exact mechanism is still uncertain (Folgado *et al* 2013). This is probably due to sharing of structural and functional similarities with other genes/ gene products unrelated to cold-responsiveness (Seppanen 2000). Moreover, all the cold-responsive genes are not nucleus-controlled as some genes imparting frost tolerance are under the control of chloroplast (Lu *et al* 2013). Also, the transgenic plant studies have accentuated the role of a group of gene products to impart partial to complete cold temperature tolerance in several plants (Seppanen and Coleman 2003). The gene action for LTR genes has been reported to vary from recessive to partially dominant to both additive and non-additive, largely additive to dominant in various crops (Hermanova *et al* 2007). Thus, most of the studies have concluded that freezing stress resistance is generally a complex trait with polygenic inheritance (Folgado *et al* 2013). In fact, the genetic machinery involved in cold-sensing is invariably present in susceptible plants also though the expression of the primary LTR genes are either at high (particularly *heat shock protein 70* gene), sub-optimal levels or absent in the susceptible plants (Seppanen and Coleman 2003). Moreover, past studies have suggested the independent genetic control of freezing tolerance in cold acclimated and non-acclimated potato plants (Stone *et al* 1993).

The LTR genes can be grouped as cold-acclimation and cold-stress tolerance genes. As discussed above, the inheritance of both types of genes is independent of each other such that there are five categories of the potato plants on basis of occurrence and/or expression of these genes i.e. freezing tolerant and able to cold-acclimate (CA), freezing tolerant and unable to CA, freezing sensitive and able to CA, freezing sensitive and unable to CA and chilling sensitive. The benefit of cold acclimation is to make the plant cold-stress ready. The CA of plant results in accumulation of cryoprotective solutes, altered pigment composition and enhanced Rubisco expression besides switching on of the LTR genes (Zhang *et al* 2002). The extent of the alterations in the biochemical and physiological processes are largely genotype specific (Barientos *et al* 1993). During the acclimation process, the LTR-genes are up-regulated simultaneously with increased freezing tolerance.

2.3.1 Gene expression studies

Incidence of frost leads to differential expression of several genes which markedly vary among the frost-hardy and susceptible plants (Aversano *et al* 2015). The variation in the expression of a total of 855 genes in plants acclimated to frost stress compared to the non-acclimated plants were also observed by Aversano *et al* 2015. Further, they reported the negative and positive correlation of 11 and 25 transcription factors respectively with respect to acclimated and non-acclimated tolerance for frost episodes. Thus, conserved sequences across distant species are likely to constrained implying similar biological function (Alföldi and Lindblad-Toh 2013).

Carvallo *et al* (2011) reported that the two closely related potato species *Solanum commersonii* and *Solanum tuberosum* vary in their abilities to cold acclimate. The expression of genes in both the plants alters in response to low temperature to same degrees with similar kinetics/ways. The sets of genes that comprised the low temperature transcriptomes and CBF regulons of the two species differ significantly. Thus, the differences in freezing tolerance of these two species may contribute to differences in cold regulatory programmes. The transgenic experiments involving the transfer of single genes encoding antifreeze proteins to freezing-sensitive plants have been reported to lower freezing temperatures by 10℃ in the test plants (Griffith and Yaish 2004). However, over-expression of a single gene conferring frost tolerance cannot render absolute frost resistance in the test plants further indicating the polygenic origin and nature of the frost tolerance trait.

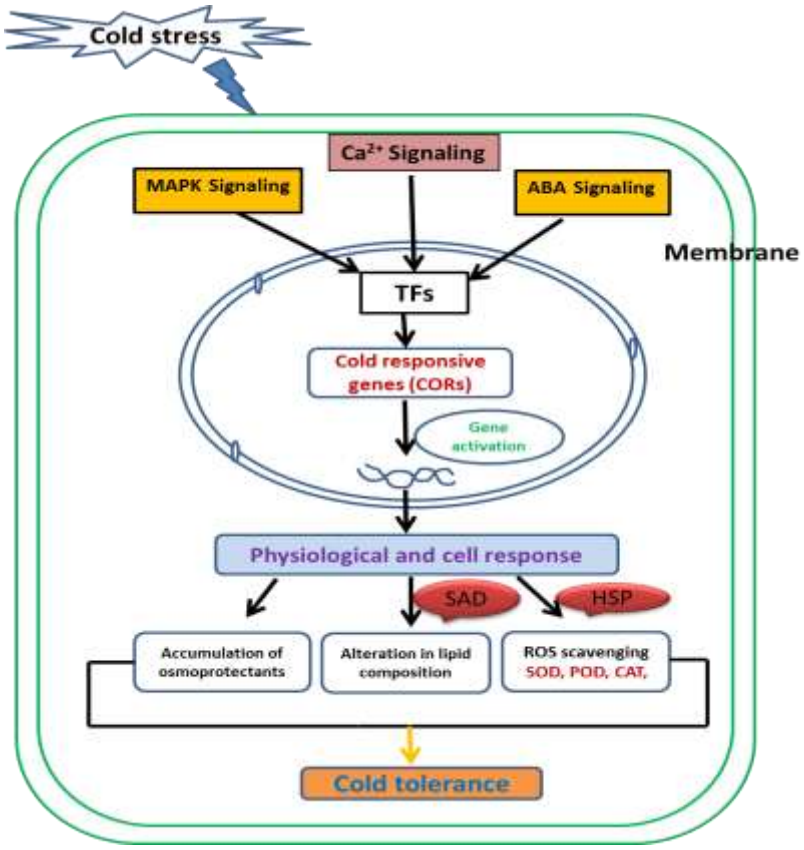


Figure 2.1 Cold tolerance mechanism of gene expression in potato

The genes and their products governing the mechanisms of frost tolerance and cold acclimation in potato are not well documented and identified. Moreover, there is very limited genetic diversity for freezing tolerance in popularly cultivated *S. tuberosum* cultivars. However, the wild potato species which grow at high altitude under low temperate conditions, such as the *S. commersonii* and *S. acaule* can tolerate freezing temperatures in range of -4.5 to -11ºC (Chen and Li 1980).

2.3.1.3 Heat shock proteins (HSPs)

The heat shock proteins (HSPs) are a group of low molecular weight cytosolic proteins (110, 90 and 70 kDa) which are induced in response to heat or elevated temperature stress (Park and Seo 2015). The HSPs are also anticipated to have a key role in protein biogenesis as protein misfolding results in induction of HSPs (Kozutsumi *et al* 1988). These HSPs (90, 110 and 70 kDa) are also up-regulated in response to low temperature stress (Folgado *et al* 2013, Seppanen 2000). In addition, the increase in chilling tolerance was closely correlated with HSP expression (Sebehat *et al* 1996). Further investigations revealed that a pre-exposure to heat shock is required for the expression of some HSPs at low temperature (Sebehat *et al* 1998).

2.3.1.2 Dehydrin

Kosová *et al* (2007) have documented that dehydrin can act as an emulsifiers or chaperones in the cells. The product of dehydrin gene protects proteins and membranes against unfavorable structural changes caused by dehydration. Baudo *et al* (1996) identified two homologous dehydrin genes scdhn1 and stdhn1 in freezing-tolerant *S. commersonii* and freezing-sensitive *S. tuberosum* cv. Bintje. The *dehydrin (dhn)* gene produces dehydrin proteins belonging to LEA-D-11/RAB gene family products responsible for enhancing desiccation tolerance in several plants (Hanin *et al* 2011, Aversano *et al* 2015). However, being primarily the structural proteins, these have a key role in imparting low temperature tolerance that involves occurrence of physiological dehydration (Hanin *et al* 2011). A study on dehydrin gene expression showed that only one barley dehydrin (*dhn5*), which was not mapped to the freezing tolerance loci, was up-regulated by low temperature under controlled acclimation conditions (Van Zee *et al* 1995). The counterparts of *dhn* genes in wheat are recognized as a set of wheat specific cold stress proteins encoded by *wcs120* gene family. During cold acclimation, the expression of *Wcs120* is regulated by phosphorylation of a negative regulatory factor (Sahran *et al* 1997).

Similarly, Seppanen and Coleman (2003) observed that in freezing tolerant genotypes the accumulation of ScgstF1 and Scdhn2 transcripts coincides with the temporarily reduction of photosynthesis. But in case of cold-acclimated and hydrogen peroxide treated plants, ScgstF1 and Scdhn2 accumulated in freezing-tolerant genotypes whereas Schsc70 transcript was more abundant in S. tuberosum. They also suggested that the signal pathways associated

with low temperature cold acclimation or H_2O_2 may overlap. Moreover, Janda *et al* (2003) have observed a significant correlation among the enzyme activities and frost tolerance in cereal crops. The glutathione-S-transferase, ascorbate peroxidase and GPx were significantly higher in the hardened plants than in the controls.

2.3.1.1 Stearoyl-acyl carrier protein desaturase (SADs)

Therefore, both nucleus and chloroplast-controlled genes have been observed to be differentially expressed on frost exposure. Li *et al* (2015) had cloned and characterized stearoyl-acyl carrier protein desaturase (SADs) from four *Solanum* species. Analysis showed that the *S. commersonii* and *S. acaule* possess freezing tolerance and cold acclimation capacity. Moreover, both the species showed similar SAD amino acid sequences but it differed from freezing sensitive *S. cardiophyllum*. Furthermore, the ScoSAD from *S. commersonii* was cloned and transformed into the cultivated potato *cv.* Zhongshu8 and the over expression of this gene in transgenic plants appreciably improved freezing tolerance. Thus, these SAD genes are also involved in maintaining the homeostasis between saturated and unsaturated fatty acids.

2.4 Genetic resources for frost tolerance in potato

The major genetic resources for frost tolerance genes in potato include the wild relatives of the cultivated *S. tuberosum.* There are many primitive and wild relatives of potato such as *S. commersonii*, *S. acaule* and *S. juzepczukii* reported to have high potential to tolerate freezing temperature even up to –6°C. *Solanum commersonii* Dunal is a well-known wild potato commonly used as materials for somatic hybridization due to various biotic and abiotic stress resistances. The first draft of *S. commersonii* (wild potato of central and South America) genome consisting of 12 chromosomes accounting for ~830 megabases was released in 2015 (Aversano *et al* 2015). This genome size was slightly smaller than the genome size of the *S. tuberosum.* Both the genomes happen to share considerable genetic similarity except some variations in the intergenic sequences (Galvez *et al* 2017). Recently, the complete chloroplast genome of *S. commersonii* consisting of a total of 113 genes (79 coded specific proteins, 30 tRNA and four rRNA genes) has also been sequenced (Cho *et al* 2016). Interestingly, though *S. commersonii* exhibits sexual incompatibility with *S. tuberosum,* the chloroplastic DNA maximum likelihood phylogenetic analysis showed it to be closely related to *S. tuberosum* (Cho *et al* 2016). This will help our understanding of the evolutionary relationship and occurrence of genetic similarity among the wild relatives of cultivated potato. A whole new database, 'PoMaMo' dedicated to accession deposit, search and mining for the sequences and other genetic information has been launched (Meyer *et al* 2005).

The research interventions for frost tolerance in potato are limited in India. A published report of Kang *et al* (2007) on field evaluation of potato cultivars and lines for frost

tolerance has shown Kufri Pushkar as one of the moderately frost tolerant cultivar. Another study involved selection of frost tolerant clones from segregating progeny of tetraploid *Solanum tuberosum ssp. andigena* (SS 2040) and diploid *Solanum spegazzinii* (SS 2040-EC460686 accession) by Luthra *et al* (2008). However, so far no frost tolerant cultivars of potato in India have been commercialized. The genetic variation for freezing tolerance is limited in cultivated potato species (*Solanum tuberosum* L.). Therefore, the frost episodes have been managed by following various agronomic interventions, such as irrigation management, adequate application of urea/ N-fertilizer.

2.5 Breeding approaches

The most common breeding approaches employed in potato improvement are hybridization and clonal selection. But one of the major difficulties associated with the traditional breeding relates to tetrasomic nature of inheritance, virtually all the potato cultivars are auto-tetraploids. High heterozygosity and severe breeding depression in parental clones require exceptionally high populations of potato seedlings to be screened in order to recover individuals for evaluation as potential clones. The development of frost tolerant/resistant potato clones was one of the oldest breeding objectives (Reddick 1930), particularly where potato is grown as a cool season crop. For a successful breeding, there is need to understand the genetics of frost resistance, its hardening characteristics and standardization of laboratory screening techniques/protocols for rapid evaluation of potential frost tolerant plants.

Use of these species as genetic resources of frost tolerance in conventional breeding generally depends upon their crossability. But due to differences in endosperm balance number (EBN) and ploidy level, these species exhibit cross incompatibility with the cultivated potato species. To overcome these barriers various techniques such as manipulations of ploidy level, bridge crosses, auxin treatments and embryo rescue can be useful for potato breeders to access promising characteristics present in wild species (Hirano 2015). Luthra *et al* (2010) have reported some promising frost tolerant relative species such as *S. spegazzinii* (diploid), *S. demissum* (hexaploid) and *S. tuberosum ssp*. *andigena* (tetraploid). Among these, the Andigena group of cultivated potato is easily and directly crossable with the other predominant cultivated group of *tuberosum*. Thus, it can be used as genetic resource in breeding. Further, the identified accessions of wild species can be used to diversify the genes of frost tolerance in cultivated potato. Palta (2014) reported several frost tolerant wild species of potato possess two desirable traits non-acclimated freezing tolerance (NAFT) and capacity to cold acclimatize (CAC). The traits were reported to be lacking in cultivated potato. These traits were genetically distinct so these must be selected individually and can incorporate into desirable genotypes. Similarly, Kleinhenz *et al* (1995) generated segregating backcross population by crossing two potato species *S. commersoni* (frost tolerant) × *S. cardiophyllum*

(frost sensitive). And then the F_1 individual was used as female parent and backcross to each parent to study the differences in leaf morpho-anatomical traits. The study suggested that stomatal index values may be used in selecting potentially frost tolerant germplasm in segregating progenies in potato.

From this review of literature, it was concluded that in addition to natural field selection criteria effective phenotyping techniques are urgently required in order to identify potential frost tolerant potato clones. Further, the gene expression profiling was also identified as a robust molecular tool for assessment of variability among the genetic resources for low temperature stress tolerance. The association of leaf morphoanatomical traits, such as stomatal index, trichome density on adaxial leaf surface and thicker palisade layers needed to be confirmed for their utilization as markers of frost hardiness in potato.

CHAPTER III

MATERIAL AND METHODS

The present study entitled, 'Generation of frost tolerant potato (*Solanum tuberosum* L.) clones through intervarietal hybridization and expression analysis of putative frost tolerant genes' was carried out in the Department of Vegetable Science, and the School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana (latitude 30º 54' N and longitude 75º 45' E; with a mean height of 247 m above sea level) during the three autumn-winter seasons of year 2018-19, 2019-2020 and 2020-2021 and as off season at Keylong (32º 34' N and longitude 77º 15' E; with a mean sea level 3090.83 m), Himachal Pradesh in 2018. The experimental site was located in the subtropical climatic zone, with winter during the months from October to March (*Rabi*). Plenty of sunshine and moderately low temperature prevailed during experimental period, which is suitable for cultivation of potato in Punjab. The hybridization work was carried out at Keylong, HP, since long days and cool weather conditions during summer at this location are favorable for induction of profuse flowering in potato.

The study was conducted as series of three different experiments. First experiment was conducted using four potato genotypes for optimization of screenings protocols under controlled environment conditions. To study the transcript profiling of cold tolerance related genes, the second experiment was carried out in selected tolerant and susceptible potato genotypes. The third experiment was carried out during the summer season, 2018 at Keylong and characterization of potential frost tolerant clones was carried during the cropping seasons 2018-19; 2019-20 and 2020-21 in Punjab. Details of materials and methods used in this study are presented below:

3.1 Optimization of controlled environment screening protocols for rapid identification of the frost tolerant clones

3.1.1 Experimental methodology

3.1.1.1 Location of work

A pot experiment was conducted during December to February, 2019-20 at Vegetable Research Farm, Department of Fruit Science, PAU, Ludhiana, Electron Microscopy and Nanoscience (EMN) Laboratory, PAU, Ludhiana and Wheat Research Farm, Plant breeding and Genetics (PBG), PAU, Ludhiana under polyhouse and controlled environmental conditions.

3.1.1.2 Plant material

Four genotypes of potato *viz.* two tolerant- Kufri Anand, J/2-19, two susceptible- Kufri Pukhraj and MS/7-645 were selected for this experiment.

Approximately 150 single pre-sprouted virus free potato tubers of each genotype were grown in plastic pots in a controlled environment/polyhouse in the second week of

December. A mixture of coco peat: vermiculite: perlite in 3:1:1 ratio was used as a substrate for growing the plants.

3.1.1.3 Treatments

All the potato plants were divided randomly into two equal sized groups and then transferred to different growing conditions to achieve cold acclimation and non-acclimation conditions. Temperature, photoperiod and relative humidity were maintained as per experimental requirements as mentioned in Figure 3.1. For freezing treatments, the plants were exposed to low temperature of (-2℃± 1) in a chamber under controlled conditions for two different time spans 1.0 h and 1.5 h. The experimental details are described in Figure 3.1.

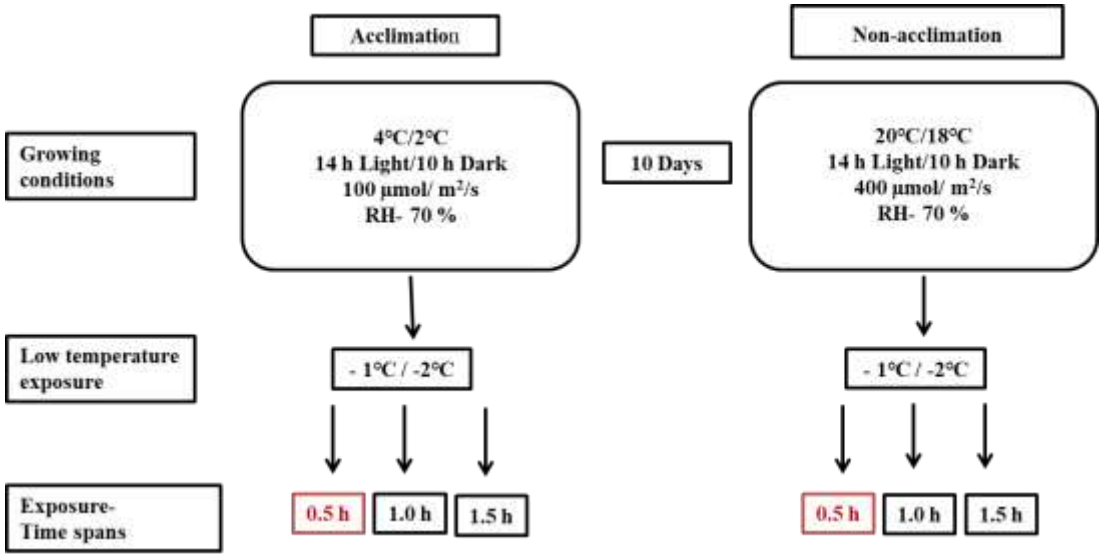


Figure 3.1: A graphical representation of experimental lay-out.

The plants were exposed to low temperature (-2℃ ±1) for two time spans 1.0 h and 1.5 h in the experiment 1 (Physiological response studies).

The plants were exposed to low temperature (-1℃ ±1) for three time spans 0.5 h, 1.0 h and 1.5 h in the experiment 2 (Gene expression studies).

3.1.1.4 Observations recorded

Leaf gas exchange parameters, [net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and water use efficiency (WUE)] and [chlorophyll fluorescence parameters, [maximum quantum yield of PSII (Fv/Fm), effective quantum yield of PSII (ΔF/Fm'), electron transport rate (ETR)] were measured by using a portable photosynthesis (LI-6400 XT, LI-COR Inc., Lincoln, NE, USA) equipped with an open-flow infrared gas analyzer at a steady state of (PAR of 100-800 µmol m^{-2} s^{-1}, reference CO_2 concentration of 400 µmol mol^{-1}, air flow rate of 500 µmol s^{-1}, and block temperature (20°C) for all measurements as described in De Fredites *et al* (2017) and Sharma *et al* (2014) at various stages *viz*. initial stage 30 days after planting (DAP), 10 days after cold acclimation and at different days after treatment during recovery days *i.e.*, 2, 7 and 15 days after treatment

(DAT).

Electrolyte leakage assay was carried out using the protocol described in (Thalhammer *et al* 2014) with some modifications. Electrolyte leakage was determined immediately after freezing treatment for 1.0 h and 1.5 h by using a conductivity meter (Model 306, Systronics, Ahmedabad, Gujarat).

$$\text{Electrolyte leakage (EL)} = \frac{\text{Initial conductivity}}{\text{Final conductivity}} \times 100$$

To study the variations in leaf morpho-anatomical characteristics associated with frost tolerance among the potato genotypes. The microscopic studies of oblique and transverse sections of the leaves was carried out using the methods as described in (Gitz and baker 2009, Yaun *et al* 2020) with slight modification. The leaf section was mounted on glass slides, covered with coverslips and viewed under Optical Microscope, (Leica DM 5000B, Germany). Parameters such as, stomatal index (%), trichome density on adaxial leaf surface (numbers per mm^2) and palisade parenchymatous cell length (µm) were recorded.

3.1.1.5 Statistical Analysis

Experimental data were subjected to analysis of variance (ANOVA) using Generalized Linear Model procedures of Statistical Analysis System (SAS Version 9.3, SAS Institute, Cary, N.C., USA) using completely randomized design with factorial arrangement (Steel and Torri 1980). The graphs were prepared in Excel 2010 (Microsoft Corporation, Richmond, WA, USA).

3.2 Transcript profiling of dehydrin (*dhn1*), heat shock protein/cognate (hsp70), and Stearoyl-acyl carrier protein desaturase (SAD) genes in selected tolerant and susceptible potato clones

3.2.1 Plant materials and treatment details

Three genotypes of potato *viz.* J-2/19, Kufri Pukhraj and MS/7-645 were selected for this experiment. Approximately 150 single pre-sprouted virus free potato tubers of each genotype were grown in plastic pots in a polyhouse in second week of December. All the potato plants were divided randomly into two equal sized groups and then transferred to different growing conditions to achieve cold acclimation and non-acclimation. Temperature, photoperiod and relative humidity were maintained as per experimental requirements. For freezing treatments, the plants were exposed to low temperature of (-1℃± 1) in a chamber under controlled conditions for three different time spans 0.5 h, 1.0 h and 1.5 h. The experimental details are described in Figure 3.1.

3.2.2 Preparation of Diethyl pyrocarbonate (DEPC)-treated consumables for RNA isolation

All glass wares and plastic wares were ensured to be RNase free by treating them with 0.1% DEPC water for overnight at room temperature. For preparing 0.1 % DEPC water,

1 ml of DEPC was dissolved in 1 litre of distilled water. For RNA isolation from potato leaves, the micro tubes (1.5 ml and 2 ml) and tips (1 ml, 200 µl and 10 µl), along with pestles and mortars, were fully immersed in DEPC water. After overnight treatment, the tips and micro tubes were filled in tip boxes and jam jars, respectively. The tip boxes and jam jars were dried in oven at 80°C for overnight and all the material was autoclaved at 121°C for 15-20 minutes.

3.2.3 Sample collection

Fresh and young leaves were collected at different time interval *i.e.,* 10 days after cold acclimation and non-acclimation; after freezing treatment at three different time spans 0.5 h, 1.0 h and 1.5 h of both cold acclimated and non-acclimated plants and collected samples were immediately stored in liquid nitrogen.

3.2.4 RNA isolation

The total RNA was isolated manually using TRizol method (Chomczynski and Sacchi, 1987) with following steps:

1. Freshly collected leaf tissues were crushed in liquid nitrogen to fine powder in a pre-chilled mortar and pestle.
2. About 100 mg powder was transferred to DEPC treated 2 ml eppendorf tubes.
3. Immediately, 1 ml of RNAiso Plus was added to crush tissue. The homogenized sample was then incubated for 5 minutes at room temperature for complete dissociation of the nucleoprotein complex.
4. Afterwards, 200 µl of chloroform was added per 1 ml of RNAiso Plus reagent. The samples were mixed on vigorous shaking using hand for 15 seconds and incubated for 2-3 minutes at room temperature.
5. Samples were centrifuged at 12000 rpm for 15 minutes at 4 ℃. The aqueous phase was pipetted out and transferred into a new tube. It was taken care to avoid drawing any of the interface or organic layer while removing the aqueous phase.
6. 500 µl of 100% isopropanol was added per 1 ml of RNAiso Plus followed by incubation at room temperature for 10 minutes and samples were centrifuged at 12000 rpm for 10 minutes at 4°C.
7. The supernatant was removed, leaving behind the RNA pellet. The pellet was washed with 1 ml of 75% ethanol per 1 ml of RNAiso Plus, used in the initial homogenization. The samples were then briefly vortexed, followed by centrifugation at 7500 rpm for 5 minutes at 4℃. The supernatant was discarded.
8. The RNA pellet was air dried for 5-10 minutes, then dissolved in 50 µl of autoclaved 0.1 % DEPC-water and stored at -80°C.

3.2.5 Assessment of quality and purity of RNA

3.2.5.1 Agarose gel electrophoresis

The RNA samples were thawed on ice for 30 minutes. The integrity and quantity of RNA was determined on 1.2% (w/v) denaturing agarose gel. The gel electrophoresis tank, combs, gel tray, measuring cylinder were washed once with 0.5% Sodium dodecyl sulfate (SDS) followed by 70% ethanol. Agarose powder (HiMedia) was dissolved in 1X 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (prepared in 0.1% DEPC water). The agarose mixture was heated until clear or transparent and cooled down bearable temperature followed by addition of 37 % formaldehyde. Ethidium bromide was also added at a final concentration of 1 µg/ml. The agarose solution was then poured into pre-casted gel tray with combs and left to solidify at room temperature. RNA Samples were heat denatured at 70°C for 10 minutes and were loaded into wells by mixing with autoclaved 50% glycerol (prepared in 0.1% DEPC water) in 1:1 proportion, *i.e.,* 5 µl of RNA and 5 µl of 50% glycerol. The gel was subjected to electrophoresis at constant voltage (80 V) for about 30 minutes. The gel was visualized, and quality of RNA was judged on the basis whether the RNA separated into distinct bands on gel (good quality) or formed a smear (degraded).

Table 3.1: Composition of MOPS buffer

S. No.	Chemical	Quantity
1	3-(N-morpholino) propane sulfonic acid	200 mM
2	Sodium acetate	80 mM
3	EDTA	10 mM

3.2.5.2 Nano drop spectrophotometer

RNA quality was also quantified using Thermo scientific Nanodrop 1000 spectrophotometer (Thermo fisher scientific Inc., Waltham, MA, USA)

3.2.5.3 Complematy DNA (cDNA) synthesis

cDNA synthesis was carried out using reverse transcription of total RNA to cDNA using first strand cDNA synthesis kit Takara Prime script (Takara Bio USA, Inc., San Jose, California, USA), and steps were performed as follows:

1. The reaction mixture (10 µl), containing 1 µl oligo dT primer (50 µM), 1 µl dNTP mixture (10 mM each), 1 µl RNA (1 µg), was prepared in a microtube.
2. It was incubated at 65°C for 5 minutes and immediately cooled on ice.
3. A total volume of 20 µl reaction was prepared using previous mixture from step 1 with 4 µl Prime Script Buffer (5X), 0.5 µl RNAase inhibitor (40 U/µl) and 1 µl Prime Script RTase (200 U/µl). The mixture was incubated at 42°C for 60 minutes followed by 70°C for 15 minutes and cooled on ice.

3.2.6 Confirmation of cDNA

cDNA synthesis was confirmed using universal primer 26S RNA by PCR (Singh *et al* 2004). The forward primer (26S r RNA F) sequence was: 5'-CACAATGATAGGAAGAGCCGAC-3' with T_m=54℃ whereas, sequence of reverse primer (26Sr RNA R) was: 5'-CAAGGGAACGGGCTTGGCAGAATC-3' and T_m= 54 °C. Single tube one step PCR was performed using an Applied Biosystems, master cycler (Thermo Fisher scientific Inc. NYSE: TMO, Waltham Massachusetts, USA). A negative control (without template DNA) was included in each amplification reaction. The PCR amplification program for elongation factor 1 alpha (*EF1α*) was same as for heat shock protein/cognate (*hsp70*), dehydrin (*dhn1*), and Stearoyl-acyl carrier protein desaturase (*SAD*) specific primers. PCR amplified products were resolved in 2.5% agarose gel and DNA fragments were then visualized under gel documentation system and photographed.

3.2.7 Relative expression analysis by qRT-PCR

qRT- PCR analysis was performed to analysed the relative expression of the integrated *EF1α* gene in control and treated plants, using *EF1α* was used as a reference gene control. Hsp70-17, *dhn1* and SAD specific qRT-PCR primers sequences design from *Integrated DNA Technology* (IDT), USA. cDNA was used as a template for expression analysis by SYBR® Premix Ex Taq™ II (Takara) (Takara Bio USA, Inc. San Jose, California, USA) using Step One-Real time PCR (Applied Biosystems), (Thermo Fisher scientific Inc. NYSE: TMO, Waltham Massachusetts, USA). Total volume of qRT-PCR reaction composition was set to 10 µl, containing 1 µl (40 ng) cDNA, 0.7 µl (0.25 µM) each primer, 5 µl (1X) SYBR Premix Ex Taq™ II. The PCR conditions were: 94°C for 5 min., 40 cycles for 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C and melt curve at 60°C for 1 minute.

The relative expression of specific genes *viz.* heat shock protein/cognate (*hsp70*), dehydrin (*dhn1*), and Stearoyl-acyl carrier protein desaturase (*SAD*) were normalized to *EF1α* gene for each set of sample and results were analyzed by 2-(ΔΔCt) method (Livak and Schmittgen 2001). According to this method:

ΔCt = Ct mean (target gene) - Ct mean (reference gene)

ΔΔ Ct =ΔCt (target sample) - ΔCt (control sample)

2-ΔΔCT represents fold change or relative gene expression in treated plants in relative to control/initial.

3.3 Generation and characterization of potential frost tolerant potato clones on the basis of leaf morpho-anatomical and physiological traits

3.3.1 Location of work

The hybridization work was carried out at Punjab Agricultural University (PAU), Research Station, Keylong, Himachal Pradesh (32º 34' N and longitude 77º 15' E; with a

mean sea level 3090.8 m). Evaluation and characterization of clones were carried out at Vegetable Research Farm, Department of Vegetable Science, PAU, Ludhiana (Plates 1 and 2)

3.3.2 Experimental methodology

3.3.2.1 Plant material

The selected parental genotypes were planted on 15th of May, 2018 at PAU Research Station, Keylong Himachal Pradesh for crossing programme. The crop was raised following the package of practices recommended by Punjab Agricultural University, Ludhiana (Anonymous 2021).

Table 3.2: Parentage and source of generated potential frost tolerant potato clones

S. No.	Cross codes	Parents	Sources
1	PAU3	J-2/19 × MS/7-645	CPRI, Shimla
2	PAU7	MS/7-645 × J-2/19	CPRI, Shimla
3	PAU9	MS/7-645 × CP-3765	CPRI, Shimla

3.3.2.2 Hybridization methodology and procedure

3.3.2.2.1 Selection of parents

On the basis of the breeding objectives, genotypes possessing frost tolerant and desirable horticultural traits were selected. The genotype J-2/19 was identified as frost tolerant as it exhibited low foliage damages (< 11%) under frost incidences in the cropping season 2016-17 at Ludhiana whereas, MS/7-645 was a high yielding (43.5 t ha^{-1}) potential genotype (Anonymous, 2013-14). Moreover, the genotype J-2/19 exhibited significantly higher stomatal and trichome density on both adaxial and abaxial leaf surfaces than the genotype MS/7-645. Similarly, CP-3765 genotype was procured from CRPI, Shimla as a frost tolerant genotype.

3.3.2.2.2 Emasculation

The mature large size unopened buds were selected for emasculation and all others floral parts were removed gently with the help of forceps in the morning hours before pollination.

3.3.2.2.3 Pollination

The pollen from a desirable parent using a mechanical vibrator was collected in eppendorf tubes. Pollination was carried out by placing the freshly collected pollen grains on stigma of female parent with the help of camel brush gently without damaging it.

3.3.2.2.4 Tagging and bagging

The pollinated buds were covered by butter paper bags and tagging was carried out to ensure the identification of the cross pollinated parents by writing all the essential information like parents and date of crossing etc.

Plate 1: Pictorial representation of hybridization work at Keylong, nursery raising and transplanting at Ludhiana

Plate 2: Evaluation of potential frost tolerant potato clones in the early clonal generations at Ludhiana

3.3.2.2.5 Berry harvesting

After 10-15 days of pollination, berries were set. And at this stage, butter paper bags were removed and each bunch of berries was covered with a thin muslin cloth bags for about 6-7 weeks till maturity to protect and prevent from damages and fruit drop. The picking of berries started from last week of September till the mid of October. Fruits were harvested when they attained greenish-yellow colour and its skin became soft. The harvested berries were stored at room temperature to enable loosening of the fruit skin.

3.3.2.2.6 Seed extraction and storage

The true potato seed extraction was accomplished in the month of October by hand crushing method. The selected berries were crushed in a polythene bag by using a wooden hammer. Then crushed material placed in water tub for one to two hours for separation of mucilaginous substances. Afterwards, pulp and debris of the berries were removed from water and poured onto standard kitchen sieve to separate the seeds. Drying of the seeds was carried out by drying in sun light. These extracted seeds were stored at a room temperature in paper bags.

3.3.2.3 Selection procedure of potential potato clones in seedling and early clonal generations

3.3.2.3.1 Seedling stage (F_1 generation)

The true potato seeds (TPS) of various entries were treated with Gibberellic acid (1000-1500 ppm) for 24 hours to break the dormancy. A mixture of coco peat, vermiculite, perlite and husk in 3:1:1:1 ratio and fully decomposed farm yard manure were used as a substrate for raising the seedlings. The seeds were sown in plug trays in second week of October and to minimize the virus infection and the seedlings were raised in low aphid conditions.

After about 25-30 days, when the seedlings were at 4-6 leaf stage, these were transplanted in the field at 20 cm intra- and 60 cm inter-row distance. The seedlings were raised following standard protocol and cultural practices (Plate 1). Weak seedlings were rogued out and each seedling was harvested separately. At harvest, the clones showing irregular tuber shape, deep eyes and undesirable tuber colour were rejected.

3.3.2.3.2 Early clonal generations

The clones were evaluated in seedling (single hill), F_1C_1 generation (short row trials) and F_1C_2 generation (duplicate row trials). The assessment of tuber yield was not carried out as the tuber size of the clones was not uniform and moreover, environmental effect was also there. From each selected seedling, five tubers were retained for evaluation in next year. The subsequent clonal generations of F_1 seedlings were labelled as F_1C_1 and F_1C_2 (Plate 2).

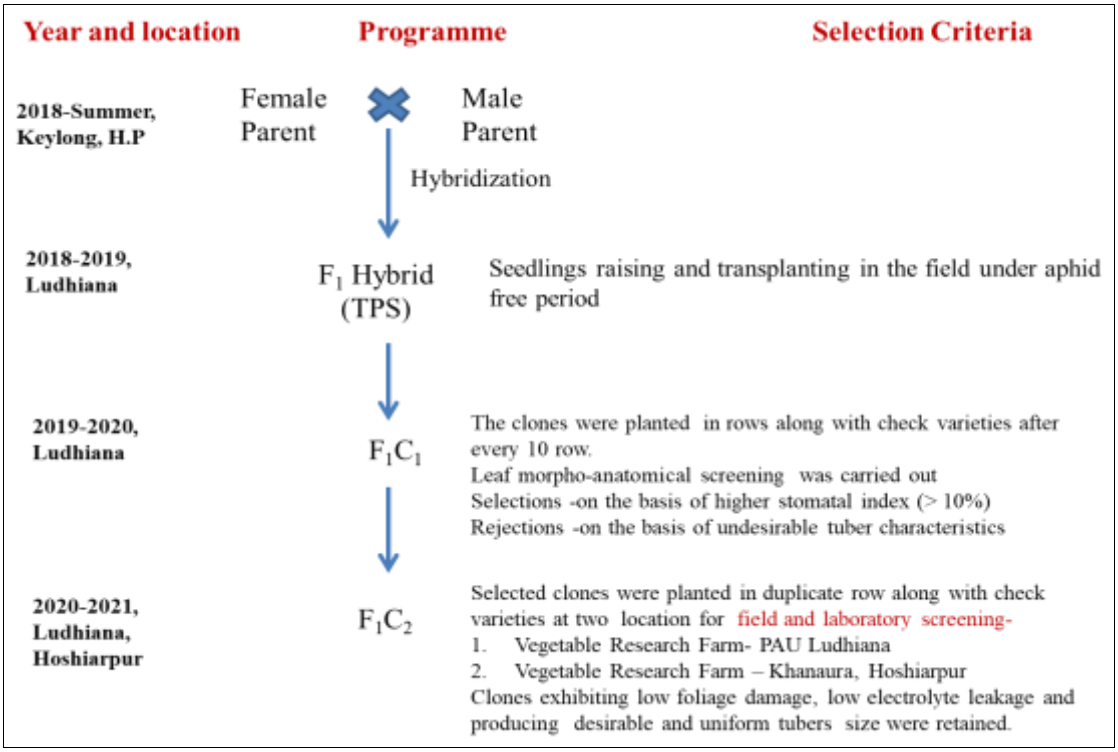


Figure 3.2: Flow chart presentation of generation and evaluation of potential frost tolerant potato clones

Table 3.3: Details of clonal evaluation and selection

Cross code	Crosses	Stage	Number of TPS/ tubers sown	No. of tubers germinated and survived	No. of tubers harvested	No. of tubers discarded at harvest	No. of tubers decayed during cold storage /sprouting	Tubers retained
PAU3	**J-2/19 × MS/7-645**	Seedling (F_1)	640	460	370	80	30	260
		F_1C_1	250	185	176	40	30	106
		F_1C_2	106	98	75	20	-	-
PAU7	**MS/7-645 × J-2/19**	Seedling (F_1)	370	300	243	90	55	98
		F_1C_1	98	61	58	15	2	41
		F_1C_2	41	39	35	-	-	-
PAU9	**MS/7-645 × CP-3765**	Seedling (F_1)	350	280	233	80	45	108
		F_1C_1	108	53	52	9	4	39
		F_1C_2	39	35	33	-	-	-

3.3.2.3.2.1 F_1C_1 generation

The selected clones were planted in rows along with check varieties after every 10 rows. At vegetative growth stage screening was carried out on the basis of leaf morpho-anatomical characters. And at harvesting stage, clones with misshaped tuber, deep eyes and crack tubers were rejected as these characters have a high repeatability over the generations.

3.3.2.3.2.2 F_1C_2 generation

In F_1C_2 generation, the clones possessing stomatal index more than 10% and desirable tuber characters were selected and planted in duplicate row along with the check varieties. The characterization and identification of potential frost tolerant potato clones were carried out at two different locations *viz.* Vegetable Research Farm- PAU Ludhiana and PAU-Vegetable Research Farm - Khanaura, Hoshiarpur.

At vegetative growth stage around 40-60 days after planting, these clones were subjected to low temperatures stress under field condition. The visual assessment for foliage damage and recovery from frost injury was performed at Khanaura according to the descriptions illustrated in Table 3.5. Subsequently, leaf samples were collected for determining the electrolyte leakage (%). At harvesting, clones producing desirable tuber characteristics *i.e.*, uniform tuber size and shapes were retained for further evaluation.

3.3.3 Evaluation and identification of potential frost tolerant clones on the basis of leaf morpho-anatomical traits

For the morpho-anatomical screening, the third or fourth fully expanded leaf counting basipetally was collected from three plants per clones. The excised leaf tissue was washed with distilled water to wash off any debris. Before sectioning, the leaflets were placed in distilled water to make the leaves turgid and fully expanded during sectioning. The leaf tissue was sectioned with a sharp surgical blade to obtain oblique sections of semi-permanent slides of appropriate (less than 5µm) thickness (Bisognin *et al* 2006). Glass slides and forceps were properly wiped off to remove any debris and dust. The thin film was peeled gently from both the adaxial and abaxial leaf surfaces using sharp blade. The fleshly peeled film was mounted in water on the glass slides and covered with a coverslips (Gitz and baker 2009). The prepared slide was observed under Optical Research Microscopy (Leica DM 5000B, Germany). The transverse section was obtained by cutting leaf from midrib in square shape, further into a very thin and fine section. The section was mounted on glass slides, covered with coverslips and viewed under Optical Microscope, (Leica DM 5000B, Germany)

Direct microscopic measurements of the thickness (length and width) of the palisade parenchyma and epidermal tissue at (20X) of the sampled leaves were recorded at several locations. The average length and width of the palisade cells per unit number of cells were

calculated in photomicrographs by direct microscopic measurements using the adaptable algorithm software of the Leica application suite program and also by using image J software (Pereira *et al* 2013, Yaun *et al* 2020).

3.3.3.1 Observations recorded

Table 3.4: Leaf morpho anatomical parameters recorded on abaxial, adaxial and transverse leaf surfaces of potato clones

S. No.	Observations	Magnification
1	Stomatal density (both surface)	20× (100µm)
2	Stomatal Index (%)	20× (100µm)
3	Trichome density (both surface)	10× (200µm)
4	Epidermal layer thickness	20× (100µm)
5	Palisade layer length	20× (100µm)
6	Palisade layer width	20 × (100µm)

3.3.4 Statistical Analysis

The collected data were subjected to analysis of variance (*ANOVA*) using Generalized Linear model procedures of SAS (Version 9.3, SAS Institute, Cary, NC, USA). The means were separated using Least Significant Difference (LSD, $p \leq 0.05$) test (Gomez and Gomez 1984). The data recorded for leaf morpho-anatomical traits were used for calculating the association between different traits by using SPSS software (Version 16.0, SPSS Institute, Chicago, IL, USA). The cluster analysis, principal component analysis (PCA) and biplot analysis were performed for genotypes and traits to study the relationships among the tested clones based on different traits by using JMP software(Version 9.3, SAS Institute, Cary, NC, USA).

3.3.5 Evaluation and identification of potential clones on the basis of their tolerance to membrane injury frost damage in field and growth and yield characterization

3.3.5.1 Frost damage

In 2020-21 cropping season, a subset of potato clones in F_1C_2 generation were evaluated in field under natural frost incidences at PAU-Vegetable research farm, Khanaura. During the months of December to January, the lowest minimum temperature was reached to 1.5℃ thus, causing foliage damages (Figure 4.28). Frost damage was visually assessed using a 0-9 scale (0= no damage; 9= all plants killed) after 2-3 days of frost incidence using methods as described in Luthra *et al* (2008) and Rizza *et al* (1994) with some modifications.

Table 3.5: Description of methods used to record frost damage (FD, %), recovery rate (RR) and frost tolerance score (FTS) in potato

Scale (0–9)	Foliage damage (%)	Scale (1–5)	Recovery rate	Scale (1–5)	Frost tolerance Score
0	No damage	1	Fast recovery (fully recovered within 5 days with undamaged primary leaf)	1	Highly tolerant
1	<10%	2	Moderate recovery (fully recovered after 7 days with damage only on tip)	2	Tolerant
2	11-20%	3	Slow recovery (fully recovered after 12 days with half leaf damaged)	3	Moderately tolerant
3	21-30%	4	Very slow (recovered only after 15 days)	4	Slightly tolerant
4	31-40%	5	No recovery	>5	Susceptible
5	41-50%				
6	51-60%				
7	61-70%				
8	71-80%				
9	>90%				

3.3.5.2 Membrane injury

Cell membranes are most sensitive to freezing stress and alterations in membrane function occur in the early stages of plant injury. Electrolyte leakage has been commonly used to detect the extent of membrane injury in stressed plants by measuring electrical conductance of a cell effusate.

Electrolyte leakage assay was carried out using the protocol described in (Cottee 2007 and Thalhammer *et al* 2014) with some modifications. A conductivity meter (Model 306, Systronics, Ahmedabad, Gujarat) was used for its determination.

$$\text{Electrolyte leakage (EL)} = \frac{\text{Initial conductivity}}{\text{Final conductivity}} \times 100$$

3.3.5.3 Growth and yield attributing traits

The growth and yield attributing traits of the clones were recorded at various stages of crop growth *viz.* plant height (cm), plant emergence (%) and plant vigour (scale 1-5), plant foliage structure (compact and semi-compact), numbers of sprouts per plant were recorded at vegetative growth stage.

Number of tubers per plant (TNPP, number of tubers per plant), tuber yield per plant (TYPP, g), tuber skin type and colour, tuber flesh colour, tuber shape and depth of tuber eyes were recorded after harvesting of tuber crop.

3.3.5.4 Statistical analysis

The collected data were subjected to analysis of variance (*ANOVA*) using Generalized Linear model procedures of SAS (Version 9.3, SAS Institute, Cary, NC, USA). The means were separated using Least Significant Difference (LSD, $p \leq 0.05$) test (Gomez and Gomez 1984). Pearson's correlation coefficient analysis was performed to study the association among growth and yield attributing traits by using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA).

CHAPTER IV

RESULTS AND DISCUSSION

This study was conducted with an overall aim of optimization of screening protocols for evaluation of frost tolerant potato clones, leaf morpho-anatomical traits associated with frost tolerance and expression profiling of putative frost tolerance genes. The investigation was conducted as series of three different experiments. The first experiment was conducted using four potato genotypes with varying ability of frost tolerance for optimization of screenings protocols under controlled conditions. The second experiment "Expression profiling of putative frost tolerance genes" was carried out in the laboratory of School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, Punjab. The third was experiment was carried out during the summer season, 2018 at PAU Research station Keylong, Himachal Pradesh for hybridization programme. The evaluation and identification of potential frost tolerant clones at its seedling and early clonal generations was carried during 2018-19; 2019-20 and 2020-21 at vegetable research farm, PAU, Ludhiana and PAU-vegetable research farm, Khanaura, Hoshiarpur.

The results have been presented and discussed experiment wise as follows:

4.1 Optimization of controlled environment screening protocols for rapid identification of the frost tolerant clones

4.2 Transcript profiling of dehydrin (*dhn1*), heat shock protein/cognate (*hsp70*), and Stearoyl-acyl carrier protein desaturase (*SAD*) genes in selected tolerant and susceptible potato clones

4.3 Generation and characterization of potential frost tolerant potato clones on the basis of leaf morpho-anatomical and physiological traits

4.1 Optimization of controlled environment screening protocols for rapid identification of the frost tolerant clones

Potato is sensitive to low temperature stress. Moreover, due to various complexities of genetic control of these traits, efforts on breeding for frost tolerance could achieve limited success (Palta and Li *et al* 1979). The genetic variability in potato germplasm for frost tolerance in India had been reported by Kang *et al* (2007) and Luthra *et al* (2008). However, the assessment of frost tolerance under natural conditions was found to be complex because of natural variability in the intensity and timing of frost events (Levitt 1980, Li and Palta 1978). Further, there is also a lack of precise controlled environment freezing tests which can be used to complement the natural field's selection criteria. Thus, rapid and reliable screening methods/techniques to characterize the available frost tolerant and susceptible potato germplasm are urgently required (Stone *et al* 1993). The study was conducted using various techniques, such as leaf gas exchange responses, chlorophyll fluorescence electrolyte leakage

assay and leaf morpho anatomical traits to optimize the screening techniques for frost tolerance in potato.

4.1.1 Leaf gas exchange response

In this study, the leaf gas-exchange parameters, such as net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and water use efficiency (WUE) of four genotypes were measured at different time spans *i.e.*, at initial stage 30 days after planting (30DAP) before any treatment, 10 days after acclimation and non-acclimation and at different days after treatment during recovery *i.e.,* 2 days after treatment (2DAT), 7 days after treatment (7DAT) and 15 days after treatment (15 DAT).

At initial stage (30DAP), the potato genotypes exhibited significant differences for P_N and g_s parameters while for C_i and WUE no significant differences were observed among the genotypes. Among the genotypes, MS/7-645 and J-2/19 exhibited the highest values for P_N and g_s respectively (Table 4.1). Furthermore, the genotype MS/7-645 recorded the highest values for C_i and WUE though the differences were not significant (Table 4.1).

Table 4.1: Differences among potato genotypes for leaf gas exchange responses at initial stage (30 DAP)

Genotype	P_N (µmol/m^2/sec)	g_s (mmol/m^2/sec)	C_i (µmol Co_2 /mol)	WUE
K. Anand	22.1	0.40	266.4	4.10
J-2/19	22.0	0.44	265.4	4.15
MS/7-645	23.4	0.41	269.1	4.28
K. Pukhraj	21.0	0.37	268.9	4.01
LSD ($p \leq 0.05$)	0.99	0.19	4.26	0.33

P_N: Net photosynthetic rate; g_s: Stomatal conductance; WUE: water use efficiency rate, C_i: Intercellular Co_2 concentration

Leaf gas exchange parameters were used as indicators for detecting effects of stress on plants, since photosynthesis is closely related to plant growth and yield traits (Rivero *et al* 2014, Sharma *et al* 2019). Photosynthesis is a critical physiological process that primarily contributes to energy and carbon inputs essential for plant development and growth (Sharma *et al* 2020, Meena *et al* 2021).

Across genotypes**,** growing conditions had significant impact on all the leaf gas exchange parameters *viz*., P_N, g_s, C_i and WUE (Table 4.2 and Plate 4). Overall, cold acclimation condition significantly reduced P_N and WUE by 65.8% and 17.5%, respectively as compared with that of non-acclimated condition. However, C_i exhibited an increase 16.5% under cold acclimation condition (Table 4.2). Across acclimation conditions, the genotypes also exhibited significant differences for P_N, g_s, C_i and WUE parameters. Genotype J-2/19

Plate 3: Field evaluation of potato genotypes for frost tolerance under natural frost incidences

Plate 4: Effect of cold acclimation conditions on potato genotypes

recorded the highest values of $P_{N,}$ g_s, C_i and WUE as compared with that of Kufri Anand and MS/7-645. While, the lowest $P_{N,}$ g_s, C_i and WUE values were recorded in Kufri Pukhraj (Table 4.2).

Table 4.2: Net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and water use efficiency (WUE) of potato genotypes as influenced by cold acclimation

Treatment	P_N (µmol/m^2/sec)	g_s (mmol/m^2/sec)	C_i (µmol Co_2 /mol)	WUE
Condition (C)				
ACC	5.28^b	0.21^b	315.7^a	2.91^b
NACC	15.47^a	0.35^a	263.6^b	3.53^a
Genotype (G)				
K. Anand	10.31^{ab}	0.28^{ab}	287.9^b	3.20^b
J-2/19	10.95^a	0.30^a	295.5^a	3.57^a
MS/7-645	10.37^{ab}	0.28^{ab}	289.7^{ab}	3.35^{ab}
K. Pukhraj	9.88^b	0.27^a	285.4^b	2.77^c
ANOVA				
C	***	NS	***	***
G	***	***	***	***
C × G	***	NS	**	***

ACC: Cold acclimation; NACC: non-acclimation; ***, ** –significant differences at $p \leq 0.001$ and 0.05, respectively; NS –not significant at $p \leq 0.05$

The conditions × genotype interactions showed significant differences for all the leaf gas exchange parameters, except g_s indicating that the leaf gas exchange responses to low temperature condition varied among the genotypes across the different growing conditions (Table 4.2). The low temperature has previously been reported to adversely affect the various physiological characteristics, particularly photosynthetic efficiency more significantly than respiration (Steffen *et al* 1989, Sharma *et al* 2020). Moreover, *Solanum tuberosum* is a frost sensitive crop species. Consequently, it reduces photosynthetic ability and yield, mainly caused by stomatal limitations, non-stomatal limitations and damages to the photosynthetic apparatus. Similar results were reported in rice (De Freitas *et al* 2019), tomato (Zhou *et al* 2018) and others crops (Sharma *et al* 2020). Thus, improving photosynthetic efficiency might help plants to resist various abiotic stresses including low temperature stress (Sharma *et al* 2020). This information will further help in understanding and exploring the various factors responsible for imparting or increasing freezing tolerance in potato genotypes.

4.1.2 Leaf gas-exchange response during recovery days

Overall, the freezing treatment resulted in significant changes in P_N and g_s under both the treatment time span 1.0 and 1.5 h (Figure 4.2). The potato genotypes exhibited significant differences for P_N and g_s parameters under both the treatment time spans.

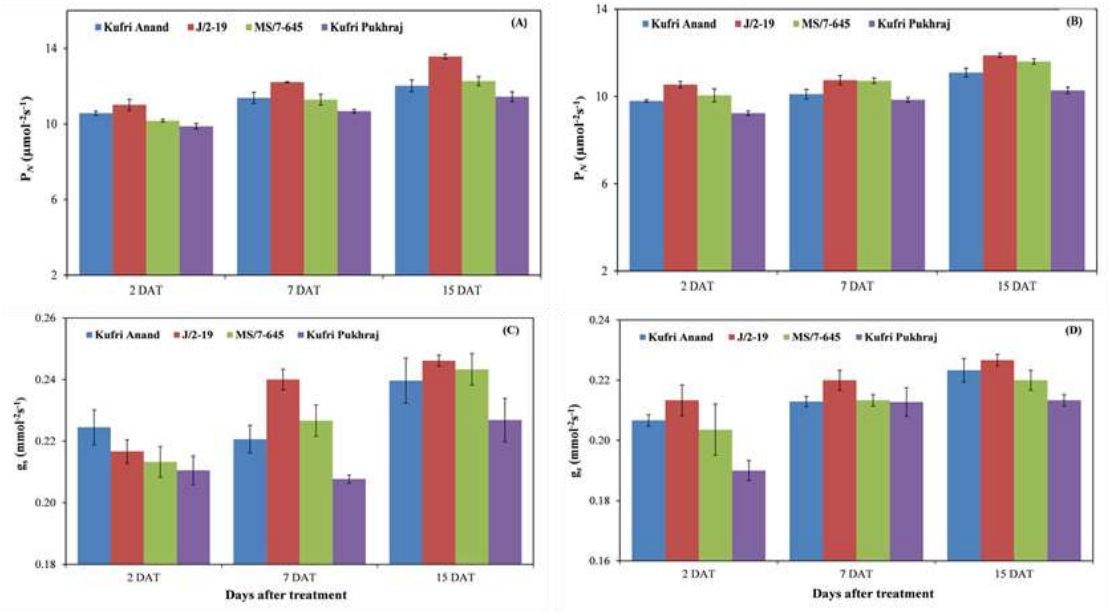


Figure 4.1: Effect of freezing treatments on the leaf gas exchange parameters measured in non-acclimated plants during recovery at different intervals [2 days after treatment (2DAT), 7 days after treatment (7DAT) and 15 days after treatment (15DAT)]. (A) & (B) –P_N for 1.0 h and 1.5 h and (C) & (D) –g_s for 1.0 h and 1.5 h

During recovery, under both treatment time spans P_N and g_s parameters followed a gradual increase over the recovery days *i.e.*, 2DAT, 7 DAT and 15DAT, however the rate of increase significantly varied within recovery days and across the genotypes (Figure 4.1). While, at 1.0 h treatment time span g_s value remained unchanged on 7th day of recovery in genotypes Kufri Anand and Kufri Pukhraj. Over the treatment time spans, on 2nd day of recovery the tolerant genotype J-2/19 recorded highest P_N and g_s values as compared with that of genotypes Kufri Anand and MS/7-645 whereas, the lowest values for both the traits were recorded in Kufri Pukhraj (Figure 4.1 and Plate 5).

On 7th day of recovery, overall the genotypes exhibited considerable increases in values for both the traits. On an average value of P_N and g_s parameters increased by 8.7% and 4.5%, respectively under both the treatment time spans as compared with the values of 2nd day. Furthermore, on 15th day of recovery a significant increase in values of P_N (15.8 %) and g_s (9.6%) was recorded on an average across the genotypes (Figure 4.1 and Plate 6).

Freezing stress primarily caused decrease in P_N and g_s (Figure 4.1), suggesting that it caused stomatal closure and prevented water loss at the expense of CO_2 for photosynthesis

Plate 5: Rejuvenation of the potato genotypes on 2nd day of recovery after freezing treatment for two spans of time (A) 1.0 h; (B) 1.5 h.

Plate 6: Rejuvenation of the potato genotypes on 15th day of recovery after freezing treatment for two spans of time (A) 1.0 h; (B) 1.5 h.

(De Freitas *et al* 2019, Sharma *et al* 2020). A significant genotypic variability for P_N and g_s traits was observed in potato genotypes which were subjected to freezing temperature at different treatment time spans (Figure 4.1). During recovery, the photosynthetic efficiency and stomatal conductance of all the genotypes were improved and hence resulted in more efficient and rapid recovery of plants. However, considerable differences in rate of recovery were observed among the genotypes. The tolerant genotype J-2/19 maintained the higher P_N and g_s values as compared to sensitive genotype Kufri Pukhraj (Figure 4.1). Thus, further information on leaf gas exchange of potato genotypes will enhance understanding of their adaptation mechanisms to low temperature conditions particularly at recovery which can be applied to implement frost tolerant breeding strategies.

4.1.3 Chlorophyll fluorescence analysis

Maximum quantum yield of PSII (Fv/Fm), effective quantum yield of PSII (ΔF/Fm'), electron transport rate (ETR) of four genotypes were measured at different time spans *i.e.*, at initial stage 30 days after planting (30DAP) before any treatment, 10 days after acclimation and non-acclimation and at different days after treatment during recovery *i.e.*, 2 days after treatment (2DAT), 7 days after treatment (7DAT) and 15 days after treatment (15 DAT) using the portable photosynthesis system LI-COR for all measurements as described in (Thornton *et al* 1996, Sharma *et al* 2019).

For chlorophyll fluorescence parameters, the differences among genotypes were also not significant for Fv/Fm, ΔF/Fm' and ETR (Table 4.3). A numerical higher values for Fv/Fm and ΔF/Fm' parameters were recorded in genotype J-2/19 as compared to that of genotypes, Kufri Anand, MS/7-645 and Kufri Pukhraj. Similarly, for electron transport rate (ETR) a numerically highest value was recorded in genotype MS/7-645 (Table 4.3).

Table 4.3: Differences among potato genotypes for chlorophyll fluorescence at initial stage (30 DAP)

Genotype	Fv/Fm	ΔF/Fm'	ETR
K. Anand	0.81	0.43	102.34
J-2/19	0.81	0.46	105.58
MS/7-645	0.80	0.44	106.26
K. Pukhraj	0.80	0.44	105.06
LSD ($p \leq 0.05$)	0.26	0.89	0.49

Fv/Fm: maximum quantum yield of PSII; ΔF/Fm': Effective quantum yield of PSII; ETR: Electron transport rate

Across genotypes, growing conditions had significant impact on Fv/Fm, Fv'/Fm' and ETR (Table 4.4). Overall, under cold acclimated condition, a significant reduction in Fv/Fm

(6.2%), ΔF/Fm' (7.0%) and ETR by (51.4%) was observed as compared with that of non-acclimation condition (Table 4.4). Similarly, in wheat, Sharma *et al* (2015) reported significant differences for Fv/Fm between stressed and unstressed conditions along with genotypic differences for others physiological parameters.

The conditions × genotype interactions did not showed significant differences for all the chlorophyll fluorescence parameters (Table 4.4). However, De Freitas *et al* (2019) and Sharma *et al* (2015) had utilized the chlorophyll fluorescence parameters along with others leaf gas exchange for phenotyping rice and wheat for various abiotic stress, respectively. Overall, genotypes did not exhibit any significant differences for all the chlorophyll fluorescence parameters, such as Fv/Fm, and ETR. However, the tolerant genotypes, J-2/19 and Kufri Anand recorded numerically higher values for ΔF/Fm' and ETR parameters as compared to that of sensitive MS/7-645. Meanwhile, the sensitive genotype Kufri Pukhraj recorded numerically lowest values for all the parameters (Table 4.4).

Table 4.4: Maximum quantum yield (Fv/Fm), effective quantum yield (ΔF/Fm'), and electron transport rate (ETR) of potato genotypes as influenced by cold acclimation

Treatment	Fv/Fm	ΔF/Fm'	ETR
Condition (C)			
ACC	0.76^{b}	0.35^{b}	25.9^{b}
NACC	0.81^{a}	0.42^{a}	53.3^{a}
Genotype (G)			
K. Anand	0.79^{a}	0.39^{a}	39.78^{a}
J-2/19	0.79^{a}	0.39^{a}	39.75^{a}
MS/7-645	0.79^{a}	0.38^{a}	39.72^{a}
K. Pukhraj	0.78^{b}	0.37^{a}	39.08^{a}
ANOVA			
C	***	***	***
G	***	NS	NS
C × G	NS	NS	NS

ACC: Cold acclimation; NACC: non-acclimation; *** –significant differences at $p \leq 0.001$; NS –not significant at $p \leq 0.05$

In addition to the well-established traditional phenotyping method, electrolyte leakage (Stone *et al* 1993), chlorophyll fluorescence had been broadly employed as a rapid and reproducible alternative method that can be used to assess the efficiency of PSII under environmental stress conditions (Prášil *et al* 2007, Mishra *et al* 2011, Mattila *et al* 2020). The

fluorescence parameter Fv/Fm is an indicator of the maximum photochemical efficiency of PSII. It is considered as an appropriate assay for plant cold sensitivity and tolerance, due to inherent tolerance or physiological acclimation. Further, it has been shown to produce results that correlate well with other methods (Rizza *et al* 2001, Mishra *et al* 2011, Badeck and Rizza 2015). Thus, to further explore the behavior of PSII in non-acclimated plants of potato under freezing stress during recovery days chlorophyll fluorescence parameters, such as Fv/Fm, ΔF/Fm' and ETR were investigated.

4.1.4 Chlorophyll fluorescence on recovery days

During recovery after exposure to freezing treatments, the effects of cold stress on non–acclimated plants were measured as chlorophyll fluorescence parameters *i.e*. Fv/Fm, ΔF/Fm' and ETR. Among genotypes, the freezing treatment did not cause significant changes in the Fv/Fm ratio, except in genotype MS/7-645. However, a significant reduction in value of Fv/Fm was observed when the treatment time span was increased from 1.0 h to 1.5 h. Furthermore, considerable variations in ΔF/Fm' and ETR were also evident among genotypes and under different treatment durations (Figure 4.2). Over the recovery days, the genotypes also exhibited considerable differences for Fv/Fm, ΔF/Fm' and ETR parameters. On the 2nd day of recovery genotype J-2/19 recorded the highest Fv/Fm, ΔF/Fm' and ETR values compared to Kufri Anand and MS/7-645. However, Kufri Pukhraj (sensitive genotype) recorded lowest values for all parameters, except for Fv/Fm parameter in 1.5 treatment hours (Figure 4.2 and Plate 5).

On 7th day of recovery, overall all the genotypes exhibited considerable increases in values of all the parameters. On an average values of Fv/Fm, ΔF/Fm' and ETR parameters were increased by 2.5%, 7.2% and 5.3%, respectively at both treatment time spans as compared with the values of 2nd day (Figure 4.2). Further, genotypes J/2-19, Kufri Anand and MS/7-645 exhibited considerably higher recovery rate as compared to that of sensitive genotype Kufri Pukhraj (Figure 4.3). Furthermore, on 15th day of recovery, a significant increases in values of Fv/Fm (3%), ΔF/Fm' (15%) and ETR by (12%) was observed on an average across the genotypes. Thus, the recovery rate of the plants was reflected by considerable increases in chlorophyll fluorescence parameters. However, it was also observed that there is a significant effect of treatment time span on the recovery time period.

The significant differences between treatment durations for the Fv/Fm, ΔF/Fm' and ETR (Figure 4.2) revealed that the photochemical apparatus was damaged by the duration of freezing exposure indicating that PSII activity in potato was not stable under freezing exposures. Similarly, Rapacz (2007) also mentioned the effects of freezing on photosynthetic apparatus in winter wheat. Indicating that frost exposure had significant impact on activity and stability of PSII in potato under low temperature stress. Similarly, in wheat significant differences for Fv/Fm between stressed and unstressed conditions along with genotypic

differences for others physiological parameters had been reported (Sharma *et al* 2015). Genotypic differences for Fv/Fm, ΔF/Fm' and ETR under freezing stress had been reported in barley (Dai *et al* 2007), winter wheat (Rapacz 2007) and Fv/Fm in wheat and oats (Rizza *et al* 2001, Sharma *et al* 2015).

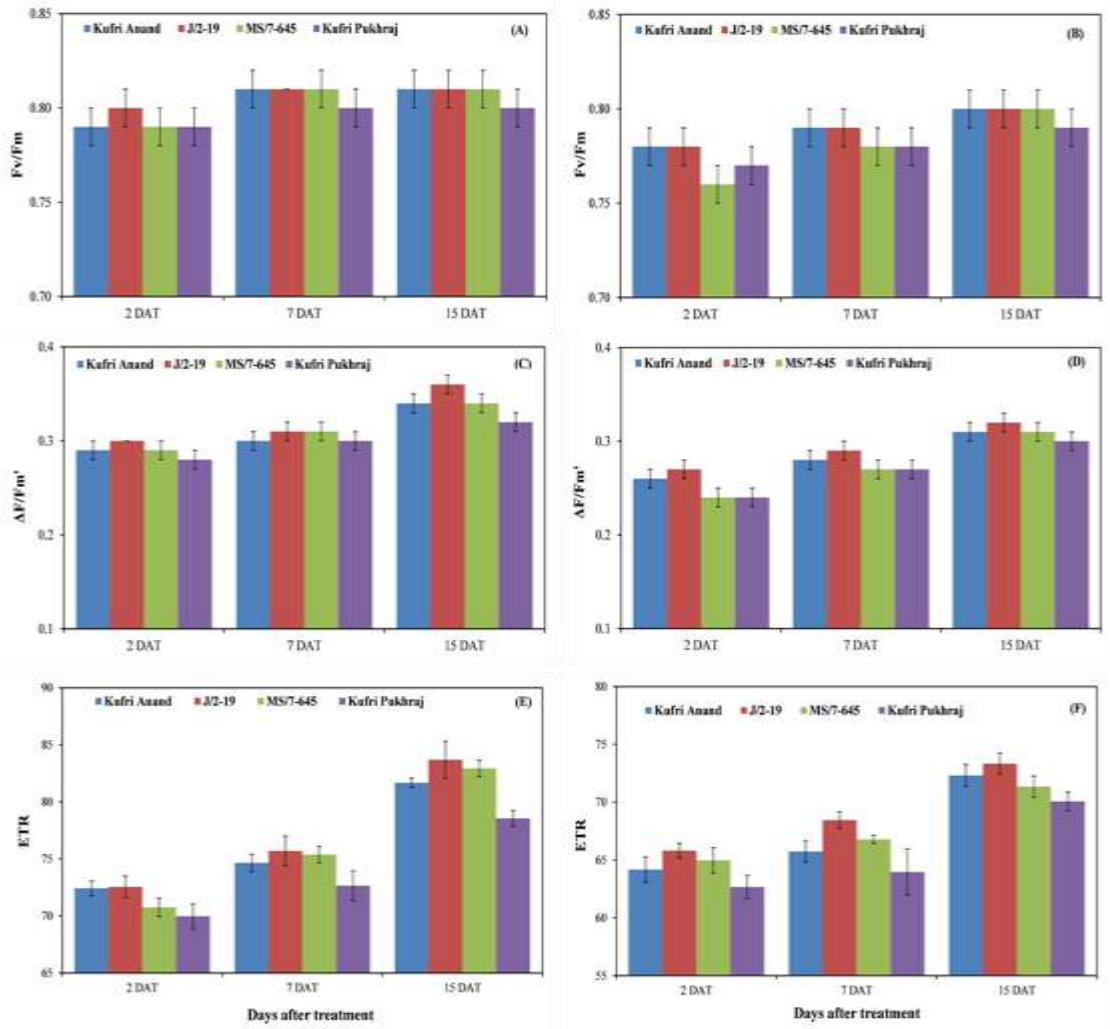


Figure 4.2: Effect of freezing treatments on the chlorophyll fluorescence parameters measured in non-acclimated plants during recovery at different intervals [2 days after treatment (2DAT), 7 days after treatment (7DAT) and 15 days after treatment (15DAT)]. (A) & (B) – Fv/Fm for 1.0 h and 1.5 h; (C) & (D) – ΔF/Fm' for 1.0 h and 1.5 h and (E) & (F) – for ETR 1.0 h and 1.5 h. Values are presented as means and SE.

In terms of recovery rate the treatment durations caused considerable differences among the genotypes. The tolerant genotype J-2/19 took less time to resume the growth back from freezing treatment as compared to sensitive genotype Kufri Pukhraj. Freezing stress caused remarkable decrease in ΔF/Fm' and ETR however, during recovery rapid increase in values of these parameters were observed. Similar results were reported in winter barley (Dai *et al* 2007) indicating that the capacity of electron transport was inhibited by freezing stress

and damage in chlorophyll reaction centers was reversible. Moreover, the recovery was more pronounced in J-2/19 and Kufri Anand at 7 DAT, but a rapid recovery rate was also observed in MS/7-645 (Figure 4.3). Furthermore, this method was useful in identifying the genotypes with intermediate behavior (MS/7-645), which at 1 h exhibited a response similar to the tolerant genotype Kufri Anand and at 1.5 h similar to the susceptible genotype (Kufri Pukhraj).

4.1.5. Membrane injury

Overall, the freezing treatment induced significant variations in the development of visual symptoms and membrane injury both in the cold acclimated as well as non–acclimated plants. Among low-temperature stress duration treatments, though visual symptoms of cold stress, such as loss in turgidity, in 1.0 h time span treatment at -2°C wilting and drooping of leaves were exhibited, but no significant injury to leaf tissue was recorded. However, with extended time span of cold treatment to 1.5 h incited significant variations in leaf damages, particularly the leaves of cold acclimated plants were entirely damaged with freezing injury.

To evaluate the effect of freezing stress on membrane injury, electrolyte leakage of both the cold acclimated and non–acclimated plants exposed to 1.0 and 1.5 h time span at -2°C was determined. The freezing treatments induced a significant variation in cell membrane injury in all the tested potato genotypes. Across the cold acclimated and non-acclimated plants conditions, treatment time span had significant impact on membrane injury (Figure 4.3). Both the cold acclimated and non–acclimated plants of genotype J-2/19 recorded significantly less electrolyte leakage content as compared to that of the genotypes, Kufri Anand, MS-7/645 and Kufri Pukhraj (Figure 4.3). Hence, the results suggested that the cell membrane injury increased with increase in exposure time of plants to cold stress conditions. Among the bio-physiological traits associated with frost we focused on the cell membrane and PSII activity of plants. Freezing stress cause a serious constraint to integrity and fluidity of membranes (Rizza *et al* 2001, Wu *et al* 2019). In plants freezing stress often induced leakage of ions from the cells and the stability of membrane can be assessed as an indicator of cellular damage (Li *et al* 1979, Prášil *et al* 2007, Shi *et al* 2019).

In current study, the results showed that that EL was lower in non-acclimated than cold acclimated plants across both treatment durations. However, the values were higher in cold acclimated than non-acclimated plants of all genotypes (Figure 4.3). The results suggested that cold acclimation does not help in enhancing the stability of cell membrane in response to low temperature stress. Rather plants become more pale and weaker than control thus, showing more membrane damage. However, the tolerant genotype J-2/19 showed more stability in membrane damage than the cold sensitive genotypes, Kufri Pukhraj and MS/7-645 (Figure 4.3).

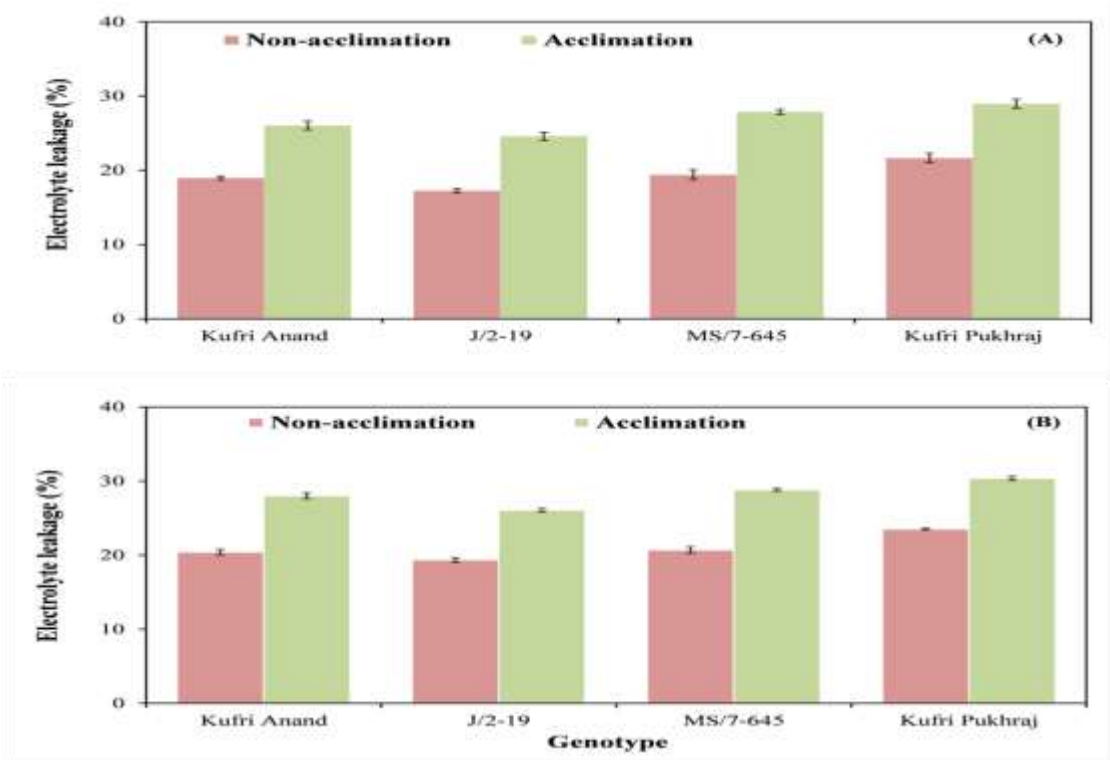


Figure 4.3: Membrane injury estimated as electrolyte leakage (%) in cold acclimated and non-acclimated plants of potato genotypes exposed to -2℃ (freezing treatment) for two spans of time (A) 1.0 h; (B) 1.5 h. Values are presented as means and SE.

Overall, across genotypes the membrane damage significantly increased with increased in time span of freezing treatment at -2°C. Indicating that freezing durations had significant impact on evaluation of genotypes for freezing tolerance as depicted by membrane damage. Wu *et al* (2019) suggested that the integrity of cell membranes was retained at low temperatures by maintaining the lipid peroxidation system, osmotic potential of cell membranes and permeability thus, leading to a reduction in the damage caused by freezing. Furthermore, measuring membrane damage made it possible to appraise differences more accurately in the extent of stress caused injury. Our results suggested that EL can be a useful tool to be integrated in a breeding programme as a testing procedure of frost tolerance.

4.1.6 Leaf morpho anatomical traits

The genotypes exhibited a significant variation in the leaf morpho-anatomical traits, such as stomatal index on the adaxial leaf surface, trichome density on adaxial leaf surface and palisade parenchymatous cell length (Figure 4.4). The genotype, CP-3765 recorded the highest (17.6%) stomatal index on adaxial leaf surface as compared to that of genotypes, MS/7-645 and J-2/19 (Figure 4.4). Previously, Palta and Li (1979) reported a higher stomatal density on adaxial leaf surface of the hardy genotypes with freezing tolerance in potato. However, Aslamarz and Vahdati (2010) recorded a significantly lower value of stomatal

density in the leaves of walnut cultivars exhibiting hardy response to frost stress. Furthermore, the stomatal density was used as a best initial screening characteristic for classifying the cultivars of *Pistiachio atlantica* for drought resistance (Belhadj *et al* 2011).

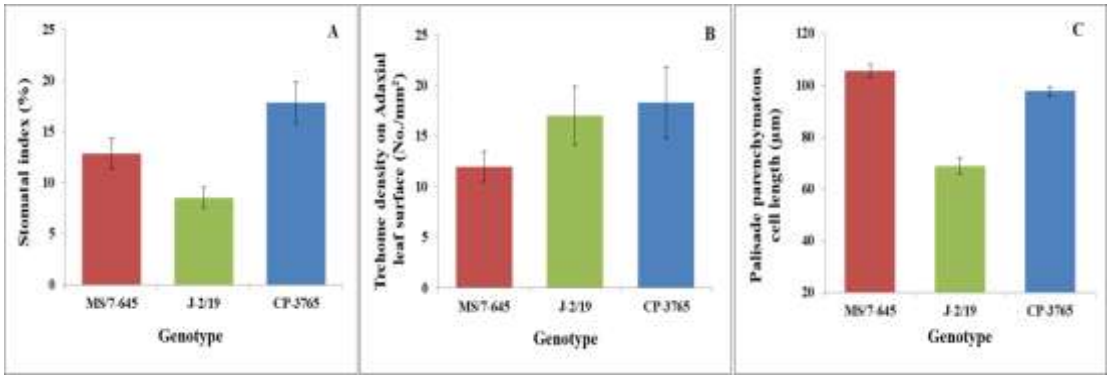


Figure 4.4: Variation in leaf morpho-anatomical traits of potato genotypes MS/7-645, J-2/19 and CP-3765. A) Stomatal index (%); B) Trichome density on adaxial leaf surface (no/mm^2) and C) Palisade parenchymatous cell length (µm). Values are presented as means and SE.

Similarly, the highest density of trichomes (18.3 no/mm^2) on the adaxial surface was recorded in the genotype CP-3765 followed by in genotype J-2/19 (17.0 no/mm^2) (Figure 4.4). The trichomes act as physical and chemical block agents and play an important role in leaf thermoregulation (Bisogenin *et al* 2006, Bickford 2016). Moreover, it is also beneficial in inducing resistance against various biotic and abiotic stresses (Wagner *et al* 2004, Kim *et al* 2012). Likewise, the genotype MS/7-645 recorded the highest (105.6 µm) palisade parenchymatous cell length. Whereas, the genotypes, J-2/19 and CP-3765 exhibited smaller and compactly arranged palisade parenchyma cells (Figure 4.4). Indicating that occurrence of variation for palisade parenchymatous cell thickness might have a potential role for imparting frost tolerance in potato genotypes (Bisgonin *et al* 2006, Kaur 2020). Moreover, the palisade parenchymatous cell dimensions are genetically transmittable characters (Estrada 1982).

Overall, the findings of this study are in consistent with earlier studies that the potato genotypes exhibit significant variations for the leaf morpho anatomical traits associated with frost tolerance. Although various screening techniques based on physiological responses associated with freezing tolerance are available. However, these techniques involve certain limitations, such as difficulties in handling of instrument, requirement of highly skilled labour, sensitivity of instrument to environmental conditions, and moreover, screening of large populations in field conditions is quite challenging and time consuming. Therefore, among the studied screening techniques the leaf morpho anatomical characteristics would be easily integrated as phenotyping technique for frost tolerance into potato breeding. Thus, this technique may possibly be utilized to identify genetic variations in the segregation populations at early stages of crop growth in the early clonal generations.

4.2 Transcript profiling of dehydrin (*dhn1*), heat shock protein/cognate (*hsp70*), and stearoyl- acyl carrier protein desaturase (SAD) genes in selected tolerant and susceptible potato clones

The three genotypes J-2/19 (tolerant), MS/7-645 (moderately susceptible) and Kufri Pukhraj (susceptible) were selected based on their response to cold stress in field condition (Ludhiana, 2016-17). Plants were grown at a temperature of (20±2℃) with a 12/13 hours photoperiod and a light intensity of approximately 600-800 µ mol $m^{-2}s^{-1}$. All the plants were divided randomly into two equal sized groups. One group for non–acclimation (NACC) and another group of plants to achieve cold acclimation (ACC), transferred in another chamber, temperature was lowered to 4±2℃. Further, after 10 days of treatments, the plants were exposed to freezing temperature of (-1℃) in dark conditions for three different time spans of 0.5h, 1.0 h and 1.5 h.

RNA was extracted from leaf of potato plants at different treatment stages. RNA quantity and quality was determined by using Nano drops and on 1.2% agarose gel (Plate 7). The cold acclimated and non-acclimated potato plants of three genotypes were analysed for expression of *HSP70, Dehydrin* and *SAD* genes through RT-PCR using gene specific primers (Table 4.5). RNA integrity was verified and revealed two predominant fragments corresponding to 28S and 18S RNA (Plate 7). As a preliminary step, cDNA was validated using 26S r RNA- based universal internal control gene specific primers showing an anticipated 534 bp fragment confirming authenticity of cDNA (Plate 8).

Table 4.5: Primer sequences of candidate reference and specific genes

S. No	Gene	Primer name	Primer sequences F/R
1	*26S r RNA*	26S r RNA	CACAATGATAGGAAGAGCCGAC CAAGGGAACGGGCTTGGCAGAATC
2	*EF1 α*	EF1 alpha	GATGGTCAGACCCGTGAACA CCTTGGAGTACTTCGGGGTG
3	*HSP70*	StHSP70-17	ACCTCTTTCCCTTGGTCTGG ACCAGGTTGATTGTCGGAGT
4	*Dehydrin*	dhn1	AAACTGACGAGTATGGCAACC CATCATCCTCGGAAGAGCTG
5	*SAD*	SAD3	CTCCTTCTGTGGAAGGTGGA TCAGGAATTTCCTTGCACCT
6	*SAD*	Qsad Qsad	GTCCGTGTTCACCACTCAGA GCGGCATGGAATGAGTAACT

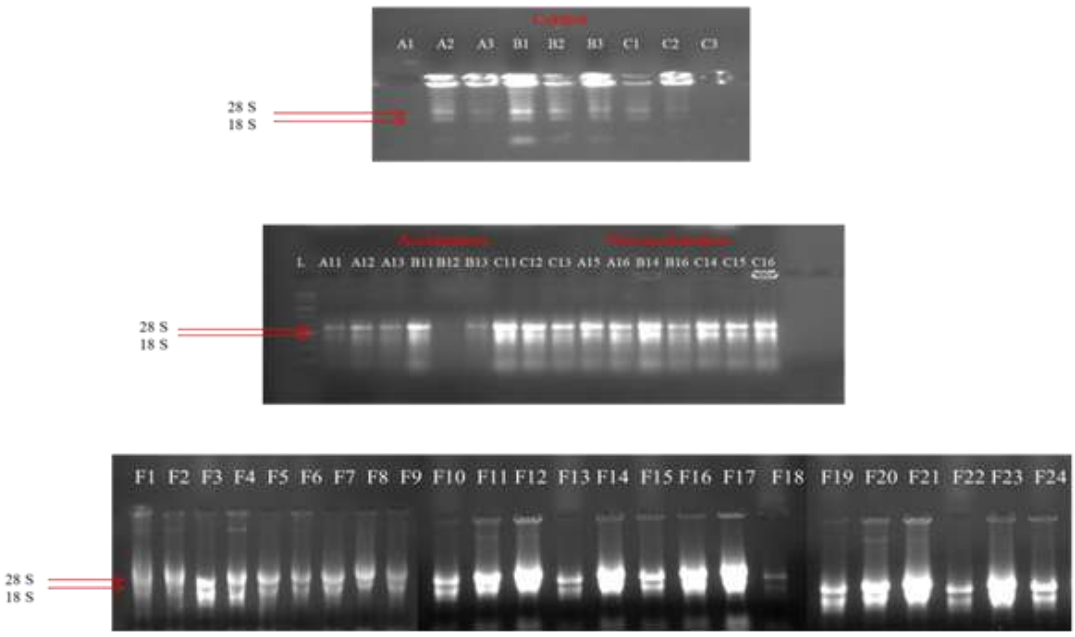


Plate 7: RNA integrity verification

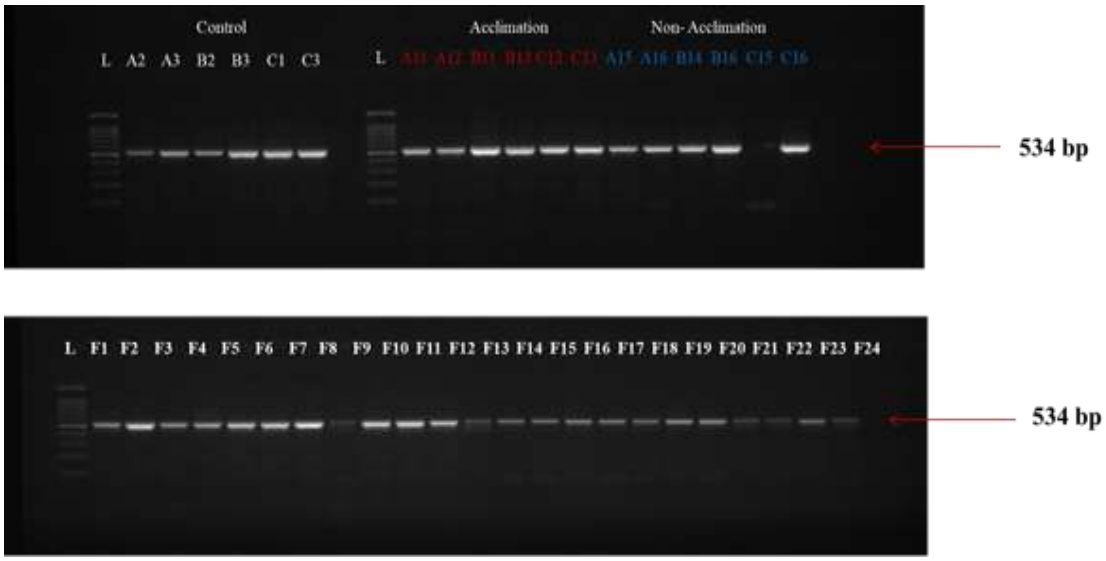


Plate 8: Confirmation of cDNA with 26S rRNA universal gene specific primers

The numbers A11- C12 and A-15-C16 represents cold acclimated and non-acclimated plants of three genotypes in replicates; F1-F24 represents low temperature (-1℃) treated plants for different time spans *i.e.*, 0.5 h, 1.0 h and 1.5 h L refers to 100 bp DNA ladder (SMOBIO, Cat No. DM1100)

4.2.1 Calibration of qRT-PCR and generation of standard curves

The standard curves for *Elongation factor 1 alpha*, *SAD* and *HSP70* were generated individually on five 10-fold dilutions (10, 0.1, 0.01, 0.001 and 0.0001 ng/reaction) of cDNAs of cold treated and non-treated plants for validation of comparative C_t (threshold cycle) method for relative gene quantitation. The results displayed that mean C_t values were linear over range of dilutions in standard curves, showing slope of -2.40 and -3.15 and reaction performance with PCR efficiency of 160.8% and 107.9%, for *SAD* and *HSP 70* gene, respectively (Figure 4.5) indicating low degree of experimental variation. The melt curve analysis for all the four genes also displayed a single peak in all genotypes that ensured high specificity of reaction (Figure 4.6).

The cDNAs, from three potato genotypes with cold treatment along with control were amplified using qRT-PCR primers for *Elongation factor 1 alpha, Dehydrin, SAD* and *HSP70*. A single peak for each primer indicated the specificity of reaction (Figure 4.6). The expression of *HSP70, Dehydrin 1, SAD* and was normalized using *Elongation factor 1 alpha*. The comparative transcript level was calculated using delta delta C_t ($\Delta\Delta C_t$) method (Livak and Schmittgen 2001). To analyze the general trend of gene expression under different treatment conditions fold change was calculated using respective control for all the genotypes.

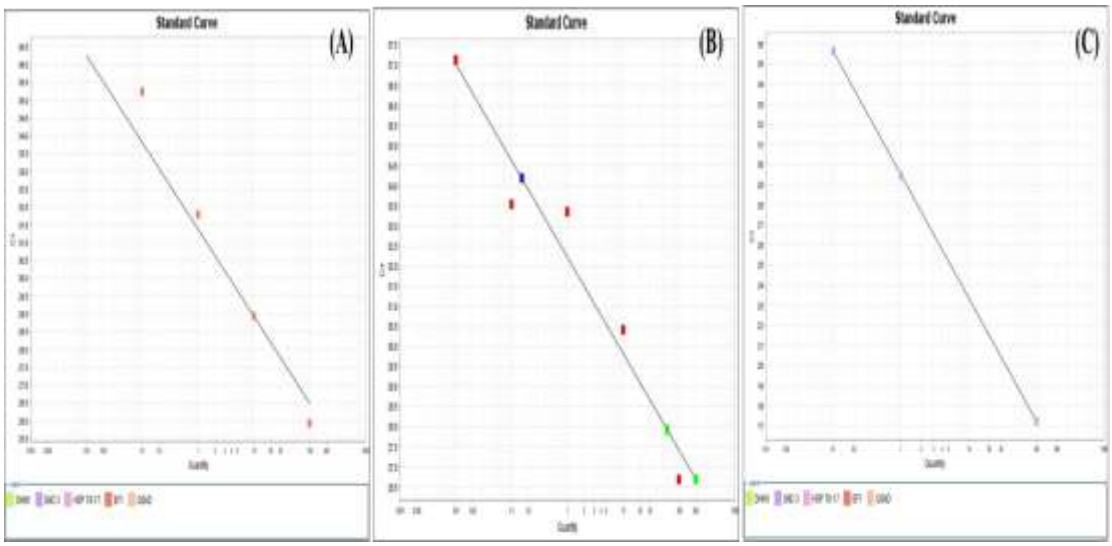


Figure 4.5: Standard curve analysis of (A) *Elongation factor 1 alpha*; (B) *hsp70* and (C) *SAD* genes

4.3.2 Gene expression studies of the three cold responsive gene(s)

The qRT-PCR experiments were conducted using qPCR Master Mix (Takara) with *Elongation factor 1 alpha* as internal reference and other gene specific primers (Table 4.5) In our present study, the effect of different growing conditions (cold acclimation and non-acclimation) and freezing treatment durations on expression level of *HSP 70*, *Dehydrin* and *SAD* genes was studied in three different potato genotypes.

MS/7-645	Kufri Pukhraj	J-2/19

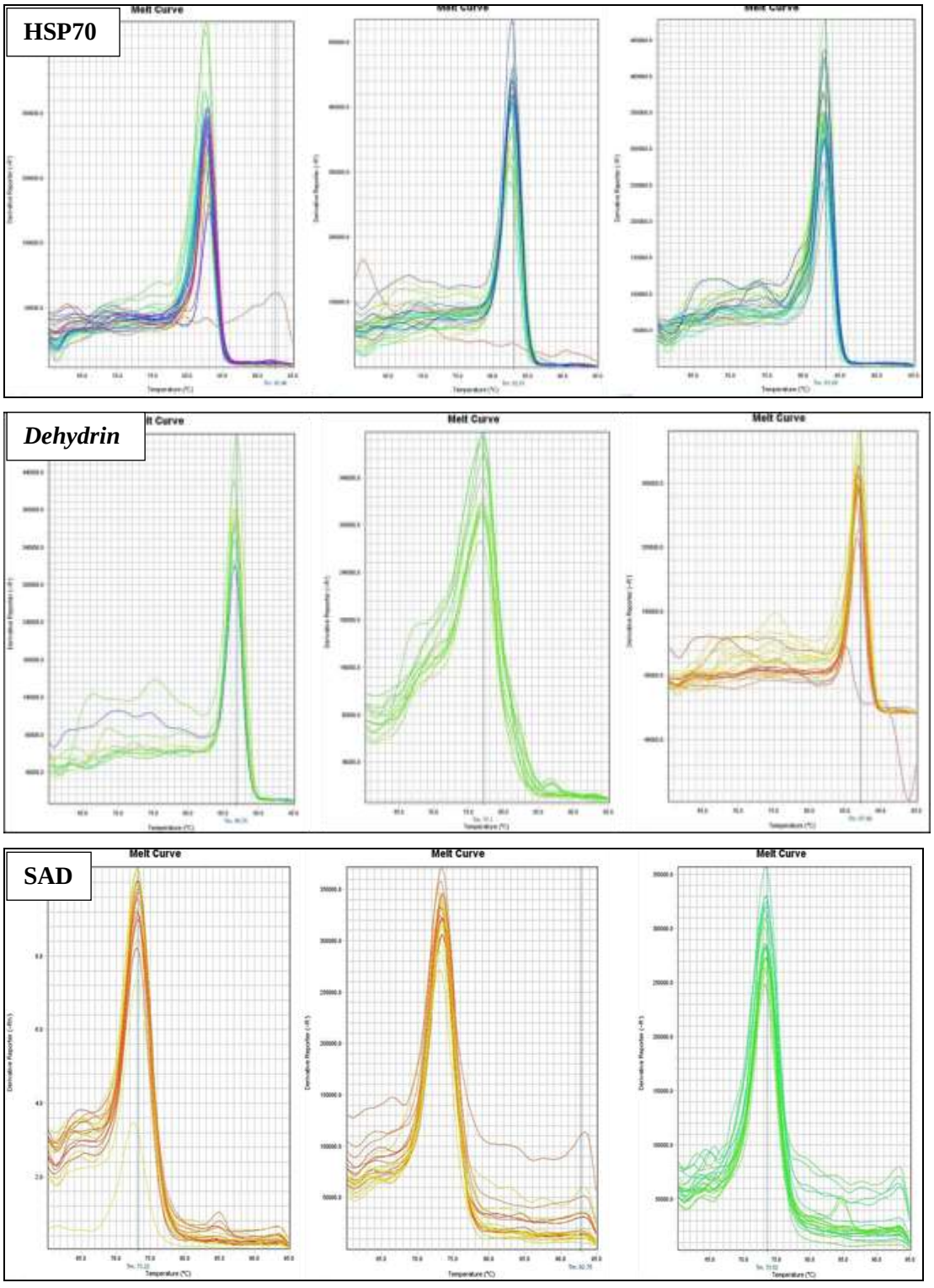


Figure 4.6: Melting curves of *hsp70*, *dehydrin* and *SAD* genes in different potato genotypes

4.3.2.1 Heat shock protein 70 (*hsp*70)

At the transcriptional level, the overall expression pattern of the Hsp70 gene was significantly upregulated in both cold acclimated and non-acclimated plants in all genotypes (Figure 4.7). After cold acclimation, the plants were subjected to freezing treatment for different time spans. At 0.5 h of treatment time span, the relative expression of Hsp70 gene was 2.16 fold higher in the tolerant genotype J-2/19 while, only 1.8 to 1.9 fold changes were observed in the susceptible genotypes Kufri Pukhraj and MS/7-645 following cold acclimation and freezing exposure (Figure 4.8).

Further, at 1.0 h of treatment time span, the expression of Hsp70 was 2.62 fold higher in the tolerant genotype J-2/19 whereas, lowest (2.36) fold change in expression level was observed in genotype Kufri Pukhraj followed by 2.42 fold changes in genotype MS/7-645 (Figure 4.8). At 1.5 h, the level of gene expression of Hsp70 in genotype J-2/19 was increases to (3.03) significantly over other treatment time spans. Similarly, the expression level of Hsp70 gene was also significantly increased to 2.91 and 2.99 fold in susceptible genotypes Kufri Pukhraj and MS/7-645, respectively (Figure 4.8). Across the genotypes, the overall expression of Hsp70 gene was found to be significantly increased over the treatment time spans in cold acclimated plants. Our results are consistent with the previous observation that upon cold exposure, the quantity of Hsp70 gene was significantly increased in both the *Solanum commersoni* and *Solanum tuberosum cv.* Desiree species. However, the fold increase was lower in *Solanum tuberosum* Desiree (Folgado *et al* 2013). Furthermore, in response to cold stress, many HSPs genes were reported to be up-regulated in various crops such as tomato (Sabehat *et al* 1998); wheat (Kosova *et al* 2013), Maize (Kollipara *et al* 2002), and Pea (Taylor *et al* 2005).

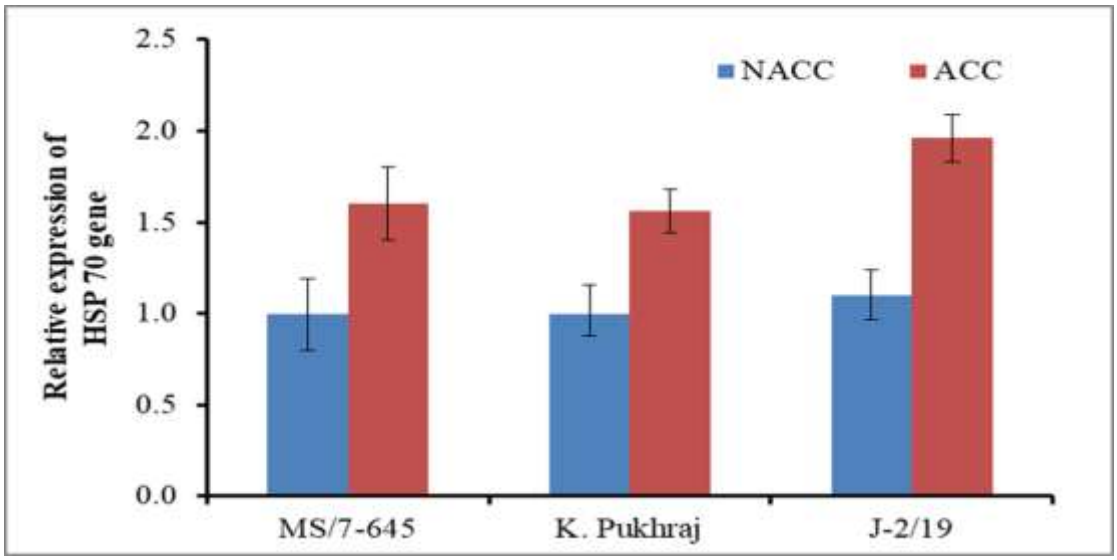


Figure 4.7: Fold change in the expression of *hsp70* gene in potato genotypes upon cold acclimation

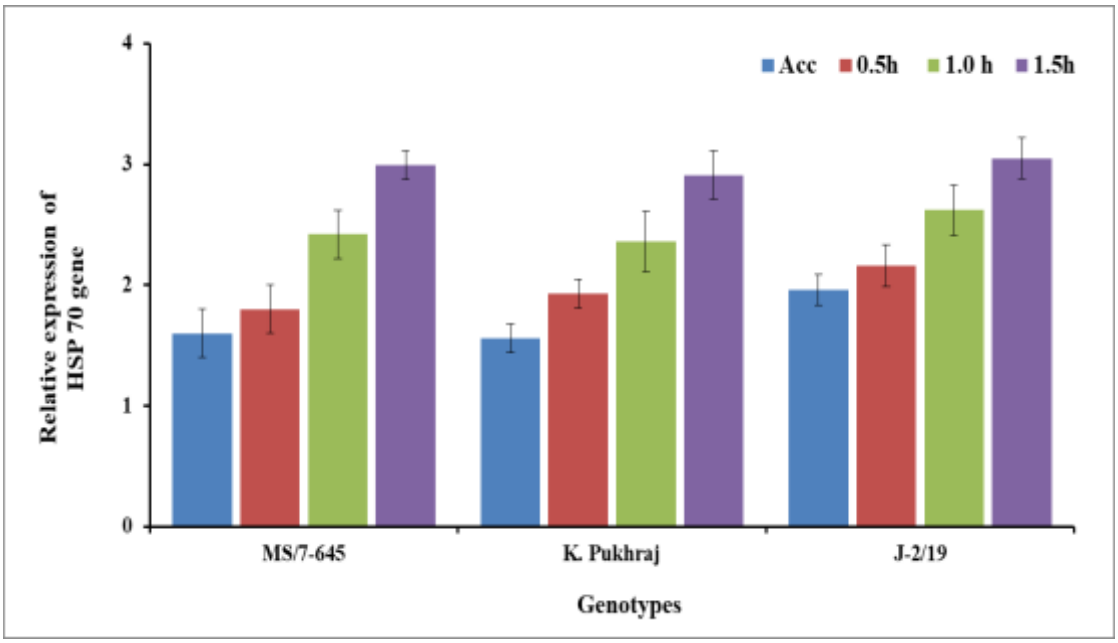


Figure 4.8: Expression profiles of *hsp70* gene in cold acclimated potato genotypes at different treatment time spans (0.5 h; 1.0 h & 1.5 h)

Heat shock proteins (HSPs), as chaperones play essential role in conferring biotic and abiotic stress tolerance in many crop plants (Breiman 2014, Park and Seo 2015, Ul Haq *et al* 2019). Moreover, HSPs are also involved in enhancing the membrane stability, detoxifies the reactive oxygen species (ROS) by positively regulating the antioxidant enzymes system (Ul Haq *et al* 2019).

4.3.2.2 Dehydrin (*dhn1*)

At the transcriptional level, the overall expression pattern of the dehydrin gene was significantly upregulated in both cold acclimated and non-acclimated plants of all the genotype (Figure 4.9). At 0.5 h of treatment time span, the relative expression of dehydrin gene was 2.46 fold higher in the tolerant genotype J-2/19 while, lower 2.08-2.23 fold changes were observed in the susceptible genotypes Kufri Pukhraj and MS/7-645, respectively following cold acclimation and freezing exposure (Figure 4.10). Further, At 1.0 h of treatment time span, the expression of dehydrin gene was 2.77 fold higher in the tolerant genotype J-2/19 whereas, lowest (2.69) fold change in expression was observed in MS/7-645 genotype followed by 2.35 fold changes in Kufri Pukhraj genotype (Figure 4.10). At 1.5 h, the level of gene expression of dehydrin in genotype J-2/19 was increased but it was not significantly higher than treatment time spans. However, the expression level of dehydrin gene was significantly increased in susceptible genotype Kufri Pukhraj but in genotype MS/7-645 its value was only increased numerically (Figure 4.10).

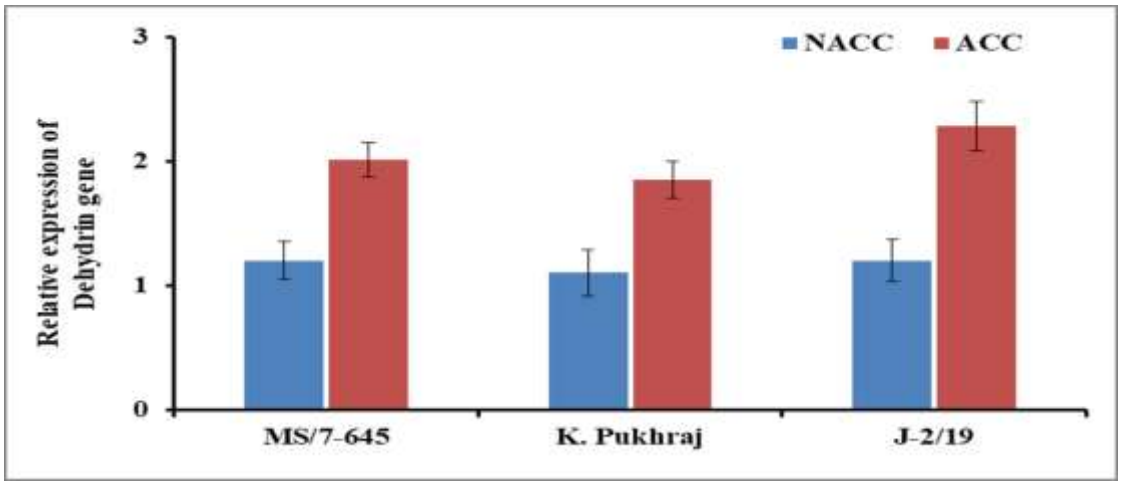


Figure 4.9: Fold change in expression of *dehydrin* gene in potato genotypes upon cold acclimation

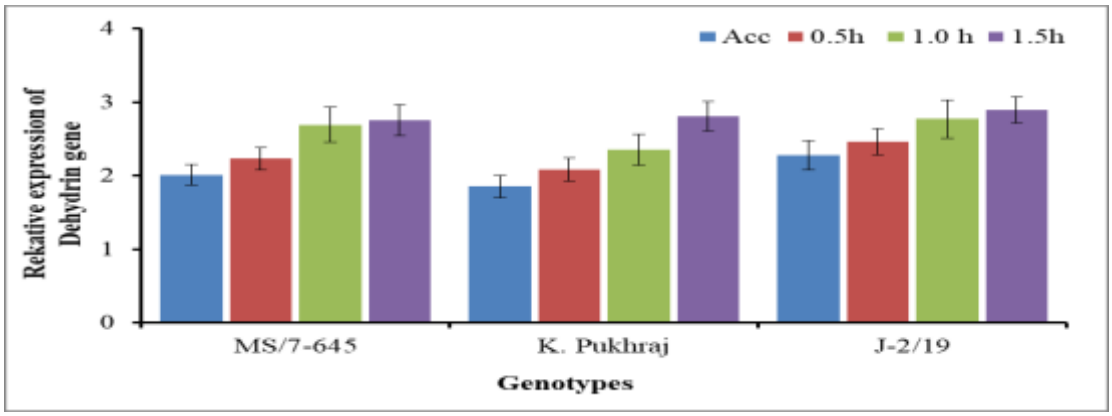


Figure 4.10: Expression profiles of *dehydrin* gene in cold acclimated potato genotypes at different treatment time spans (0.5 h, 1.0 h & 1.5 h)

Our results suggested that higher expression of dehydrin gene was observed in cold acclimated plants upon freezing exposure as compared to the non-acclimated plants at each of the treatment durations. Similarly, Karlsson and Palta (1999) suggested that in response to cold treatment the dehydrin expression was increased in both the cold acclimating (*Solanum commersoni*, *Solanum acaule*) and non-acclimating (*Solanum tuberosum*) species of potato. However, no quantitative relationships were found between NART, ACC with concentration of dehydrin gene. So, increased in expression of dehydrin gene can be a general environmental stress response. A similar finding was reported by Baudo *et al* (1996) in potato and our results were in consistent with these studies.

Despite their expression during the late seed maturation stages, they are also involved in plant response to a number of abiotic stresses including cold by providing dehydration tolerance in plants (Hanin *et al* 2011, Charfeddine *et al* 2017). This gene also acts as cryoprotectants and chaperons to protect the enzymes from reactive oxygen species (ROS) during low temperature stress. Furthermore, in Arabidopsis overexpression of chimeric genes

leads to accumulation of dehydrin genes equal to or higher than in cold acclimated plants and the transgenic plants exhibited lower LT50 values and improved survival when exposed to freezing stress (Puhakainen 2004).

4.3.2.3 Stearoyl-acyl carrier protein desaturase (*SADs*) gene

The results suggested that at the transcriptional level, the overall expression pattern of the *SAD* gene was upregulated in both the cold acclimated and non-acclimated plants of all the genotypes. However, significant differences were observed among the genotypes under different growing conditions (Figure 4.11).

At the transcriptional level, at 0.5 h of treatment time span the relative expression of *SAD* gene was 4.03 fold significantly higher in the tolerant genotype J-2/19, while lowest 1.83 fold change was observed in the susceptible genotype Kufri Pukhraj. However, the genotype MS/7-645 recorded 3.03 fold changes following cold acclimation and freezing exposure (Figure 4.12). Further, at 1.0 h of treatment time span, the expression was significantly 4.43 fold higher in the tolerant genotype J-2/19 whereas, lowest (1.97) fold change in expression was observed in Kufri Pukhraj (Figure 4.12). At 1.5 h, the level of gene expression of SAD in genotype J-2/19 was increased but it was not significantly higher than the other treatment time spans. However, in susceptible genotypes MS/7-645 and Kufri Pukhraj the expression level of SAD gene was either reduced or remained unchanged, respectively (Figure 4.12).

Our results suggested that higher expression of SAD gene in cold acclimated and freezing exposed plants in potato genotypes relative to control (non-acclimated) plants at each of the treatment durations. As, this gene are believed to be directly involved in maintaining of homeostasis of saturated and unsaturated fatty acids in plasma membrane thus, protecting and maintaining stability of cell plasma membrane during low temperature stress.

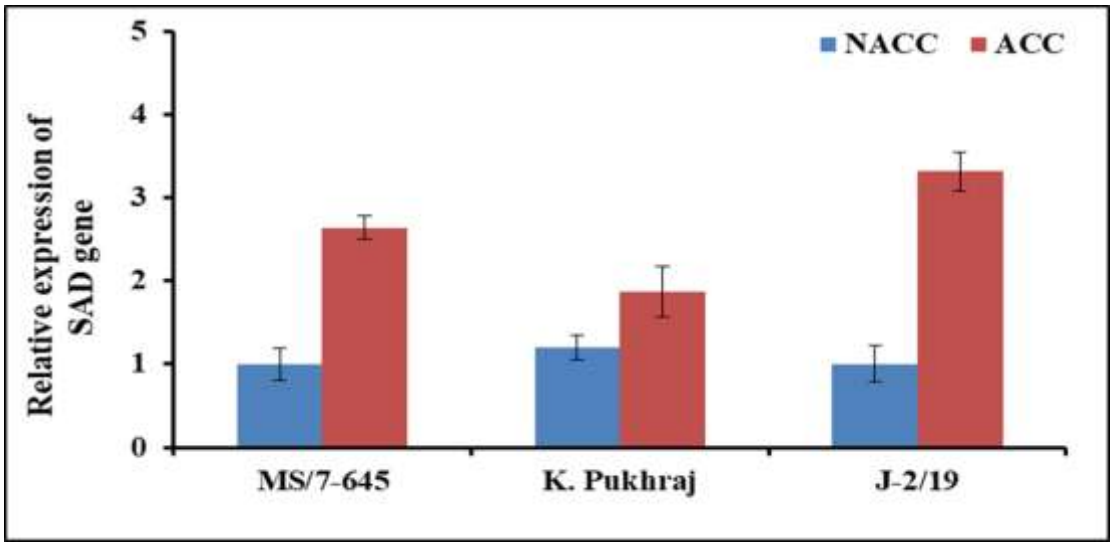


Figure 4.11: Fold change in expression of *SAD* gene in potato genotypes upon cold acclimation

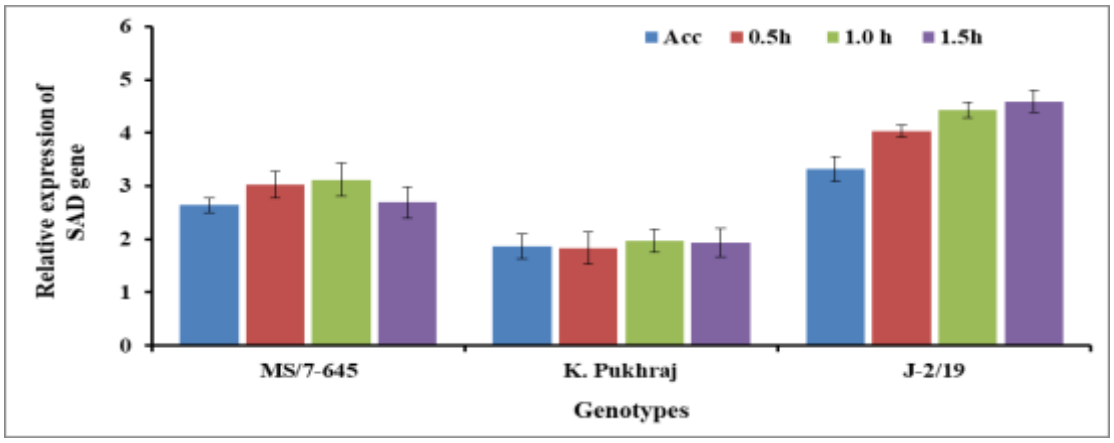


Figure 4.12: Expression profiles of *SAD* gene in cold acclimated potato genotypes at different treatment time spans (0.5 h, 1.0 h & 1.5 h)

Our results are in confirmation with Li *et al* (2015) in potato in which the SAD gene from *Solanum commersonii* (ScoSAD) was cloned and transferred in *Solanum tuberosum cv.* Zhongshu 8 and consequently the overexpression of the gene in the transgenic plants showed significant freeze tolerance. Similarly, Bevilacqua *et al* (2015) identified OsFAD2 (fatty acid desaturase 2) gene in rice that contribute to cold tolerance by maintaining fluidity of cell membrane. Furthermore, the sikSACPD gene identified in *Saussurea involucrate* species involved as a candidate SAD gene for enhancing cold tolerance in tobacco and others crop plants (Liu *et al* 2015). The freezing tolerance and expression of SACOD gene was greater in *S. involucrate* than the Fab2 gene in Arabidopsis. Thus, the expression of sikSACOD gene increased with decreased in temperature. Moreover, the transgenic tobacco plants (FAB2: SikSACPD) showed more resistance as compared with that of wild and another transgenic plants (FAB2:FAB2). Further, under cold stress treatment there was 5-20% increase in oleic acid (C18:1) proportion in the leaves of SikSACPD transgenic plants (Liu *et al* 2015) and the level of membrane fatty acid desaturation is associated with its cold acclimation in this species.

4.3 Generation and characterization of potential frost tolerant potato clones on the basis of leaf morpho-anatomical and physiological traits

4.3.1 Characterization of clones on the basis of leaf morpho-anatomical traits

The clones were evaluated in seedling stage (single hill); F_1C_1 generation (short row trials) and F_1C_2 generation (duplicate row trials). In F_1C_1 generation the selected clones were planted in rows along with check varieties after every 10 rows. At vegetative growth stage around 40-60 days after planting characterization was carried out on the basis of leaf morpho-anatomical traits *viz.*, stomatal density, trichome density, dimensions of palisade parenchymatous and epidermal cell. Similarly at harvesting stage, the clones with misshaped tubers, deep eyes and crack tubers were rejected, as these characters have a high repeatability over the generations.

Stomata are the inter-cellular cavities between two adjoining guard cells and the primary structures involved in water loss and gas exchange in plants. Genetic differences in stomatal density on both the leaf surfaces may have potential in breeding for increased photosynthetic and water-use efficiency. Moreover, the stomata exhibit enormous morphological and mechanical diversity which directly affect the leaf gas exchange properties of the genotype (Bisognin *et al* 2006). While, trichomes are specialized leaf epidermal structures that play a critical role in providing resistance to a variety of biotic and abiotic stresses by functioning as physical and chemical block agents (Bisognin *et al* 2006). Further, the trichome related characteristics, such as trichome density, types and their dimensions are the most important factors for the protecting of the plants from various biotic and abiotic stresses (Bisognin *et al* 2006).

The anatomical traits, such as palisade parenchymatous and epidermal cells numbers and their dimensions are the important characteristics which are highly associated with frost tolerance in potato crop and useful traits in the selection of frost tolerant / resistant genotypes as reported by Palta and Li (1979) and Chen *et al* (1980). In addition to higher stomatal index, the multiple and thicker palisade layers are also reported to positively associated with freezing tolerant members in *Solanum* tuber bearing species (Palta and Li 1979, Tiwari *et al* 1986).

4.3.1.1 Leaf morpho-anatomical characterization of clones derived from a cross J-2/19 × MS/7-645 (PAU3)

In the clones derived from cross J-2/19 × MS/7-645, the stomatal density (numbers per mm^2) on adaxial surface in the studied clones varied from 10.0 to 110 with mean value of 42.5. Similarly, the stomatal density (numbers per mm^2) on abaxial surface in the studied clones varied from 70.0 to 308.3 with mean value of 167.8 was recorded (Table 4.6). Further, on adaxial leaf surface the epidermal cell density (numbers per mm^2) was varied from 188.3 to 568.3 with mean value 324.7. Stomatal index varied from 3.8% to 25.5% with mean value 11.5% (Table 4.6).

Further, cell plot analysis indicated that the highest stomatal index (25.5%) was recorded in PAU3-477 whereas; lowest (3.8%) was recorded in PAU3-399 (Figure 4.13 and Plate 9). In the studied clones, results showed that the trichome density (numbers per mm^2) ranged from 5.0 to 20.0 and 5.0 to 41.7 on adaxial and abaxial leaf surfaces with the mean values 9.8 and 20.2, respectively (Table 4.6 and Plate 10). Similarly, the box plot analysis indicated that among the studied leaf morphological traits maximum variability was observed in stomatal density on abaxial leaf surface, epidermal cell density on adaxial leaf surface followed by stomatal ratio. Similarly for trichome related traits maximum variability was indicated by trichome density on abaxial leaf surface whereas, minimum variability recorded by trichome ratio (Figure 4.14).

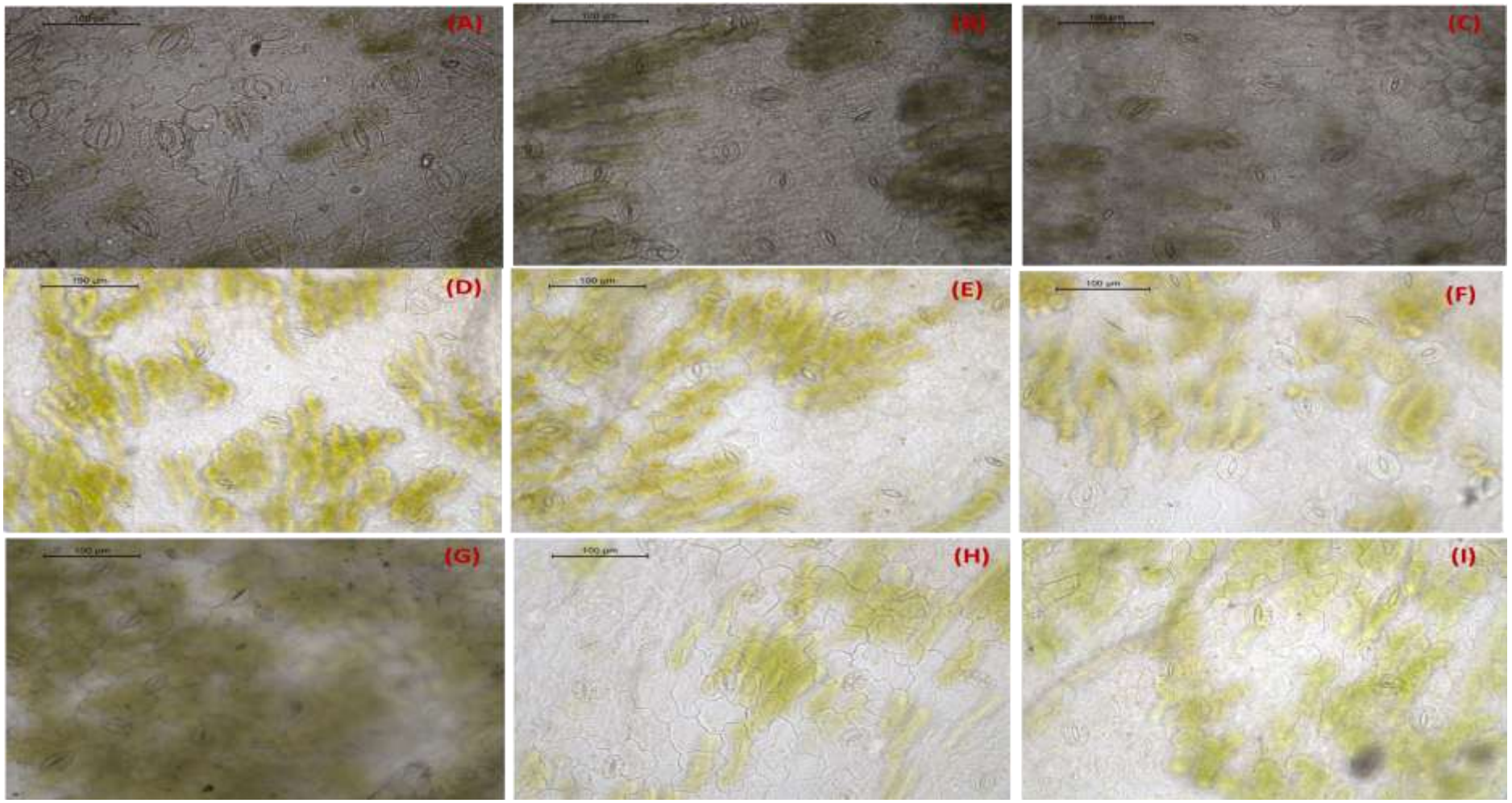


Plate 9: Screening of potato clones on the basis of stomatal index (%)

A) PAU3-477; B) PAU3-483; C) PAU3-591; D) PAU7-960; E) PAU7-981; F) PAU7-987; G) PAU9-1302; H) PAU9-1257; I) PAU9-1255

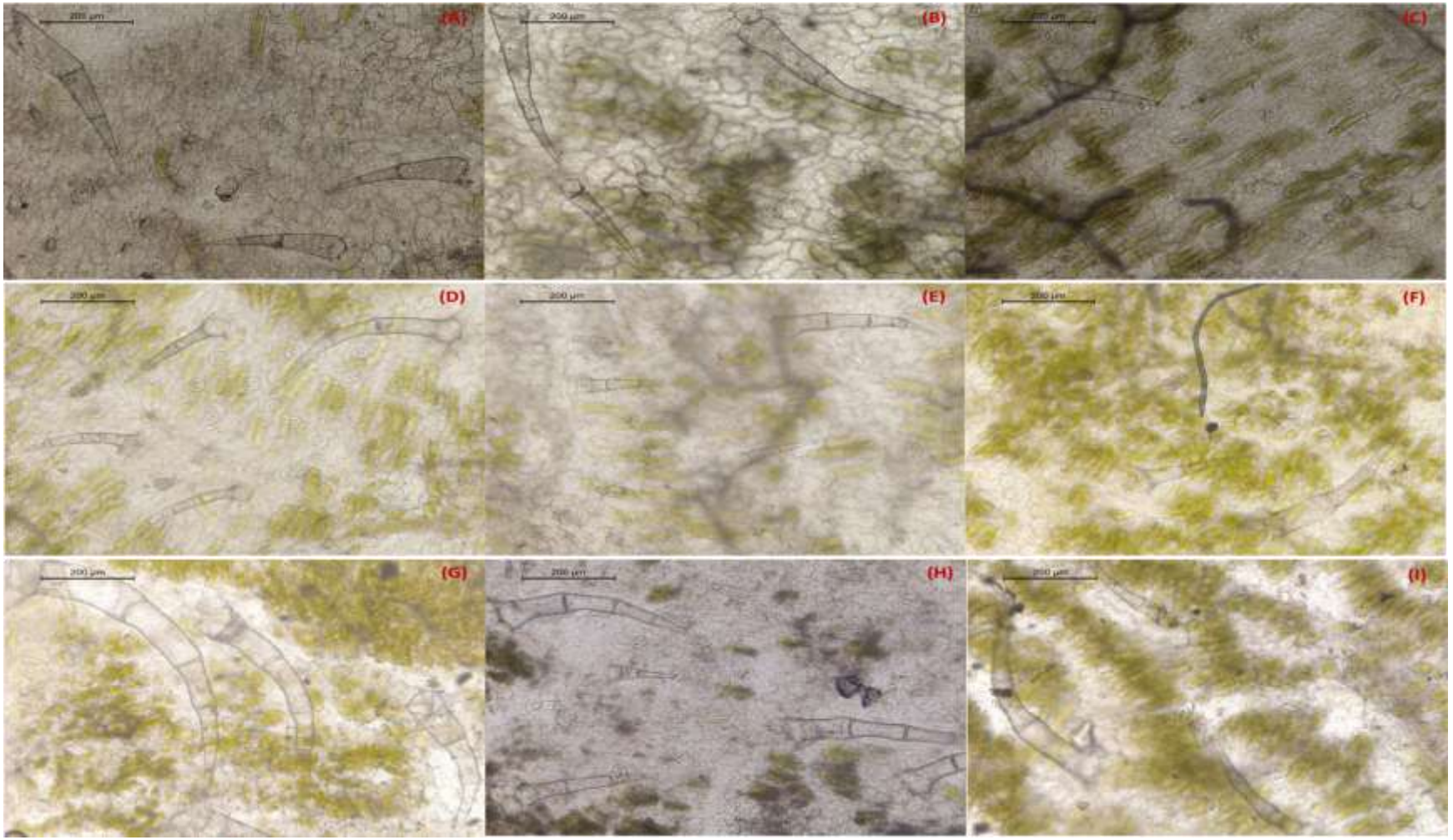


Plate 10: Screening of potato clones on the basis of trichome density on adaxial leaf surface

A) PAU3-501; B) PAU3-444; C) PAU3-427; D) PAU7-988; E) PAU7-983; F) PAU7-1009; G) PAU9-1306; H) PAU9-1278; I) PAU9-1261

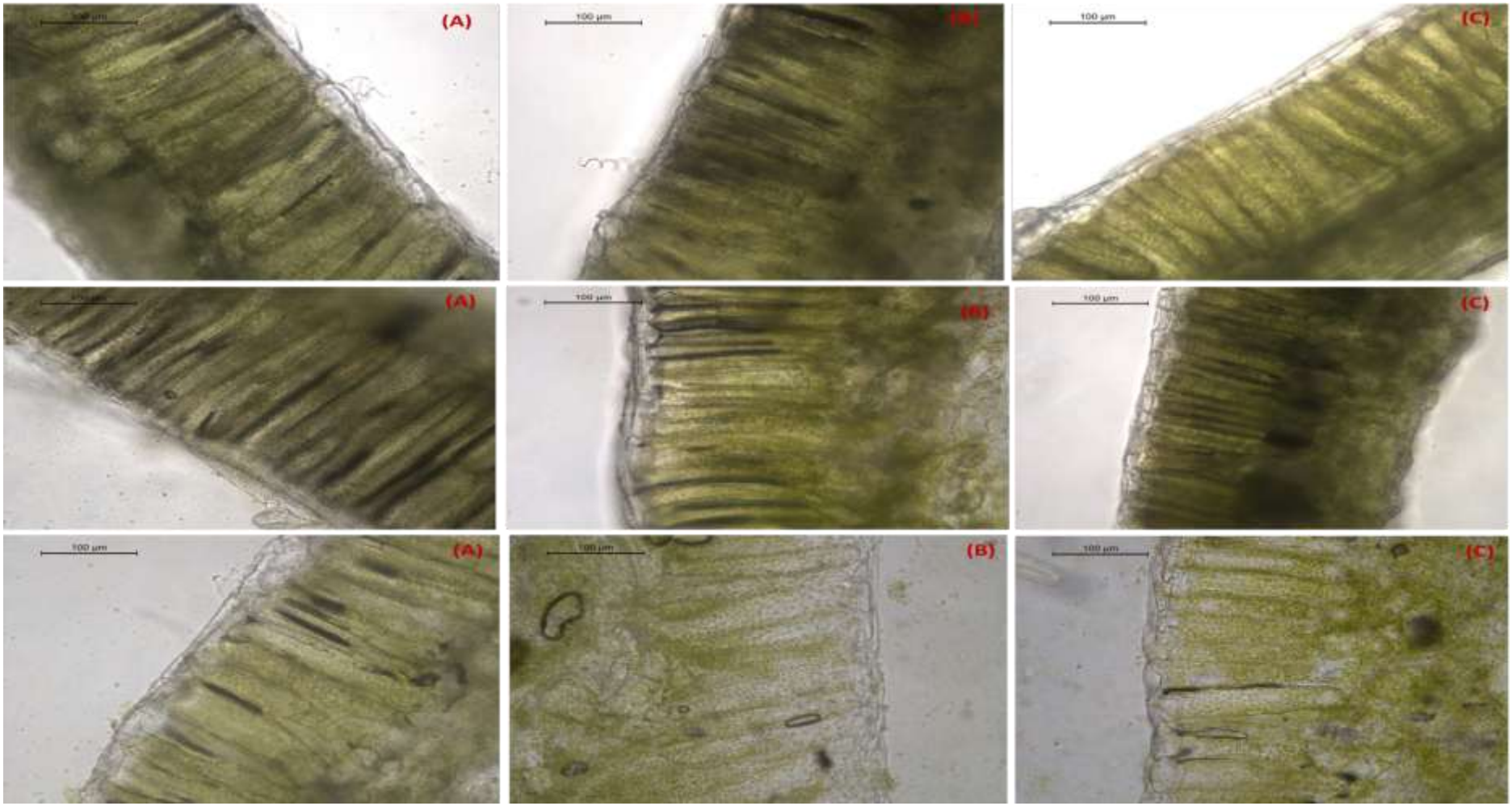


Plate 11: Screening of potato clones on the basis of palisade parenchymatous cell length

A) PAU3-498; B) PAU3-590; C) PAU3-391; D) PAU7-955; E) PAU7-1015; F) PAU7-986; G) PAU9-1266; H) PAU9-1269; I) PAU9-1255

Table 4.6: Descriptive statistics of leaf morpho-anatomical traits of clones derived from crosses, J-2/19 × MS/7-645 (PAU3); MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9)

Traits	Cross	N	Min.	Max.	Range	Mean	S.D	S.E	Skewness
SDAD	PAU3	168	10.0	110.0	100.0	42.5	17.2	1.3	0.9
	PAU7	57	15.0	95.0	80.0	47.8	15.0	2.0	0.5
	PAU9	47	21.7	80.0	58.3	46.7	14.5	2.1	0.3
EDAD	PAU3	168	188.3	568.3	380.0	324.7	70.7	5.5	0.5
	PAU7	57	180.0	678.3	498.3	328.8	99.2	13.3	1.5
	PAU9	47	161.7	770.0	608.3	336.4	97.5	14.4	1.8
SI	PAU3	168	3.8	25.5	21.7	11.5	3.7	0.3	0.6
	PAU7	57	4.8	21.4	16.6	13.0	3.8	0.5	0.4
	PAU9	47	5.0	21.8	16.8	12.4	3.6	0.5	0.2
SDAB	PAU3	168	70.0	308.3	238.3	167.8	47.1	3.6	0.5
	PAU7	57	46.6	261.6	215.0	164.0	37.1	5.0	-0.2
	PAU9	47	75.0	335.0	260.0	197.4	54.4	8.0	0.7
SR	PAU3	168	0.1	0.6	0.5	0.3	0.1	0.0	0.6
	PAU7	57	0.1	0.8	0.7	0.3	0.1	0.0	1.5
	PAU9	47	0.1	0.5	0.4	0.2	0.1	0.0	0.8
TDAD	PAU3	168	5.0	20.0	15.0	9.8	3.6	0.3	0.7
	PAU7	57	5.0	33.3	28.3	12.0	6.3	0.8	1.5
	PAU9	47	6.7	25.0	18.3	13.9	5.2	0.8	0.5
TDAB	PAU3	168	5.0	41.7	36.7	20.2	7.1	0.5	0.7
	PAU7	57	8.3	41.6	33.3	26.8	7.6	1.0	-0.1
	PAU9	47	8.3	53.3	45.0	22.0	8.4	1.2	1.1
TR	PAU3	168	0.2	1.7	1.5	0.5	0.2	0.0	1.5
	PAU7	57	0.1	1.3	1.1	0.5	0.3	0.0	1.2
	PAU9	47	0.2	2.5	2.3	0.7	0.5	0.1	1.8
PPL	PAU3	168	83.3	188.2	104.9	137.6	23.3	1.8	0.0
	PAU7	57	88.1	239.7	151.6	140.3	29.8	4.0	0.6
	PAU9	47	98.5	205.8	107.3	155.8	25.4	3.7	0.0
PPW	PAU3	168	17.1	36.2	19.1	24.1	3.4	0.3	0.4
	PAU7	57	16.3	42.7	26.4	25.0	6.0	0.8	0.9
	PAU9	47	18.8	31.6	12.8	25.1	3.0	0.4	-0.1
EL	PAU3	168	21.9	52.2	30.3	36.1	5.1	0.4	0.2
	PAU7	57	21.9	50.9	29.0	37.5	5.7	0.8	-0.1
	PAU9	47	26.4	49.6	23.2	34.3	4.7	0.7	0.7
EW	PAU3	168	13.2	28.0	14.8	18.7	2.5	0.2	0.5
	PAU7	57	14.2	27.9	13.7	20.3	3.6	0.5	0.6
	PAU9	47	14.6	29.6	15.0	21.6	3.0	0.4	0.6

Legend: SDAD: Stomatal density on adaxial leaf surface (numbers/mm^2), EDAD: Epidermal cell density on adaxial leaf surface (numbers/mm^2), SI: Stomatal index (%), SDAB: Stomatal density on abaxial leaf surface (numbers/mm^2), SR: Stomatal ratio, TDAD: Trichome density on adaxial leaf surface, TDAB: Trichome density on abaxial leaf surface, TR: Trichome ratio, PPL: Palisade cell length (µm); PPW: Palisade cell width (µm); EL: Epidermal cell length (µm); EW: Epidermal cell width (µm)

Similarly, in terms of leaf anatomical traits of clones the length and width of palisade parenchymatous cells varied from 83.3 µm to 188.2 µm and 17.1µm to 36.2 µm with mean values 137.6 µm and 24.1 µm, respectively (Table 4.6 and Plate11). Further, the epidermal cell dimensions length varied from 21.9 µm to 52.2 µm with mean value 36.1 µm and width of epidermal cell varied from 13.2 µm to 28.0 µm with mean value 18.7 µm (Table 4.6). Further, the box plot analysis revealed that among the studied anatomical traits the maximum variability was observed in palisade parenchyma cell length followed by epidermal cell length, whereas minimum was recorded for epidermal cell width (Figure 4.14).

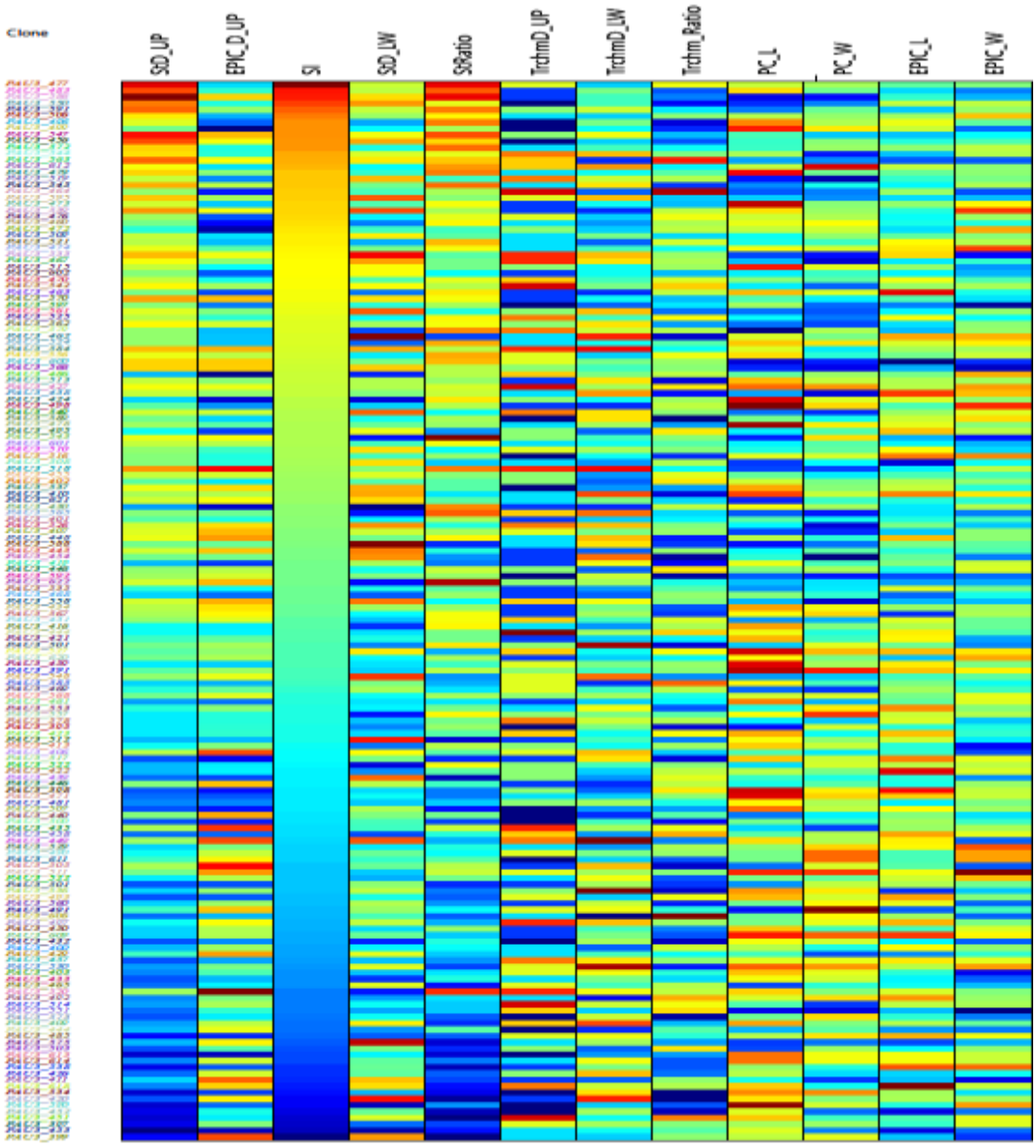


Figure 4.13: Cell plot analysis of 168 potato clones derived from a cross, J-2/19 × MS/7-645 (PAU3) for various morpho-anatomical traits

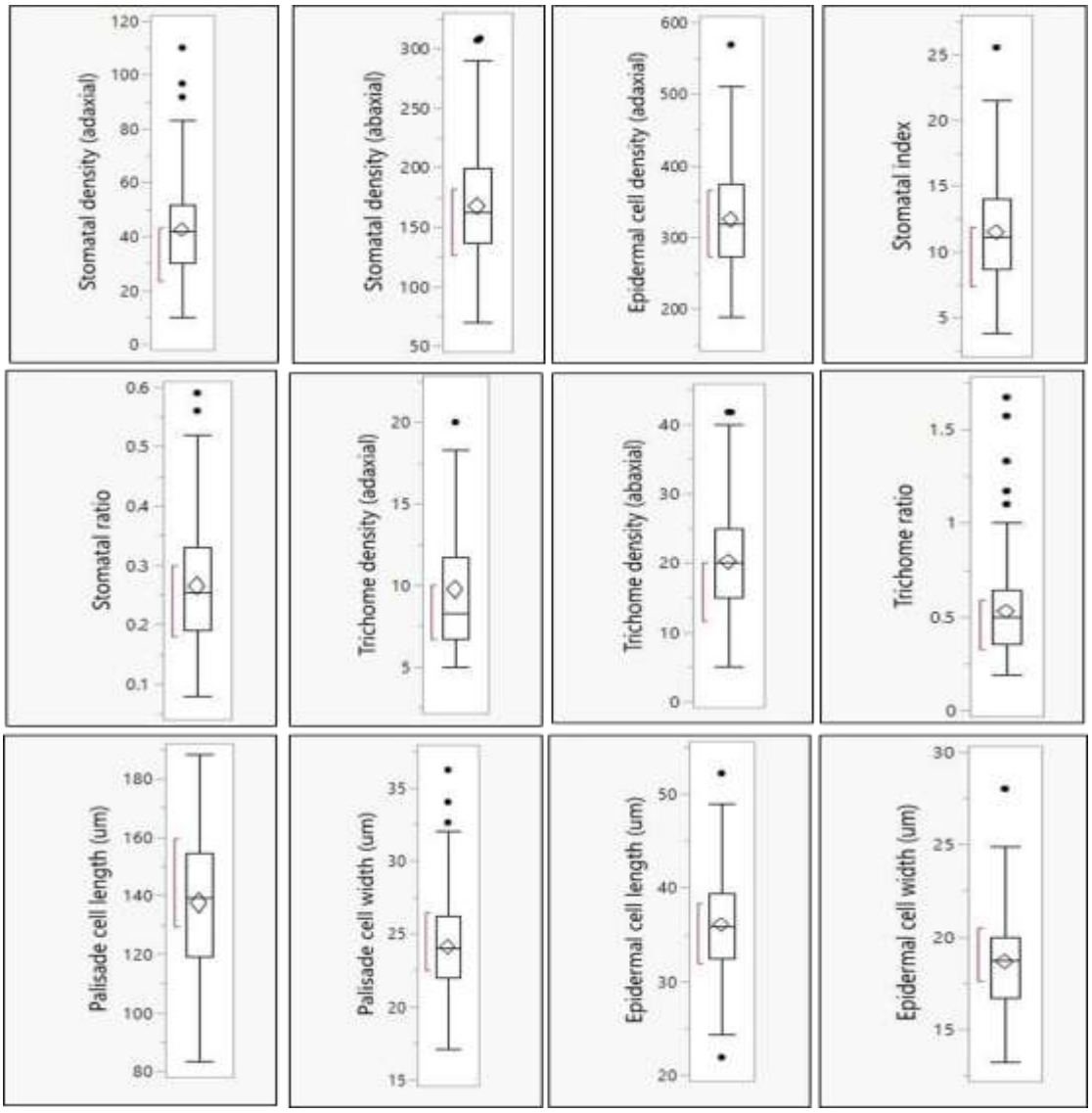


Figure 4.14: Box plot analysis of various morpho-anatomical traits of potato clones derived from a cross, J-2/19 × MS/7-645 (PAU3)

4.3.1.2 Leaf morpho-anatomical characterization of clones derived from cross a MS/7-645 × J-2/19 (PAU7)

In the clones derived from a cross MS/7-645 × J-2/19 (PAU7), the stomatal density (numbers per mm^2) in the studied clones varied from 15.0 to 95 and 46.0 to 261.6 on adaxial and abaxial leaf surfaces with mean values 47.8 and 164.0, respectively (Table 4.6). Further, the epidermal cell density on adaxial leaf surface was varied from 180.0 to 678.3 with mean value 328.8 and stomatal index varied from 4.8 % to 21.4 % with mean value 13.0% (Table 4.6). Further, cell plot analysis indicated that the highest stomatal index (21.4%) was reported in PAU7-960 whereas; lowest (4.8%) was recorded in PAU7-957 (Figure 4.15 and Plate 9). Likewise, results showed that the trichome density varied from 5.0 to 33.3 and 8.3 to 41.6 on adaxial and abaxial leaf surfaces with the mean values 12.0 and 26.8, respectively (Table 4.6 and Plate 10). Similarly, the box plot analysis revealed that the among the studied leaf

morphological traits maximum variability was recorded in stomatal index, trichome density on abaxial leaf surface whereas, minimum variability was observed in traits, such as stomatal ratio and epidermal cell density on adaxial leaf surface (Figure 4.16).

Further, the length and width of palisade parenchyma cells of the clones varied from 88.1 µm to 239.7 µm and 16.3 µm to 42.7 µm with mean values 140.3 µm and 25.0 µm, respectively (Table 4.6 and Plate 11). Further, the epidermal cell dimensions length was varied from 21.9 µm to 50.9 µm with mean value 37.5 µm and width of epidermal cell varied from 14.2 µm to 27.9 µm with mean value 20.3µm (Table 4.6). Further, the box plot analysis indicated the maximum variability for epidermal cell width whereas, minimum for palisade parenchyma cell width (Figure 4.16).

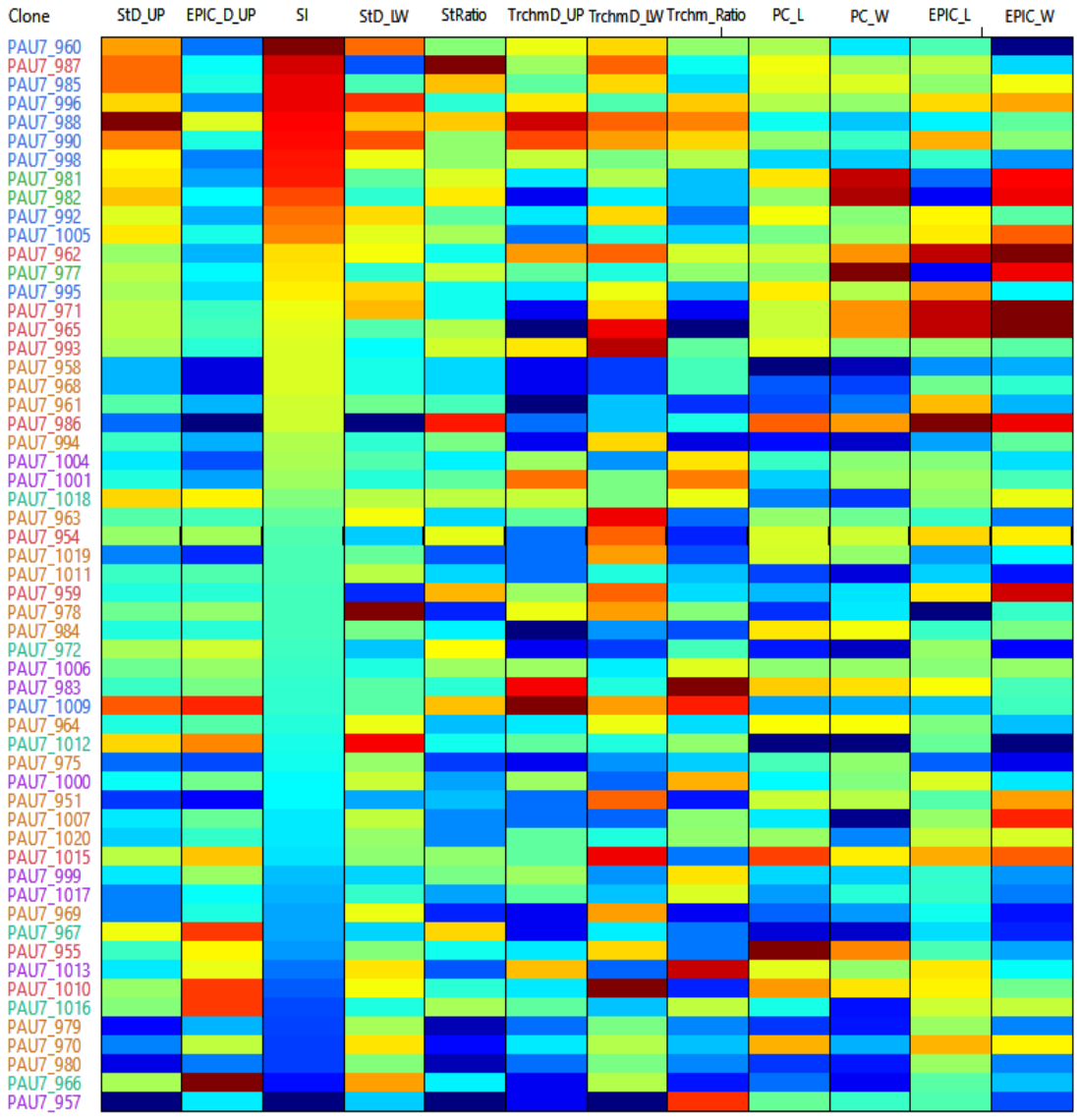


Figure 4.15: Cell plot analysis of 57 potato clones derived from a cross, MS/7-645 × J-2/19 (PAU7) for various morpho-anatomical traits

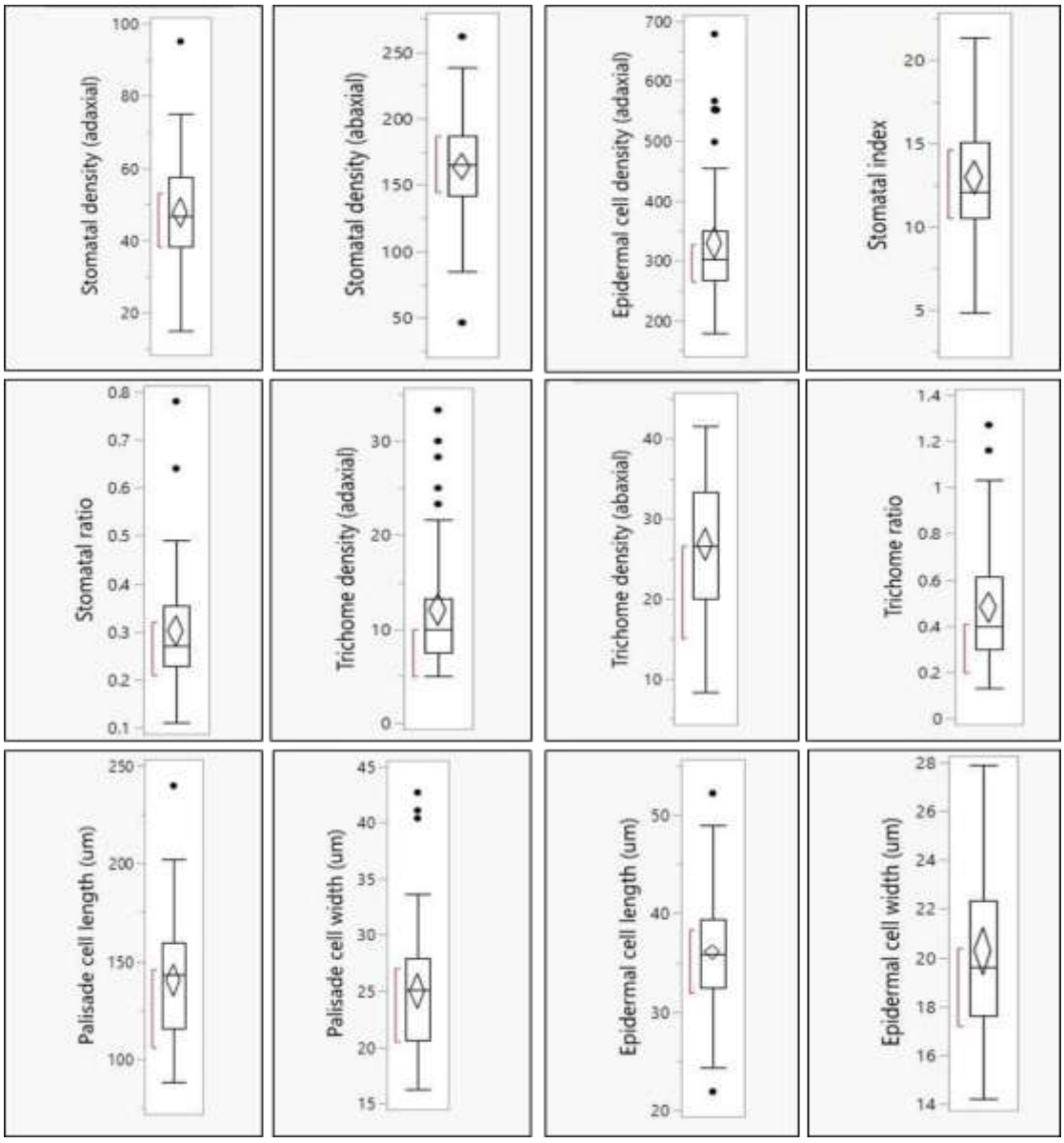


Figure 4.16: Box plot analysis of various morpho-anatomical traits of potato clones derived from a cross, MS/7-645 × J-2/19 (PAU7)

4.3.1.3 Leaf morpho-anatomical characterization of potato clones derived from a cross MS/7-645 × CP-3765 (PAU9)

In clones derived from MS/7-645 × CP-3765 (PAU9), the stomatal density (numbers per mm^2) in the studied clones varied from 21.7 to 80.0 and 75.0 to 335.0 on adaxial and abaxial leaf surfaces with mean values 46.7 and 197.4 respectively. Further, the epidermal cell density on adaxial leaf surface was varied from 161.7 to 770.0 with mean value 336.4 and stomatal index varied from 5.0 % to 21.8% with a mean value 12.4% (Table 4.6). Further, cell plot analysis indicated that the highest stomatal index (21.8%) was reported in PAU9-1302 followed by (19.6%) in PAU9-1298 whereas, lowest (5.0%) was recorded in PAU9-1278 (Figure 4.17 and Plate 9). Results showed that the trichome density varied from 6.7 to 25.0 and 8.3 to 53.3 on adaxial and abaxial leaf surfaces with the mean values 13.9 and 22.0,

respectively (Table 4.6 and Plate 10). Similarly, the box plot analysis indicated that the maximum variability was shown for traits, such as stomatal density abaxial and stomatal index whereas; minimum variability was recorded by trichome ratio (Figure 4.18).

Further, the length and width of palisade parenchyma cells of the clones varied from 98.5 µm to 205.8 µm and 18.8 µm to 31.6 µm with mean values 155.8 µm and 25.1 µm, respectively (Table 4.6 and Plate 11). Further, the epidermal cell dimensions length varied from 26.4 µm to 49.6 µm with mean value 34.4 µm and width of epidermal cell varied from 14.6 µm to 29.6 µm with mean value 21.6 µm (Table 4.6). Further, the box plot analysis indicated the maximum variability for palisade parenchyma cell width whereas, minimum for epidermal cell length (Figure 4.18).

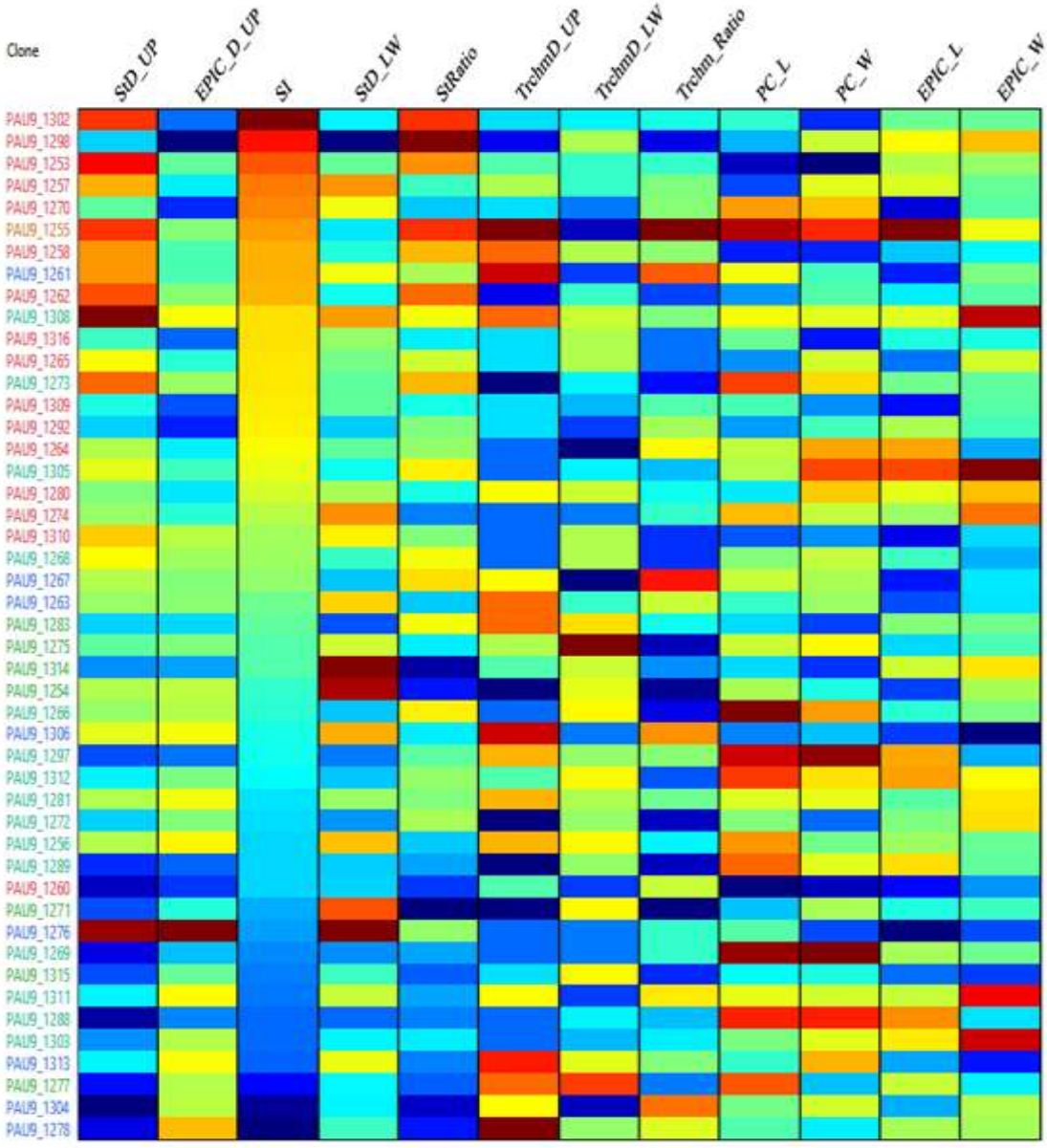


Figure 4.17: Cell plot analysis of 47 potato clones derived from a cross, MS/7-645 × CP-3765 (PAU9) for various morpho-anatomical traits

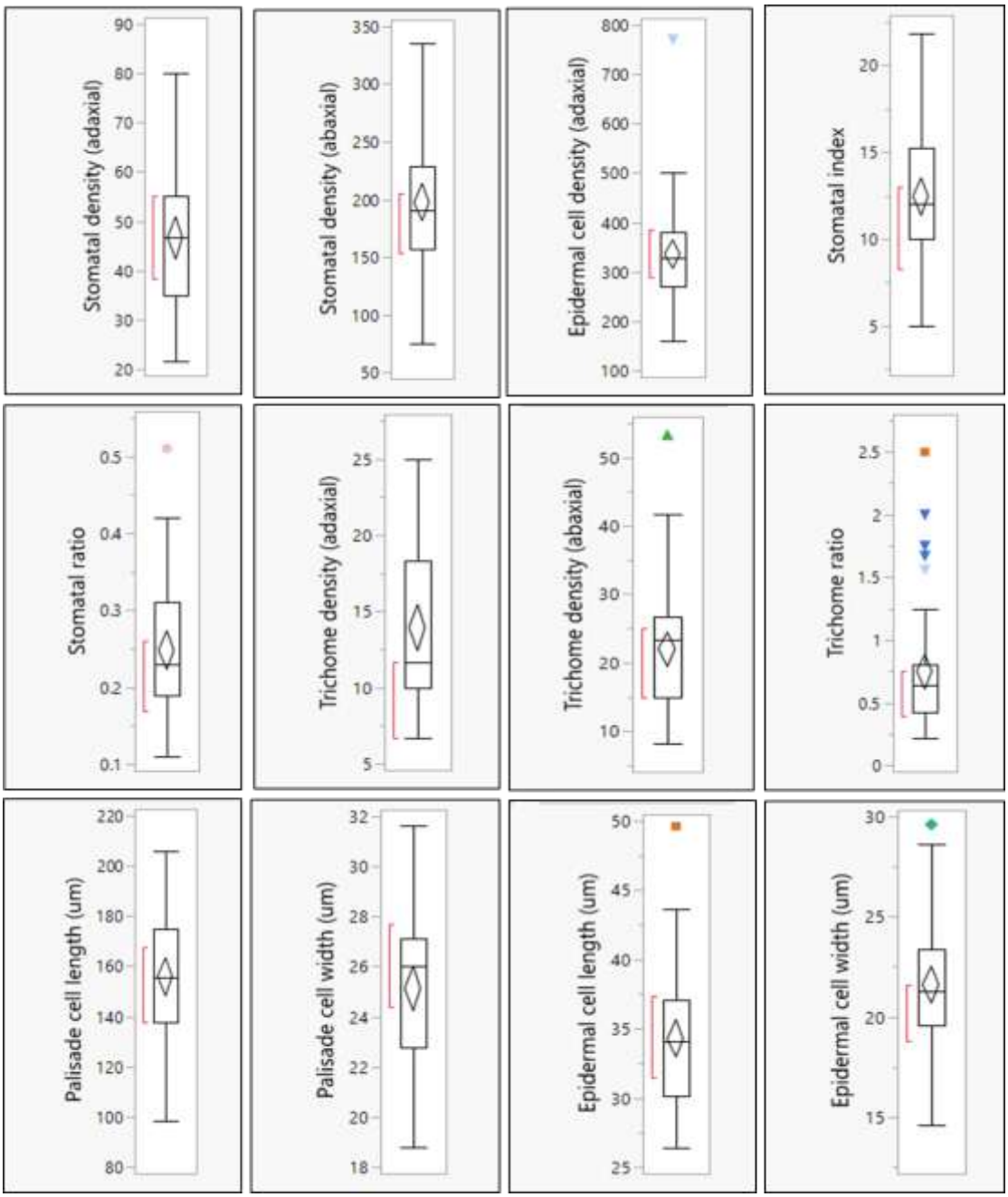


Figure 4.18: Box plot analysis of various morpho-anatomical traits of potato clones derived from a cross, MS/7-645× CP-3765 (PAU9)

Significant variations for all the leaf morpho-anatomical traits were observed among the studied clones derived from three different crosses. Our findings are in consistent with the previous studies that there is association of the leaf morpho-anatomical traits with the frost tolerance behaviour of potato genotypes/species. Thus, these traits can potentially be used for the characterization/screening of segregating progenies/ potato clones for frost tolerance (Kleinhenzl *et al* 1995). The greater stomatal index may facilitate better gas exchange in leaves of potato, as stomatal resistance is considered as a main limitation to CO_2 flow into a leaf (Taiz and Zeiger 1991, Kleinhenzl *et al* 1995,). Similarly, Kleinhenzl *et al* (1995) also

reported that higher stomatal index on adaxial leaf surface was positively correlated with frost tolerance in potato. Further, small cell size, thick cell walls and low stomatal density on adaxial leaf surface have been reported to be associated with frost tolerance (Chen *et al* 1980, Estrada 1982, Li and Fennell, 1985). However, this relationship does not hold true for all potato species because contradictory results have also been reported. It is concluded that cell size is probably a minor factor in relation to frost tolerance. In potato leaves, the variability in the number of palisade layers has also been reported (Palta and Li 1979). Two layers of palisade parenchyma in frost-hardy ones, while only one layer in less cold hardy was observed and this traits was also reported to be genetically transmittable (Estrada 1982).

4.3.2 Cluster analysis

Cluster analysis was performed using a hierarchical clustering heatmap and the clones derived from three different crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) were grouped into different clusters based on the their proximate composition of various leaf morpho-anatomical traits indicating the presence of wide genetic variability.

4.3.2.1 Cluster analysis of clones derived from cross J-2/19 × MS/7-645 (PAU3)

The hierarchical clustering heatmap analysis of 168 potato clones was performed and the clones were distributed in 13 clusters (Figure 4.19). The corresponding clones in each cluster are presented in Table 4.7 and their characteristics are described as below. Cluster I consists of sixteen clones accounting for 9.5% of the total clones. It comprised of clones with highest epidermal cell density on adaxial leaf surface and the lowest palisade parenchymatous cell length. The clones possessed lower values for traits, such as epidermal cell length and stomatal index (%). However, the cluster also comprised of clones with higher value for stomatal density on adaxial leaf surface and trichome density on both leaf surfaces (Figure 4.19, Table 4.8 and Table 4.9). Cluster II comprised of fifteen clones accounting for 8.9% of the total clones. This cluster consists of clones having intermediate values for stomatal index, palisade parenchymatous cell length, epidermal cell length and width. Higher values for trichome density on both leaf surfaces and lowest palisade parenchymatous cell width were recorded (Figure 4.19, Table 4.8 and Table 4.9). Hence, these clones may impart frost tolerance as the higher trichome density and compactly arranged palisade parenchyma cells are associated frost tolerance (Kleinhenz *et al* 1995, Campitelli *et al* 2013). Moreover, the occurrence of greater number of trichomes on the leaf surfaces can ensure better maintenance of leaf temperature as trichomes play a vital role in thermoregulation (Gates 1980, Kim *et al* 2012, Campitelli *et al* 2013,).

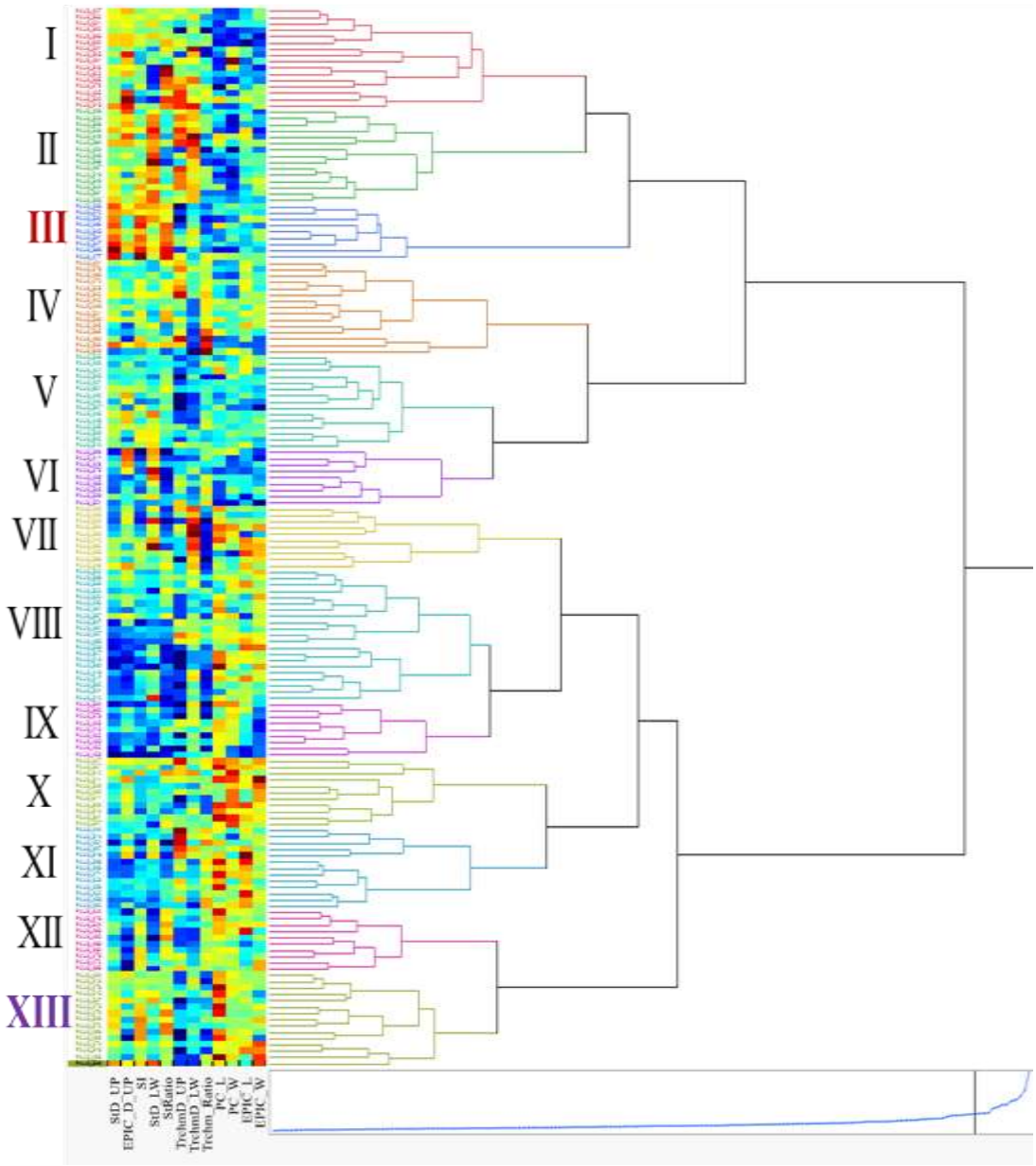


Figure 4.19: The hierarchical clustering heatmap of 168 potato clones derived from a cross, J-2/19 × MS/7-645 (PAU3)

Cluster III comprised of 5.4% of total clones which are identified with the highest values for stomatal density on adaxial leaf surface, stomatal index and stomatal ratio but intermediate values for palisade parenchymatous cell length and width and epidermal cell length and width. Therefore, these clones might be identified as highly potential frost tolerant clones on the basis of leaf morpho-anatomical traits (Figure 4.19, Table 4.8 and Table 4.9). It can be anticipated that such higher stomatal index on adaxial leaf surface will results in greater stomatal conductance and photosynthesis rates leading to formation of more sugars which thus will enhance the solute concentration of the cell cytoplasm (Pyakurel and Wang 2014). The higher amount of sugars so formed will decrease the freezing point of the tissue thereby imparting frost tolerance (Deryabin *et al* 2007, Naz *et al* 2014). Among all the studied

clones, PAU3-477 recorded highest stomatal index (25%) and also exhibited bigger stomatal cavities. Although, some studies reported that increase in the stomatal density can be compensated by diminished size of the stomatal cavity (Pyakurel and Wang 2014, Sun *et al* 2014). Cluster IV consists of fifteen clones accounting for 8.9% of total clones and it comprised of clones having higher trichome density on adaxial leaf surface and higher trichome ratio (0.95). The clones of this cluster are also identified with intermediate values for palisade parenchymatous cell length and width, epidermal length and width and stomatal index (Figure 4.19, Table 4.8 and Table 4.9). Cluster V consists of fifteen clones accounting for 8.9% of total clones and comprised clones with intermediate values for traits, such as stomatal index; stomatal and trichome ratio except for trichome density on abaxial leaf surface (Figure 4.19, Table 4.8 and Table 4.9).

Cluster VI comprised of 5.4% of total clones which are identified with the lowest stomatal ratio and palisade parenchymatous cell length. Intermediate values for trichome ratio and trichome density on adaxial leaf surface was recorded. However, the clones recorded lower values for traits, such as stomatal index, palisade parenchymatous cell width, epidermal cell length and width (Figure 4.19, Table 4.8 and Table 4.9). Cluster VII had clones accounting of 5.9% of total clones comprised with intermediate values for stomatal index, epidermal density on adaxial leaf surface, trichome density on adaxial leaf surface and palisade parenchymatous cell length and width. Higher values for epidermal cell length and width were recorded by these clones. However, the highest trichome density on abaxial leaf surface and lowest trichome ratio was observed in this cluster (Figure 4.19, Table 4.8 and Table 4.9). Cluster VIII comprises of 12.5% of total clones and the highest numbers of clones were placed under this group. The clones were identified by having lower values for stomatal index, stomatal ratio, stomatal density on adaxial leaf surface and trichome density on both the leaf surfaces. However, intermediate values for trichome ratio, palisade parenchymatous cell length and width, epidermal cell length and width were recorded in this group (Figure 4.19, Table 4.8 and Table 4.9).

Cluster IX this group comprised of 5.4% of total clones which are identified by clones having lowest stomatal density on adaxial leaf surface, stomatal index, stomatal ratio and epidermal cell width. Lower value for trichome density on abaxial leaf surface was recorded and intermediate values for traits, such as palisade parenchymatous cell length and width (Figure 4.19, Table 4.8 and Table 4.9). Cluster X comprised of 6.6% of total clones identified by intermediate values for traits, such as stomatal index, stomatal ratio and trichome density on both leaf surfaces. Highest palisade parenchymatous cell width (30 µm) and epidermal cell width (22.1 µm) were recorded by these clones (Figure 4.19, Table 4.8 and Table 4.9). Cluster XI comprised of 7.7% total clones with lower values for stomatal index and stomatal ratio and intermediate values for palisade parenchymatous cell length and width

and epidermal cell width. However, the clones in this cluster recorded the highest values for traits, such as trichome density on adaxial leaf surface, trichome ratio and epidermal cell length (Figure 4.19, Table 4.8 and Table 4.9).

Table 4.7: Grouping of 168 potato clones derived from a cross, J-2/19 × MS/7-645 (PAU3) into 13 clusters on the basis of various leaf morpho-anatomical traits

Cluster	No. of clone(s)	Name of clone(s)
I	16	PAU3-407, PAU3-562, PAU3-621, PAU3-592, PAU3-566, PAU3-600, PAU3-501, PAU3-505, PAU3-491, PAU3-543, PAU3-622, PAU3-565, PAU3-579, PAU3-435, PAU3-520, PAU3-518
II	15	PAU3-406, PAU3-526, PAU3-558, PAU3-533, PAU3-449, PAU3-584, PAU3-454, PAU3-548, PAU3-588, PAU3-467, PAU3-519, PAU3-549, PAU3-544, PAU3-561, PAU3-595
III	9	PAU3-456, PAU3-570, PAU3-550, PAU3-483, PAU3-545, PAU3-591, PAU3-547, PAU3-586, PAU3-477
IV	15	PAU3-431, PAU3-578, PAU3-469, PAU3-470, PAU3-525, PAU3-542, PAU3-402, PAU3-446, PAU3-521, PAU3-556, PAU3-495, PAU3-585, PAU3-464, PAU3-563, PAU3-606
V	15	PAU3-535, PAU3-400, PAU3-421, PAU3-395, PAU3-401, PAU3-601, PAU3-440, PAU3-444, PAU3-597, PAU3-443 PAU3-446, PAU3-455, PAU3-509, PAU3-602, PAU3-510
VI	9	PAU3-399, PAU3-411, PAU3-429, PAU3-575, PAU3-439, PAU3-465, PAU3-503, PAU3-508, PAU3-551
VII	10	PAU3-409, PAU3-426, PAU3-420, PAU3-530, PAU3-536, PAU3-410, PAU3-462, PAU3-438, PAU3-589, PAU3-448
VIII	21	PAU3-522, PAU3-568, PAU3-553, PAU3-522, PAU3-620, PAU3-450, PAU3-461, PAU3-567, PAU3-397, PAU3-481, PAU3-457, PAU3-485, PAU3-538, PAU3-507, PAU3-613, PAU3-590, PAU3-419, PAU3-517, PAU3-500, PAU3-501, PAU3-512
IX	9	PAU3-534, PAU3-560, PAU3-573, PAU3-403, PAU3-413, PAU3-432, PAU3-433, PAU3-452, PAU3-453
X	11	PAU3-427, PAU3-527, PAU3-612, PAU3-511, PAU3-529, PAU3-490, PAU3-611, PAU3-609, PAU3-614, PAU3-391, PAU3-557
XI	13	PAU3-444, PAU3-514, PAU3-451, PAU3-497, PAU3-618, PAU3-398, PAU3-493, PAU3-471, PAU3-423, PAU3-459, PAU3-422, PAU3-458, PAU3-492
XII	10	PAU3-424, PAU3-416, PAU3-524, PAU3-463, PAU3-430, PAU3-468, PAU3-460, PAU3-476, PAU3-472, PAU3-486
XIII	15	PAU3-434, PAU3-513, PAU3-478, PAU3-515, PAU3-537, PAU3-473, PAU3-479, PAU3-506, PAU3-572, PAU3-496, PAU3-499, PAU3-474, PAU3-516, PAU3-498, PAU3-539

Table 4.8: Mean values of group identified by cluster analysis for stomata related traits in potato clones in F_1C_1 generation of J-2/19 × MS/7-645 (PAU3) cross Values are presented as Mean ± SD.

Cluster	Stomatal Density		Epidermal cell	Stomatal index (%)	Stomatal Ratio
	Adaxial	Abaxial	Adaxial		
I	52.5 ±9.6	150.5 ±41.9	410.6 ±76.0	11.5 ±2.1	0.37±0.1
II	54.4 ±9.0	241.0 ±27.7	367.2 ±61.9	13.1 ±2.6	0.23 ±0.0
III	84.8 ±12.8	200.6 ±21.6	354.6 ±53.3	19.5 ±3.2	0.43 ±0.1
IV	45.7 ±13.6	160.3 ±25.9	305.6 ±47.0	12.9 ±2.6	0.29 ±0.1
V	42.3 ±7.7	168.8 ±35.2	332.3 ±57.5	11.5 ±2.4	0.26 ±0.0
VI	28.9 ±7.9	208.9 ±46.6	355.6 ±84.0	7.8 ±2.4	0.15 ±0.0
VII	38.0 ±12.1	203.2 ±51.3	343.8 ±47.5	10.0 ±3.1	0.20 ±0.1
VIII	27.5 ± 9.2	146.0 ±38.2	280.3 ±56.0	8.8 ±1.7	0.20 ±0.1
IX	22.8 ±8.0	127.9 ±32.4	281.4 ±42.6	7.3 ±1.7	0.18 ±0.1
X	39.5 ±10.3	152.7 ±28.8	350.0 ±42.3	10.2 ±2.8	0.27 ±0.1
XI	27.7 ±5.7	148.7 ±29.9	297.7 ±54.6	8.6 ±1.7	0.20 ±0.1
XII	36.9 ±6.6	111.7 ±24.5	233.0 ±32.3	13.7 ±1.9	0.34 ±0.1
XIII	54.1 ±9.4	170.8 ±34.0	307.0 ±57.5	15.1 ±2.4	0.32 ±0.1

Table 4.9: Mean values of group identified by cluster analysis for trichome density, trichome ratio and other anatomical traits in potato clones in F_1C_1 generation of J-2/19 × MS/7-645 (PAU3) cross Values are presented as Mean ± SD.

Cluster	Trichome density		Trichome ratio	Palisade parenchyma cell		Epidermal cell	
	Adaxial	Abaxial		Length (µm)	Width (µm)	Length (µm)	Width (µm)
I	11.5 ±3.5	25.0 ±6.4	0.48 ±0.2	107.7 ±15.6	22.9 ±4.7	31.6 ±4.0	17.1 ±1.9
II	13.2 ±3.1	29.3 ±4.6	0.46 ±0.1	120.7 ±17.0	19.9 ±2.2	34.8 ±3.2	17.5 ±1.7
III	7.8 ±2.4	19.8 ±4.3	0.40 ±0.2	123.2 ±20.1	22.5 ±2.0	33.5 ±2.4	18.0 ±1.4
IV	12.5 ±3.1	14.3 ±4.9	0.95 ±0.3	128.0 ±14.9	22.5 ±2.1	37.2 ±3.4	18.0 ±1.7
V	7.1 ±1.9	14.5 ±3.9	0.51 ±0.1	126.3 ±14.7	23.1 ±1.3	34.8 ±3.7	17.3 ±1.5
VI	9.4 ±2.0	15.4 ±3.4	0.64 ±0.2	115.6 ±17.3	21.9 ±2.6	30.3 ±2.9	16.2 ±1.8
VII	9.0 ±2.4	33.2 ±5.0	0.27 ±0.1	149.2 ±16.3	24.1 ±3.1	38.5 ±6.0	20.1 ±2.2
VIII	7.9 ±2.6	20.3 ±4.3	0.39 ±0.1	147.1 ±16.2	25.1 ±1.8	37.4 ±4.7	20.2 ±1.6
IX	7.2 ±2.5	21.7 ±3.4	0.35 ±0.2	148.5 ±8.8	25.6 ±3.2	32.4 ±5.0	15.6 ±0.8
X	10.6 ±3.7	20.2 ±4.6	0.54 ±0.2	158.5 ±17.7	30.5 ±2.1	39.4 ±3.4	22.1 ±2.5
XI	13.5 ±3.9	17.6 ±5.5	0.79 ±0.2	154.2 ±16.6	24.7 ±2.6	43.0 ±4.6	19.3 ±0.9
XII	9.0 ±2.2	14.2 ±3.2	0.64 ±0.1	151.0 ±12.8	25.5 ±1.4	35.8 ±5.8	19.5 ±2.0
XIII	7.0 ±1.6	17.1 ±4.3	0.43 ±0.1	162.6 ±16.8	25.7 ±1.3	37.7 ±2.6	20.3 ±3.0

Cluster XII comprised of 5.9% total clones having higher values of stomatal index, stomatal ratio, trichome ratio, and length and width of palisade parenchymatous and epidermal cells. However, lower value of trichome density on adaxial leaf surface (Figure 4.19, Table 4.8 and Table 4.9). Cluster XIII comprised of 8.9% of total clones and the clones are identified with the highest values for palisade parenchymatous cell length (162 µm). In addition, the higher mean values for important traits, such as stomatal density on adaxial leaf surface (54.1); stomatal index (15.1%) and stomatal ratio (0.32) were recorded. Intermediate values for palisade parenchymatous cell width, epidermal cell length and width. These clones might be identified as potential frost tolerant clones on the basis of palisade parenchymatous cell length and higher mean stomatal index (Figure 4.19, Table 4.8 and Table 4.9). The stomata and leaf intercellular space are considered as a main resistance sources to the diffusion of CO_2 into a leaf (Kleinhenz *et al* 1995). The higher stomatal index on adaxial leaf surface will result in greater stomatal conductance and ultimately higher photosynthetic rate in plants (Pyakurel and Wang 2014). Additionally, the greater trichome density will enhance the absorption of solar radiation leading to accumulation of leaf heat and create layer of air forming cushion surrounding the leaf to impart frost tolerance (Gates 1980, Campetalli *et al* 2013, Peng *et al* 2015).

4.3.2.2 Cluster analysis of clones derived from cross MS/7-645 × J-2/19 (PAU7)

The hierarchical clustering heatmap analysis of 57 potato clones was performed and the clones were distributed in 6 clusters based on various leaf morpho-anatomical traits (Figure 4.20). The corresponding clones in each cluster are presented in Table 4.10 and their characteristics are described as below. Cluster I consists of 19.3% of the total clones, which are defined by /identified with the highest stomatal ratio, palisade parenchymatous and epidermal cell length and trichome density on abaxial leaf surface. Intermediate values for stomatal density and epidermal cell density on adaxial leaf surface and trichome density on adaxial leaf surface were recorded (Figure 4.20, Table 4.11 and Table 4.12). Cluster II consists of three clones accounting for 5.2% of the total clones. It comprised of clones having the highest values for palisade parenchymatous and epidermal cells width and stomatal ratio, whereas lowest value for epidermal cell length. Similarly, lower values for traits, such as trichome density on both leaf surfaces, trichome ratio and palisade parenchymatous cell length (Figure 4.20, Table 4.11 and Table 4.12).

Cluster III comprised of 17.5% of total clones which are identified with the highest stomatal density on adaxial leaf surface and stomatal index, trichome density on adaxial leaf surface and trichome ratio. These clones recorded higher values for traits, such as trichome density on abaxial leaf surface, length and width of palisade parenchymatous and epidermal cells (Figure 4.20, Table 4.11 and Table 4.12).

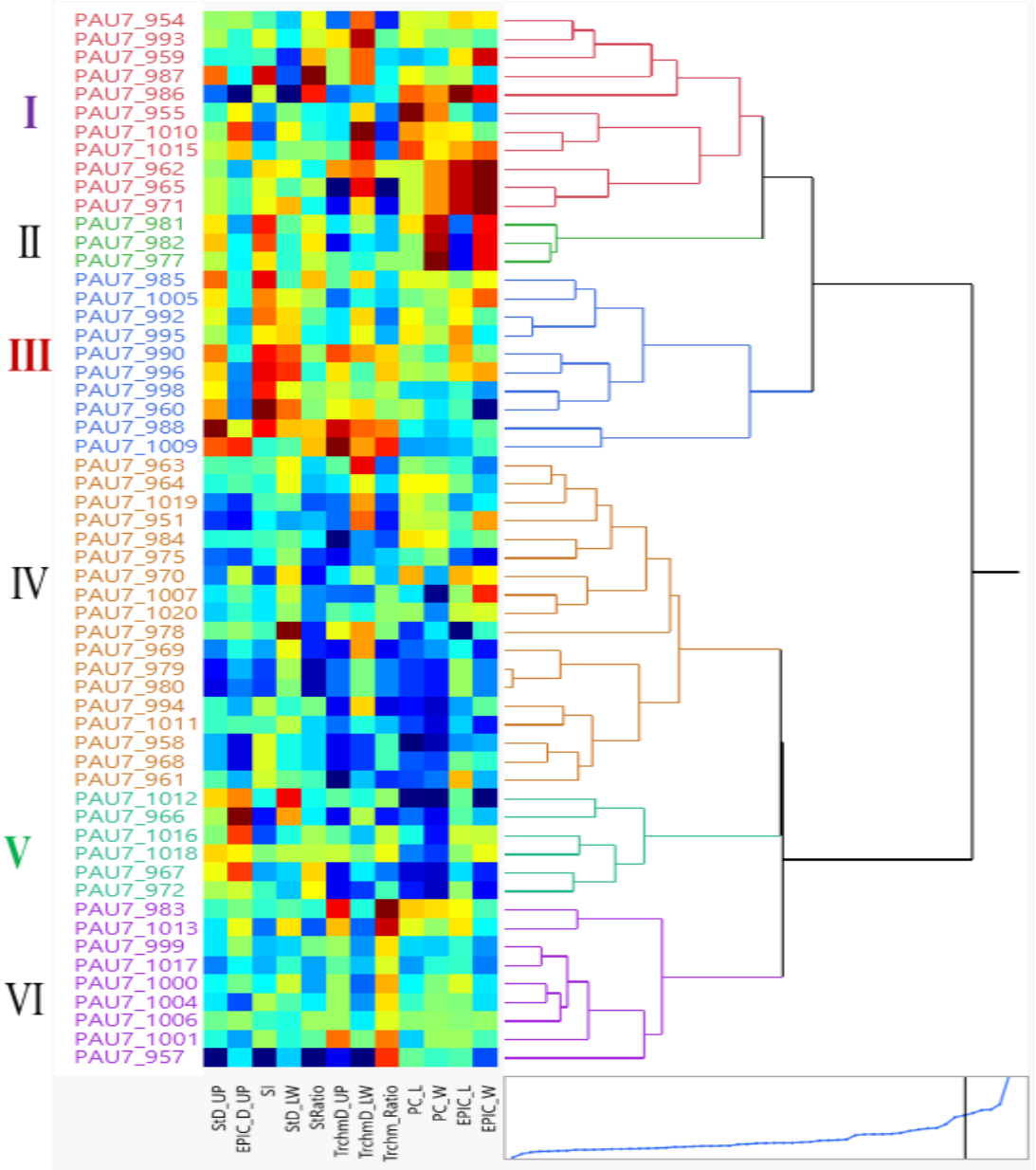


Figure 4.20: The hierarchical clustering heatmap analysis of 57 potato clones derived from a cross, MS/7-645 × J-2/19 (PAU7)

Therefore, these clones might be identified as potential frost tolerant clones on the basis of leaf morpho-anatomical traits as these traits are associated with frost tolerance in potato (Palta and Li 1979). The higher stomatal index and greater numbers of trichomes on adaxial leaf surface are important factors for protecting the plants from various biotic and abiotic stresses (Bisognin *et al* 2006, Campitelli *et al* 2013). These traits are directly involved in CO_2 diffusion and leaf temperature regulations in plants (Gates 1980, Kleinhenz *et al* 1995). Cluster IV comprised of eighteen clones accounting for 31.5% of total clones. These groups of clone were identified with the lowest stomatal, epidermal cell and trichome density on adaxial on leaf surface. Intermediate values for stomatal density on abaxial leaf surface. Similarly, lower values for stomatal index, length and width of palisade parenchymatous and

epidermal cells were recorded (Figure 4.20, Table 4.11 and Table 4.12). According to mean performance for leaf morpho-anatomical traits in this cluster, the clones might possess little or no frost tolerance. The clones exhibited lower values for the frost tolerance associated characters (Palta and Li 1979). Cluster V consisted of six clones accounting for 10.5% of total clones and the group of clones are identified with the highest values for epidermal cell density on adaxial leaf surface. Lowest values were recorded for traits, such as stomatal index (10.1) and palisade parenchymatous cell length and width. Intermediate values for trichome ratio and stomatal density on both leaf surfaces were also recorded (Figure 4.20, Table 4.11 and Table 4.12).

Table 4.10: Grouping of 57 potato clones derived from a cross, MS/7-645 × J-2/19 (PAU7) into six clusters on the basis of various leaf morpho-anatomical traits

Cluster	No. of clone(s)	Name of clone(s)
I	11	PAU7-954, PAU7-993, PAU7-959, PAU7-987, PAU7-986, PAU7-955, PAU7-1010, PAU7-1015, PAU7-962, PAU7-965, PAU7-971
II	3	PAU7-981, PAU7-982, PAU7-977
III	10	PAU7-985, PAU7-1005, PAU7-992, PAU7-995, PAU7-990, PAU7-996, PAU7-998, PAU7-960, PAU7-988, PAU7-1009
IV	18	PAU7-963, PAU7-964, PAU7-1019, PAU7-951, PAU7-984, PAU7-975, PAU7-970, PAU7-1007, PAU7-1020, PAU7-978, PAU7-969, PAU7-979, PAU7-980, PAU7-994, PAU7-1011, PAU7-958, PAU7-968, PAU7-961
V	6	PAU7-1012, PAU7-966 PAU7-1016, PAU7-1018, PAU7-967, PAU7-972
VI	9	PAU7-983, PAU7-1013, PAU7-999, PAU7-1017, PAU7-1000, PAU7-1004, PAU7-1006, PAU7-1001, PAU7-957

Cluster VI comprised of nine clones having the lowest epidermal cell width and trichome density on abaxial leaf surface and highest trichome ratio (0.87). Similarly, lower values for various traits, such as stomatal density on abaxial leaf surface, stomatal index, stomatal ratio, length and width of palisade parenchymatous and epidermal cell (Figure 4.20, Table 4.11 and Table 4.12). However, clones recorded higher value for trichome density on adaxial leaf surface (15.9). Overall, the stomatal index (%) was used as a main parameter for selection of potato clones for frost tolerance in the present study. In this cross, the clones showed stomatal index value more than 10% in all the clusters.

Table 4.11: Mean values of groups identified by cluster analysis for stomata related traits in potato clones in F_1C_1 generation of MS/7-645 × J-2/19 (PAU7) cross Values are presented as Mean ± SD.

Cluster	No. of clone(s)	Stomatal density		Epidermal cell	Stomatal Index	Stomatal Ratio
		Adaxial	Abaxial	Adaxial		
I	11	50.0 ±10.5	140.4 ±48.7	341.0 ±100.9	13.3 ±3.3	0.40 ±0.17
II	3	60.0 ±6.0	148.9 ±6.7	282.2 ±14.9	17.5 ±1.8	0.40 ±0.04
III	10	67.6 ±12.2	193.3 ±25.8	317.3 ±96.7	18.0 ±2.8	0.35 ±0.09
IV	18	36.0 ±7.7	169.8 ±30.2	280.6 ±48.0	11.4 ±2.2	0.21 ±0.05
V	6	56.1 ±6.5	168.0 ±47.3	514.7 ±105.4	10.1 ±2.3	0.35 ±0.08
VI	9	37.0 ±9.2	151.1 ±22.8	314.4 ±48.8	10.5 ±2.7	0.24 ±0.07

Table 4.12: Mean values of groups identified by cluster analysis for trichome density, trichome ratio and other anatomical traits in potato clones in F_1C_1 generation of MS/7-645 × J-2/19 (PAU7) cross Values are presented as Mean ± SD.

Cluster	No. of clones	Trichomes density		Trichomes ratio	Palisade cell		Epidermal cells	
		Adaxial	Abaxial		Length (µm)	Width (µm)	Length (µm)	Width (µm)
I	11	11.5 ±5.0	34.7±5.8	0.33±0.13	171.6 ±32.1	29.8 ±3.9	43.5 ±5.3	23.6 ±4.0
II	3	9.4 ±2.6	24.4±3.5	0.40±0.09	152.6 ±15.0	41.4 ±1.2	26.7 ±2.0	26.1 ±0.1
III	10	17.8 ±8.8	30.1±3.9	0.58 0.25	144.4 ±17.2	24.4 ±2.4	38.5 ±4.2	20.0 ±2.9
IV	18	8.5 ±2.8	25.9±7.5	0.34±0.10	129.0 ±27.7	21.9 ±4.1	35.0 ±5.2	19.1 ±2.7
V	6	9.7 ±3.6	22.5±4.8	0.45±0.16	107.6 ±14.5	18.0 ±1.1	36.9 ±2.4	17.9 ±3.1
VI	9	15.9 ±6.7	18.8±5.1	0.87±0.23	137.6 ±19.1	25.3 ±2.4	38.0 ±2.3	18.5 ±1.3

4.3.2.3 Cluster analysis of clones derived from cross MS/7-645 × CP-3765 (PAU9)

The hierarchical clustering heatmap analysis of 47 potato clones was performed and the clones were distributed in 5 cluster groups based on various leaf morpho-anatomical traits (Figure 4.21). The corresponding clones in each cluster are presented in Table 4.13 and their characteristics are described as below.

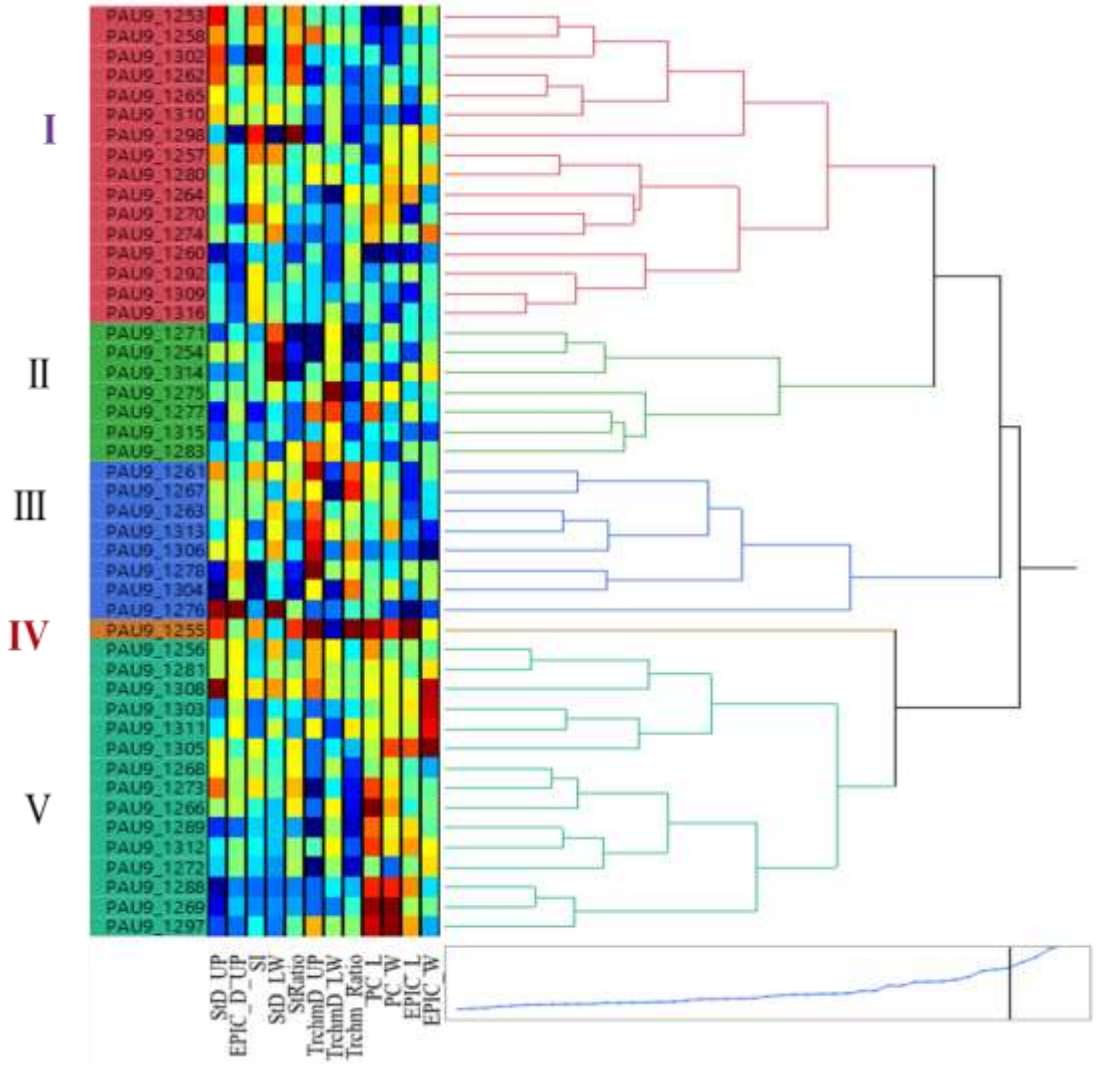


Figure 4.21: The hierarchical clustering heatmap analysis of 47 potato clones derived from a cross, MS/7-645 × CP-3765 (PAU9)

Cluster I consists of sixteen clones accounting for 34.0% of the total clones. It comprised of clones with the higher stomatal density on adaxial leaf surface and stomatal index. The lowest values were recorded for palisade parenchymatous cell length, epidermal cell density and trichome density on adaxial leaf surface. Lower values for traits, such as epidermal cell length and palisade parenchymatous cell width. Those clone with intermediate value for trichome density on abaxial leaf surface (Figure 4.21, Table 4.14 and Table 4.15). Hence, these clones might be identified as low temperature tolerant clones. The stomatal index has been utilized as selection criteria in potato as this trait was found to be highly associated with frost tolerance (Kleinhenz *et al* 1995, Palta and Li 1979). Moreover, stomatal density will directly affect the leaf gas exchange properties of plants (Bisognin *et al* 2006). Cluster II comprised of fifteen clones accounting for 7.0% of the total clones. This group is identified with the lowest stomatal index, stomatal density on adaxial leaf surface and stomatal ratio and palisade parenchymatous cell width. The highest values for stomatal

density and trichome density on abaxial leaf surface were recorded. Lower values for palisade parenchymatous cell length, epidermal cell length and width (Figure 4.21, Table 4.14 and Table 4.15). Cluster III comprised of 17.0% of total clones which are identified with the highest epidermal cell density on adaxial leaf surface and stomatal ratio. These clones recorded the lowest epidermal cell length (30.1) and width (19.2). The clones in this cluster observed intermediate values for traits, such as palisade parenchymatous cell length and width and trichome density on adaxial leaf surface (Figure 4.21, Table 4.14 and Table 4.15).

Table 4.13: Grouping of 47 potato clones derived from a cross, MS/7-645 × CP-3765 (PAU9) into five clusters on the basis of various leaf morpho-anatomical traits

Cluster	No. of clone(s)	Name of clone(s)
I	16	PAU9-1253, PAU9-1258, PAU9-1302, PAU9-1262, PAU9-1265, PAU9-1310, PAU9-1298, PAU9-1257, PAU9-1280, PAU9-1264, PAU9-1270, PAU9-1274, PAU9-1260, PAU9- 1292, PAU9-1309, PAU9-1316
II	7	PAU9-1271, PAU9-1254, PAU9-1314, PAU9-1275 , PAU9-1277, PAU9-1315, PAU9-1283
III	8	PAU9-1261, PAU9-1267, PAU9-1263, PAU9-1313, PAU9-1306, PAU9-1278, PAU9-1304, 18091276
IV	1	PAU9-1255
V	15	PAU9-1256, PAU9-1281, PAU9-1308, PAU9-1303, PAU9-1311, PAU9-1305, PAU9-1268, PAU9-1273, PAU9-1266, PAU9-1289, PAU9-1312, PAU9-1272, PAU9-1288, PAU9-1269, PAU9-1297

Cluster IV comprised of only one clone *i.e.* PAU9-1255 having the highest stomatal density on adaxial leaf surface, stomatal index, stomatal ratio, trichome density on adaxial leaf surface, length and width of palisade parenchymatous and epidermal cells. The lowest stomatal density and trichome density on abaxial leaf surface (Figure 4.21, Table 4.14 and Table 4.15). It can be anticipated that the higher stomatal density and stomatal index on adaxial leaf surface will help in improving the leaf gas exchange properties of plants under adverse environmental conditions (Kleinhenz *et al* 1995, Campitelli *et al* 2013). Moreover, boundary layer resistance and intercellular space are also considered to be involved in carbon dioxide diffusion in plants (Kleinhenz *et al* 1995). Thus, PAU9-1255 can be identified as a potential frost tolerant clone. Cluster V consists of fifteen clones accounting for 31.9% of total clones and the clones possessed intermediate values for all leaf morpho-anatomical traits except for trichome density on adaxial leaf surface (Figure 4.21, Table 4.14 and Table 4.15).

Table 4.14: Mean values of groups identified by cluster analysis for stomata related traits in potato clones in F_1C_1 generation of MS/7-645 × CP-3765 (PAU9) cross Values are presented as Mean ± SD.

Cluster	No. of clone(s)	Stomatal density		Epidermal cell	Stomatal Index (%)	Stomatal Ratio
		Adaxial	Abaxial	Adaxial		
I	16	51.1 ±12.8	191.3 ±45.3	274.2 ±58.1	15.7 ±2.7	0.28 ±0.1
II	7	37.1 ±7.9	232.2 ±80.9	326.2 ±48.3	10.3 ±2.0	0.18 ±0.1
III	8	47.5 ±18.3	223.3 ±58.4	442.5 ±145.9	10.0 ±3.8	0.22 ±0.1
IV	1	68.3 ± 0	161.7 ± 0	341.7 ± 0	16.6 ± 0	0.42 ± 0
V	15	44.4 ±15.2	176.2 ±40.0	350.5 ±74.3	11.0 ±2.4	0.25 ±0.1

Table 4.15: Mean values of group identified by cluster analysis for trichome density, trichome ratio and other anatomical traits in potato clones in F_1C_1 generation of MS/7-645 × CP-3765 (PAU9) cross Values are presented as Mean ± SD.

Cluster	No. of clone(s)	Trichome density		Trichome Ratio	Palisade parenchyma cell		Epidermal cells	
		Adaxial	Abaxial		Length (µm)	Width (µm)	Length (µm)	Width (µm)
I	16	12.2±3.0	19.5±5.5	0.68±0.2	137.0±22.8	23.9±3.1	33.4±4.1	21.4±2.1
II	7	13.3±5.5	34.5±9.6	0.39±0.2	151.4±18.6	23.6±2.2	33.1±2.9	20.9±2.1
III	8	19.6±4.9	16.7±6.8	1.31±0.5	151.1±11.9	24.6±2.1	30.1±2.7	19.2±2.8
IV	1	25.0±0	10.0±0	2.50±0	200.9±0	29.5±0	49.6±0	23.4±0
V	15	12.3±4.7	22.4±5.5	0.57±0.3	177.5±17.6	27.2±2.5	37.2±3.4	23.3±3.7

Overall, the segregating progenies derived from the three different crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) exhibited significant variations among the studied clones for all the twelve leaf morpho anatomical traits and cluster analysis classified the clones in different clusters according to their proximity in traits. Our findings are in consistent with (Kleinhenz *et al* 1995, Palta and Li 1979) that the potato genotypes/ species exhibit high variability for leaf morpho anatomical traits. Moreover, these traits can be used for the screening of potato segregating populations for frost tolerance even during non-frost years.

4.3.3 Principal component analysis (PCA)

Principal component analysis showed that the % of total variance determined by different principal components (PCs) and the correlation among the traits and these PCs contributed both negatively and positively in differential variability.

4.3.3.1 Principal component analysis (PCA) of clones derived from cross J-2/19 × MS/7-645 (PAU3)

The PCA obtained from the 168 potato clones for leaf morpho-anatomical traits are illustrated in Table 4.16 and Figure 4.22. Three PCs elucidated 64% of the total variation recorded in the clones (Table 4.16). The first two PCs were the most prominent, with a cumulative contribution to the total variation of 43.5%. In the PC1, stomatal density on adaxial leaf surface (0.48), stomatal index (0.361), stomatal ratio (0.321), epidermal density on adaxial leaf surface (0.281), stomatal density on the abaxial leaf surface (0.312) and trichome density on adaxial leaf surface (0.143) recorded the highest variability (Table 4.16). However, length and width of palisade parenchymatous and epidermal cells contributed negatively to the PC1 (−0.366, −0.339, −0.256 and −0.211), respectively (Table 4.16). Similarly, the maximum variability was observed for stomatal ratio (0.479), stomatal index (0.440) and stomatal density on adaxial leaf surface (0.271) in the PC2, whereas the trichome density on abaxial leaf surface (0.436) recorded higher variability than other traits in the PC3 (Table 4.16 and Figure 4.22).

Table 4.16: Principal components analysis matrix of leaf morpho-anatomical traits of 168 potato clones derived from a cross, J-2/19 × MS/7-645 (PAU3)

Traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigenvalue	3.15	2.06	1.72	1.49	1.06	0.79	0.59
% Variance	26.28	17.19	14.35	12.48	8.84	6.63	4.96
Cumulative % variance	26.28	43.47	57.83	70.31	79.16	85.79	90.75
Stomatal density adaxial	0.481	0.271	0.195	0.069	-0.095	0.139	0.103
Epidermal density adaxial	0.281	-0.241	0.150	0.189	0.371	0.667	-0.004
Stomatal index	0.362	0.441	0.128	-0.014	-0.298	-0.208	0.138
Stomatal density abaxial	0.246	-0.328	0.170	0.190	-0.592	0.247	0.210
Stomatal ratio	0.321	0.479	0.087	-0.057	0.372	-0.043	-0.045
Trichome density adaxial	0.143	-0.051	-0.286	0.648	0.238	-0.245	0.093
Trichome density abaxial	0.124	-0.332	0.436	0.277	0.263	-0.470	0.075
Trichome ratio	-0.003	0.204	-0.630	0.320	-0.062	0.194	0.119
Palisade length	-0.366	0.211	0.228	0.109	-0.038	0.097	0.523
Palisade width	-0.339	0.225	0.241	0.033	0.271	0.237	0.346
Epidermal length	-0.256	0.150	0.161	0.447	-0.238	-0.095	-0.003
Epidermal width	-0.212	0.256	0.285	0.329	-0.119	0.190	-0.709

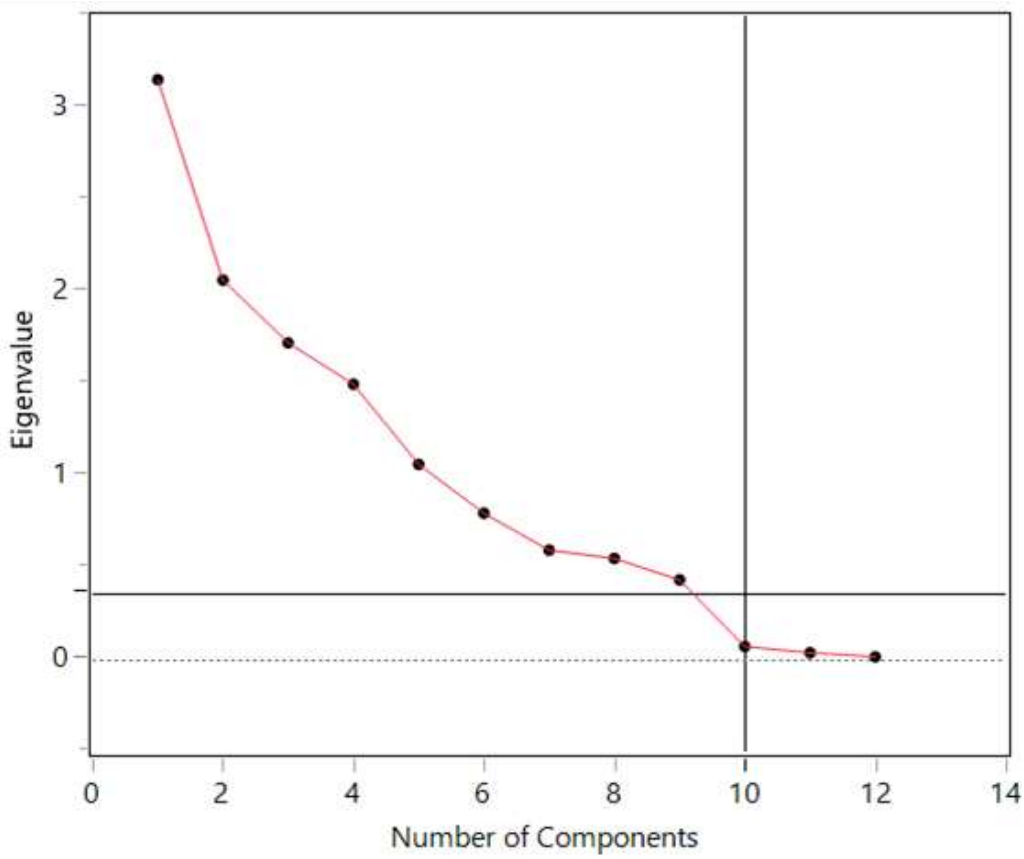


Figure 4.22: Scree plot of clones derived from a cross, J-2/19 × MS/7-645 (PAU3)

4.3.3.2 Principal component analysis (PCA) of clones derived from cross MS/7-645 × J-2/19 (PAU7)

The PCA obtained from the 57 potato clones for leaf morpho-anatomical traits are illustrated in Table 4.17 and Figure 4.23. Three PCs explained 58.0% of the total variation observed in the clones (Table 4.17). With a cumulative contribution to the total variation of 45.5%, the first two PCs were the most important. In the PC1, stomatal density on adaxial leaf surface (0.37), stomatal index (0.39), stomatal ratio (0.38), and trichome density on abaxial leaf surface (0.31), length (0.32) and width (0.37) of palisade parenchymatous cells recorded the highest variability (Table 4.17). Further, the epidermal density on adaxial leaf surface (-0.045) and stomatal density on the abaxial leaf surface (-0.053) contributed negatively to the PC1 (Table 4.17). Similarly the maximum variability was observed for stomatal density on adaxial (0.40), stomatal density on abaxial leaf surface (0.32), trichome density on adaxial leaf surface (0.49) and trichome ratio (0.40) in the PC2, whereas the epidermal cell density on adaxial leaf surface (0.37), stomatal density on abaxial leaf surface (0.49) and trichome density on abaxial leaf surface (0.50) observed higher variability as compared with other traits in the PC3 (Table 4.17 and Figure 4.23).

Table 4.17: Principal components analysis matrix of leaf morpho-anatomical traits of 57 potato clones derived from a cross, MS/7-645 × J-2/19 (PAU7)

Traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigenvalue	3.16	2.29	1.51	1.45	1.27	0.88	0.64
% Variance	26.32	19.13	12.58	12.08	10.61	7.38	5.41
Cumulative % variance	26.32	45.46	58.04	70.12	80.73	88.11	93.52
Stomatal density adaxial	0.378	0.400	0.110	-0.252	0.057	0.004	0.221
Epidermal density adaxial	-0.045	0.284	0.372	0.107	0.601	-0.250	0.297
Stomatal index	0.396	0.136	-0.153	-0.340	-0.389	0.201	0.033
Stomatal density abaxial	-0.053	0.324	0.492	0.142	-0.458	0.092	0.290
Stomatal ratio	0.384	0.062	-0.256	-0.335	0.427	-0.011	-0.083
Trichome density adaxial	0.201	0.495	-0.167	0.335	-0.033	0.010	-0.262
Trichome density abaxial	0.315	0.035	0.507	-0.030	0.023	0.054	-0.528
Trichome ratio	-0.018	0.400	-0.459	0.452	-0.023	-0.061	0.029
Palisade length	0.327	-0.250	0.135	0.403	-0.001	-0.229	-0.268
Palisade width	0.377	-0.257	-0.041	0.197	-0.196	-0.486	0.148
Epidermal length	0.172	-0.176	0.045	0.370	0.229	0.767	0.060
Epidermal width	0.360	-0.259	-0.030	0.144	0.015	0.063	0.570

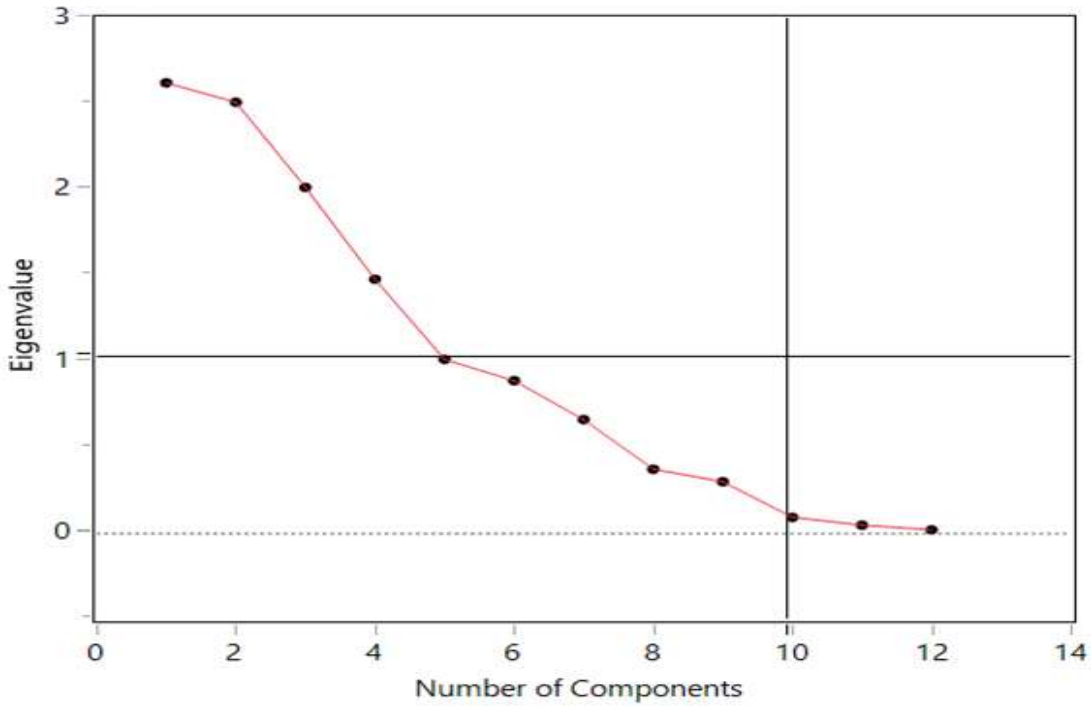


Figure 4.23: Scree plot of clones derived from a cross, MS/7-645 × J-2/19 (PAU7)

4.3.7 Principal component analysis (PCA) of clones derived from cross MS/7-645 × CP-3765 (PAU9)

The PCA obtained from the 47 potato clones for morpho-anatomical traits are illustrated in Table 4.18 and Figure 4.24. Three PCs explained 59.5% of the total variation recorded in the clones (Table 4.18).

With a cumulative contribution to the total variation of 42.7%, the first two components were the most important. The PC1, PC2, and PC3 contributed 21.8 %, 20.1% and 16.8% variability, respectively. Different parameters contributed both positively and negatively to different principal components (PCs). In the PC1, stomatal ratio (0.38), palisade parenchymatous cell width (0.34), epidermal cell length (0.45) and stomatal index (0.29) recorded the highest variability whereas, the epidermal density on adaxial leaf surface (-0.30) contributed negatively to the PC1 (Table 4.18). Similarly the maximum variability was observed for stomatal density on adaxial leaf surface (0.51), stomatal index (0.50), and stomatal ratio (0.40) in the PC2. Further, the epidermal cell density on adaxial leaf surface (0.35) and trichome ratio (0.62) recorded higher variability than the other traits in the PC3 (Table 4.18).

Table 4.18: Principal components analysis matrix of leaf morpho-anatomical traits of 47 potato clones derived from a cross, MS/7-645 × CP-3765 (PAU9)

Traits	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigenvalue	2.62	2.51	2.01	1.48	1.01	0.88	0.65
% Variance	21.85	20.91	16.77	12.31	8.41	7.38	5.49
Cumulative % variance	21.85	42.76	59.53	71.85	80.27	87.66	93.16
Stomatal density adaxial	0.062	0.511	0.149	0.406	0.016	-0.158	0.054
Epidermal density adaxial	-0.306	0.019	0.348	0.452	0.060	-0.125	-0.516
Stomatal index	0.290	0.495	-0.142	-0.002	-0.031	-0.033	0.402
Stomatal density abaxial	-0.390	0.124	0.122	0.401	-0.305	0.155	0.486
Stomatal ratio	0.386	0.402	-0.034	0.064	0.259	-0.220	-0.312
Trichome density adaxial	-0.057	0.009	0.515	-0.114	0.563	0.235	0.242
Trichome density abaxial	-0.171	-0.147	-0.341	0.288	0.673	0.007	0.219
Trichome ratio	0.111	0.083	0.619	-0.285	-0.054	0.081	0.065
Palisade length	0.255	-0.364	0.170	0.302	-0.026	-0.400	0.099
Palisade width	0.338	-0.341	0.145	0.196	-0.135	-0.309	0.282
Epidermal length	0.454	-0.194	0.055	0.151	0.132	0.295	0.021
Epidermal width	0.294	-0.047	-0.052	0.369	-0.159	0.694	-0.186

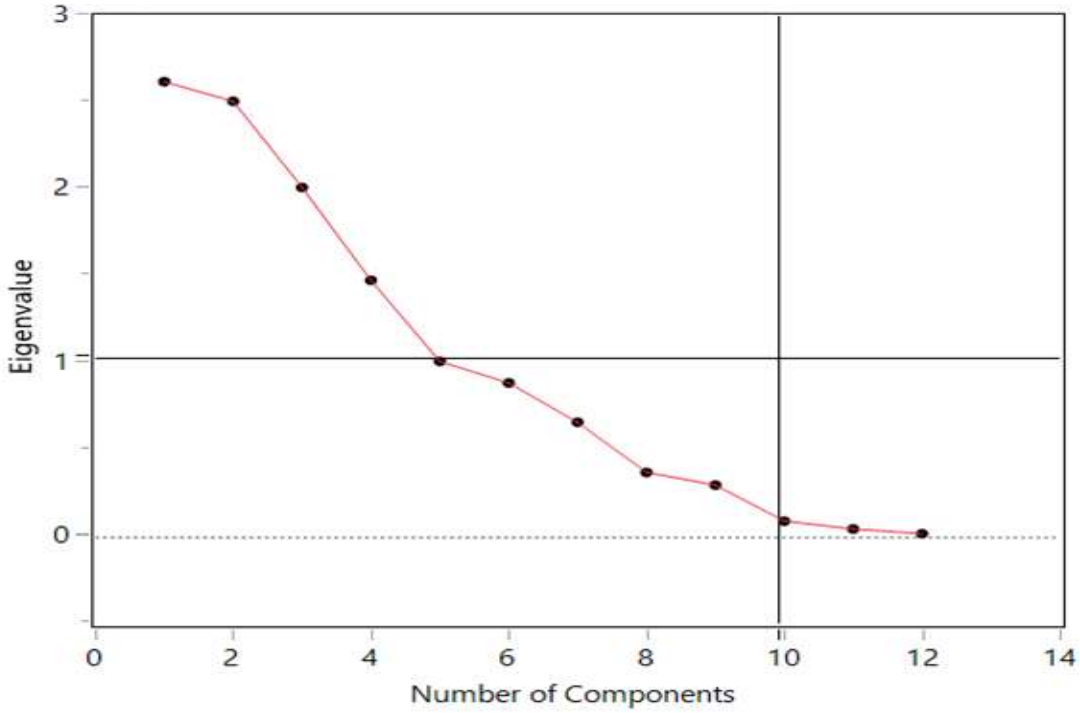


Figure 4.24: Scree plot of clones derived from a cross, MS/7-645 × CP-3765 (PAU9)

Overall, from the results it was observed that the PCA analysis is an effective approach to evaluate the relative contribution of each studied traits in determining the phenotypic variation. Studies in different crops such as walnut (Panahi *et al* 2021), potato (Daiz-Valencia *et al* 2021), onion (Gedam *et al* 2021) and sweet potato (Rosero *et al* 2020) are in consistent with our findings.

4.3.4 Biplot analysis

The genotype by trait (GT) biplot analysis further revealed the association among the different traits and clones for low temperature stress

Genotype by trait (GT) biplot was performed from a two-way matrix of twelve leaf morpho-anatomical traits and 168 potato clones derived from cross PAU3 (Figure 4.25). An acute angle between different traits in the same direction indicated a high association between the corresponding traits for classifying clones. Clones superior for a particular trait were placed closer and along the vector line direction. In biplot analysis, the clones were mostly concentrated on the positive side of both, PC1 and PC2. The clones, PAU3-456, PAU3-570, PAU3-550, PAU3-483, PAU3-545, PAU3-591, PAU3-547, PAU3-586, PAU3-477, PAU3-467 and PAU3-483 were more inclined in the direction of stomatal ratio and stomatal index (Figure 4.25). Similarly, the clones, PAU3-496, PAU3 537, PAU3-545, PAU3-467 and PAU3-483 were more inclined in the direction of palisade parenchymatous length. Moreover, the clones, PAU3-486, PAU3-621, PAU3-537, PAU3-545, PAU3-467 and PAU3-483 were more inclined in the direction of trichome density on adaxial leaf surface (Figure 4.25).

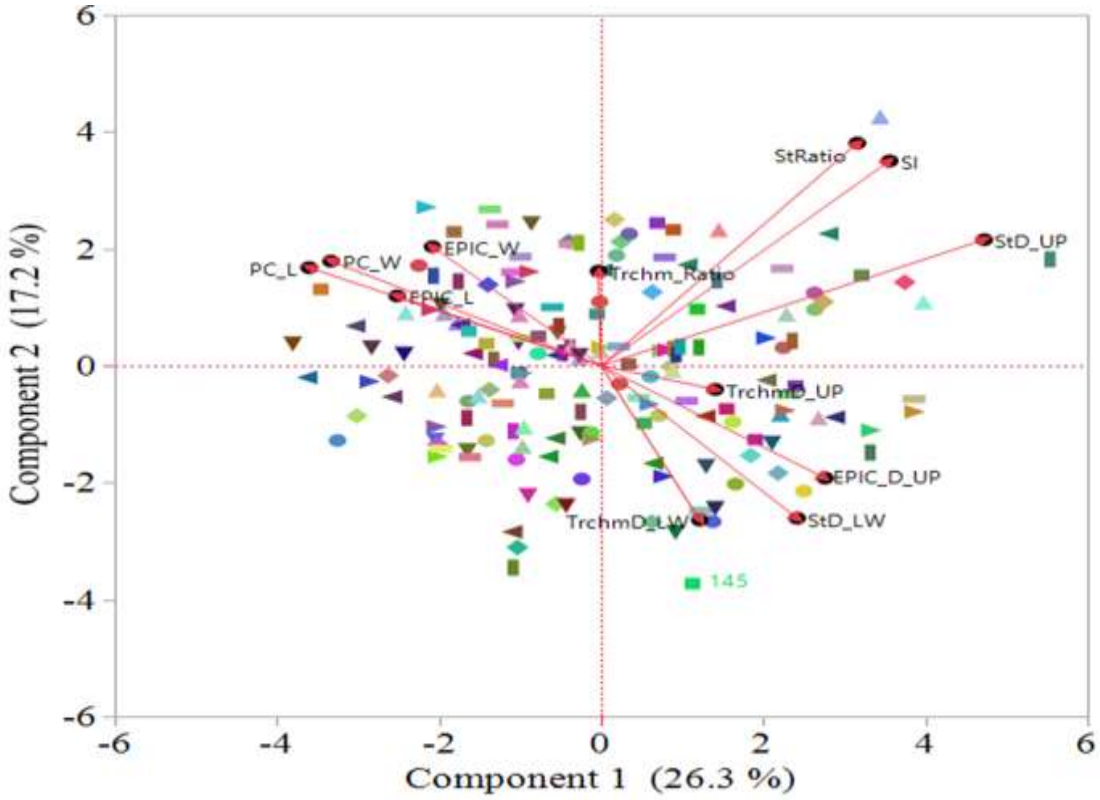
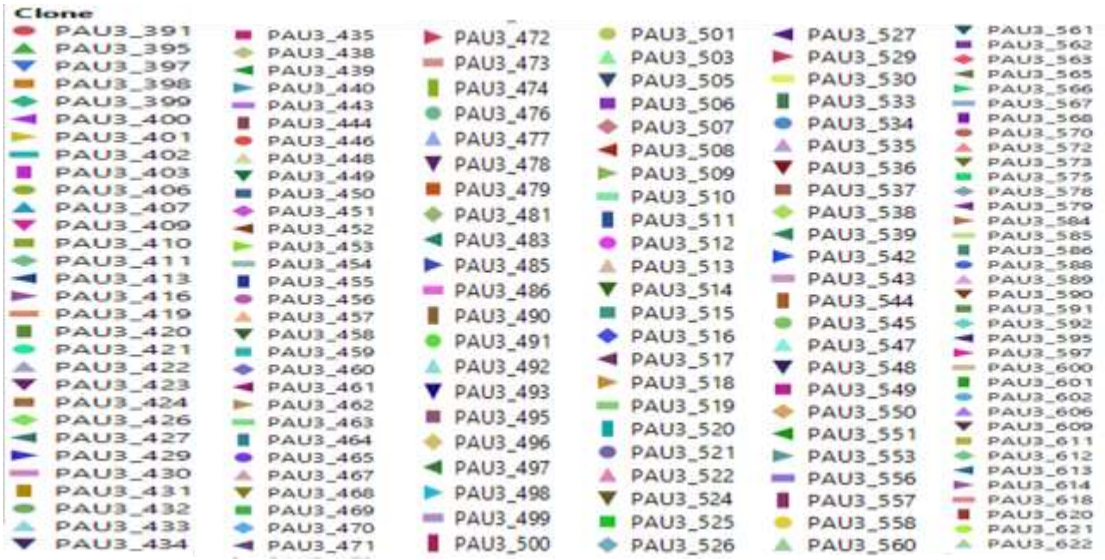


Figure 4.25: Genotype by trait biplot of 168 potato clones derived from a cross, PAU3 based on the leaf morpho-anatomical traits



StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

In this study the clones, PAU3-477, PAU3-483 and PAU3-533 were placed closer to frost associated trait (stomatal index) at a considerable distance from the origin. Stomatal index is a suitable index to identify promising frost tolerant clones (Aslamarz and Vahdati

2010). Higher stomatal density is advantages for greater stomatal conductance and hence higher photosynthesis in plants (Pyakurel and Wang 2014). Similarly, the clones, PAU3-496, PAU3-537 and PAU3-545 are placed closer to palisade parenchyma length and at far distance from the origin. The clones and traits that lie far away from the origin have better breeding potential than other clones (Gedam *et al* 2021) Thus, these clones can be utilized as promising tolerant potato clones in potato breeding program.

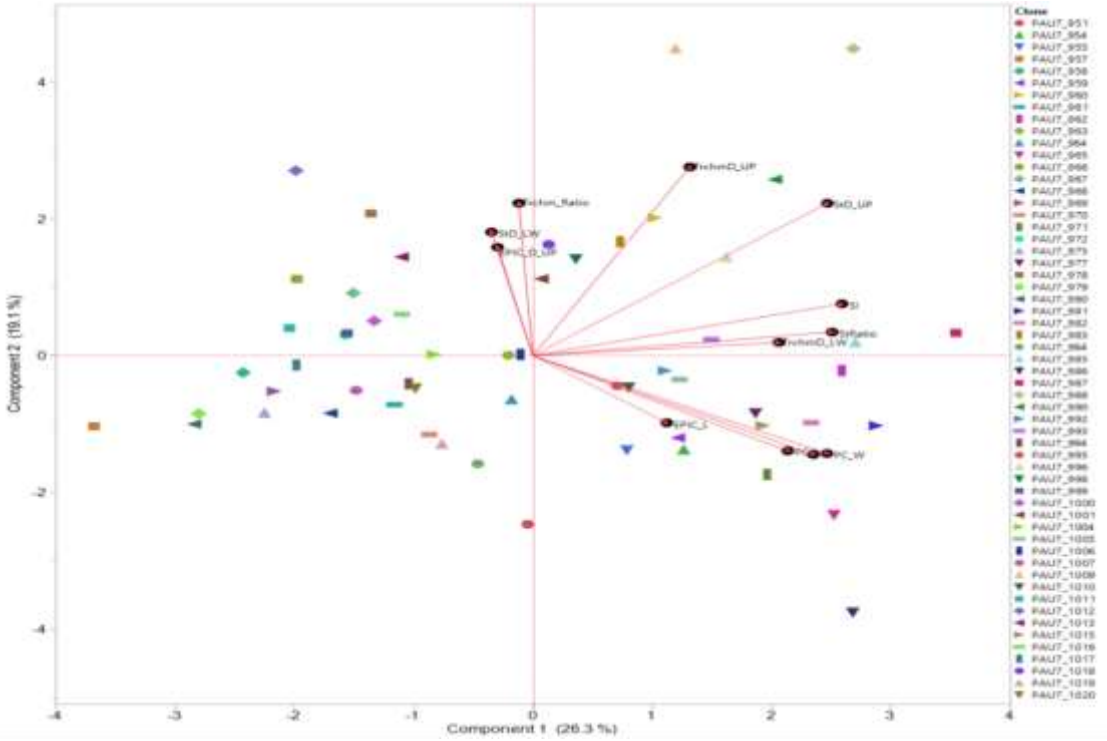


Figure 4.26: Genotype by trait biplot of 57 potato clones derived from a cross, PAU7 based on the leaf morpho-anatomical traits

StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

Genotype by trait (GT) biplot was created from a two-way matrix of twelve leaf morpho-anatomical traits and 57 potato clones derived from PAU7 cross (Figure 4.26). An acute angle between different traits in the same direction indicated a high association between corresponding traits for classifying clones. Clones superior for a particular trait were plotted closer and along the vector line direction. In biplot analysis, the clones were mostly concentrated on the positive side of both, PC1 and PC2. The clones, such as PAU7-993, PAU7-982, PAU7-985, PAU7-987 and PAU7-996 were more inclined in the direction of stomatal ratio, stomatal index and stomatal density on adaxial leaf surface (Figure 4.26). Further, the clones, PAU7-951, PAU7-1020, PAU7-1010, PAU7-992, PAU7-977, PAU7-965,

PAU7-1005, PAU7-981 and PAU7-971 were more inclined in the direction of anatomical traits such as length and width of palisade parenchyma and epidermal cells. Further, the clones, PAU7-960, PAU7-983, PAU7-998, PAU7-1001 and PAU7-1018 were more inclined in the direction of trichome traits (Figure 4.26). In this study the clones, PAU7-985, PAU7-987 and PAU7-996 were placed closer to frost associated trait (stomatal index) at a considerable distance from the origin. To identify promising frost tolerant clones stomatal index was used a suitable screening marker for frost tolerance in potato and walnut (Palta and Li 1979, Aslamarz and Vahdati 2010). Higher stomatal index on adaxial leaf surface is advantages for greater stomatal conductance and photosynthesis under adverse environmental conditions (Kleinhenz *et al* 1995, Pyakurel and Wang 2014). Similarly, the clones, PAU7-951, PAU7-1020, PAU7-1010, PAU7-992, PAU7-1005, PAU7-981 and PAU7-971 were placed closer to palisade parenchyma length and at distanced from the origin (Figure 4.26). The clones and traits that lie far away from the origin have better breeding potential than other clones (Gedam *et al* 2021) Thus, these clones and traits can be utilized as potential frost tolerant potato clones and promising traits for frost tolerance breeding program in potato crop.

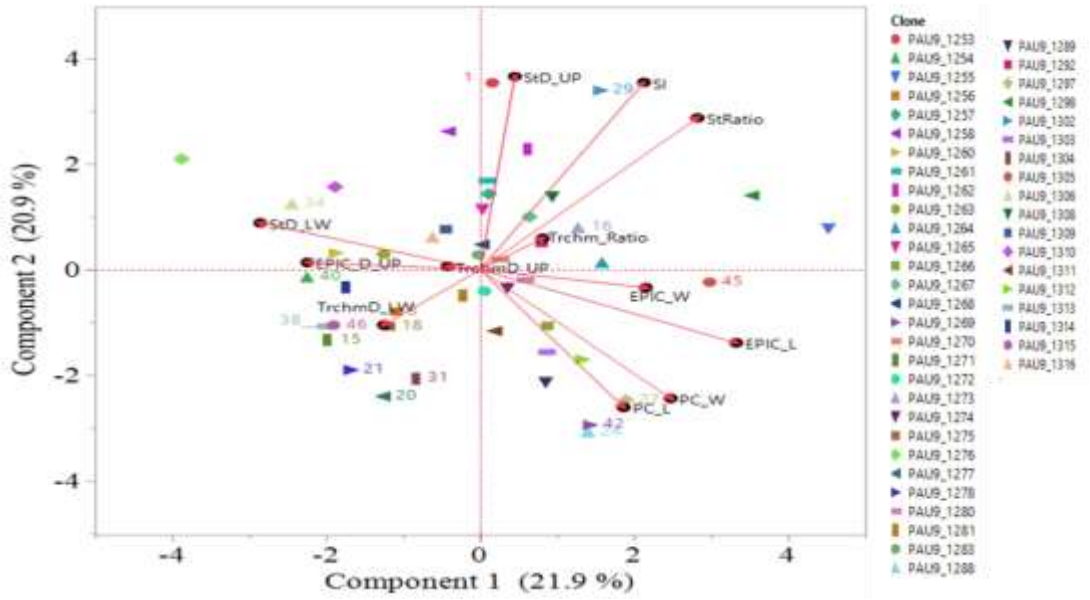


Figure 4.27: Genotype by trait biplot of 47 potato clones derived from a cross, PAU9 based on the leaf morpho-anatomical traits

StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

Genotype by trait (GT) biplot was created from a two-way matrix of twelve parameters and 47 potato clones. A smaller angle between different traits in the same direction indicated a high association between corresponding traits for classifying clones.

Clones superior for a particular trait were plotted closer and along the vector line direction. In biplot analysis, the clones were mostly concentrated on the positive side of both, PC1 and PC2. The clones such as PAU9-1308, PAU9-1257, PAU9-1302, PAU9-1273 and PAU9-1270 highly inclined to stomatal ratio and stomatal index (Figure 4.27). Similarly, clones such as PAU9-1253, PAU9-1262, PAU9-1261, PAU9-1267 and PAU9-1268 were more inclined in the direction of stomatal density on adaxial leaf surface. Further, the clones PAU9-1312, PAU9-1266, PAU9-1303, PAU9-1269, PAU9-1288, PAU9-1274/1289, PAU9-1311, PAU9-1305, PAU9-1280 and PAU9-1272 were more inclined in the direction of length and width of palisade and epidermal cells (Figure 4.27). Moreover, the clone PAU9-1292 was more inclined in the direction of trichome density on adaxial leaf surface and trichome ratio. In this study the clones, PAU9-1308, PAU9-1257, PAU9-1302 and PAU9-1273 were placed closer to frost associated trait (stomatal index) at a considerable distance from the origin (Figure 4.27). Stomatal index was used as an effective anatomical screening marker to identify potential frost tolerant clones in walnut and potato (Kleinheinz *et al* 1995, Aslamarz and Vahdati 2010). Similarly, the clones, PAU9-1312, PAU9-1266, PAU9-1303, PAU9-1269 and PAU9-1288 were placed closer to palisade parenchyma length and at distanced from the origin. Greater numbers of stomata on adaxial leaf surface, compact and thicker palisade parenchymatous cell are directly involved in stomatal conductance in plants. As these traits are main sources of resistance to carbon dioxide diffusion into leaf in plants (Kleinheinz *et al* 1995). The clones and traits that lie far away from the origin have better breeding potential than other clones (Gedam *et al* 2021) Thus, these clones can be utilized as promising tolerant potato clones in potato breeding program.

Overall, the biplot analysis indicated that stomatal indexes, stomatal density on adaxial leaf surface and stomatal ratio was closely associated with each other in all three crosses. Moreover, these traits were placed at a considerable distance from the origin and can be considered as adaptive promising traits for frost tolerance breeding in potato for screening large segregating populations. Similarly, for screening large population biplot studies was reported by Daiz- Valencia *et al* (2021), Gedam *et al* (2021) and Panahi *et al* (2021) in potato, onion and walnut crops, respectively.

4.3.4 Characterization and evaluation of potato clones in F_1C_2 generation

In F_1C_1 generation, on the basis of stomatal index 92, 39 and 33 clones from the segregating populations of crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) were selected, respectively. Simultaneously, on the basis of tuber characteristics, a set of 55 clones derived from three different crosses were also selected. In F_1C_2 generation, for further evaluation and clonal advancement these selected clones were planted at two different locations:-

Location–I (L1) – Vegetable Research Farm, PAU-Ludhiana

Location –II (L2) –PAU- Vegetable Research Farm, Khanaura, Hoshiarpur

4.3.4.1 Field evaluation and membrane injury at Ludhiana

During the cropping season 2020-2021, the selected clones derived from three different crosses were planted in rows with check varieties after every 10 rows for its evaluation. The crop was planted in the month of October and raised by following the package of practices recommended by Punjab Agricultural University, Ludhiana.

In F_1C_2 generation, assessment of cell membrane stability was performed in terms of electrolyte leakage in potato clones after low temperature exposure in the months of December and January. The minimum temperatures recorded at the automatic weather stations of Ludhiana by the Department of Climate Change and Agricultural metrology, PAU, Ludhiana (Figure 4.28).

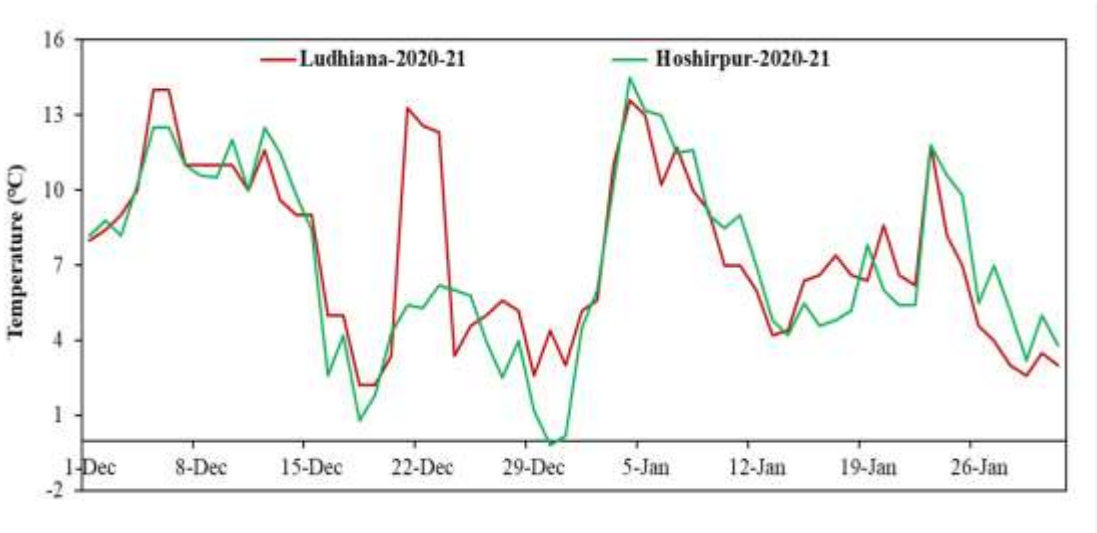


Figure 4.28: Minimum temperatures during December to January months at Ludhiana and Hoshiarpur during cropping season 2020–21

Significant variations were observed among the clones for percent electrolyte leakage. In the clones derived from cross J-2/19 × MS/7-645, the percent electrolyte leakage in the studied clones varied from 4.6 to 11.2 with mean value 6.8 (Table 4.18 and Figure 4.29). Similarly, in the clones derived from cross MS/7-645 × J-2/19, the percent electrolyte leakage in the studied clones varied from 7.7 to 15.7 with mean value 12.1 (Table 4.19 and Figure 4.29). Further, in the clones derived from MS/7-645 × CP-3765, the percent electrolyte leakage in the studied clones varied from 8.3 to 16.4 with mean value 12.1 (Table 4.19 and Figure 4.29).

Frost or freezing injury has been thought to involve cell membrane as one of the most common signs of injury. On exposure to freezing temperature the ice formation on plant tissues caused dehydration (extracellular freezing) and consequently leading to loss of integrity of cell membrane and leakages of ions which is presumably a result of mechanical

stress (Li and Fennell 1985) Thus, ultimately affecting the integrity and organization of cell ultrastructure (Li *et al* 1979, Wu *et al* 2019).

Table 4.19: Descriptive statistics of electrolyte leakage traits of potato clones derived from crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) at Ludhiana

Crosses	N	Minimum	Maximum	Range	Mean	S.D	S.E	Skewness	Kurtosis
PAU3	76	6.9	16.0	9.1	10.0	1.8	0.2	1.0	1.6
PAU7	39	7.7	15.7	7.9	12.1	1.8	0.3	-0.6	0.0
PAU9	32	8.3	16.4	8.1	12.0	2.1	0.4	0.2	-0.8

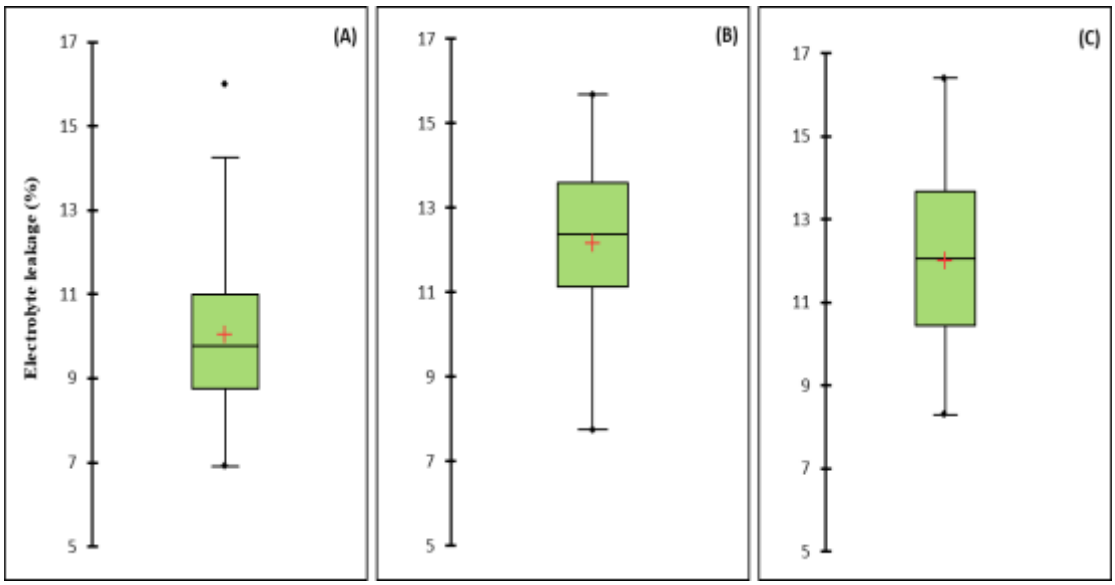


Figure 4.29: Electrolyte leakage (%) of potato clones derived from different crosses at Ludhiana A) PAU3, B) PAU7 and C) PAU9 after low temperature exposure in F_1C_2 generation

It is apparent from the results that there is significant genotypic variability in the studied clones in terms of percent electrolyte leakage (Table 4.19 and Figure 4.28). Which may help in determining the potential frost tolerant potato clones and furthermore these clones can be evaluated and exploited in breeding programe for frost tolerance. Similarly, Stone *et al* (1993) revealed that the assessment of percent electrolyte leakage was carried out to determine the cell membrane injury and frost tolerance among the F_1 progenies, backcross generations and their parents in potato. Further, Lyons (1973) reported that the cell membrane system suffered frost damage in plants, and that the primary cause of freezing injury is damage to the cell membrane system, which leads to changes in cell membrane permeability. Therefore, plant freezing resistance is closely related to the integrity of the bio-membrane system. Furthermore, it was confirmed that the maintaining of integrity and stability of the bio

membranes is an important way to enhance cold resistance in various crops such as potato (Wu *et al* 2019) and walnut (Panahi *et al* 2021).

4.3.4.2 Biplot analysis

The genotype by trait biplot revealed the association among different traits and clones and further association of the clones with particular traits. The genotype by trait biplot analysis was performed from a two-way matrix of twelve leaf morpho-anatomical traits, electrolyte leakage and clones derived from different crosses J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9).

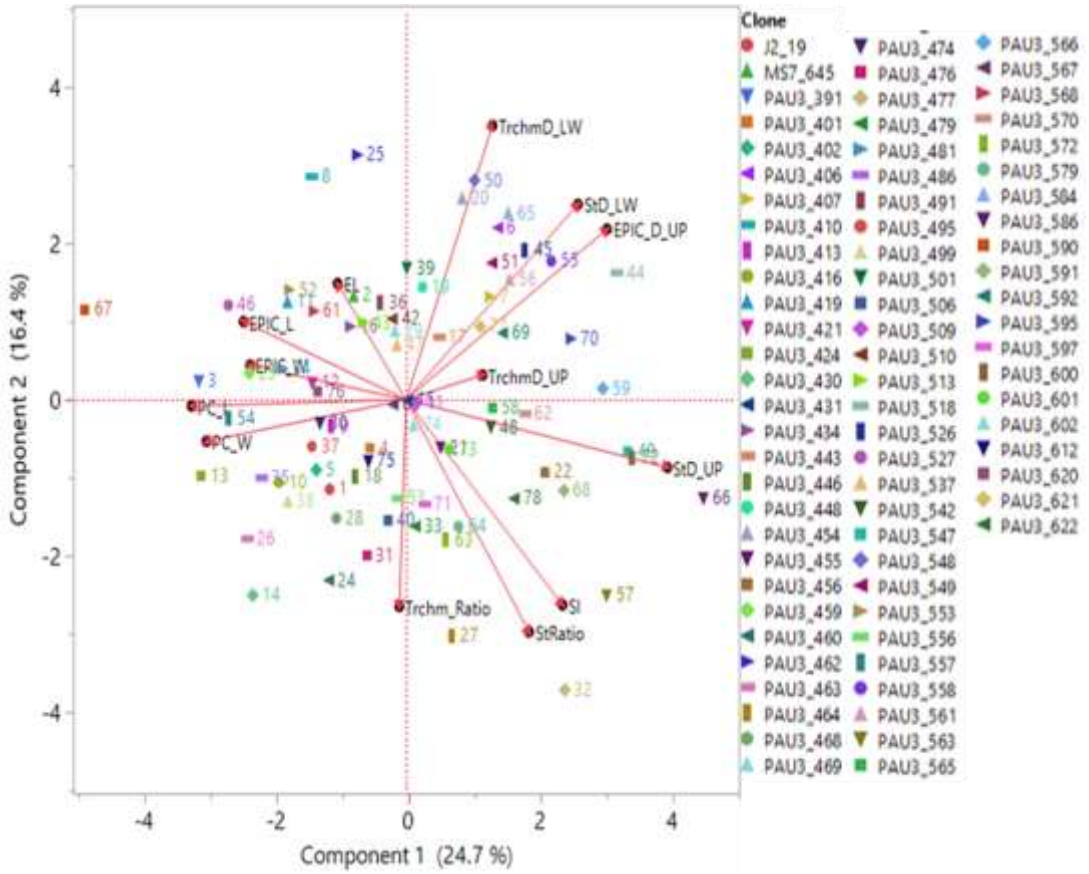


Figure 4.30: Genotype by trait biplot of potential frost tolerant potato clones derived from a cross, PAU3 based on the leaf morpho-anatomical traits and electrolyte leakage

StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

Genotype by trait (GT) biplot was performed from a two-way matrix of twelve leaf morpho-anatomical traits, electrolyte leakage and clones derived from cross J-2/19 × MS/7-645 (Figure 4.30). The genotype by trait biplot analysis further revealed the association between different traits and clones for low temperature stress.

An acute angle between different traits in the same direction indicated a high association between the corresponding traits for classifying clones. Clones superior for a particular trait were plotted closer and along the vector line direction. In biplot analysis, the clones were mostly concentrated on the positive side of both, PC1 and PC2 (Figure 4.30). The clones, PAU3-563, PAU3-586, PAU3-477 and PAU3-483 were more inclined in the direction of stomatal ratio and stomatal index. These traits are located in opposition direction of electrolyte leakage and hence, negatively correlated with electrolyte leakage. Therefore, clones might possess tolerance to low temperature stress and stomatal index can be used as an effective screening marker. Our findings are in consistent with Kleinhenz *et al* (1995). Similarly, the clones, PAU3-456, PAU3-464, PAU3-477, PAU3-479, PAU3-506, PAU3-563, PAU3-572, PAU3-579, PAU3-586, PAU3-597, PAU3-591, PAU3-622, PAU3-424, PAU3-602, PAU3-570, PAU3-455, PAU3-600 and PAU3-547 were plotted in the opposite direction of electrolyte leakage (Figure 4.30). Indicating that these clones exhibited low ion leakage under low temperature exposure in the field conditions. Thus, these clones can be identified as promising frost tolerant clones. Cell membrane is the primary site of injury in cold stress. The degree of cell membrane injury can evaluate through electrolyte leakages assessment (Li *et al* 1979, Stone *et al* 1993, Aslamarz and Vahdati 2010). While, PAU3-491, PAU3-434, PAU3-531 and MS/7-645 were more inclined in the direction of electrolyte leakage and so these clones can be identified as sensitive on the basis of more ion leakages. Moreover, the clones PAU3-424, PAU3-557, PAU3-421 and PAU3-391 were more inclined in the direction of palisade parenchyma cell length (Figure 4.30).

Genotype by trait (GT) biplot was created from a two-way matrix of twelve leaf morpho-anatomical traits, electrolyte leakage and potato clones (Figure 4.31). An acute angle between different traits in the same direction indicated a high association between corresponding traits for classifying clones. Clones superior for a particular trait were plotted closer and along the vector line direction. The clones, PAU7-993, PAU7-982, PAU7-985, PAU7-987 and PAU7-1015 were more inclined to stomatal ratio and stomatal index. The clones, PAU7-958, PAU3-961, PAU3-964, PAU3-972, PAU7-975, PAU7-994, PAU7-999, PAU7-1000, PAU7-1007, PAU7-1006, PAU7-1011 and J-2/19 were plotted in opposite direction of electrolyte leakage (Figure 4.31). Indicating that these clones exhibited low ion leakage under low temperature exposure in the field conditions. Thus, these clones can be identified as promising clones for frost tolerance. The electrolyte leakage assessment can be used as an effective indicator to evaluate the degree of cell membrane injury and hence, low temperature tolerance in plants (Aslamarz and Vahdati 2010, Liu *et al* 2013). However, the clones, PAU7-990 and PAU7-996 were more inclined in the direction of electrolyte leakage and stomatal density on adaxial leaf surface (Figure 4.31). These two traits showed very strong association and the associated clones possessed higher ion leakage along with higher

adaxial stomatal density. Thus, these clones might be sensitive to low temperature stress. Further, the clones PAU7-965, PAU7-982, PAU7-1005, PAU7-981 and PAU7-971 were more inclined in the direction of anatomical traits, such as length and width of palisade parenchymatous and epidermal cells. Similarly, the clones PAU7-998, PAU7-1009 and PAU7-1018 were more inclined in the direction of trichome density on adaxial leaf surface (Figure 4.31).

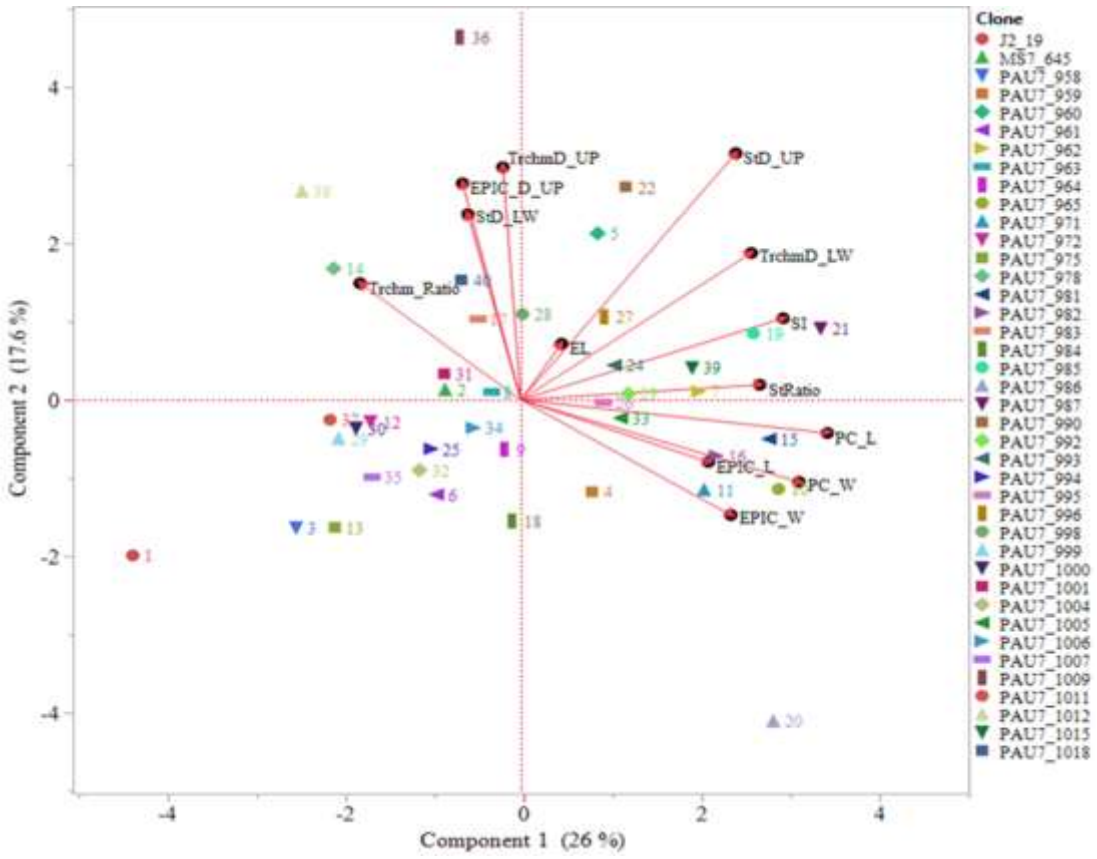


Figure 4.31: Genotype by trait biplot of potential frost tolerant potato clones derived from a cross, PAU7 based on the leaf morpho-anatomical traits and electrolyte leakage

StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

Genotype by trait (GT) biplot was created from a two-way matrix of twelve leaf morpho-anatomical traits, electrolyte leakage and potato clones (Figure 4.32). An acute angle between different traits in the same direction indicated a high association between corresponding traits for classifying clones. Clones superior for a particular trait were plotted closer and along the vector line direction. The clones such as PAU9-1255 and PAU9-1264 highly inclined to stomatal ratio and stomatal index. The clones, PAU9-1263, PAU9-1261,

PAU9-1266, PAU9-1274, PAU9-1281, PAU9-1309, PAU9-1304, PAU9-1312, PAU9-1270 and PAU9-1280 were plotted in opposite direction of electrolyte leakage. Indicating that these clones exhibited low ion leakage under low temperature exposure in the field conditions (Figure 4.32). Thus, these clones can be identified as promising clones for frost tolerance breeding programe on the basis of low electrolyte leakage. To detect cell membrane injury under low temperature stress electrolyte leakage has been commonly used (Tsarouhas *et al* 2000, Aslamarz and Vahdati 2010, Liu *et al* 2013). However, clones such as PAU9-1253, PAU9-1262 and PAU9-1273 were more inclined in the direction of stomatal density on adaxial leaf surface and electrolyte leakage (Figure 4.32). These two traits showed very strong association and the associated clones possessed higher percent of leakage along with higher adaxial stomatal density. Further, the clone PAU9-1267 and PAU9-1305 were more inclined in the direction of trichome density on adaxial leaf surface and trichome ratio (Figure 4.32).

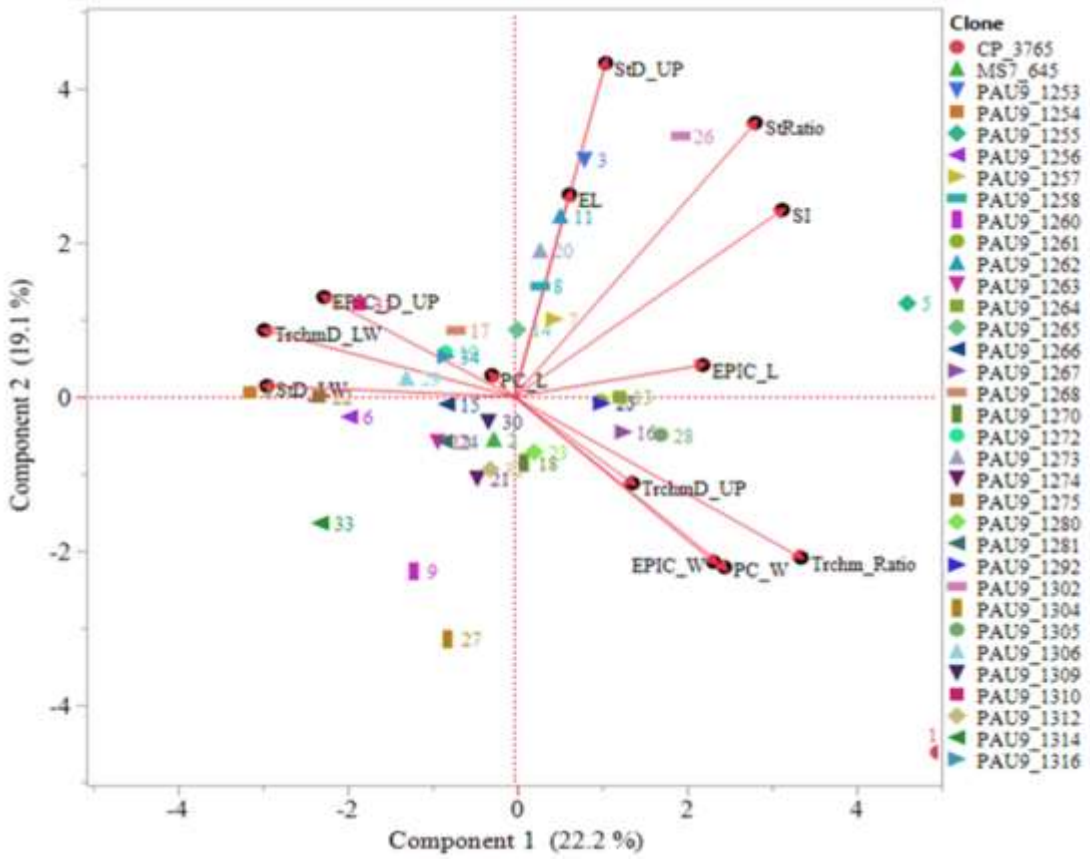


Figure 4.32: Genotype by trait biplot of potential frost tolerant potato clones derived from a cross, PAU9 based on the leaf morpho-anatomical traits and electrolyte leakage

StD_UP= Stomatal density on adaxial leaf surface
EPIC_UP= Epidermal cell density on adaxial leaf surface
StD_LW= Stomatal density on abaxial leaf surface
TrchmD_UP= Trichome density on adaxial leaf surface
TrchmD_LW= Trichome density on abaxial leaf surface
SR= Stomatal ratio
TR: Trichome ratio
PCL= Palisade cell length
PCW= Palisade cell width
EPIC_L= Epidermal cell length
EPIC_W: Epidermal cell width
SI= Stomatal index

4.3.4.3 Field evaluation and foliage damage at Khanaura, Hoshiarpur

The experimental materials consists of a set of 55 clones derived from three different crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645× CP-3765 (PAU9) on the basis of their tuber characteristics (Plates 12 and 13). During the cropping season 2020–21, the selected clones along with the check genotypes namely J/2-19, Kufri Anand, CP-3765, CP-3838, Kufri Jyoti, MS/7-645 and Kufri Pukhraj were planted in the month of October at PAU-Vegetable Research Farm, Khanaura. The clones were planted in rows along with check genotypes after every 10 rows for its evaluation. The crop was raised by following the package of practices recommended by Punjab Agricultural University, Ludhiana.

In December, two severe frost episodes were observed at Vegetable Research Farm (VRF), Khanaura, Hoshiarpur. The first episode on the nights of 18th and 19th December 2020 with the minimum (0.8–1.8℃) and maximum (15.5–19.8°C) air temperature. Subsequently, the second frost episode was observed after 10 days, on the nights of 29th to 31st December 2020 with the minimum and maximum air temperatures ranging from 0.2–1.2℃ and 9–13℃ respectively (Figure 4.28). However, surface temperature might have reached below 0°C resulting in severe frost giving white carpet appearance on the plants in the early morning hours. Such a severe frost incidence provided a rare opportunity for screening of the potato germplasm including varieties and breeding lines for frost tolerance (Kang *et al* 2007, Luthra *et al* 2008). During these periods, potato plants were damaged by natural freezing and frost. Plants were in the early growth stage (60 days after planting) were exposed to low freezing temperatures for 3 to 4 h. The extent of damage to potato plants was assessed 2-3 days after freezing and frost damage (FFD) occurred. The genotypes and clones were categorized into different groups based on their degrees/percentage of damaged.

The genotypes with ≤ 10%, 11–20% and 21–30% foliage damage were considered as tolerant, moderately tolerant and slightly tolerant, respectively. These genotypes exhibited ability for resuming the vegetative growth after the endurance of frost incidence. The genotypes with >30% foliage damage were considered frost susceptible and the recovery rate in such genotypes was observed to be slow (Rizza *et al* 2001, Luthra *et al* 2007, Chang *et al* 2014). The effect of natural frost damage on growth and recovery of plants was assessed 2–3 days after the events under field conditions at the vegetable research farm (VRF), Khanaura, Hoshiarpur. Various visual symptoms of frost effect such as yellowing and burning like appearance; frost injured leaves become highly fragile and loss turgidity; blackish discolorations on leaf blade and terminal leaves and Ice crystal formation on the leaves in early morning hours were observed (Plates 14 and 15).

In the present study, the field investigations recorded that no clones were severely damaged/injured. However, a significant variability was observed among potato genotypes

and clones for the foliage damage, visual symptoms development/ appearance and recovery rate. The results also showed a clear distinction between the clones derived from different crosses with different responses to frost. The foliage damage scoring showed that the clones PAU3-460, PAU3-537, PAU7-981, PAU7-999, PAU9-1259, PAU9-1314, PAU9-1257 and PAU9-1309 along with the genotypes J-2/19, CP-3838, CP-4206 showed no symptoms of foliage damage and exhibited a good level of frost tolerance and recovery rate (Figures 4.33; 4.34; 4.35; 4.36 and Plate 16). By contrast, the clones such as PAU3-413, PAU3-500, PAU3-518, PAU7-993 and PAU9-1263 and genotypes, Kufri Pukhraj and Kufri Jyoti showed high level of foliage damage (>30 %), yellowing and leaf burning symptoms. However, clones PAU3-409, PAU3-416, PAU3-621, PAU7-959, PAU7-980, PAU9-1262, PAU9-1267 and PAU9-1315 showed intermediate foliage damage and only slight yellowing of leaf margins. Notably, all the studied clones showed some degree of foliage damage except seven clones (Figures 4.33; 4.34; 4.35 and 4.36).

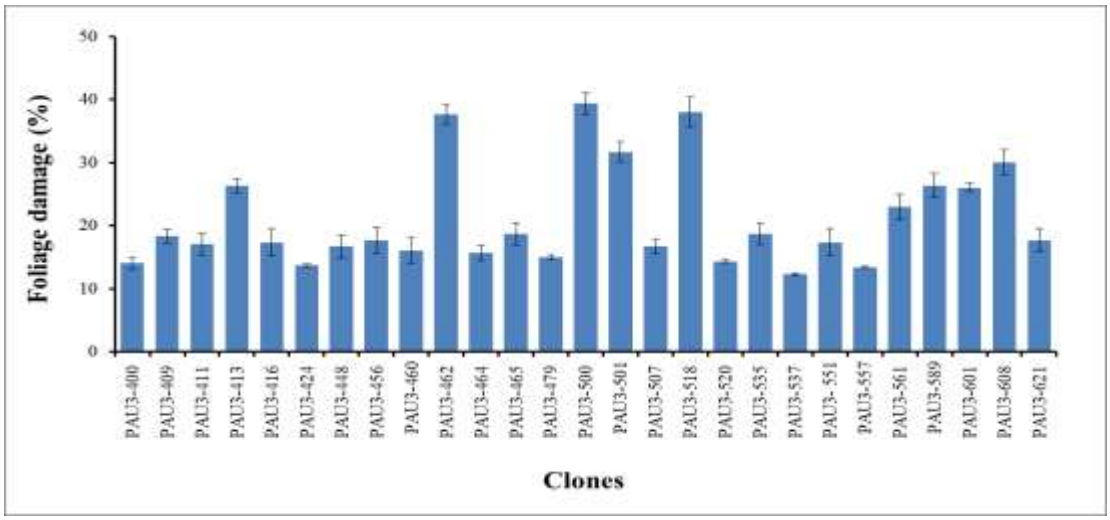


Figure 4.33: Percent foliage damage in clones derived from J-2/19 × MS/7-645 cross

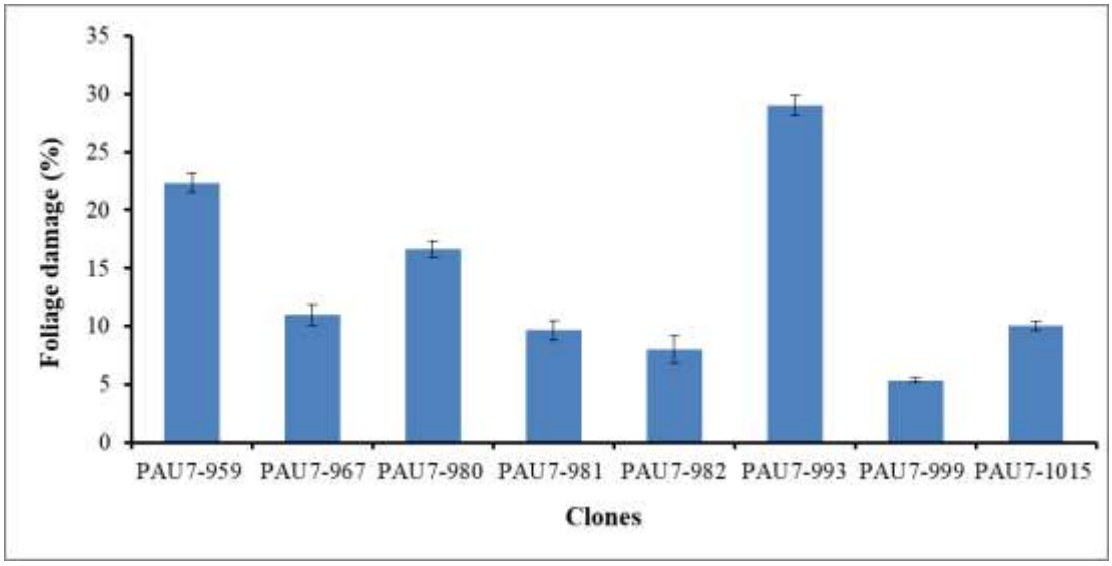


Figure 4.34: Percent foliage damage in the clones derived from MS/7-645 × J-2/19 cross

Plate 12: Selection of clones on the basis of tuber characteristics at harvesting in F_1C_1 generation

Plate 13: Rejection of clones on the basis of tuber characteristics at harvesting in F_1C_1 generation

Plate 14: Frost damages under field condition

Plate 15: Ice crystal formation and foliage damage under natural frost event

Plate 16: Field evaluation and selection of potato clones for frost tolerance under natural frost incidences at Vegetable research farm, Khanaura-Hoshiarpur (2020-21)

A) PAU3-460; B) PAU3-537; C) PAU3-981; D) PAU7-999; E) PAU9-1257; F) PAU9-1259; G) PAU9-1314; H) PAU9-1309

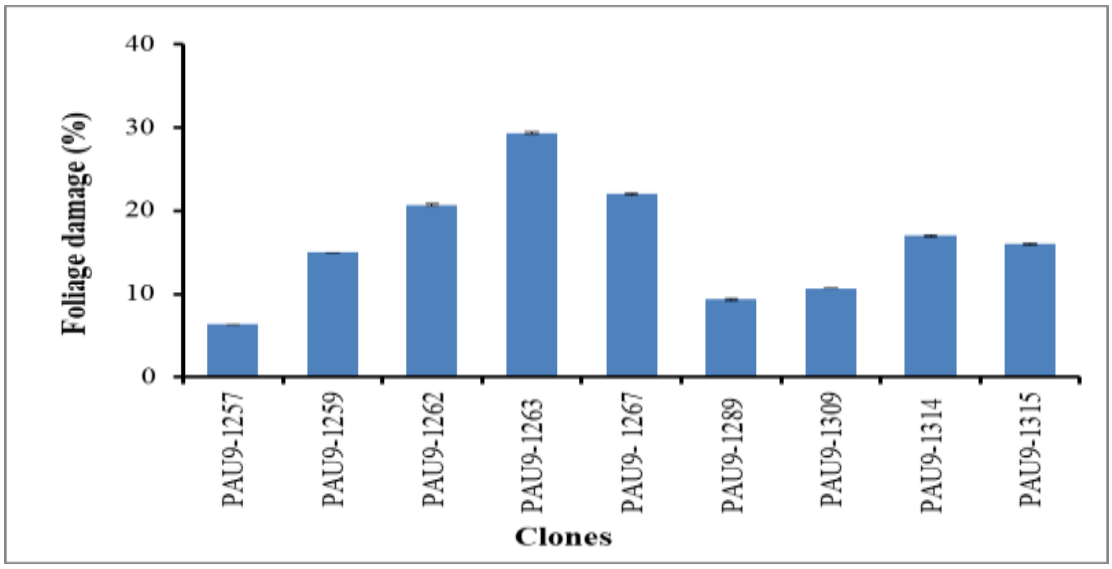


Figure 4.35: Percent foliage damage in the clones derived from MS/7-645 × CP-3765 cross

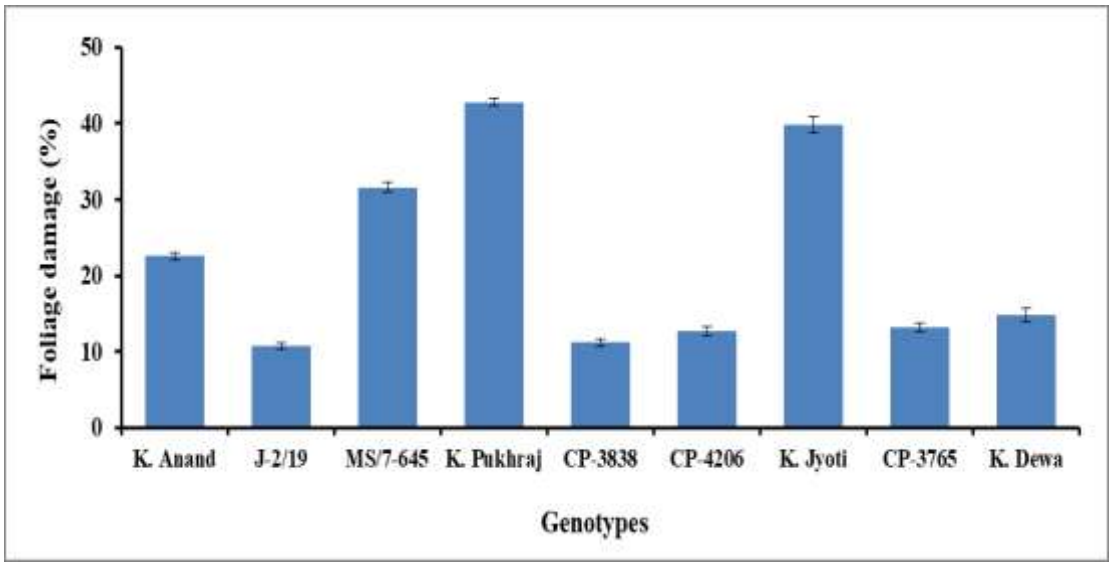


Figure 4.36: Percent foliage damage in genotypes

Furthermore, the genotypes, Kufri Anand, CP-3838, CP-3765, CP-4206 and J-2/19 exhibited a good level of frost tolerance as well as fast recovery rate, while moderate to high level of susceptibility to frost incidence was found in the genotypes MS/7-645, Kufri Jyoti and Kufri Pukhraj. Further, the recovery rate was better in the genotype MS/7-645 was better as compared with that of Kufri Jyoti and Kufri Pukhraj. Our results are consistent with winter field observations in various crops such as potato (Luthra *et al* 2008); wheat (Rizza *et al* 2001). Foliage damage and recovery rate were visually assessed using a 0–9 scale and 1–5 scale respectively according to Rashid and Iqbal (2007), Luthra *et al* (2008), Kang *et al* (2007) and Rizza *et al* (2001) with slight modifications.

The plants generally stop growing at < -0.8°C, sustain freezing damage at < -1.5°C (Xu *et al* 2016), and are frozen to death at < -3°C (Wu *et al* 2019). Delay in the development of the canopy and initiation of tubers are the main effect of natural freezing and frost damage in potato (Chang *et al* 2014). Similarly, Chang *et al* (2014) reported that the recovery rate by plants were in terms of their ability to rejuvenate after exposure to natural frost incidences. Our findings are in consistent with Chang *et al* (2014) that the foliage damage had direct effect on the canopy development rate. Moreover, there were significant differences in regrowth among the plants with no damage, mild damage and plants with moderate and severe damage.

4.3.4.4 Membrane injury

In F_1C_2 generation, in addition to foliage damage, the selected clones and genotypes were evaluated on the basis of membrane injury, their growth and yield, and tuber characteristics. The clones and genotypes were assessed by evaluation of membrane injury through electrolyte leakage method.

In the clones derived from cross J-2/19 × MS/7-645, the percent electrolyte leakage in the studied clones varied from 11.4 to 18.6 with mean value 14.8 (Table 4.20 and Figure 4.37) as indicated by box plot analysis. In the clones derived from cross MS/7-645 × J-2/19, the percent electrolyte leakage in the studied clones varied from 11.8 to 16.8 with mean value 13.5 (Table 4.20 and Figure 4.37). In clones derived from cross MS/7-645 × CP-3765 the percent electrolyte leakage in the studied clones varied from 10.5 to 15.3 with mean value of 12.8 (Table 4.20 and Figure 4.37).

Table 4.20: Descriptive statistics of electrolyte leakage trait of potato clones derived from crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) at Khanaura, Hoshiarpur

Crosses	N	Minimum	Maximum	Range	Mean	S.D	S.E	Skewness	Kurtosis
PAU3	32	11.4	18.6	7.2	14.8	1.8	0.3	0.1	-0.7
PAU7	7	11.8	16.8	5.1	13.5	1.7	0.7	0.8	0.2
PAU9	9	10.5	15.3	4.7	12.8	1.6	0.6	0.2	-1.0

Evaluation of potato for freezing and frost is necessitates for the understanding of how it cause damages to crop and how crop responds to it. Foliage damages may vary from mild symptoms in young terminal leaves to complete killing of plants (Chang *et al* 2014). However, the evaluation of crop response to environmental stress are more frequently determined by the effect on yield in various crops *i.e.*, such as onion (Gedam *et al* 2021), potato (Wellik *et al* 1981). Although, the extent of frost damages/injury and yield losses is influenced by various factors such as freezing rate, duration of exposure to low temperature,

lowest temperature reached, thawing rate, post thawing conditions ice nucleation and growth stage in which the foliar damage occurred (Levitt 1980, Stone *et al* 1993). Thus, subsequently all these factors will determine severity of the damage. However, if frost damage kills the plants/crop before tubers reach the desired size, yield losses tend to be relatively high (Rich 1983).

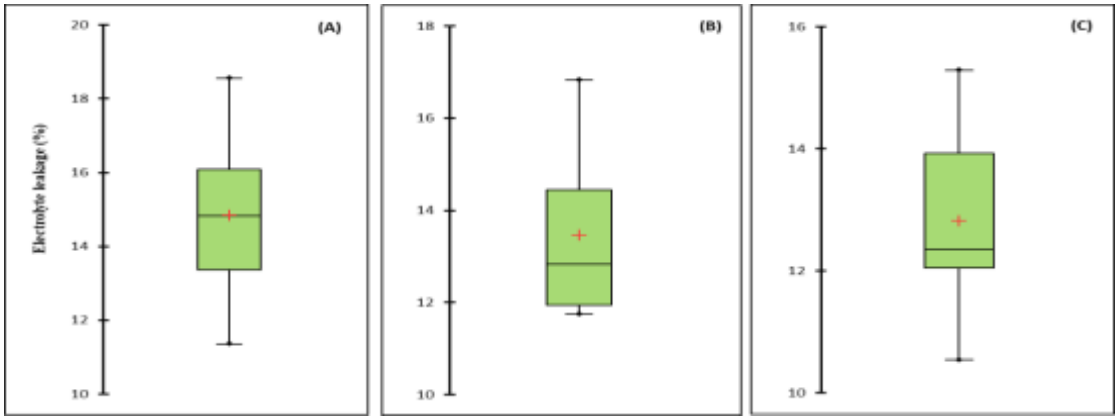


Figure 4.37: Electrolyte leakage (%) of potato clones derived from different crosses at Khanaura A) PAU3, B) PAU7 and C) PAU9 after low temperature exposure in F_1C_2 generation

4.3.5 Characterization of potato clones on the basis of field performance

4.3.5.1 Growth and yield attributing traits

4.3.5.1.1 Plant emergence (%)

The clones derived from the cross J-2/19 × MS/7-645 (PAU3), plant emergence (%) varied from 16.7 to 100 with mean value 50.8. Similarly, in the clones derived from MS/7-645 × J-2/19 (PAU7), plant emergence varied from 25.0 to 100 with mean value 66.6. Further, for MS/7-645 × CP-3765 (PAU9), the plant emergence varied from 20.0 to 100 with mean value 55.5 (Table 4.21).

Further, the box plot analysis indicated that the clones derived from J-2/19 × MS/7-645 cross showed maximum variability than the clones derived from other two crosses (Figure 4.37, 4.38 and 4.39). Similarly, Nagar *et al* (2019) reported that plant emergence in the studied genotypes was ranged from 88-95% at 30 days after planting (30DAP). Kufri Jyoti and Kufri Khayti recorded higher than 93.5% of plant emergence as compared to other cultivars. In Turkey, the Marifona potato cultivar yielded higher (86.5%) of plant emergence as compared to Hermes potato cultivar (Altuntas and Asilturk 2011).

4.3.5.1.2 Plant vigour

The plant vigour was measured on the visual observation in (1-5 scale) at 60 DAP. Similarly, the plant vigour was assessed on the scale (1-5), in the clones derived from J-2/19 × MS/7-645 (PAU3) it was varied from 1 to 5 with mean value 2.6. The clones derived from MS/7-645 × J-2/19 (PAU7), plant vigour varied from 1.5 to 5.0 with mean value 3.1. Further,

MS/7-645 × CP-3765 (PAU9), plant vigour varied from 1.0 to 5.0 with mean value 3.7 (Table 4.21).

Further, the box plot analysis indicated that the clones derived from J-2/19 × MS/7-645 cross showed maximum variability than the clones derived from other two crosses (Figures 4.38; 4.38 and 4.39).

4.3.5.1.3 Plant height (cm)

It was measured by using measuring tape for three randomly selected plants from each genotype. For plant height, clones varied significantly across the crosses (Table 4.21). In the clones derived from J-2/19 × MS/7-645 (PAU3) cross, plant height in the studied clones varied from 23.3 to 60.2 with mean value 41.5. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7), plant emergence varied from 37.3 to 75.5 with mean value 49.1. Further, MS/7-645 × CP-3765 (PAU9), plant emergence varied from 28.3 to 67.3 with mean value 48.6 (Table 4.21).

Table 4.21: Descriptive statistics of growth and yield attributing traits of clones of derived from three crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP-3765 (PAU9) at Ludhiana during cropping season 2020-21

Traits	Cross	N	Min.	Max.	Range	Mean	S.D	S.E	Skewness	Kurtosis
PE	PAU3	86	16.7	100.0	83.3	50.8	25.7	2.77	0.7	-0.5
	PAU7	39	25.0	100.0	75.0	66.6	20.4	3.3	0.1	-0.8
	PAU9	33	20.0	100.0	80.0	55.5	29.0	5.1	0.3	-1.2
PV	PAU3	86	1.0	5.0	4.0	2.6	1.0	0.2	-0.5	-0.4
	PAU7	39	1.5	5.0	3.5	3.1	0.9	0.2	0.3	-0.5
	PAU9	33	1.0	5.0	4.0	3.7	1.0	0.2	-0.8	0.1
PH	PAU3	86	23.3	60.2	36.8	41.5	8.6	0.93	0.2	-0.9
	PAU7	39	37.3	75.5	38.2	49.1	7.9	1.3	1.2	2.3
	PAU9	33	28.3	67.3	39.0	48.6	9.3	1.6	0.0	-0.5
NSPP	PAU3	86	1.0	5.0	4.0	2.0	1.0	0.10	1.0	0.6
	PAU7	39	1.0	4.3	3.3	2.4	0.8	0.1	0.0	-0.2
	PAU9	33	1.0	4.7	3.7	2.3	0.9	0.2	0.7	0.4
NTPP	PAU3	86	3.0	33.0	30.0	10.8	6.1	0.66	1.5	2.7
	PAU7	39	2.0	30.0	28.0	14.0	5.9	1.0	0.3	0.2
	PAU9	33	3.0	25.5	22.5	11.9	5.1	0.9	0.3	0.2
TYPP	PAU3	86	125.0	1540.0	1415.0	661.6	304.8	32.87	0.7	0.1
	PAU7	39	260.0	2400.0	2140.0	822.0	419.6	68.1	1.6	4.0
	PAU9	33	90.0	1630.0	1540.0	871.1	305.6	54.0	0.0	0.6

Legend: PE: Plant emergence (%), PV: Plant vigour, PH: Plant height (cm),
NSPP: Numbers of sprouts per plant, NTPP: Numbers of tubers per plant, TYPP: Tuber yield per plant

Further, the box plot analysis indicated that the maximum variability were recorded in the clones derived from J-2/19 × MS/7-645 cross than the clones derived from other two crosses (Figures 4.38; 4.39 and 4.40).

Anoumaa *et al* (2017) also reported highly significant differences for plant height between traditional and modern cultivars of potato in Western Highlands region of Cameroon. Similarly, Nasir and Toth (2021) recorded reduced in plant height (99.0 to 96 cm) in Demon cultivar while, no significant effect was observed in Hopehely under drought stress.

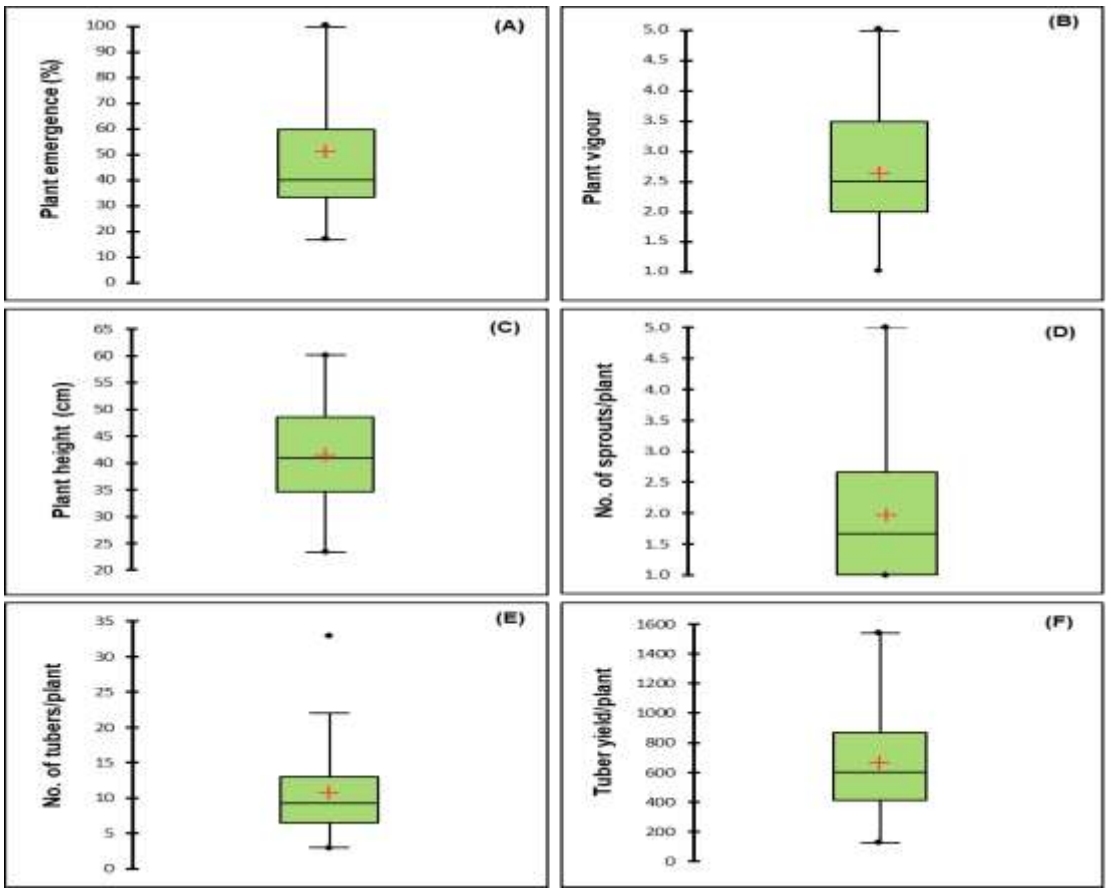


Figure 4.38: Box plot analysis of the potato clones derived from J-2/19 × MS/7-645 cross in F_1C_2 generation at Ludhiana during cropping season 2020-21 A) Plant emergence (%); B) Plant vigour; C) Plant height (cm); D) No. of sprouts per plant; E) No. of tubers per plant and F) Tuber yield per plant

4.3.5.1.4 Number of sprouts per plant

For number of sprouts per plants, the clones derived from J-2/19 × MS/7-645 (PAU3), number of sprouts/plants varied from 1.0 to 5.0 with mean value 2.0. Similarly, in the clones derived from MS/7-645 × J-2/19 (PAU7), number of sprouts per plant varied from 1.0 to 4.3 with mean value 2.4. Further, MS/7-645 × CP-3765 (PAU9), number of sprouts per plant varied from 1.0 to 4.7 with mean value 2.3 (Table 4.21).

Further, the box plot analysis revealed that the maximum variability was shown in number of sprouts per plant by the clones derived from J-2/19 × MS/7-645 cross than the

clones derived from other two crosses (Figures 4.38, 4.39 and 4.40). Our findings are in consistent with (Anoumaa *et al* 2017) Anoumaa *et al* (2017) they also reported highly significant phenotypic differences among the genotypes for number of sprouts per plant.

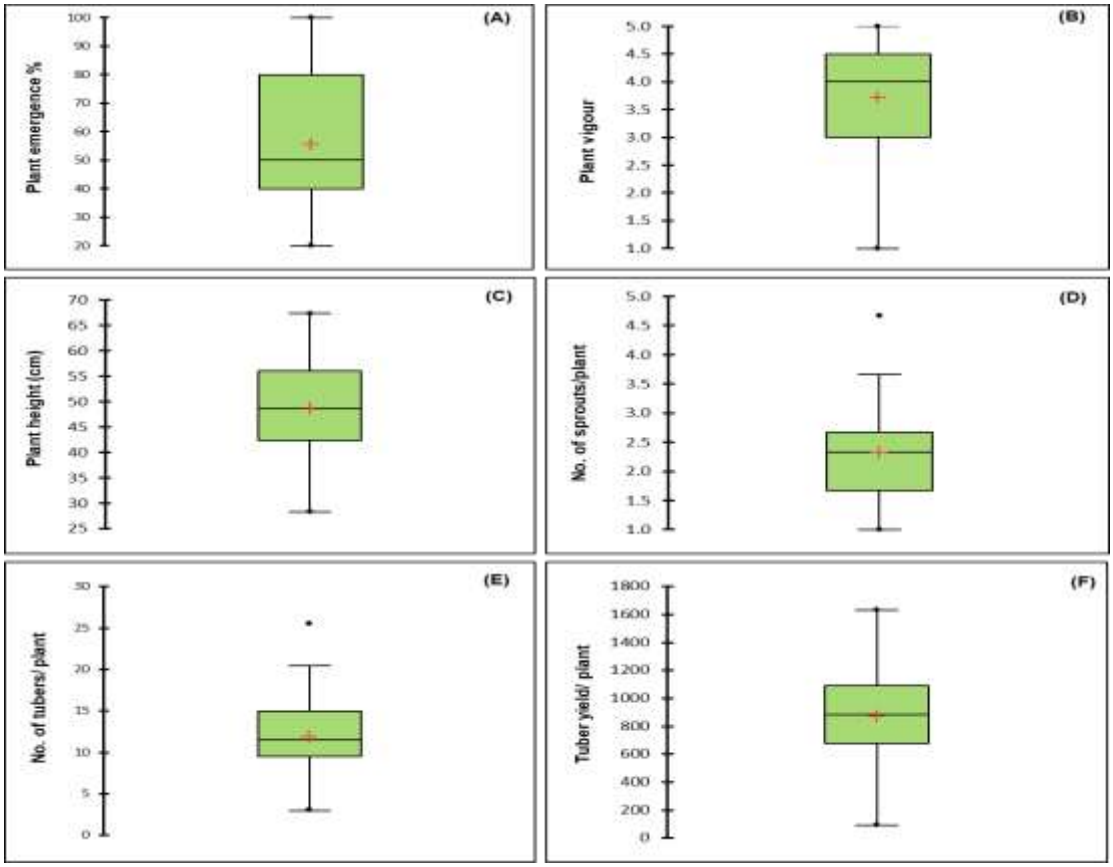


Figure 4.39: Biplot analysis of the clones derived from MS/7-645 × J-2/19 cross in F_1C_2 generation at Ludhiana during cropping season 2020-21 A) Plant emergence (%); B) Plant vigour; C) Plant height (cm); D) No. of sprouts per plant; E) No. of tubers per plant and F) Tuber yield per plant

4.3.5.1.5 Number of tubers per plant

Number of tubers per plant is an important trait which directly contributes to tuber yield per plant. In the studied clones, results showed that the numbers of tubers per plant (g) ranged from 3.0 to 33.0, 2.0 to 30.0 and 3.0 to 25.5 with the mean values of 10.8, 14.0, and 11.9 in J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7), and MS/7-645 × CP-3765 (PAU9) crosses respectively (Table 4.21).

It was observed that the clones, PAU3-496 (33), PAU3-448 (30.5) and PAU3-407 (29) had significantly more number of tubers per plant than overall mean. Similarly, PAU7-961(30.0) had the highest number of tubers per plant followed by PAU7-972 (26.0) (Table 4.20). Further, the clones PAU9-1316 (25), PAU9-1281 (20) had significantly more number of tubers per plant than over all mean. Further, the box plot analysis indicated that the maximum variability was indicated by number of tubers per plant in the clones derived from

J-2/19 × MS/7-645 cross than the clones derived from other two crosses (Figures 4.38, 4.39 and 4.40). Our findings are in consistent with Luthra *et al* (2018) also reported large variation in number of tubers per plant in segregating population of coloured potato clones.

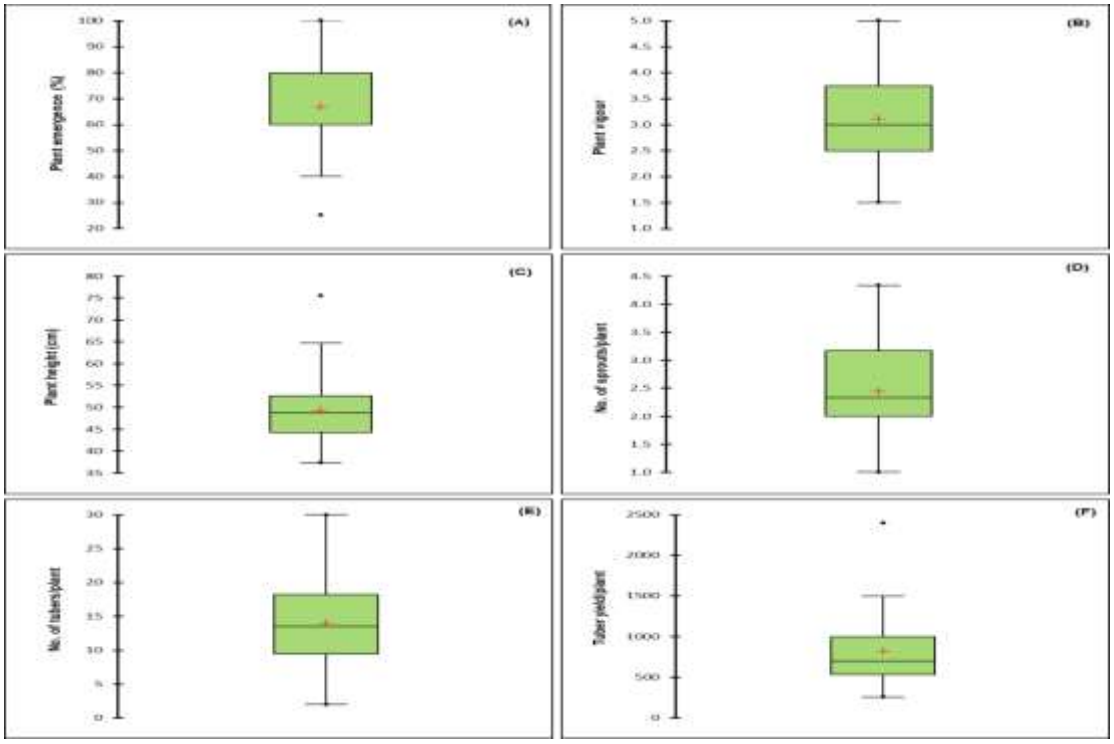


Figure 4.40: Biplot analysis of the clones derived MS/7-645 × CP-3765 cross in F_1C_2 generation at Ludhiana during cropping season 2020-21 A) Plant emergence (%); B) Plant vigour; C) Plant height (cm); D) No. of sprouts per plant; E) No. of tubers per plant, and F) Tuber yield per plant

4.3.5.1.6 Tuber yield per plant (g)

The clones derived from J-2/19 × MS/7-645 (PAU3), tuber yield per plant in the studied clones varied from 125 to 1540 with mean value 1415. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7) cross, tuber yield per plant varied from 260 to 2400 with mean value 822.0. Further, MS/7-645 × CP-3765 (PAU9), tuber yield per plant varied from 90 to 1630 with mean value 871.0 (Table 4.21). It was observed that the clones, PAU3-424 (1540), PAU3-407 (1425) and PAU3-431 (1300) had significantly more number of tubers per plant than overall mean. Similarly, PAU7-961 (2400) had the highest tuber yield per plant followed by PAU7-972 (1725) (Figures 4.38; 4.39 and 4.40).

Further, the clones PAU9-1316 (1630 g), PAU9-1309 (1375 g) had significantly more tuber yield per plant than the overall mean. Further, the box plot analysis indicated that the clones derived from MS/7-645 × CP-3765 showed the maximum variability for tuber yield per plant than the clones derived from other crosses J-2/19 × MS/7-645 and MS/7-645 × J-2/19 (Figure 4.38, 4.39 and 4.40). Similarly, Mahgoub *et al* (2015) and Anoumaa *et al* (2017)

also reported significant difference among the studied cultivars for yield characteristics such as tuber yield per plant. The tuber yield per plant and numbers of tuber per plant are affected by the timing of tillage systems. Marfonia and Hermes recorded mean values of 1100 g and 848.9 g, respectively for tuber yield per plants (Altuntas and Asilturk 2011).

The variation observed among the potato clones are graphically represented for various growth and yield attributing traits *viz.*, plant emergence, plant vigour, plant height (cm), number of sprouts per plant, number of tubers per plant and tuber yield per plant (g) of different crosses J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7), and MS/7-645 × CP-3765 (PAU9) are illustrated in Figures 4.38; 4.39 and 4.40, respectively.

4.3.5.2 Correlations among growth and yield component traits

The worth of several independent secondary traits involved in the selection program can be determined based on their significant association with the dependent traits like yield. The correlation coefficients for various growth and yield based traits such as plant emergence, plant vigour, plant height, number of sprouts per plant, number of tubers per plant and tuber yield per plant of clones derived from different crosses J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7), and MS/7-645 × CP-3765 (PAU9) are illustrated in Table 4.22, Table 4.23 and Table 4.24. Tuber yield are complex and quantitatively inherited trait and influenced genetically and environmentally. Due to these selections may be difficult and time consuming to improve yield directly. Therefore, identification and use of highly correlated characters are more appropriate Anoumaa *et al* (2017).

Table 4.22: Correlation matrix (r) describing association of growth and yield attributing traits of 76 potato clones (PAU3) evaluated for frost tolerant under field condition

	Plant emergence (%)	Plant vigour	Plant height (cm)	No. of sprouts/ plant	No. of tubers /plant	Tuber yield/ plant
Plant emergence (%)	1					
Plant vigour	.255*	1				
Plant height (cm)	0.047	.599**	1			
No. of sprouts/plant	.336**	.535**	.299**	1		
No. of tubers /plant	0.022	.313**	.233*	.332**	1	
Tuber yield/plant	0.032	.551**	.522**	.431**	0.618**	1

* = Significant at 5% level of probability, ** = highly significant at 1% level of probability

The present study showed a very high correlation between growth and yield components of different crosses. In case of cross PAU3, tuber yield per plant showed highly significant and positive associations with plant vigour (0.551**), plant height (0.522**), number of sprouts per plant (0.431**) and number of tuber per plant (0.618**) whereas, plant emergence showed non-significant positive association. Similarly, the plant vigour and number of sprouts per plant found to be significantly and positively correlated with all the growth and yield traits. Similar findings were reported by Anoumaa *et al* (2017).

Table 4.23: Correlation matrix (r) describing association of growth and yield attributing traits of 39 potato clones (PAU7) evaluated for frost tolerant under field condition

	Plant emergence (%)	Plant vigour	Plant height (cm)	No. of sprouts/ plant	No. of tubers /plant	Tuber yield/ plant
Plant emergence (%)	1					
Plant vigour	.538**	1				
Plant height (cm)	-0.26	0.151	1			
No. of sprouts/plant	0.106	0.023	-0.103	1		
No. of tubers /plant	0.097	0.331*	-0.002	0.225	1	
Tuber yield/plant	-0.004	0.264	0.108	0.093	0.755**	1

* = Significant at 5% level of probability, ** = highly significant at 1% level of probability

In the clones derived from PAU7 cross, plant emergence showed highly significant and positive correlation with plant vigour whereas, plant height and tuber yield per plant were found to be negatively correlated with plant emergence. However, the number of sprouts per plant and number of tuber per plant showed positive association but non-significant. Further, the number of tuber per plant showed highly significant and positive association with tuber yield per plant. Similarly, the plant vigour showed positive correlation with all the studied traits.

In the clones derived from cross PAU9, similarly, the number of tuber per plant was found to significantly and positively correlate with number of tuber per plant. Likewise, the plant vigour showed highly significant and positive association with traits such as plant height, number of sprouts per plant and number of tubers per plant whereas, number of tuber per plant was positively correlated but not significantly. However, plant emergence was found

to be negatively associated with plant height and tuber yield per plant. Our findings are consistent with Nasir and Toth (2021). They also observed significant increase in tuber yield with increase in number of tubers per plant.

Table 4.24: Correlation matrix (r) describing association of growth and yield attributing traits of 33 potato clones (PAU9) evaluated for frost tolerant under field condition

	Plant emergence (%)	Plant vigour	Plant height (cm)	No. of sprouts/plant	No. of tubers / plant	Tuber yield/ plant
Plant emergence (%)	1					
Plant vigour	0.394*	1				
Plant height (cm)	-0.098	.572**	1			
No. of sprouts/plant	0.250	.562**	0.183	1		
No. of tubers /plant	0.244	0.249	0.057	-0.007	1	
Tuber yield/plant	-0.085	0.407*	0.416*	0.065	0.724**	1

* = Significant at 5% level of probability, ** = highly significant at 1% level of probability

4.3.5.3 Morphological and tuber characteristics

4.3.5.3.1 Foliage type

The clones derived from J-2/19 × MS/7-645 (PAU3) cross, twenty (22.9%) were compact while sixty seven (77.0%) possessed semi-compact foliage type. Similarly, in clones derived from MS/7-645 × J-2/19 (PAU7), three (7.6%) were compact while twenty three (58.9%) possessed semi-compact foliage type. Others (33.3%) were different. Further, in clones derived from MS/7-645 × CP-3765 (PAU9), seven (21.2%) were compact while twenty six (78.7%) possessed semi-compact foliage type (Tables 4.25; 4.26 and 4.27).

4.3.5.3.2 Tuber shape

Tuber shape is an important tuber trait for consumer preference. It plays important role particularly during selections in early generations and becomes a desirable quality tuber traits. Great variability was observed for tuber shapes *viz.*, oval, oval round, round, oblong and others. The clones derived from J-2/19 × MS/7-645 (PAU3) cross, twenty eight (32.2%) were oval round; seventeen (19.5%) possessed oval tuber shape, eight (9.2 %) possessed round tuber shape, and five clones (5.7 %) were oblong.

Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7) cross, twelve (30.7%) were oval round; ten (25.6 %) possessed oval tuber shape, six (15.4 %) possessed round shape, four (10.3 %) and two clones (5.1%) possessed oblong and irregular tuber shape respectively (Table 4.25; 4.26 and 4.27). Further, the clones derived from MS/7-645 × CP-3765 (PAU9) cross, thirteen (39.4%) were oval round; ten (30.3%) of clones possessed oval shape tuber, two (6.0%) possessed round tuber shape, while three (9.0%) were oblong. Tuber shape is a genotypic trait which is influenced by environmental condition and crop duration (Kumar and Minhas 2015). By using the subjective morphological descriptors and tuber length and width measurements the assessment of tuber shape variation in potato crop were carried out by Lindqvist-Kreuze *et al* 2015. Further, they classified and identified different morphological tuber shapes within the population.

4.3.5.3.3 Skin type

The tuber skin type was classified as smooth and rough in potato. Out of 87 clones seven (8.0 %) possessed rough skin type while eighty (92.0%) possessed smooth skin type in the clones derived from J-2/19 × MS/7-645 (PAU3) cross. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7), four (10.3%) possessed rough skin type while thirty five (89.7%) possessed smooth skin type. Further, the clones derived from MS/7-645 × CP-3765 (PAU9), two clones (6.0%) were rough while thirty one (93.9%) possessed smooth skin type (Tables 4.25; 4.26 and 4.27).

4.3.5.3.4 Depth of tuber eyes

The clones derived from J-2/19 × MS/7-645 (PAU3) cross, forty two (48.3%) possessed shallow tuber eyes while twenty eight (32.1%) possessed medium tuber eyes type and only ten (11.5 %) of clones possessed deep tuber eyes. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7), twenty three (58.9%) possessed shallow tuber eyes while nine (23.1%) possessed medium tuber eyes type and only seven (17.9%) of clones possessed deep tuber eyes. Further, the clones derived from MS/7-645 × CP-3765 (PAU9) cross, twenty three (69.7%) possessed shallow tuber eyes while nine (27.3%) possessed medium tuber eyes and only one (3.0 %) of clones possessed deep tuber eyes (Table 4.25; 4.26 and 4.27). Similarly, Lindqvist-Kreuze *et al* 2015 also used subjective morphological descriptors for assessment of variation in tuber eye depth.

4.3.5.3.5 Tuber skin colour

The clones derived from J-2/19 × MS/7-645 (PAU3) cross, thirty two (36.8%) possessed cream white type tuber skin colour; thirty clones (34.4 %) were yellow skin colour whereas, twenty three (26.4%) possessed cream tuber skin colour. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7), ten (25.6%) possessed cream white type tuber skin colour; fourteen (35.9%) were yellow skin colour whereas, fifteen (38.5 %) possessed cream

tuber skin colour. Further, the clones derived from MS/7-645 × CP-3765 (PAU9), five (15.2%) possessed cream white type tuber skin colour; fifteen (45.5%) were yellow skin colour whereas, thirteen (39.4%) possessed cream tuber skin colour (Tables 4.25; 4.26 and 4.27).

4.3.5.3.6 Tuber flesh colour

Tuber flesh colour is an important tuber characteristic in perspective to potato processing industry. Wide range of flesh coloured have been reported from traditional white and yellow to red-purple which are rich in anthocyanins (Luthra *et al* 2018; Cima *et al* 2020). The clones derived from J-2/19 × MS/7-645 (PAU3) cross, thirty four (39.0%) possessed yellow flesh color; thirty four clones (39.0%) were cream colour whereas, eighteen (20.6%) possessed white flesh color. Similarly, the clones derived from MS/7-645 × J-2/19 (PAU7), nine (23.1%) possessed yellow flesh color; twenty three clones (58.9%) were cream colour whereas, six (15.4%) possessed white flesh color. Further, the clones derived from MS/7-645 × CP-3765 (PAU9), eleven (33.3%) possessed yellow flesh color; nineteen clones (57.6%) were cream colour whereas, one (3.0%) possessed white flesh color. (Tables 4.25; 4.26 and 4.27). In consistent with our findings, Luthra *et al* 2018 reported wide variation for various tuber characteristics *viz*., tuber flesh color; tuber skin color and tuber shape in segregating population of potato.

Table 4.25: Morphological traits of potential frost tolerant potato clones derived from J-2/19 × MS/7-645 cross

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Eye depth	Tuber skin colour	Flesh colour
1	PAU3-407	Semi compact	Oval round	Smooth	Medium	White-cream	Cream
2	PAU3-424	Semi compact	Pear Shaped	Smooth	Shallow	Yellow	Yellow
3	PAU3-431	Semi compact	Elongated	Smooth	Shallow	Cream	Yellow
4	PAU3-501	Semi compact	Pear Shaped	Smooth	Shallow	Yellow	Yellow
5	PAU3-518	Semi compact	Oval round	Smooth	Medium	Cream	Yellow
6	PAU3-527	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
7	PAU3-535	Compact	Oval	Smooth	Shallow	Cream	Yellow
8	PAU3-568	Semi compact	Elongated	Smooth	Shallow	Yellow	Yellow
9	PAU3-620	Semi compact	Round	Smooth	Shallow	White-cream	Yellow
10	PAU3-391	Semi compact	Oval round	Smooth	Medium	Yellow	Yellow
11	PAU3-401	Semi compact	Oval	Smooth	Shallow	Yellow	Cream
12	PAU3-402	Open canopy	Oval	Smooth	Shallow	Yellow	Yellow
13	PAU3-406	Semi compact	Oval round	Smooth	Deep	Cream	White
14	PAU3-410	Semi compact	Flat oval	Smooth	Medium	Cream	Cream
15	PAU3-413	Semi compact	Oval	Smooth	Medium	White-cream	Yellow
16	PAU3-416	Semi compact	Oval round	Smooth	Medium	White-cream	Yellow
17	PAU3-419	Open canopy	Oval round	Smooth	Medium	White-cream	Cream
18	PAU3-421	Semi compact	Irregular	Smooth	Shallow	Yellow	White
19	PAU3-430	Semi compact	Oval	Smooth	Shallow	White-cream	Cream
20	PAU3-434	Semi compact	Irregular	Smooth	Deep	Yellow	Yellow
21	PAU3-443	Semi compact	Oval round	Smooth	Medium	Yellow	Yellow
22	PAU3-448	Semi compact	Oval round	Smooth	Deep	Cream	Yellow

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Eye depth	Tuber skin colour	Flesh colour
23	PAU3-454	Semi compact	Oval round	Smooth	Deep	Cream	Yellow
24	PAU3-455	Semi compact	Oval	Smooth	Shallow	Yellow	Yellow
25	PAU3-456	Semi compact	Oval round	Smooth	Medium	Cream	Cream
26	PAU3-459	Semi compact	Oval round	Smooth	Medium	Cream	Yellow
27	PAU3-460	Semi compact	Oval round	Smooth	Shallow	Cream	Yellow
28	PAU3-462	Open canopy	Irregular	Rough	Shallow	White-cream	Yellow
29	PAU3-463	many stem	Oval round	Smooth	Medium	Cream	Cream
30	PAU3-464	Semi compact	Oval round	Smooth	Medium	Yellow	White
31	PAU3-468	Compact	Irregular	Smooth	Shallow	White-cream	Cream
32	PAU3-469	Semi compact	Oval round	Smooth	Deep	Cream	Yellow
33	PAU3-470	Compact	Oblong	Smooth	Medium	White-cream	Cream
34	PAU3-472	Open canopy	Oval round	Smooth	Shallow	Cream	Yellow
35	PAU3-473	Semi compact	Oval	Smooth	Medium	White-cream	White
36	PAU3-474	Semi compact	Oval	Smooth	Medium	Cream	Cream
37	PAU3-476	Semi compact	Irregular	Smooth	Medium	Yellow	White
38	PAU3-477	Semi compact	Oblong	Smooth	Protruding	White-cream	Yellow
39	PAU3-478	Semi compact	Oval	Smooth	Shallow	Cream	Cream
40	PAU3-479	Semi compact	Oval round	Smooth	Shallow	Cream	Cream
41	PAU3-481	Compact	Oval round	Smooth	Shallow	White-cream	Yellow
42	PAU3-483	Compact	Oval	Smooth	Shallow	White-cream	White
43	PAU3-486	Semi compact	Round	Rough	Shallow	Cream	Cream
44	PAU3-495	Semi compact	Oblong	Smooth	Shallow	Cream	White
45	PAU3-496	Semi compact	Oblong	Smooth	Medium	White-cream	Cream

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Eye depth	Tuber skin colour	Flesh colour
46	PAU3-499	Semi compact	Irregular	Smooth	Medium	Yellow	Cream
47	PAU3-506	Compact	Oval round	Smooth	Medium	White-cream	White
48	PAU3-509	Compact	Oval round	Smooth	Shallow	White-cream	Cream
49	PAU_510	Semi compact	Oval round	Smooth	Medium	White-cream	Cream
50	PAU3-513	Semi compact	Oblong	Rough	Protruding	Yellow	Yellow
51	PAU3-525	Semi compact	Oval	Smooth	Deep	White-cream	Cream
52	PAU3-526	Compact	Oblong	Smooth	Protruding	White-cream	Cream
53	PAU3-537	Compact	Oval	Smooth	Shallow	White-cream	Cream
54	PAU3-539	Semi compact	Oblong	Rough	Shallow	White-cream	White
55	PAU3-542	Compact	Flattened	Smooth	Shallow	White-cream	White
56	PAU3-544	Compact	Elongated	Smooth	Shallow	Cream	White
57	PAU3-446	Compact	Oval	Smooth	Shallow	Yellow	Yellow
58	PAU3-547	Semi compact	Oval round	Smooth	Deep	White-cream	Yellow
59	PAU3-548	Semi compact	Round	Smooth	Shallow	White-cream	White
60	PAU3-549	Semi compact	Oblong	Smooth	Medium	Cream	White
61	PAU3-553	Semi compact	Oval	Smooth	Shallow	Yellow	Yellow
62	PAU3-556	Semi compact	Irregular	Rough	Medium	Yellow	Yellow
63	PAU3-557	Semi compact	Irregular	Smooth	Medium	Yellow	Cream
64	PAU3-558	Semi compact	Oval	Smooth	Shallow	White-cream	Cream
65	PAU3-561	Compact	Elongated	Rough	Shallow	Yellow	Yellow
66	PAU3-563	Compact	Oval round	Smooth	Shallow	Cream	Cream
67	PAU3-565	Compact	Round	Smooth	Shallow	White-cream	Cream
68	PAU3-566	Semi compact	Irregular	Smooth	Protruding	Yellow	Cream

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Eye depth	Tuber skin colour	Flesh colour
69	PAU3-567	Semi compact	Oval round	Smooth	Shallow	Yellow	Cream
70	PAU3-570	Semi compact	Oval round	Smooth	Medium	White-cream	White
71	PAU3-572	Semi compact	Oblong	Smooth	Shallow	Yellow	Yellow
72	PAU3-578	Semi compact	Oval round	Smooth	Shallow	White-cream	White
73	PAU3-579	Compact	Round	Smooth	Shallow	Yellow	White
74	PAU-584	Compact	Oval round	Smooth	Deep	Yellow	White
75	PAU3-586	Semi compact	Irregular	Rough	Medium	Yellow	Yellow
76	PAU3-589	Semi compact	Oblong	Rough	Shallow	Yellow	Yellow
77	PAU_591	Semi compact	Oval round	Smooth	Medium	Yellow	Yellow
78	PAU3-592	Semi compact	Oblong	Smooth	Shallow	Yellow	White
79	PAU3-595	Semi compact	Round	Smooth	Shallow	White-cream	Cream
80	PAU3-597	Semi compact	Oval round	Smooth	Deep	Yellow	White
81	PAU3-600	Semi compact	Round	Smooth	Deep	White-cream	Cream
82	PAU3-601	Semi compact	Oval	Smooth	Medium	White-cream	Cream
83	PAU3-602	Semi compact	Irregular	Rough	Protruding	Cream	Cream
84	PAU3-612	Semi compact	Irregular	Smooth	Medium	White-cream	Cream
85	PAU3-621	Compact	Round	Smooth	Shallow	Cream	Cream
86	PAU3-622	Compact	Oval	Smooth	Shallow	Cream	Cream
87	PAU3-491	Compact	Oblong	Smooth	Shallow	Cream	Cream

Table 4.26: Morphological traits of potential frost tolerant potato clones derived from MS/7-645 × J-2/19 cross

S. No.	Clones	Foliage Type	Tuber shape	Skin type	Depth of tuber eyes	Tuber skin color	Flesh color
1	PAU7-959	Semi compact	Oval round	Smooth	Medium	White-cream	Cream
2	PAU7-962	Semi compact	Oval	Smooth	Shallow	Cream	Cream
3	PAU7-963	Many stem	Oval flat	Smooth	Shallow	Yellow	Cream
4	PAU7-964	Open canopy	Oblong	Smooth	Shallow	White-cream	Yellow
5	PAU7-965	Many stem	Flattened	Smooth	Medium	White-cream	Cream
6	PAU7-971	Open canopy	Oval	Smooth	Shallow	Yellow	Yellow
7	PAU7-978	Many stem	Oval	Smooth	Shallow	Yellow	Yellow
8	PAU7-981	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
9	PAU7-982	Semi compact	Oblong	Smooth	Protruding	White-cream	Yellow
10	PAU7-983	Open canopy	Round	Smooth	Shallow	Cream	Yellow
11	PAU7-984	Open canopy	Oblong	Smooth	Shallow	Yellow	White
12	PAU7-985	Semi compact	Pear shaped	Smooth	Shallow	White-cream	Yellow
13	PAU7-987	Semi compact	Oval	Smooth	Shallow	Yellow	White
14	PAU7-990	Semi compact	Oval round	Smooth	Shallow	Cream	Yellow
15	PAU7-992	Semi compact	Oval round	Smooth	Deep	White-cream	Yellow
16	PAU7-993	Semi compact	Oval round	Smooth	Deep	White-cream	Yellow
17	PAU7-994	Semi compact	Round	Smooth	Shallow	Cream	Yellow
18	PAU7-995	Semi compact	Round	Smooth	Shallow	Cream	White
19	PAU7-996	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow

S. No.	Clones	Foliage Type	Tuber shape	Skin type	Depth of tuber eyes	Tuber skin color	Flesh color
20	PAU7-998	Open canopy	Round	Smooth	Deep	White-cream	Yellow
21	PAU7-999	Semi compact	Round	Rough	Medium	Cream	Yellow
22	PAU7-1000	Semi compact	Irregular	Smooth	Medium	Cream	Cream
23	PAU7-1001	Semi compact	Irregular	Rough	Deep	White-cream	Yellow
24	PAU7-1004	Compact	Oblong	Smooth	Protruding	Yellow	Cream
25	PAU7-1005	Limp foliage	Oval round	Smooth	Deep	Yellow	Cream
26	PAU7-1006	Open canopy	Oval	Smooth	Medium	Yellow	Yellow
27	PAU7-1007	Compact	Oval	Smooth	Shallow	Yellow	Yellow
28	PAU7-1009	Open canopy	Oval	Smooth	Shallow	Cream	White
29	PAU7-1012	Semi compact	Oval round	Rough	Shallow	Cream	Yellow
30	PAU7-1015	Semi compact	Oval round	Smooth	Shallow	Cream	White
31	PAU7-1018	Semi compact	Oval	Smooth	Shallow	Cream	Yellow
32	PAU7-958	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
33	PAU7-960	Compact	Oval	Rough	Shallow	Yellow	Yellow
34	PAU7-961	Semi compact	Round	Smooth	Medium	Yellow	Yellow
35	PAU7-961A	Semi compact	Oval	Smooth	Medium	White-cream	Cream
36	PAU7-972	Semi compact	Oblong	Smooth	Medium	Yellow	Yellow
37	PAU7-975	Open canopy	Oval round	Smooth	Deep	Cream	Cream
38	PAU7-986	Compact	Oval round	Smooth	Deep	Cream	White
39	PAU7-1011	Semi compact	Oval	Smooth	Medium	Cream	White

Table 4.27: Morphological traits of potential frost tolerant potato clones derived from MS/7-645 × CP-3765 cross

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Depth of tuber eyes	Tuber skin colour	Flesh colour
1	PAU9-1253	Compact	Pear shaped	Smooth	Medium	Cream	Yellow
2	PAU9-1255	Semi compact	Oval round	Smooth	Deep	Yellow	Yellow
3	PAU9-1256	Compact	Oval	Smooth	Shallow	Cream	Yellow
4	PAU9-1257	Semi compact	Oblong	Smooth	Shallow	Cream	Cream
5	PAU9-1258	Semi compact	Oval	Smooth	Medium	Yellow	Cream
6	PAU9-1260	Compact	Oval	Smooth	Shallow	White-cream	Yellow
7	PAU9-1261	Compact	Oblong	Smooth	Medium	Yellow	Cream
8	PAU9-1262	Compact	Oval round	Smooth	Shallow	Cream	Yellow
9	PAU9-1263	Semi compact	Oval round	Smooth	Medium	White-cream	Cream
10	PAU9-1264	Semi compact	Flat oval	Smooth	Shallow	Yellow	Cream
11	PAU9-1265	Semi compact	Oval round	Smooth	Shallow	White-cream	Cream
12	PAU9-1267	Semi compact	Oval	Smooth	Medium	Cream	Yellow
13	PAU9-1268	Semi compact	Oval Flat	Smooth	Medium	White-cream	Yellow
14	PAU9-1270	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
15	PAU9-1273	Semi compact	Round	Smooth	Shallow	Cream	Cream
16	PAU9-1274	Compact	Oval round	Smooth	Shallow	Cream	Cream
17	PAU9-1275	Semi compact	Oval round	Rough	Shallow	Cream	Yellow

S. No.	Clones	Foliage type	Tuber shape	Skin Type	Depth of tuber eyes	Tuber skin colour	Flesh colour
18	PAU9-1280	Semi compact	Oval Flat	Smooth	Medium	Yellow	Yellow
19	PAU9-1292	Semi compact	nd	nd	nd	nd	Nd
20	PAU9-1302	Semi compact	Oval round	Smooth	Shallow	Cream	Cream
21	PAU9-1308	Semi compact	Oval round	Smooth	Shallow	Yellow	Cream
22	PAU9-1309	Semi compact	Oval	Smooth	Shallow	Yellow	Yellow
23	PAU9-1306	Compact	Oval round	Smooth	Shallow	Yellow	Yellow
24	PAU9-1310	Semi compact	Oblong	Smooth	Shallow	Yellow	Yellow
25	PAU9-1312	Semi compact	Oval	Smooth	Shallow	Cream	Yellow
26	PAU9-1314	Semi compact	Oval	Smooth	Shallow	White-cream	Yellow
27	PAU9-1254	Semi compact	Round	Smooth	Medium	Cream	White
28	PAU9-1266	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
29	PAU9-1272	Semi compact	Oval	Smooth	Shallow	Yellow	Yellow
30	PAU9-1281	Semi compact	Oval	Rough	Shallow	Cream	Cream
31	PAU9-1305	Semi compact	Oval round	Smooth	Shallow	Yellow	Yellow
32	PAU9-1316	Semi compact	Oval	Smooth	Medium	Yellow	Yellow
33	PAU9-1304	Semi compact	Oval round	Smooth	Medium	Cream	Yellow

nd: Data not available

CHAPTER V

SUMMARY

The present study entitled, 'Generation of frost tolerant potato (*Solanum tuberosum* L.) clones through intervarietal hybridization and expression analysis of putative frost tolerant genes' was carried out in the Department of Vegetable Science, Electron Microscopy and Nanoscience Laboratory (EMN), Department of Soil Science, and School of Agriculture Biotechnology, Punjab Agricultural University (PAU), Ludhiana (latitude 30º 54' N and longitude 75º 45' E; with a mean height of 247 m above sea level) during the autumn-winter season of year 2018-19, 2019-2020 and 2020-2021 and summer season at Keylong, (32º 34' N and longitude 77º 15' E; with a mean sea level 3090.8 m)Himachal Pradesh in 2018. The experimental site was located in the subtropical climatic zone with winter during the months from October to March (*Rabi* season). In addition to the optimization of controlled environment screening protocols for phenotyping of frost tolerant potato clones, confirmation of morpho-anatomical traits associated with frost tolerance and transcript profiling of low temperature responsive genes in the potato genotypes, the main aim of the present study was to generate and characterize the potential frost tolerant potato clones possessing desirable horticultural traits.

In the first experiment, the phenotyping of the four potato genotypes, two tolerant (J-2/19 and Kufri Anand) and two sensitive (MS/7-645 and Kufri Pukhraj) exhibiting contrasting genetic variations for foliage damage under natural frost occurrence during cropping season 2016-17 at Ludhiana were selected for further validation and phenotyping under environmentally controlled conditions. Plants of these genotypes were incubated at different cold acclimation conditions and were exposed to freezing temperature (-2℃) for two treatment time spans, 1.0 and 1.5 hour. The leaf gas exchange and chlorophyll fluorescence were measured using a portable photosynthesis system. Membrane injury was also estimated by measuring electrolyte leakage using conductivity meter. The genotype, J-2/19 exhibited the highest P_N, g_s and WUE values under acclimated growing condition. Further, this genotype also exhibited less electrolyte leakage under both the cold acclimated as well as non-acclimated conditions indicating that J-2/19 was able to maintain more stable and intact cell membrane under low temperature stress. Even during the recovery periods after freezing temperature treatment, for all the chlorophyll fluorescence and leaf gas exchange parameters the genotype J-2/19 recorded highest values as compared with those of genotypes, Kufri Anand, MS/7-645 and Kufri Pukhraj. Across genotypes and recovery days significant differences for $P_{N,}$ g_s, ΔF/Fm' and ETR were observed under both the treatment time spans, 1.0 and 1.5 hours. These findings corroborated the previous reports regarding the use of

electrolyte leakage, leaf gas exchange and chlorophyll fluorescence as a potential screening techniques with certain limitations, such as difficulties in handling and sensitivity of instrument to environmental conditions.

In the second experiment, genetic variability of the potato genotypes was also explored by using the transcript profiling of three cold responsive gene(s) *viz. hsp70, dehydrin* and *SAD* genes in three different potato genotypes J-2/19, MS/7-645 and Kufri Pukhraj. These genotypes showed differential responses with respect to growing conditions and treatment durations. At the transcriptional level, the low temperature responsive genes- *SAD, dehydrin* and *hsp70* in different potato genotypes, J-2/19 (tolerant) and MS/7-645 and Kufri Pukhraj (sensitive) displayed a trend of up regulation in cold acclimated plants compare to non-acclimated ones. However, this increase was more pronounced in tolerant genotype as compared with the susceptible genotypes. Among the studied genes, *SAD* exhibited an increased level of expression in the tolerant genotype (J-2/19) in all freezing treatment time spans as compared to *dehydrin* and *hsp70* genes.

In the third experiment, generation and characterization of potential frost tolerant clones were carried out. During the summer of 2018, potential frost tolerant potato clones were generated by using diverse parental lines. Three crosses, J-2/19 × MS/7-645 (PAU3), MS/7-645 × J-2/19 (PAU7) and MS/7-645 × CP- 3765 (PAU9) were attempted at PAU Research Station, Keylong, Himachal Pradesh. The identification and characterization of these potential frost tolerant clones were accomplished as a series of studies in two clonal generations (F_1C_1 and F_1C_2) during the 2019-2020 and 2020-2021 cropping seasons at PAU, Ludhiana.

In F_1C_1 generation, a total of 168, 57 and 47 potential clones from different crosses PAU3, PAU7 and PAU9, respectively were evaluated on the basis of the leaf morpho-anatomical traits associated with frost tolerance in potato. Data on various parameters, such as stomatal and trichome density on both adaxial and abaxial leaf surfaces, stomatal index on adaxial leaf surface, palisade parenchymatous and epidermis cell thickness were recorded. Various statistical approaches, *viz.* cluster analysis, genotype by trait biplot and principal component analysis were used as reliable methods to reduce the complexity of data sets. Further, these tools were also used to characterize the genetic divergence among the studied clones and to determine the association between the key traits and potential frost tolerant potato clones.

On the basis of proximate composition of these leaf morpho-anatomical traits, the hierarchical heatmap clustering was performed and the generated clones of PAU3, PAU7 and PAU9 crosses were divided into 13, 6 and 5 clusters, respectively. By using stomatal index as

a screening marker, those groups of clones possessing higher stomatal index (SI) (more than 10%) were selected for further evaluation under natural frost conditions.

During the season 2020-21, in F_1C_2 generation the selected clones were further evaluated for their physiological and field performance at two locations in Punjab *i.e.*, Vegetable Experimental Farm, PAU, Ludhiana and PAU Vegetable Research Farm, Khanaura, Hoshiarpur. During the months of December and January, the average minimum temperature of Punjab was reached to -0.2℃ during the nights of frost incidences. Incidentally, two severe frost episodes were observed at the PAU Vegetable Research Farm, Khanaura. These frost incidences provided a rare opportunity for screening of generated potato clones for frost tolerance. A large and significant variability was observed for foliage damage, recovery rate and electrolyte leakage (%) among the clones from different crosses at both the locations.

A crosswise genotype by trait (GT) biplot analysis was performed to study the association among the electrolyte leakage behaviour of potato clones and leaf morpho-anatomical traits associated with frost tolerance. In the clones derived from crossPAU3, a close association was recorded among the stomatal index, stomatal ratio, and adaxial stomatal and trichome density. However, the obtuse angle among these traits and electrolyte leakage indicated a negative association, indicating that the high values of these traits would lead to low electrolyte leakage and, thus imparting frost tolerance to the respective potato clones. The clones, PAU3-477, PAU3-563, PAU3-479, PAU3-464, PAU3-424, PAU3-483, PAU3-456, PAU3-602, PAU3-570, PAU3-455, PAU3-600, PAU3-547, PAU3-622 and PAU3-586 exhibited strong association with stomatal index. Owing to their placement in GT biplot in the opposite direction of electrolyte leakage, these clones were identified as frost tolerant ones. Similarly, in other two crosses, PAU7 and PAU9, the clones, PAU7-958, PAU3-961, PAU3-964, PAU3-972, PAU7-975, PAU7-994, PAU7-999, PAU7-1000, PAU7-1007, PAU7-1011, PAU9-1263, PAU9-1261, PAU9-1266, PAU9-1274, PAU9-1281, PAU9-1309, PAU9-1304, PAU9-1312, PAU9-1270 and PAU9-1280 were identified as the promising frost tolerant ones.

In addition to the screening for frost tolerant traits, the clones were also evaluated for horticultural traits. On the basis of combined selection, PAU3-460, PAU3-537, PAU7-98, PAU7-999, PAU9-1259, PAU9-1257, PAU9-1314 and PAU9-1309 were identified as the promising frost tolerant clones possessing low foliage damage (%) and desirable tuber characteristics.

In conclusion, the information generated in the current study will be useful for phenotyping the potato clones against low temperature stress. In addition to electrolyte leakage assay, the chlorophyll fluorescence and leaf gas exchange parameters (P_N and g_s) can potentially be used for screening of frost tolerant potato genotypes, but with certain

limitations. Further, the leaf morpho-anatomical traits can be potentially used as preliminary screening technique of large segregating populations for frost tolerance even during the frost free seasons. Furthermore, the potato clones generated in this study exhibited tolerance to frost and possessed desirable tuber characteristics along with high yield potential. A total of 28 clones with 11, 10 and 8 clones from J-2/19 × MS/7-645, MS/7-645 × J-2/19 and MS/7-645 × CP-3765 crosses, respectively were identified. These clones are recommended for their further evaluation and selection at multilocations in frost prone regions for their potential utilization as frost tolerant cultivars. Furthermore, in order to elucidate the unexplained reason for tolerance against frost in potato the variations in biochemical traits associated with low temperature stress can also be explored.

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APPENDICES

Table 1 Coordinates and soil physiochemical characteristics of the experimental site at PAU, Ludhiana

Characteristics	Units	Ludhiana
Coordinates		Latitude 30º 54’ N, longitude 75º 45’ E
Altitude	meter	247 a.m.s.l.
Landscape		Western plains
Soil Texture		Sandy loam
Soil Characteristics		
pH		7.3
EC	m mhos/cm	0.26
OC	%	0.58
P	ppm	39.1
S	ppm	16.18
K	ppm	138.8
Ca	ppm	220.1
Mg	ppm	114.3
Fe	ppm	9.59
Cu	ppm	1.6
Mn	ppm	4.16
Z	ppm	2.19
Mo	ppm	Trace
B	ppm	0.26

Above mean sea level (a.m.s.l.); Electric conductivity (EC); Organic Carbon (OC); Phosphorus (P); Sulphur (S); Potassium (K); Calcium (Ca); Magnesium (Mg); Iron (Fe); Copper (Cu); Manganese (Mn); Zinc (Zn); Molybdenum (Mo); Boron (B)

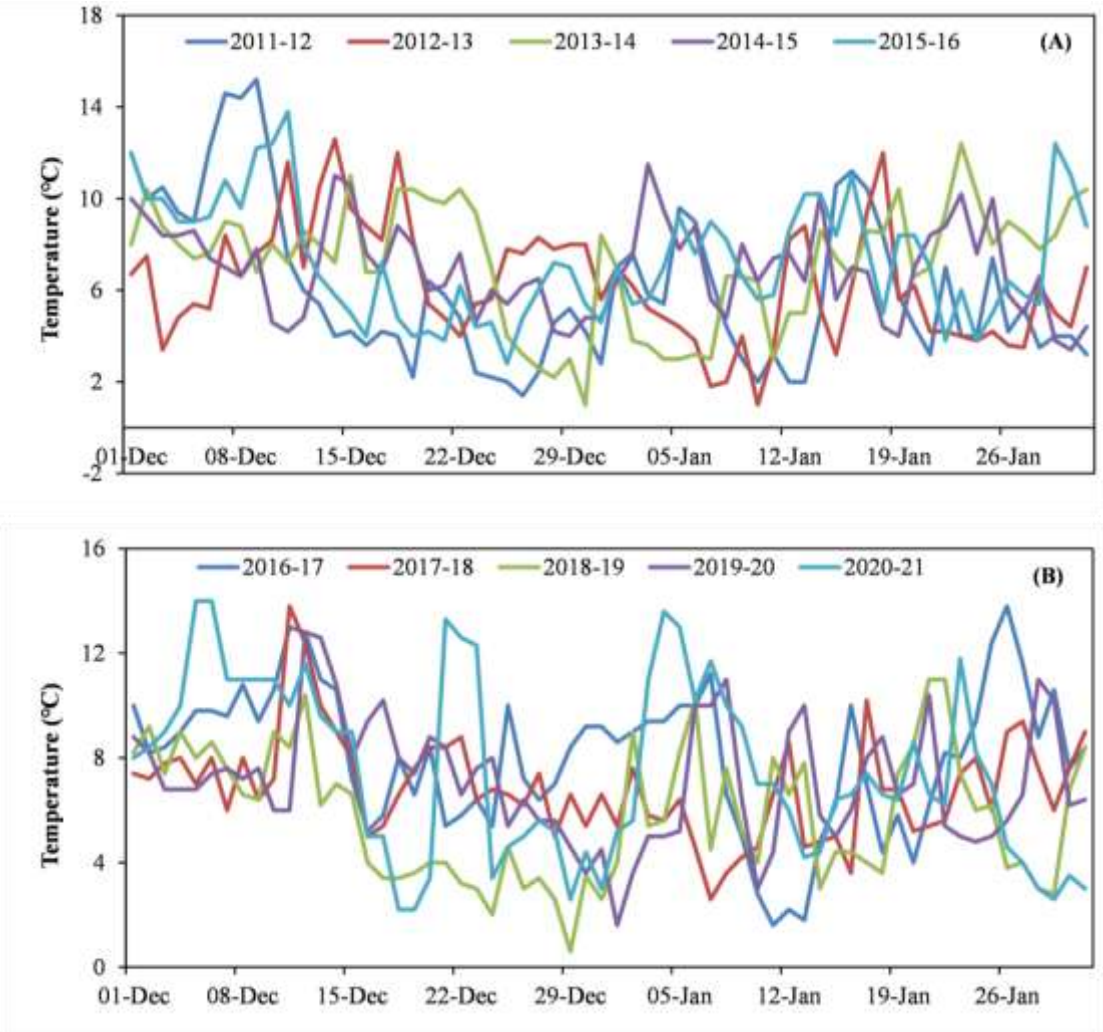

Figure 1A&B: Minimum temperature during December to January months of 5 years at three different locations Ludhiana (A- 2011–2016, B- 2016–2021)

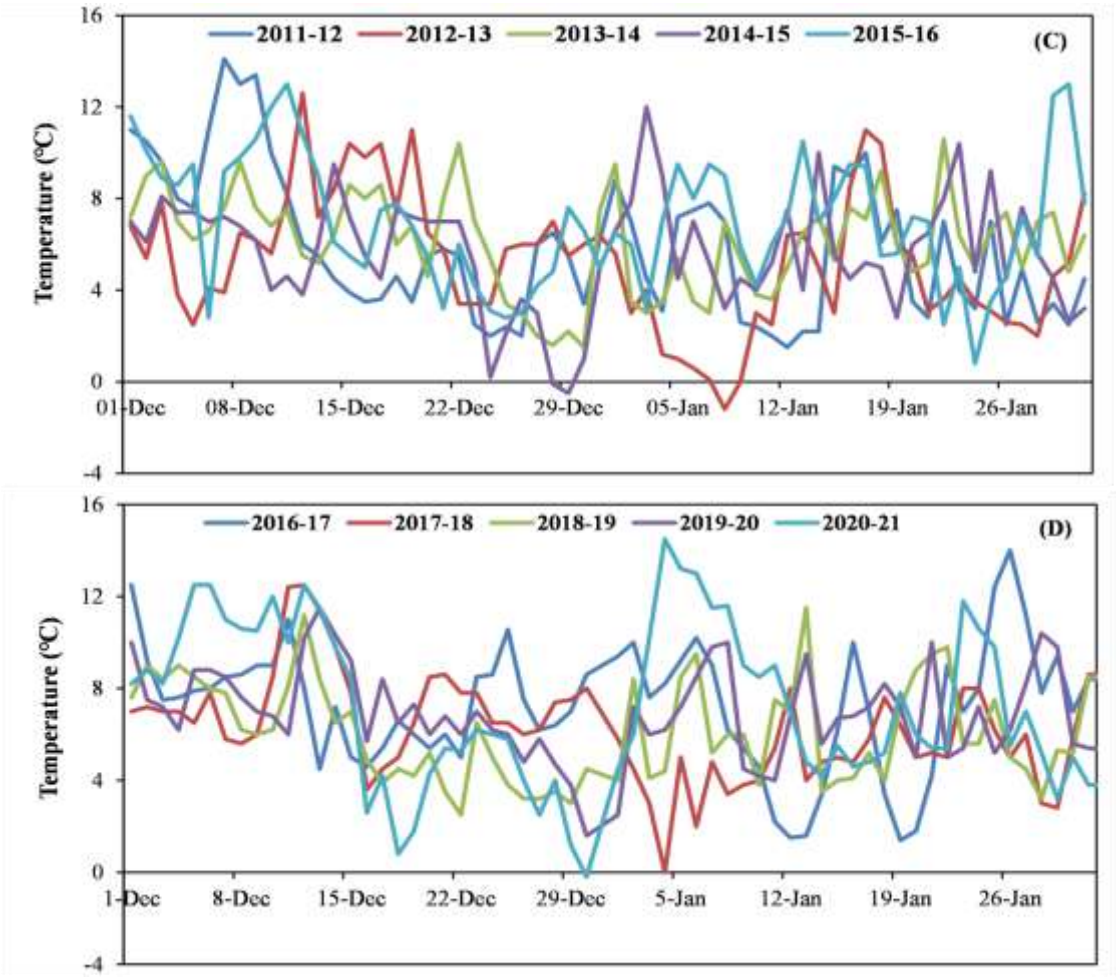

Figure 1C&D: Minimum temperature during December to January months of 5 years at Hoshiarpur (C- 2011–2016, D- 2016–2021)

Table 2 Weekly Maximum and Minimum temperature at two locations- Ludhiana and Hoshiarpur during cropping season 2020-21

Standard metrological week	Ludhiana		Hoshiarpur	
	Temperature (℃)		Temperature (℃)	
	Maximum	Minimum	Maximum	Minimum
01 Oct – 07 Oct	33.9	18.9	35.9	18.9
08 Oct – 14 Oct	34.1	18.3	35.6	18.1
15 Oct – 21 Oct	33.0	15.0	34.6	16.7
22 Oct – 28 Oct	31.1	13.4	32.8	14.7
29 Oct – 04 Nov	29.1	11.3	30.4	11.9
05 Nov – 11 Nov	28.5	10.3	29.3	12.4
12 Nov – 18 Nov	24.8	10.9	25.5	10.7
19 Nov – 25 Nov	21.5	8.4	21.6	9.4
26 Nov – 02 Dec	24.2	8.8	24.6	8.9
03 Dec – 09 Dec	24.6	11.4	25.2	10.8
10 Dec – 16 Dec	17.5	9.3	19.0	9.5
17 Dec – 23 Dec	18.6	7.3	19.4	4.0
24 Dec – 31 Dec	15.8	4.2	15.7	2.9
01 Jan – 07 Jan	17.6	10.0	19.3	10.4
08 Jan – 14 Jan	14.1	6.8	14.3	7.7
15 Jan – 21 Jan	15.8	5.2	17.1	5.6
22 Jan – 28 Jan	17.9	6.4	18.1	7.9
29 Jan – 04 Feb	20.7	6.3	20.1	6.8
05 Feb – 11 Feb	21.3	7.4	22.7	8.5
12 Feb – 18 Feb	22.5	10.8	27.0	12.2
19 Feb – 25 Feb	27.0	12.2	27.8	9.7
26 Feb – 04 Mar	27.9	11.9	30.1	12.8

VITA

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