# Effects of Silver Nitrate on Growth Characteristics of Micropropagated Potato (*Solanum tuberosum* L.) Plantlets

*A Dissertation*
*submitted in partial fulfillment of the requirement*
*for the award of the degree of*
*Masters of Science in Biotechnology*

**Under the guidance of**
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# CERTIFICATE

This is to certify that the thesis entitled **"Effects of silver nitrate on growth characteristics of micropropagated potato (*Solanum tuberosum* L.) plantlets"** submitted by Ramanpreet Kaur (Roll no: 301101026) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, to Thapar University (Deemed University), Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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# DECLARATION

I hereby declare that the work which is being presented in this thesis **"Effects of silver nitrate on growth characteristics of micropropagated potato (*Solanum tuberosum* L.) plantlets"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. N. Das,** Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

Date: 15 July 2013

Place: Patiala

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# Contents

# Abbreviations

| | |
|---|---|
| SAM | S-adenosylmethionine |
| ACC | 1-aminocyclopropane-1-carboxylate |
| ACO | ACC oxidase |
| ETR1 | Ethylene Response 1 |
| ETR2 | Ethylene Response 2 |
| ERS1 | Ethylene Response Sensor 1 |
| ERS2 | Ethylene Response Sensor 2 |
| EIN4 | Ethylene Insensitive 4 |
| RAN1 | Response To Antagonist 1 |

# Abstract

Potato (*Solanum tuberosum* L.) is an economically important food crop and ranks only after wheat and rice in terms of productivity. Due to its increasing demand, there is need to increase its production. For potato crop improvement molecular breeding is one of the best method, by which genetically engineered better potato varieties can be obtained. The success of plant genetic engineering relies on several factors which include an efficient tissue culture system, for regeneration of plants from cultured cells and tissues. Growth and development of cells cultured *in vitro* are largely dependent on the presence of phytohormones, including ethylene in the culture environment. Ethylene produced by plant tissues grown *in vitro* may accumulate in large quantities in the culture vessels, and hence is likely to influence growth and development in such systems. Modifying the accumulation of ethylene in the head-space of culture vessels and altering its biological action are important in maximizing the desired *de novo* morphogenic pathway. Ethylene biosynthesis and action can be regulated by various methods. Silver ions treatment is one of the best chemical method, which inhibits its action by inhibiting its receptors. In most of plants, ethylene is perceived by a family of five membrane-bound receptors. The coordination of a copper ion with receptor is necessary for ethylene to bind, resulting in a conformational change of the receptor and the initiation of the ethylene signal transduction pathway. Interestingly, silver ions binds to the receptors in place of copper ion of ethylene, so ethylene responses block in the presence of silver. Many authors have reported the effectiveness of silver nitrate in tissue culture system.

The objective of this study was to investigate the effects of varying silver nitrate concentrations (0.5, 1.0, 2.0, 3.0 mg $L^{-1}$) on the growth characteristics of micropropagated microplants of six Indian and one exotic potato cultivars. The nodal shoot segments from each cultivar transferred on medium of all silver nitrate concentrations and after four weeks results collected. The common effects of silver nitrate were – (1) increases in leaf size, (2) reduction in shoot height, (3) reduced number of roots (4) promotion of root elongation and greening and (5) shoot and root colour became dark green and became thick also. Then these silver nitrate treated microplants transferred on MS-basal medium. It was observed that these silver nitrate treated microplants resumed their original growth characteristics after two months.

# Chapter 1
# Introduction

Potato (*Solanum tuberosum* L.) is the world's most important non-grain food crop. After wheat and rice, potato is the most important food crop, ranks as a staple food. Its world-wide production is around 374 million tons in 2011. Potato is the most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops i.e. cassava, sweet potato and yams accounting for 90% of total world production. Field-grown potato plants and harvested potato tubers are shown in Fig. 1 and Fig. 2, respectively. Potato is a highly nutritious crop as it is rich source of proteins, carbohydrates, vitamin C and potassium. Therefore, it plays a major role with regard to global food security. The Andeans of Peru and Bolivia seem to have known this over 8000 years ago when they started cultivating the potato. Natives of these regions figured out how to grow impressive yields of potatoes under the most unsupportive conditions. On the hills there, the micro-climate and soil conditions change remarkably from ridge to ridge and terrace to terrace. These environmental conditions led them to grow number of different varieties and hence maintained the genetic diversity. In India, potato crop was brought by Europeans along with many other plants and fruits. Now India ranks third in terms of area of potato cultivation, and it is the second largest country in terms of production, around 42 million tons in 2011.

**Fig 1.** Potato plants

**Fig 2.** Potato Tuber

There is great potential of exporting potatoes from India both for seed and table purposes to our neighboring countries of South-East Asia and to Middle East countries. Potatoes can even be exported

to some of the European countries during March-May when fresh potatoes are not available in these countries. And also in recent years potato processing industries have come up in a big way requiring varieties containing low sugars and high dry-matter for preparation of specific value-added products like chips, French fries, cubes and other dehydrated products.

In India, there are large number of potato cultivars used for production of both vegetable and processing potatoes. These varieties are suitable to different agro-climatic zones of our country. Some important varieties are Kufri Jyoti, Kufri Chandramukhi, Kufri Anand, Kufri Chipsona-1, Kufri Chipsina-2, Kufri Ashoka, Kufri Pukhraj. Due to increasing population, demands and shrinking agriculture land there is great need to improve yield and nutritional quality of potatoes in India. The Central Potato Research Institute (CPRI), Shimla was established in 1949, took a leading role for the improvement of potato crop through conventional breeding techniques.

## 1.1. Taxonomy

The genus *Solanum* consists of approximately 2000 members. The haploid no. of chromosomes in potato are 12. These potato species varies in their ploidy levels, ranging from diploid (2n = 2x = 24) to hexaploid (2n = 6x = 72) including triploids, tetraploids, and pentaploids (Spooner et al.. 2005). The most common cultivated species of potato i.e., *tuberosum (*a *tetraploid* with 48 chromosomes) is a hybrid between the diploid species S. *stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen, 1979).

## 1.2. Growth phases of potato plant under field condition

Potato growth has been divided into **five phases** which are described below:

***First phase***- sprouts emerge from the seed potatoes and root growth begins.

***Second phase***- photosynthesis begins as the plant develops leaves and branches.

***Third phase***- stolons develop from lower leaf axils on the stem and grow downwards into the ground and on these stolons new tubers develop as swellings of the stolon. This phase is often (but not always) associated with flowering.

***Fourth phase***- Tuber bulking occurs when the plant begins investing the majority of its resources in its newly formed tubers. At this stage, several factors are critical to yield: optimal soil moisture and temperature, soil nutrient availability and balance, and resistance to pest attacks.

***Final phase***- maturation: the plant canopy dies back, the tuber skins harden, and their sugars convert to starches.

Tuber formation halts when soil temperatures reach 27 °C(81°F); hence potatoes are considered as a **cool-season crop**.

## 1.3. Mode of propagation

Potato plant is propagated through tubers (vegetative or asexual propagation), by planting tubers, pieces of tubers, cut to include at least one or two eyes. The tubers meant for propagation are known as "seed tubers" or "seed potatoes" as shown in Fig. 3. The main advantage of vegetative propagation is that a good potato clone can be maintained with a high degree of genetic purity. But there is disadvantage also that there is gradual degeneration of a clone by progressive accumulation of deadly viruses and seed borne pathogens in the tubers and they are carried over by repeated multiplications. For this reason, successful potato cultivation and production depends upon the availability of disease free high quality seed tubers. Here in India where there is tropical and subtropical warm climates and an abundance of various vectors e.g. aphids, mites, thrips, white flies, etc. for virus transmission are present it is most impotant to produce potato varieties resistant to viral, bacterial, nematode and fungal infections. These diseases effect the potato tubers in terms of yield and consumer acceptance. Examples of viral pathogens are potato viruses X and Y. *Streptomyces scabies* and *Erwinia carotovora* represent two bacterial pathogens of tubers that cause serious losses. The most serious losses occur due to fungal pathogens, such as *Phythophthora infestans* that causes.

There is also an another technique for propagation of potato. All new potato varieties are grown from seeds, also called "true potato seed" or "botanical seed" (Fig. 4) obtained by finely chopping the fruit and soaking it in water, the seeds separate from the flesh by sinking to the bottom after about a day. True potato seeds (TPS) are raised in nursery. The seedlings of TPS raised in nursery beds may either be transplanted in the field or left in the nursery for producing seedling tubers. The planting density depends upon the location, method of planting, purpose for which the crop is raised, etc. These seeds are an alternative means of propagation where production of 'seed potatoes' is not feasible. For usual cultivation, true potato seeds are not popularly used; but it is important with respect to potato breeding perspectives.

**Fig 3.** Seed potato

**Fig 4.** True potato seeds

## 1.4. Improvement of potato crop

The demand for potato crop has been increasing due to overgrowing population and its high nutritional quality. It has a bright future as a crop that is set to replace rice as a staple in the Asian rice-consuming countries. It requires less amount of water compared to other basic food products, without compromising the nutrition value. Potato, therefore, is increasingly being promoted as the foremost solution for meeting the increased food demand for an estimated 6 billion world population by 2030. Also, Food and Agriculture Organization of the United Nations has acknowledged potato as the food for the future in order to fight global poverty and hunger. Therefore it is need to improve the potato crop in terms of productivity, nutritional quality and disease resistance. Researchers and scientists are working towards facilitating higher and sustainable crop yields per hectare that are free from disease and pests. Crop improvement strategies like conventional breeding and molecular breeding has been used from a long time.

***1.4.1. Conventional Breeding*:** Conventional breeding develops new plant varieties by the process of crossing of wild varieties and selection of the desired traits in the progeny. It helps to achieve expression of genetic material which is already present within a species. It employs natural processes, such as sexual and asexual reproduction. Desirable characteristics from different parent plants by crossing them together could also be combined in the offsprings (F' plants), followed by vegetative propagation of the F' plants to form clones. These clones and their tuber progenies are then screened gradually over several years for favorable combinations of agronomic traits. Commercial cultivars are tetraploid and heterozygous. Due to these reasons some cultivars do not flower easily, or their fertility

get reduced, and some are pollen sterile. Moreover, conventional breeding is laborious and time consuming process.

***1.4.2. Molecular Breeding*:** Molecular breeding involves manipulating the plant genes so that a new and better variety can be developed. It is done for the same reasons as conventional breeding. The key difference is that instead of randomly mixing genes, which occurs as a result of a sexual cross, a specific gene, which is associated with a desirable trait, is selected and inserted directly into the existing plant cultivar. This can save time and reduces the chance of undesirable traits in the genetically modified plant. This approach allows the breeders to use genes from unrelated plants and sometimes other organisms to develop a improved variety. Genes of agricultural importance, for example viral, fungal or insect resistance genes have been inserted into the genomes of several crop plants (Schaff, 1991). As discussed earlier potato is a tetraploid and heterozygous, its improvement by breeding and selection is very difficult and it is a time consuming process. So applied molecular genetics has become a popular tool to obtain disease and pest resistance in potatoes (Mullins et al. 2006). There are many methods that have potential for the production of genetically modified plants also known as transgenic plants and can broadly be divided in to two categories namely indirect and direct gene transfer (Schaff, 1991).

Most widely used method for the production of transgenic plants is the *Agrobacterium*-mediated gene transfer system. It is considered to be an indirect method as the gene of interest first has to be transferred to the bacterium and then to the plant. *Agrobacterium tumefaciens* infects the plant cells *in vitro* and transfers the gene of interest to the plant genome (Hansen & Chilton, 1999). In contrast, direct transformation occurs when the foreign DNA is delivered into plant cells by the use of protoplasts, stimulating fusion by electroporation or chemical treatment, or subjecting the plant tissue in a particle gun to a shower of high velocity particles coated with the gene of interest (De Block, 1993).

## 1.5. Plant tissue culture

The success of plant genetic engineering relies on several factors which include an efficient tissue culture system. For plant improvement, cell and tissue culture techniques like shoot generation and rooting are important in the realization of their potential. Potato is amenable to a number of tissue culture techniques, like *in vitro* propagation via shoot cultures, regeneration of whole plants from protoplasts, under sterile conditions and an appropriate culture medium. Virus elimination (meristem

culture) and clonal mass propagation (micro-propagation) are widely used techniques and has the most prominent application in potato. Micropropagation helps in large-scale asexual multiplication of potato cultivars. Mostly nodal segment culture in which axillary and terminal buds grow into new plants is commonly used for shoot multiplication. The method involves culturing of nodal explants of disease-free micro-plants on semisolid or liquid culture medium. Medium normally contain a mixture of major and minor salts, vitamins, sugar (as a carbon source) and plant growth regulators. The most widely used media formulations are based on that of Murashige and Skoog (1962), which is available commercially. Plants can also be regenerated from different plant tissues (roots, leaves, tubers, stems) and cell types. There are different pathways of regeneration such as:

- Direct organogenesis from explants
- Indirect organogenesis from explants via callus
- Direct embryogenesis from explants
- Indirect embryogenesis from explants via callus

***Organogenesis*** is a process in which roots or shoots are being induced to differentiate from a cell or cell clusters. It is a developmental pathway. Morphogenesis could occur directly from the explants or indirectly *via* the formation of de-differentiated callus.

The regeneration of complete plant via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plant. For plant transformation, many different techniques such as *Agrobacterium*-mediated transformation, particle gun bombardment, and micro-injection, PEG treatment of protoplast and electroporation of protoplast can be used. However, *Agrobacterium*-mediated transformation and particle gun bombardment are the most extensively used methods.

### 1.5.1. Associated problems during tissue culture

To protect clonal propagated and regenerated tissue cultures, tubes or bottles should be protected from contamination and harsh outer atmosphere. For this polypropylene caps are widely used to close the culture tubes or bottles, but there are some abnormalities are observed in potato micro-plant cultures when these caps are used. This may be due to the suppression of gaseous exchange leading to the accumulation of ethylene inside the culture tubes or bottles. Because tissue culture by necessity is done

within closed containers, the gaseous environment may be very different from that experienced by non-enclosed plants. *In vitro* studies have indicated that ethylene can affect callus growth, shoot regeneration and somatic embryogenesis in vitro (Purnhauser et al. 1987; Songstad et al. 1988; Roustan et al. 1989; Roustan et al. 1990; Biddington, 1992; Pua and Chi, 1993). The physiological fucntions of ethylene in plants are discussed in the following section.

## 1.6. Introduction to the phytohormone: Ethylene

Phytohormones regulate germination, growth, metabolism, and other physiological activities in plants. These hormones helps the plants to quickly respond to abiotic and biotic stresses. There are five major phytohormones - auxins, cytokinins, gibberellins, and abscisic acid and ethylene. Ethylene was one of the first hormone identified and the only gaseous hormone (Abeles et al.. 1992). Depending on the plant species, ethylene can play different roles in developmental processes. For example, in deep water rice ethylene stimulates growth, yet in the plant model organism *Arabidopsis thaliana*, ethylene inhibits growth (Hattori Y et al. 2009; Bleecker AB et al. 1988). The main functions of ethylene are as follows:

- promotes ripening in many fruits
- senescence of leaves and flowers
- stimulates the release of dormancy
- shoot and root growth and differentiation
- stimulates flower opening
- stimulates leaf and fruit abscission

## 1.7. Ethylene biosynthesis

Plants are able to produce ethylene from essentially all parts of higher plants. The reactions involved in biosynthesis of ethylene is well described in the Yang cycle (Yang et al. 1984) shown in Fig. 5. The two key steps in ethylene biosynthesis are, conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and the oxidative cleavage of ACC to form ethylene (Chae et al. 2005). These two steps occur through the activity of ACC synthase and ACC oxidase (ACO), respectively (Chae et al. 2005). Regulation of ethylene production can be controlled by modulating the

levels and activity of both ACC synthase and ACO. The conversion of SAM to ACC by ACC synthase is regarded as the rate-limiting step of the ethylene biosynthesis pathway (Liu et al. 2004). The amount of ethylene produced, depends on the tissue-type as well as the abiotic and biotic cues. For an instance, ethylene levels increase during germination and positively regulate seed germination (Bleecker AB et al. 1988).

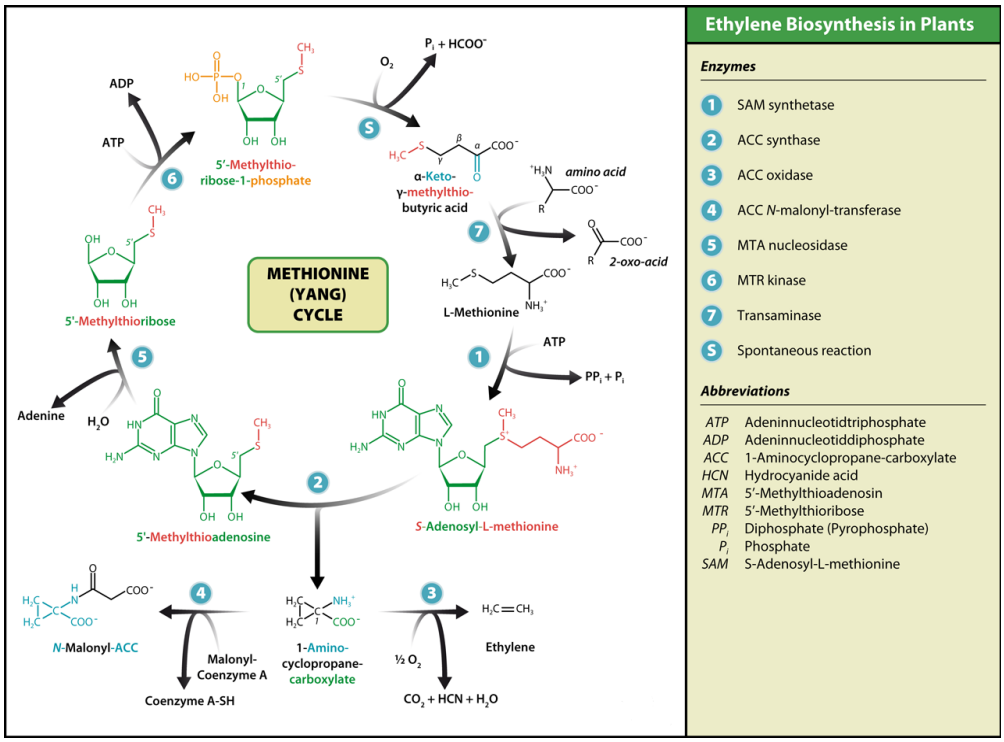



**Fig 5.** Schematic view of Ethylene biosynthesis (Wang KC-L,Li H, Ecker JR (2002),Plant cell S131- S151).

## 1.8. Overview of ethylene signal transduction

In ethylene signaling pathway, the receptors plays an important role. Plant ethylene receptors are related to bacterial two- component regulators. They are endoplasmic reticulum (ER)-associated integral membrane proteins (Chen et al. 2002) with protein kinase activities (Gamble et al. 1998; Moussatche and Klee, 2004). The receptors are disulfide- linked dimers. In Arabidopsis, a family of five disulfide-linked homodimer receptors located in the endoplasmic reticulum membrane: ETR1 and ETR2, ERS1,ERS2 and EIN4 (Chang et al. 1993; Hua et al. 1995; Hua and Meyerowitz, 1998; Sakai et al. 1998). Based on gene and protein structures, these five ethylene receptors have been divided into two subfamilies, the subfamily I receptors, ETR1 and ERS1, are the most homologous to histidine

kinases. The subfamily II members, ETR2, EIN4 and ERS2, have lost most of the amino acids critical for histidine kinase activity and instead possess serine kinase activity (Moussatche and Klee, 2004). They also contain an extra potential membrane-spanning domain at the amino terminus. All receptor isoforms contain an N-terminal ethylene-binding domain with three transmembrane α-helices, a GAF domain is present to mediate receptor-receptor interactions, and a kinase domain is also present (Binder et al. 2012). Ethylene binding occurs at the N-terminal transmembrane domain of the receptors. Histidine kinase acts as the sensor that autophosphorylates an internal histidine residue in response to environmental signals, and a response regulator that activates the downstream components upon receiving a phosphate from the histidine residue of the sensor on its aspartate residue (Wurgler-Murphy and Saito, 1997). A metal cofactor copper ion binds in the binding domain formed at the interface of the two monomers is required for ethylene to bind to the receptor (Rodriguez FI et al. 1999; Schaller et al. 1995). A metal transporter RAN1 is responsible for delivering of copper to the receptors (Hirayama T et al. 1999; Woeste et al. 2000).

Genetic analysis in tomato and Arabidopsis has shown that the receptors act as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al. 2000). In the absence of the hormone, receptors actively suppress ethylene responses. Upon ethylene binding, that suppression is removed and the response occurs. Experimentally, it has been shown that reduction of receptor content increases ethylene sensitivity (Cancel and Larsen, 2002; Hall and Bleecker, 2003; Hua and Meyerowitz, 1998; Tieman et al. 2000), while increased receptor content has the opposite effect (Ciardi et al. 2000).

## 1.9. Inhibition of ethylene signaling by silver ions

Possible mechanisms of ethylene inhibition by silver ions are described by many researchers. $AgNO_3$ inhibits ethylene action by means of silver ions by reducing the receptor capacity to bind ethylene (Yang, 1985), which would result in higher titers of ethylene in the tissues, thus inhibiting the earlier steps of its own pathway. Miyazaki and Yang (1987) reported the influence of putrescine and $AgNO_3$ on the competitive utilization of SAM. Bais et al. (2000b) also postulated that the utilization of SAM by putrescine for its conversion to spermidine would possibly result in a lower availability of SAM for ethylene biosynthesis (Fig. 6). The ethylene receptor, ETR1, contains one ethylene-binding site per homodimer and binding is mediated by a single copper ion (Cu) present in the ethylene-binding site.

The replacement of the copper co-factor by silver also serves to lock the receptor into a conformation such that it continuously represses ethylene responses (Zhao et al. 2002). The introduction of ethylene antagonists into the culture media affects the level of ACC, thereby affecting ethylene levels (Gong et al. 2005). Silver ions are 70% larger than copper ions and the bulkiness of the silver ion blocks the necessary conformational change in response to ethylene (Binder BM et al. 2007). Or the second possibility is that the silver-receptor complex is not as stable as the copper-receptor complex, due to which dissociation of ethylene become quicker.

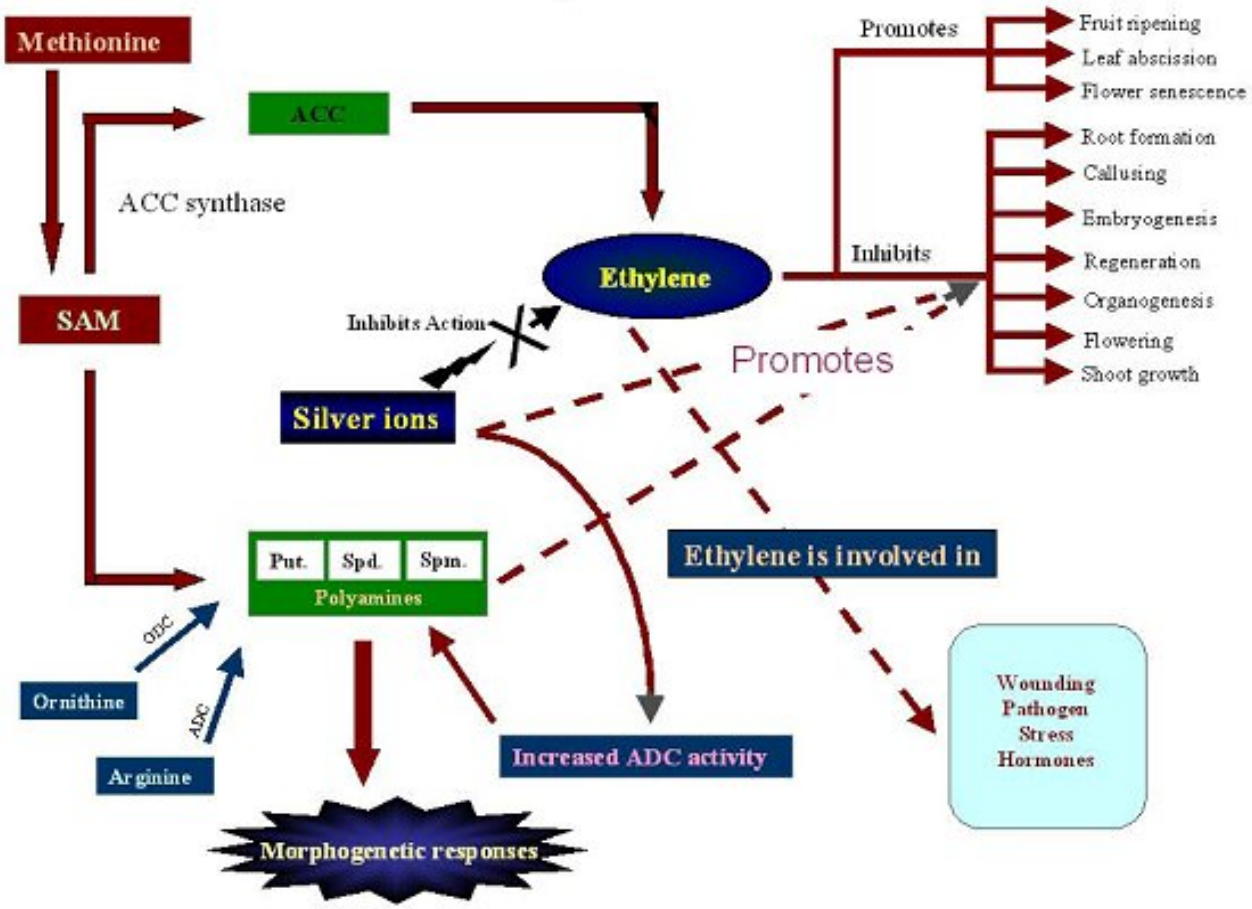



**Fig 6**. Schematic view of inhibition of ethylene action by silver ions (Kumar V et al., 2009)

# Chapter 2
# Literature Survey

The effect of ethylene has been widely studied on many plant species by many researchers by time to time. Ethylene is produced by cultured plant cells, tissues and organs. Major production of this phytohormone occurs *in vitro* as a result of wounding during explanting or on subculture. Ethylene produced by plant tissues grown *in vitro* may accumulate in large quantities in the culture vessels, particularly from rapidly growing non-differentiated callus or suspension cultures, and hence is likely to influence growth and development in such systems (Biddington, 1992). The gaseous nature of ethylene allows for diffusion through the intercellular spaces, where it can act as a signal of damage, stress, or physical contact, leading to the appropriate coordinated cellular response.

There has been increasing evidence that growth and differentiation of plant cells and tissues *in vitro* can be affected by ethylene (Chi et al. 1990). Inhibition of ethylene biosynthesis has been shown to enhance plant regeneration from callus cultures of *Triticum aestivum* (Purnhauser et al. 1987) and *Zea mays* (Vain et al. 1989), enhance shoot regeneration from cotyledon and hypocotyl cultures of *Brassica*. In case of potato, the potato tissue cultures are extremely sensitive to ethylene, and the growth of potato shoots is retarded in closed culture vessels (Hussey and Stacey, 1981, 1984).

## 2.1. Effect of silver ions on ethylene biosynthesis

Interestingly, a large number of reports are accumulating on the utility of silver ions in tissue culture and other applications, with significant contributions towards the development of plant biotechnology and transgenic research. Silver nitrate and silver thiosulfate has been used by researchers from a long time as silver ions source. Addition of $AgNO_3$ to the culture media greatly improved the regeneration of both dicot and monocot plant tissue cultures (Beyer, 1976c; Duncan et al. 1985; Davies, 1987; Purnhauser et al. 1987; Songstad et al. 1988; Chi and Pua, 1989; Veen and Over Beek, 1989; Bais et al. 2000A; Giridhar et al. 2003). In recent years, $AgNO_3$ has been employed in tissue culture studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations (Beyer, 1976a).

## 2.2. Application of silver nitrate in plant tissue culture

Silver nitrate used for various variety of plants for example potato leaf explants produced

shoot-forming callus only on medium with $AgNO_3$ (De Block, 1988). Likewise, it enhanced shoot production in cotyledon and seedling explants of several *Brassica* genotypes (Chi and Pua, 1989; Chi et al. 1990). $AgNO_3$ also enhanced shoot formation in *Brassica oleracea* (Sethi et al. 1990). It also increased shoot production in maize callus (Songstad et al. 1988). Also the use of $AgNO_3$ enhanced shoot regeneration in chile pepper (Hyde and Phillips, 1996). Ethylene inhibited shoot regeneration from cotyledon cultures of sunflower and addition of $AgNO_3$ to the medium enhanced regeneration (Chraibi et al. 1991). Work with Brussels sprouts (*B. oleracea* var. *gemmifera*) suggests that endogenous ethylene may inhibit callus growth: $AgNO_3$, was found to be essential for maintaining callus cultures as well as improving regeneration (Williams J, Biddington NL, 1990) .

Studies shown that although ethylene may promote callus growth but it may also inhibit shoot production. Thus callus proliferation was increased in *Brassica oleracea* hypocotyl cultures by the ethylene precursors SAM and ACC whereas shoot initiation was increased by $AgNO_3$ (Sethi U, Basu A and Mukherjee SC,1990). $AgNO_3$ promoted shoot regeneration in wheat callus culture, and reversed the inhibitory effect of ethylene and 2,4-D on morphogenesis. In carrot cell cultures, $AgNO_3$ promoted embryogenesis and inhibited ethylene production suggesting that embryo production was promoted by reduced ethylene biosynthesis (Roustan JP, Latche A and Fallot J 1990). The production of embryogenic calli in *Hevea brasiliensis* could be increased either by avoiding the accumulation of ethylene in the culture vessel, removing ethylene with mercuric perchlorate, inhibiting ethylene production with AOA or by the addition of $AgNO_3$, to the medium (Auboiron E, Carron MP and Michaux-Ferriere N,1990) .

In Brussels sprouts (*B. oleracea,* var. *gemmifera*) anther culture, ethephon and ACC inhibited embryo production and $AgNO_3$, promoted it, particularly with non- or poorly-responsive cultivars (Biddington NL, Sutherland RA and Robinson RT,1988). Studies on anther culture of some species indicate that ethylene affects microspore embryogenesis (Babbar and Gupta, 1986; Cho and Kasha, 1989; Reynolds, 1987; Dunwell, 1979; Biddington et al. 1988). Biddington et al. (1988) have reported silver nitrate to promote embryogenesis in anther cultures of Brussel sprouts by blocking the inhibitory effect of ethylene.

Beyer (1976) reported that when Ag(I), applied foliarly as $AgNO_3$, effectively blocked the ability of exogenously applied ethylene to elicit the classical "triple" response in intact etiolated peas (*Pisum sativum* cv. Alaska); stimulate leaf, flower, and fruit abscission in cotton (*Gossypium hirsutum* cv. Stonevilie 213); and induce senescence of orchids (Hybrid white Cattleya, Louise Georgeianna). The most outstanding antiethylene properties of Ag(I) are its persistence, specificity, and its lack of phytotoxicity at effective concentrations.

Hyde and Phillips (1996) reported that silver nitrate promotes shoot development and plant regeneration in chile pepper (*capsicum annuum* L.) via organogenesis. Here, cotyledon used as explants. Medium contained-MS salts, L2 vitamins, 3% glucose, 0.8% agar, 2mg/l BA, 2mg/l GA, 5mg/l $AgNO_3$, 5.7 pH. Treatment with silver nitrate was necessary for multiple shoot production and elongation to occur in the culture and was most effective when compared with media without $AgNO_3$. Shoots transferred to a rooting medium developed roots, increasing the overall rooting efficiency to 70-72%. Most rooted shoots grew well and produced viable seeds when grown in the greenhouse. Medium contained BA was most efficient.

Eapen S and George L, (1997) cultured peduncle segments of B. *juncea*, B. *campestris*, B. *napus*, B. *nigra* and B. *carinata* on Murashige and Skoog medium supplemented with benzyladenine and 1-naphthalene acetic acid produced shoot buds. Supplementation of the media with 30 µm silver nitrate or silver thiosulfate enhanced the frequency of shoot regeneration. The regenerated shoots could be rooted at a frequency of 95% and transferred to soil where 75% survived and set seed.

At silver concentration 50 µmol/L silver nitrate in *Solanum tuberosum in vitro* (Mader JC, 1999) silver ions blocked ethylene action resulted in (1) a total inhibition of tuberization within the 45 days of observation, (2) increase in leaf size, (3) reduction in shoot height and hairiness, (4) suppression of root hair formation and (5) promotion of root elongation and greening.

Turhan H, (2004) reported effect of silver nitrate on potato (*Solanum tuberosum L.*). The results showed that MS basal medium supplemented with $AgNO_3$ resulted in an inhibitory effect on ethylene gas produced by potato plantlets. The response of the cultivars used showed genotypic dependence to

different $AgNO_3$ concentrations. In general the best result for all cultivars were obtained from 5 or 10 μM $AgNO_3$. On the other hand the higher $AgNO_3$ concentrations (25 and 50 μM ) can be also used for cultivar Nicola and Desiree that initially had branching and abnormal plantlet growth.

Buyukalaca S et al. (2004) showed the responses of pepper anthers to different concentrations of silver nitrate (5, 10, 15 and 20 mg $l^{-1}$) and in dependence of the donor plant growing conditions were studied for haploid embryo production via anther culture. U-247 and U-238 genotypes, were used as plant materials. Flower buds were collected from the plants grown either in greenhouse or in open field conditions. Haploid embryos were obtained from all the concentrations tested, but with different production rates. The highest embryo formation (45.7 embryos per 100 anthers) was obtained from the genotype U-247 in the medium containing 15 mg $l^{-1}$ silver nitrate. The anthers taken from the plants grown in the green-house produced more embryos than that of the open field conditions.

Gao F et al. (2005) reported the role of silver nitrate in the regulation of direct root and shoot regeneration from various explants of sweet potato (*Ipomoea batatas* L.). A rapid and high frequency direct shoot regeneration procedure was achieved from stem and lamina explants. The magnitude of the response to silver nitrate varied among different explants and silver nitrate was found to have a significant effect on shoot regeneration. With the addition of 8.0mg $l^{-1}$ silver nitrate on MS medium, 73.3% of lamina explants could directly regenerate shoots. Their study suggests that the addition of silver nitrate can promote shoot regeneration from various explants.

The immature embryos of four common wheat (*Triticum aestivum L.*) genotypes were evaluated for their tissue culture response to ethylene antagonist, silver nitrate, added to callus-inductive and subculture media at six concentrations (L. M. Wu et al. 2006). The addition of $AgNO_3$ significantly improved embryogenic callus frequency and callus growth, but reduced the necrosis and almost did not affect callus induction frequencies. In general, 10 mg/l concentration may be considered most favorable for embryogenesis and prevention of necrosis; at the same time, it did not reduce callus induction and promoted callus growth.

The following section deals with a brief compilation of published research pertaining to the effect of silver nitrate in plant morphogenesis.

- Somatic Embryogenesis - The use of silver nitrate improved somatic embryogenesis in several plant species such as *buffalograss* (Fei et al. 2000), *Coffea* sp. (Fuentes et al. 2000; Giridhar et al. 2004), carrot (Nissen, 1994), white spruce (Kong and Yeung, 1994), *Triticum durum* (Fernandez et al. 1999), and *Zea mays* (Vain Hort and Flament, 1989; Vain Hort et al. 1989; Songstad et al. 1991).

- Multiple shoot induction and shoot regeneration - Silver nitrate is known to promote multiple shoot formation in different plants. In vitro shoot formation was improved by incorporating silver nitrate in the culture medium. Ganesh and Sreenath (1996) reported in vitro sprouting of apical buds of coffee under the influence of $AgNO_3$. The addition of N6-benzyladenine with $AgNO_3$ greatly enhanced the rate of sprouting. At low concentration, $AgNO_3$ was found to cause delayed senescence resulting in improved growth of the proliferated shoots in *Coffea canephora* (Fuentes et al. 2000). $AgNO_3$ enhanced in vitro shoot growth of C. *arabica* and C. *canephora* (Giridhar et al. 2003).

Shoot regeneration of Chinese radish *Cv* Red coat was improved when cultured in media supplemented with 20-30 µM $AgNO_3$ (Pua et al. 1996). *Brassica* sp. are poorly responsive to tissue culture manipulations (Narasimhulu and Chopra, 1988). *B. campestris* produces high levels of ethylene causing abnormal growth and development of the plant in tissue culture conditions (Lentini et al. 1988), and also inhibits *de novo* shoot regeneration *in vitro* (Chi et al. 1990; Chi et al. 1991; Palmer, 1992; Pua and Chi, 1993). The cotyledons and hypocotyls of 7 cultivars belonging to *B. campestris* spp. chinensis, spp. pekinensis and spp. parachinensis exhibited improved shoot regeneration on culture media supplemented with growth regulators and $AgNO_3$.

- In vitro rooting - Addition of 40 µM $AgNO_3$ resulted in root initiation and elongation in Decalepis hamiltonii (Bais et al. 2000a; Reddy et al. 2001). The effect of $AgNO_3$ on rooting and shooting was elucidated in Vanilla planifolia (Giridhar et al. 2001). Maximum number of shoots and highest shoot length was obtained on medium containing 20 µM $AgNO_3$. $AgNO_3$ not only induced shoot multiplication but also influenced rooting of vanilla explants. The plantlets obtained on medium containing 40 µM $AgNO_3$ exhibited 100% survival. Silver nitrate also

induced rooting and flowering in vitro on the rare, rhoeophytic woody medicinal plant, Rotula aquatica Lour. Dipping of the basal end of shoots in NAA (2.69 μM) and silver nitrate (11.7 μM) solution improved rooting efficiency (Sunandakumari et al.. 2004).

- Fruit ripening - Ethylene plays a crucial role in initiating and accelerating the ripening-related process. Treatment of tomato with silver ions has been shown to inhibit ethylene action and fruit ripening (Hobson et al. 1984). Furthermore, if silver ions were applied at stages of ripeness well after the breaker stage, ripening can be arrested (Tucker and Brady, 1987). The growth regulator 1-methylcyclopropane (1- MCP), like silver ions, is an extremely effective antagonist for plants or harvested plant products (Serek et al. 1995A; Serek et al. 1995b; Serek et al. 1995c; Sisler et al. 1996).

- Leaf abscission - Ethylene that stimulated leaf abscission in cotton is blocked by the silver ion (Beyer, 1976c). Without $AgNO_3$, all the leaves had abscised on the 7th day in ethylene. Plants treated with increasing concentrations of $AgNO_3$ and placed in ethylene showed progressively less leaf abscission. Treatment with 25 mg/l of $AgNO_3$ reduced the time required to reach 100% leaf abscission by 2 days. Silver nitrate treatment also reduced the growth retarding effects of ethylene. Other similar experiments with mature cotton plants have demonstrated a similar ability of $AgNO_3$ to prevent young fruit and flower abscission (Beyer, 1976c).

**2.3. Role of Silver Thiosulfate in Plant Tissue Culture**

Effect of silver thiosulphate (Ethylene inhibitor) on shoot regeneration using axillary bud explants of *Solanum nigrum* was studied by T.M. Sridhar et al. (2011). Ethylene inhibitor silver thiosulphate favoured the shoot morphogenesis. Highest frequency of regeneration (95%), maximum number of shoots (4.2) was achieved with 40 μM/L STS, added to MS medium. The optimum range of STS concentrations recorded was between 10-40 μM/L. At higher concentration adventitious root formation was observed and successful field establishment was also achieved. Ethylene inhibits the shoot morphogenesis and also affects the root formation. $Ag^+$ ions inhibit ethylene action in a wide variety of ethylene induced responses in plants by reducing the receptor capacity to bind ethylene. Thus, silver thiosulphate may be useful as a media supplement to develop efficient protocols for *in vitro* propagation of Solanum nigrum as it favours the shoot and root formation. Similarly, more published

reports are there describing the use of $Ag_2S_2O_3$ in plant tissue culture.

# Chapter 3
## Origin of the problem

In India, there are large number (more than 50) of potato varieties developed through conventional breeding. Some important potato varieties grown widely in India are Kufri Chandramukhi, Kufri Jyoti, Kufri Bahar, Kufri Badshah, Kufri Lauvkar, Kufri Sutlej, Kufri Sindhuri, Kufri Jawahar, Kufri Lalima, Kufri Pukhraj, Kufri Pushkar, Kufri Anand, Kufri Frysona, Kufri Surya, Kufri Khyati, Kufri Chipsona-1, Kufri Chipsona-2 and Kufri Chipsona-3. These cultivars vary with regard to maturation, yield, texture, disease resistance, starch and soluble sugar content, extent of low temperature sweetening and other attributes. As discussed earlier, with increasing population and growing demands in the processing sectors, there is a great need to improve the yield, nutritional qualities, disease resistance and processing attributes of potato tubers. In other words the available potato cultivars of our country could be further improved genetically i.e. through transgenics, for desirable traits. Since conventional breeding is more time-consuming and arduous task. Again transgenic approach for making designer crops heavily depend on successful regeneration of the explant after *Agrobacterium*-mediated co-cultivation techniques. With regard to regeneration, the concerned media supplemented with proper $Ag^{+}$ concentration could be useful. Since many researchers world-wide reported the effect of silver ions on the repression of ethylene in tissue culture system of number of plants. Because silver ions are involved in ethylene signaling pathway as revealed by literature survey. It is of interest to see how the silver ions influence the growth characteristics of the micropropagated plants, particularly in potato. Such type of studies were not carried out in the Indian potato cultivars, as evident from literature survey.

The aim of the present study is to see the effects of varying concentrations of silver ions on Indian potato cultivars during micropropagation (particularly to observe their growth characteristics). Since gaining this knowledge is a pre-requisite for the subsequent regeneration. In this study, a total of seven potato cultivars, that included six Indian varieties namely, Kufri Chipsona-I, Kufri Chipsona-II, Kufri Jyoti, Kufri Chandramukhi, Kufri Ashoka, Kufri Pukhraj and one exotic cultivar Desiree were chosen to see the growth characteristics during their micropropagation.

Keeping this in view, the following objectives were framed:

## *Objectives of the study*

**I** To carry out micropropagation of potato plants in MS media supplemented varying concentrations of silver ions

**II** To observe the effects of silver ions on the growth characteristics of potato plantlets

**III** To see the growth patterns of the plantlets after withdrawing silver ions

# Chapter 4
# Materials and Methods

## 4.1. Materials

### 4.1.1. Procurement of potato germplasm and other materials:

The germplasm of six Indian potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), KufriJyoti (KJ), Kufri Chandramukhi (KCM), Kufri Ashoka (As), Kufri Pukhraj (PR) and one exotic cultivar Desiree(De) were procured from Central Potato Research Institute (CPRI), Shimla, India and are routinely maintained in our laboratory on MS basal medium.

| Potato Cultivars | Year of release | Salient features | Areas of adaptation |
|---|---|---|---|
| **Kufri Chipsona-1** | 1998 | Medium maturing; tuber white, medium to large ,oval with fleet eyes; resistant to late blight, suitable for processing | North Indian plains |
| **Kufri Chipsona-2** | 1998 | Medium maturing; tuber white, medium round-oval with fleet eyes; resistant to late blight, tolerant to frost, suitable for processing | North Indian plains |
| **KufriJyoti** | 1968 | Medium maturing; tuber white, large, oval with fleet eyes; resistant to wart; moderately resistant to early and late blight. | North Indian plains and hills, South Indian hills |
| **Kufri Chandra-mukhi** | 1968 | Early maturing; tuber white, large, oval with fleet eyes; susceptible to late blight. | North Indian plains & plateau regions |
| **Kufri Ashoka** | 1996 | Early maturing; tuber white, large oval-long with fleet eyes | North Indian plains |
| **Kufri Pukhraj** | 1998 | Early maturing; large tubers, white, oval, slightly tapered, smooth skin, fleet eyes, yellow flesh | North Indian plains and hills, South Indian hills |
| **Desiree** | 1962 | Late maturing; Tuber medium oval-round red skinned tuber with fleet eyes, low to medium resistance to late blight | Origin at Netherlands |

The required chemicals were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi, and HiMedia Laboratories Mumbai. All salts and additives were purchased from HiMediaLabs Limited, India and growth hormones from sigma chemicals, USA. Glasswares and Plasticwares were purchased from Borosil and Tarsons Products Pvt. Ltd.

### 4.1.2. Composition and stock preparations for Murashige and Skoog (MS) basal medium:

**MS major salts:**

| S. No. | MS Major Salts | MS Basal conc ($mgL^{1}$) | Amount required for 100X stock ($g\ L^{-1}$) | Use of stock for 1 L medium (mL) |
|---|---|---|---|---|
| 1. | $KNO_3$ | 1900.0 | 190.0 | 10.0 |
| 2. | $NH_4NO_3$ | 1650.0 | 165.0 | 10.0 |
| 3. | $MgSO_4.7H_20$ | 370.0 | 37.0 | 10.0 |
| 4. | $CaCl_2.2H_2O$ | 440.0 | 44.0 | 10.0 |
| 5. | $KH_2PO_4$ | 170.0 | 17.0 | 10.0 |

**Note**: All the MS major salts stock solutions to be prepared separately.

**MS minor salts:**

| S. No. | MS Minor Salts | MS Basal Conc. (mg $L^{-1}$) | Amount required for 1000X stock (g $L^{-1}$) | Use of stock for 1 L medium (mL) |
|---|---|---|---|---|
| 1. | $H_3BO_4$ | 6.20 | 6.20 | 1.0 |
| 2. | $MnSO_4.4H_2O$ | 22.30 | 22.30 | 1.0 |
| 3. | $ZnSO_4.7H_2O$ | 8.60 | 8.60 | 1.0 |
| 4. | $Na_2MoO_4.2H_2O$ | 0.25 | 0.25 | 1.0 |
| 5. | $CuSO_4.5H_2O$ | 0.025 | 0.025 | 1.0 |
| 6. | $CoCl_2.6H_2O$ | 0.025 | 0.025 | 1.0 |
| 7. | KI | 0.83 | 0.83 | 1.0 |
| 8. | $Fe_2EDTA.2H_2O$ (sodium salt) | 30.0 | 30.0 | 1.0 |

**MS Vitamins:**

| S. No. | Name of Vitamins | MS Basal Conc. (mg $L^{-1}$) | Amount required for 1000X stock (mg $mL^{-1}$) | Use of stock for 1 L medium (mL) |
|---|---|---|---|---|
| 1. | Nicotinic Acid | 0.5 | 0.5 | 1.0 |
| 2. | Pyridoxine HCl | 0.5 | 0.5 | 1.0 |
| 3. | Thiamine HCl | 0.1 | 0.1 | 1.0 |
| 4. | Glycine | 2.0 | 2.0 | 1.0 |
| 5. | Myo-inositol | 100.0 | 100.0 | 1.0 |

**Note**: Preparation of MS basal medium included major salts, minor salts, vitamins, $Fe_2EDTA.2H_2O$, 3.0% sucrose, 0.7-0.8%. agaragar. The pH of medium was set to 5.8 using 0.01N HCl or 0.01N NaOH.

**4.1.3. Silver nitrate -** Silver nitrate was dissolved in water to make a stock of 6.0 $mgL^{-1}$ .

## 4.2. Methodology

### 4.2.1. Maintenance of potato germplasm (routine micropropagation)

The high-yielding Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, KufriJyoti, Kufri Chandramukhi, Kufri Pukhraj, Kufri Ashoka as used in this study were procured from Central Potato Research Institute (CPRI), Shimla, India. These cultivars vary with regard to their genetic makeup, maturation time and growth in different agro-climatic zones of the Indian subcontinent. All the cultivars early are medium maturing. All these cultivars along with Desiree (a late maturing exotic cultivar) were routinely micropropagated in our laboratory under controlled conditions (16 h light/8 h dark, 25-27ºC, 70% relative humidity) for four to five weeks on MS basal medium.

### 4.2.2. Micropropagation of potato plants in varying silver nitrate concentrations

The basal medium MS supplemented with four different concentrations of $AgNO_3$ i.e. 0.5 mg, 1.0 mg, 2.0 mg and 3.0 mg $L^{-1}$. For each potato cultivar, nodal stem segments were transferred to the above media along with control (without silver nitrate). Each culture bottle was inoculated with 7 to 8 nodal stem segments. After four weeks onwards growth characteristics such as shoot length, root length, number of nodes, number of leaves, leaf size, overall rooting patterns were noted.

### 4.2.3. Subculturing of silver nitrate-treated potato plantlets on MS medium

To observe the behavior of silver nitrate changed the normal physiological characters of the cultivars, the nodal segments from each cultivar from each concentration shifted on MS-basal medium containing bottles. It compared with control cultivar's cultures to check the level of silver nitrate effects.

# Chapter 5
## Results and Discussion

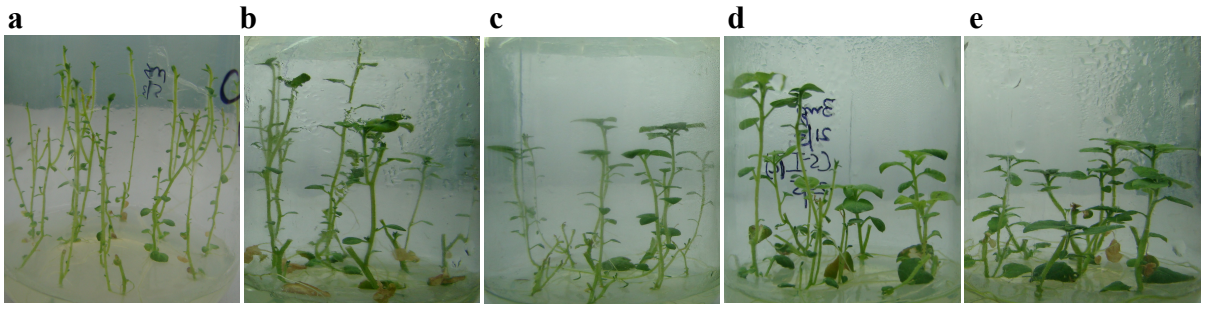
The aim of the present study is to know the effects of varying concentrations of silver nitrate on growth characteristics of micropropagated plantlets of different potato cultivars. The relevant data were noted after four weeks of culturing. The various growth parameters such as shoot length, number of nodes, number of leaves, root length, number of roots, their morphology on different concentrations of silver nitrate (0.5, 1.0, 2.0 and 3.0 mg $L^{-1}$) were compared with that of untreated plantlets (grown without silver nitrate). At the same time cultivar-wise variation was also noticed in response to silver ions. Moreover, silver nitrate-treated potato plantlets were further shifted to normal MS media to see their growth patterns. All these results are presented and discussed in the following sections.

### 5.1. Effects of varying concentrations of silver nitrate on potato cultivars

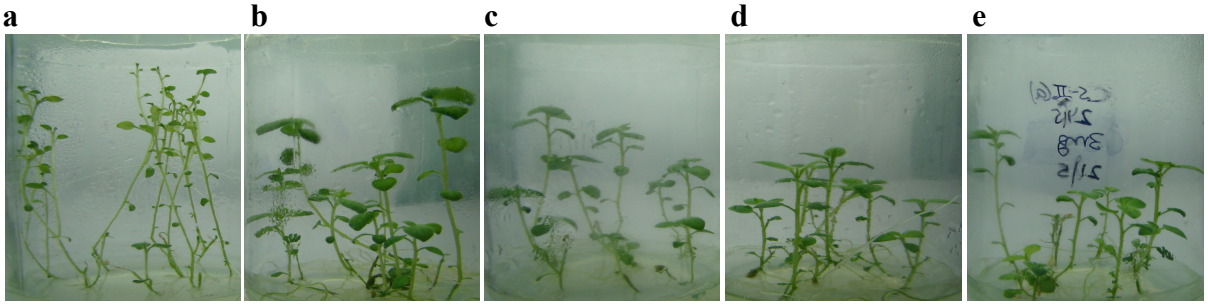
All the potato cultivars namely Kufri Chipsona-1, Kufri Chipsona -2, Kufri Jyoti, Kufri Chandramukhi, Kufri Ashoka, Kufri Pukhraj and one exotic cultivar Desiree, are different from each other in many desirable attributes such as yield, adaptability, maturation period, quality and tolerance for various stresses. It may be noted that under normal condition, they show some differences in their growth patterns if micropropagated on the simple MS-basal medium. In this study, around 4-week old nodal stem segments from each cultivar were transferred to normal MS-basal medium supplemented with varying concentrations of silver nitrate. Interestingly, the varying silver nitrate concentrations were found to influence the overall growth patterns of the potato plantlets as shown in Fig. 7 A-G. In most of the cases, silver ions influenced mainly the following attributes: (a) increase in leaf size, (b) decrease of shoot length (c) change in rooting pattern (d) change from light green to dark green potato plantlets probably due to intense pigmentation. All the data are provided in the following sections.

| Cultivar | Control | $AgNO_3$ Concentrations (mg $L^{-1}$) 0.5 | 1.0 | 2.0 | 3.0 |
|---|---|---|---|---|---|

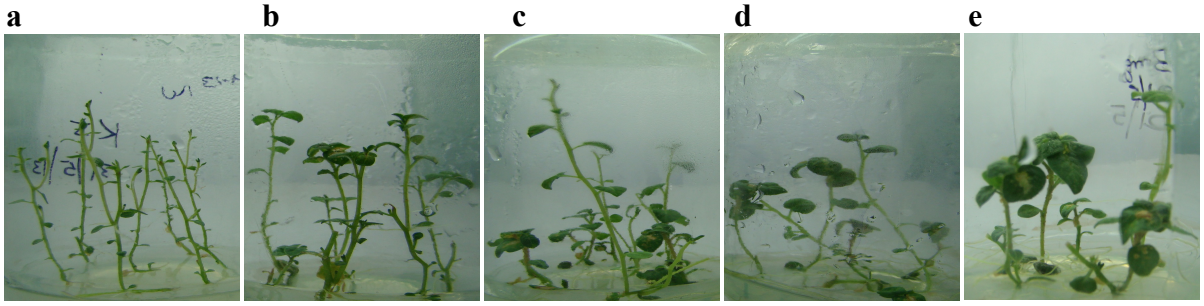
**A. Kufri Chipsona-1**



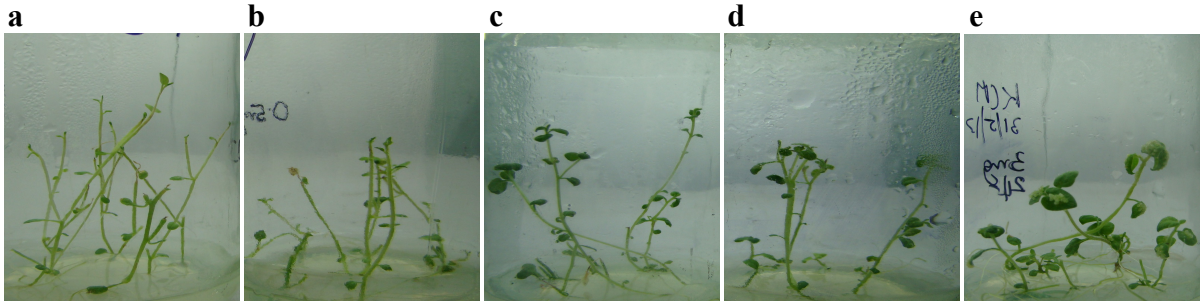

**B. Kufri Chipsona-2**




**C. Kufri Jyoti**

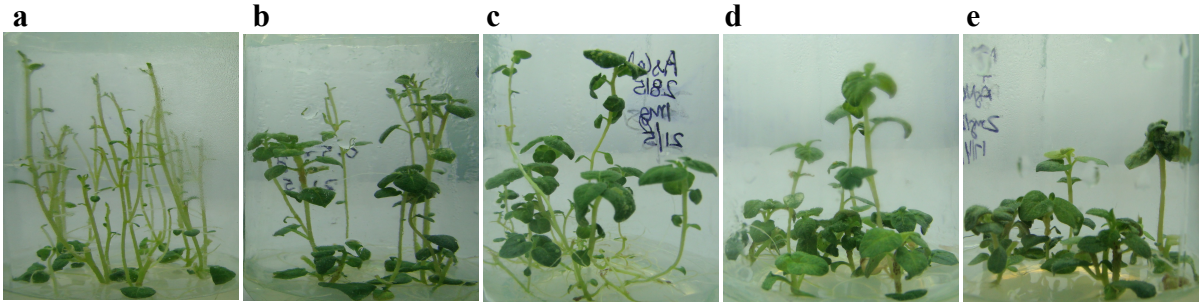



**D. Kufri Chandramukhi**

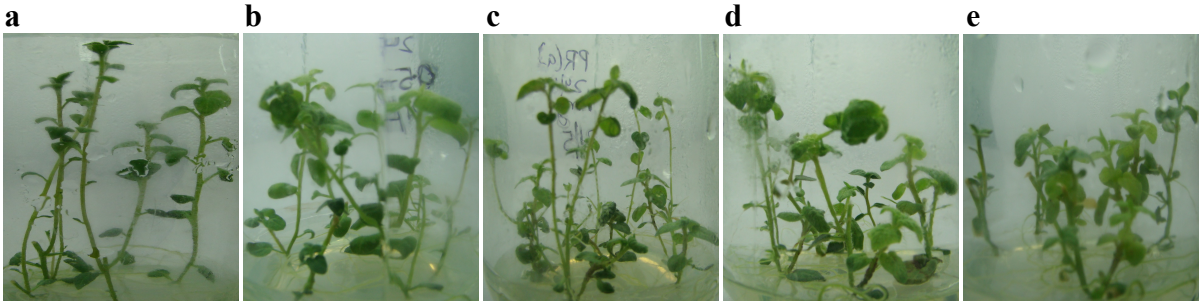


| Cultivar | Control | 0.5 | 1.0 | 2.0 | 3.0 |
|---|---|---|---|---|---|
| | | $AgNO_3$ Concentrations (mg $L^{-1}$) | | | |

**E. Kufri Ashoka**




**F. Kufri Pukhraj**




**G. Desiree**

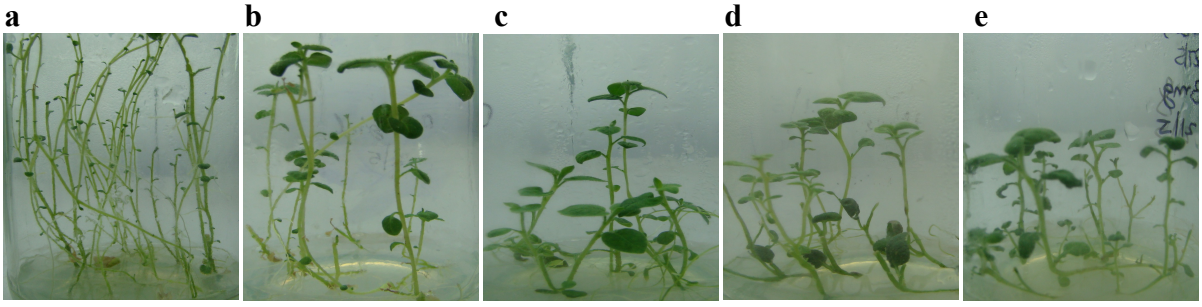



**Fig 7**. Effects of silver nitrate during micropropagation of different potato cultivars. Topmost panel refers to varying concentrations of silver nitrate. **A** Kufri Chipsona-1, **B** Kufri Chipsona-2, **C** Kufri Jyoti, **D** Kufri Chandramukhi, **E** Kufri Ashoka, **F** Kufri Pukhraj, **G** Desiree. Each row refers to particular potato cultivar : **a** control (no $AgNO_3$), **b** 0.5 mg $L^{-1}$, **c** 1.0 mg $L^{-1}$, **d** 2.0 mg $L^{-1}$, **e** 3.0 mg $L^{-1}$ $AgNO_3$ in MS media

## 5.2. Influence of silver nitrate on the growth characteristics of potato plantlets

### a) Shoots

Tissue cultured plantlets of potato cultivars are usually thin and long on MS-basal medium. The addition of silver nitrate to the MS-basal medium decreased shoot length when compared with the control ones (without silver nitrate) nearly in all the cultivars. Moreover, the shoot length decreased gradually with increase in silver ion concentration of the medium (0.5, 1.0, 2.0 and 3.0 mg $L^{-1}$) in case of all the cultivars as shown in Table. 1. Kufri Ashoka showed the shortest shoots among all the cultivars. The least effect was observed in case of Kufri Pukhraj. The cultivars like Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti and Desiree showed moderate effects.

Another important observation was the thickening of the shoots along with decrease in shoot length. The colour of shoots also became more green with increasing silver nitrate concentrations in medium as compare to control. Table 1 showing the data obtained from shoot length of all cultivars.

**Table. 1** Effect of silver nitrate on shoot length (cm)

| | **$AgNO_3$ Concentrations (mg $L^{-1}$)** | | | | |
|---|---|---|---|---|---|
| **Potato Cultivars** | **Control** | **0.5** | **1.0** | **2.0** | **3.0** |
| **Kufri Chipsona-1** | 7.7 ± 0.15 | 7.5 ± 0.20 | 6.5 ± 0.25 | 4.8 ± 0.26 | 3.7 ± 0.20 |
| **Kufri Chipsona-2** | 8.7 ± 0.15 | 7.6 ± 0.31 | 6.6 ± 0.20 | 4.6 ± 0.15 | 3.6 ± 0.21 |
| **Kufri Jyoti** | 7.3 ± 0.20 | 6.8 ± 0.30 | 5.7 ± 0.25 | 4.8 ± 0.10 | 3.5 ± 0.11 |
| **Kufri Chandramukhi** | 7.0 ± 0.36 | 6.4 ± 0.15 | 5.5 ± 0.15 | 4.4 ± 0.20 | 3.3 ± 0.10 |
| **Kufri Ashoka** | 8.2 ± 0.20 | 7.4 ± 0.26 | 5.6 ± 0.15 | 4.2 ± 0.32 | 3.1 ± 0.26 |
| **Kufri Pukhraj** | 8.0 ± 0.25 | 6.9 ± 0.10 | 5.8 ± 0.30 | 4.8 ± 0.10 | 3.7 ± 0.20 |
| **Desiree** | 8.3 ± 0.10 | 7.1 ± 0.11 | 5.6 ± 0.20 | 4.6 ± 0.15 | 3.5 ± 0.11 |

**b) Leaves**

Leaf area of all the potato cultivars was increased near about two to three folds with increase in concentration of silver nitrate in medium as compared to the control ones as shown in Fig. 7. The most prominent effect of silver ions was observed on leaf area of cultivar Kufri Ashoka whereas the leaf area of Kufri Pukhraj remain unaffected.

The number of leaves also significantly decreased in all the cultivars. Conversely, the control plantlets consists of more number of leaves than the silver nitrate treated microplants as shown in Table 2. Kufri Ashoka showed least number of leaves whereas the number of leaves were not much reduced in case of Kufri Pukhraj.

**Table. 2** Effect of silver nitrate on number of leaves

| | **$AgNO_3$ Concentrations (mg $L^{-1}$)** | | | | |
|---|---|---|---|---|---|
| **Potato Cultivars** | **Control** | **0.5** | **1.0** | **2.0** | **3.0** |
| **Kufri Chipsona-1** | 9.7 ± 0.5 | 10 ± 0.81 | 8.7 ± 0.50 | 7.5 ± 0.57 | 6.7 ± 0.50 |
| **Kufri Chipsona-2** | 13 ± 0.81 | 9.7 ± 0.50 | 8.2 ± 0.50 | 5.7 ± 0.50 | 5.5 ± 0.57 |
| **Kufri Jyoti** | 9.7 ± 0.95 | 9.2 ± 0.50 | 6.7 ± 0.50 | 5.5 ± 0.57 | 5.2 ± 0.50 |
| **Kufri Chandramukhi** | 8.2 ± 0.50 | 6.5 ± 0.57 | 6.0 ± 0.81 | 5.7 ± 0.50 | 4.7 ± 0.50 |
| **Kufri Ashoka** | 11.7 ± 1.25 | 10.2 ± 0.50 | 8.2 ± 0.50 | 5.5 ± 0.57 | 4.5 ± 0.57 |
| **Kufri Pukhraj** | 11.2 ± 0.95 | 10.5 ± 0.86 | 9.5 ± 0.57 | 8.7 ± 0.5 | 7 ± 0.81 |
| **Desiree** | 15 ± 0.81 | 11.2 ± 0.95 | 8.5 ± 0.57 | 7.5 ± 0.57 | 6.5 ± 0.57 |

The difference in leaf colour was also observed in all the cultivars. The microplants grown on varying silver nitrate concentrations showed dark green leaves as compared to the microplants grown on MS basal medium. The darkening of leaves was increased with increase in silver nitrate concentrations in the medium. Cultivars treated with increasing concentrations of silver nitrate showed progressively less leaf abscission and senescence even after long time period.

**c) Roots**

In case of roots two main parameters were observed – root length and number of roots.

*Root length:* Silver ions showed promoting effect on root elongation as shown in Table. 3. Longest roots were observed in Kufri Chipsona-1 and Kufri Pukhraj at higher concentration of silver nitrate.

**Table. 3** Effect of silver nitrate on root length (cm)

| | **$AgNO_3$ Concentrations (mg $L^{-1}$)** | | | | |
|---|---|---|---|---|---|
| **Potato Cultivars** | **Control** | **0.5** | **1.0** | **2.0** | **3.0** |
| **Kufri Chipsona-1** | 4.8 ± 0.25 | 7.0 ± 0.25 | 8.5 ± 0.30 | 9.7 ± 0.20 | 10.6 ± 0.30 |
| **Kufri Chipsona-2** | 5.2 ± 0.15 | 6.7 ± 0.20 | 7.7 ± 0.20 | 8.7 ± 0.15 | 9.8 ± 0.15 |
| **Kufri Jyoti** | 4.1 ± 0.26 | 6.5 ± 0.20 | 7.6 ± 0.20 | 8.6 ± 0.20 | 9.8 ± 0.25 |
| **Kufri Chandramukhi** | 5.0 ± 0.25 | 6.5 ± 0.05 | 7.5 ± 0.10 | 8.7 ± 0.20 | 9.4 ± 0.15 |
| **Kufri Ashoka** | 5.5 ± 0.26 | 6.8 ± 0.20 | 7.8 ± 0.15 | 8.9 ± 0.15 | 10.1 ± 0.20 |
| **Kufri Pukhraj** | 5.6 ± 0.32 | 7.1 ± 0.15 | 8.1 ± 0.20 | 9.3 ± 0.10 | 10.5 ± 0.15 |
| **Desiree** | 4.3 ± 0.15 | 5.5 ± 0.30 | 6.3 ± 0.20 | 7.6 ± 0.15 | 8.8 ± 0.15 |

*Number of roots:* Although silver nitrate enhanced the root length of all the cultivars but the number of roots decreased. The number of roots dropped gradually from the medium having concentrations 0.5 to 3 mg $L^{-1}$ respectively. It is shown in the Table 4.

**Table. 4** Effect of silver nitrate on number of roots

| | **$AgNO_3$ Concentrations (mg $L^{-1}$)** | | | | |
|---|---|---|---|---|---|
| **Potato Cultivars** | **Control** | **0.5** | **1.0** | **2.0** | **3.0** |
| **Kufri Chipsona-1** | 8.6 ± 0.57 | 8.3 ± 0.57 | 7.6 ± 1.15 | 6.3 ± 0.57 | 5.0 ± 0.81 |
| **Kufri Chipsona-2** | 10.3 ± 0.57 | 8.3 ± 1.15 | 7.3 ± 0.57 | 6.0 ± 1.00 | 4.5 ± 0.57 |
| **Kufri Jyoti** | 8.0 ± 1.00 | 7.3 ± 0.57 | 6.6 ± 1.15 | 5.6 ± 1.15 | 4.2 ± 0.50 |
| **Kufri Chandramukhi** | 7.6 ± 0.57 | 7.3 ± 0.57 | 6.6 ± 0.57 | 5.3 ± 0.57 | 3.7 ± 0.50 |
| **Kufri Ashoka** | 10.3 ± 0.57 | 8.0 ± 1.00 | 7.0 ± 1.00 | 6.3 ± 0.57 | 5.2 ± 0.50 |
| **Kufri Pukhraj** | 11.3 ± 0.57 | 9.3 ± 0.57 | 8.3 ± 0.57 | 7.0 ± 1.00 | 5.5 ± 0.57 |
| **Desiree** | 8.6 ± 1.52 | 7.3 ± 0.57 | 6.6 ± 1.15 | 5.0 ± 1.00 | 3.7 ± 0.50 |

Apart from silver nitrate effects on root length and root number, there was also another effect on root colour was observed. The roots develop green pigment in the presence of silver ions in the medium. Like shoots, thickening of the roots was also observed.

**d) Nodes**

With increasing silver nitrate concentration in medium (0.5 to 3 mg $L^{-1}$) number of nodes of all cultivars decreased, it may be due to shortened shoot length. Kufri Ashoka showed least number of nodes among all cultivars. The effect of silver nitrate on number of nodes per microplant is shown in Table 5.

There was also a significant enhancement of nodal buds was observed. The results suggest beneficial effects of silver nitrate on *in vitro* development of axillary buds.

**Table. 5** Effect of silver nitrate on number of nodes

| | **$AgNO_3$ Concentrations (mg $L^{-1}$)** | | | | |
|---|---|---|---|---|---|
| **Potato Cultivars** | **Control** | **0.5** | **1.0** | **2.0** | **3.0** |
| **Kufri Chipsona-1** | 7.0 ± 0.80 | 7.5 ± 0.57 | 6.0 ± 0.81 | 4.7 ± 0.50 | 4.2 ± 0.50 |
| **Kufri Chipsona-2** | 11.0 ± 0.80 | 8.0 ± 0.81 | 6.5 ± 0.57 | 4.5 ± 0.57 | 4.0 ± 0.81 |
| **Kufri Jyoti** | 7.5 ± 0.57 | 6.5 ± 0.57 | 5.5 ± 0.57 | 3.7 ± 0.50 | 3.5 ± 0.57 |
| **Kufri Chandramukhi** | 6.0 ± 0.81 | 5.0 ± 1.15 | 4.2 ± 0.50 | 3.5 ± 0.57 | 3.2 ± 0.50 |
| **Kufri Ashoka** | 10.5 ± 0.57 | 8.7 ± 0.50 | 5.7 ± 0.95 | 3.7 ± 0.50 | 2.2 ± 0.50 |
| **Kufri Pukhraj** | 9.0 ± 0.81 | 8.7 ± 0.50 | 6.5 ± 0.57 | 5.7 ± 0.50 | 4.7 ± 0.50 |
| **Desiree** | 13.0 ± 0.81 | 9.0 ± 0.81 | 6.2 ± 0.95 | 5.5 ± 0.57 | 4.5 ± 0.57 |

Silver nitrate was found to influence the overall morphological features of the potato plantlets such as decreased shoot length, number of leaves, nodes and roots but it promoted leaf area and root elongation. The visibility of these growth parameters were quite prominent, and increased proportionately with the increase of silver nitrate concentrations ( i.e. from 0.5 $mgL^{-1}$ to 3.0 $mgL^{-1}$ ). The data as noted in this study were quite consistent with the findings for the other cultivars such as Maris Bard, Desiree, Nicola and Russet Burbank (Turhan H, 2004).

$AgNO_3$ has been known to inhibit ethylene action (Beyer, 1976a) . In case of **leaves**, cell expansion is strongly inhibited by exogenous ethylene (Kieber et al. 1993) which is essential for leaf growth (McManus MT, 2012). Dominant mutations or blockage in the ethylene receptor ETR1 results in larger leaves due to an increase in cell size (Horiguchi et al. 2006). Silver ion is capable of specifically blocking the action of exogenously applied ethylene in classical responses such as abscission, senescence and growth retardation (Beyer, 1976c) . So, in silver nitrate case the addition of silver

nitrate inhibits the binding of ethylene to the ethylene receptor which further inhibits ethylene signaling. As a consequence cell expansion occurs to a large level and leaf size increases. It may be a reason for huge size of leaves on silver nitrate medium.

In case of **shoots**, we know that Gibberellic acid helps in elongation. GA action then loosens the cell walls, thereby facilitating enhanced cell elongation (Weller et al. 1994; Lopez-Juez et al. 1995). It is well established that ethylene can interact with GA to modify elongation growth (Kende et al. 1998). When ethylene action blocks by silver nitrate, the ethylene levels get decreased, this might be a reason for short shoot length.

In **roots** also, we know that ethylene causes exaggeration of the apical hook, shortening and thickening of the hypocotyls, and inhibition of root growth, a phenomenon known as the triple response (Abeles et al., 1992). So when silver nitrate blocks ethylene signaling, this may be a reason for increasing root length in silver nitrate containing medium with respect to control (without silver nitrate).

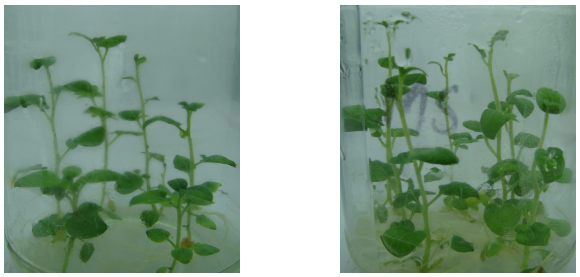
## 5.2. Effects of silver nitrate treated microplants on MS medium

To know the extent up to which silver nitrate affected the cultivars, the nodal shoot segments of each cultivar from each concentration (0.5, 1.0, 2.0 and 3.0 mg $L^{-1}$) transfered on the basal MS medium. After four weeks, behavior of silver nitrate treated microplants grown on this medium of all cultivars were observed and results were collected. In the starting growth phase of all the cultivars, silver nitrate treated microplants showed nearly same growth pattern like on silver nitrate medium. All the cultivars showed slow growth in the initial phase. But slowly as the development of these microplants continued, they started to show characteristics like the original cultivars. The continued vegetative growth of microplants caused the silver nitrate effects on these plantlets to be vanished. The microplants regained their normal leaf size, number of leaves and nodes and shoot length. These observations stated that silver nitrate effects on growth characteristics of all cultivars was not permanent, it gets disappeared after a certain level growth of these silver nitrate treated microplants. It is showed in following figures.

**5.2.1. Changes in growth characteristics of cultivars during first observation-** After four weeks of transferring the silver nitrate treated microplants on MS-basal medium, some cultivars started to show the change in growth behavior of microplants (Fig. 8). They showed original growth behaviors of cultivars on their tip parts. Others cultivars not started to show these behaviors till that time.

**A. Kufri Chipsona-1**

**a** **b**



**B. Kufri Chipsona-2**

**c** **d** **e**

**C. Kufri Pukhraj**

**f** **g** **h** **i**

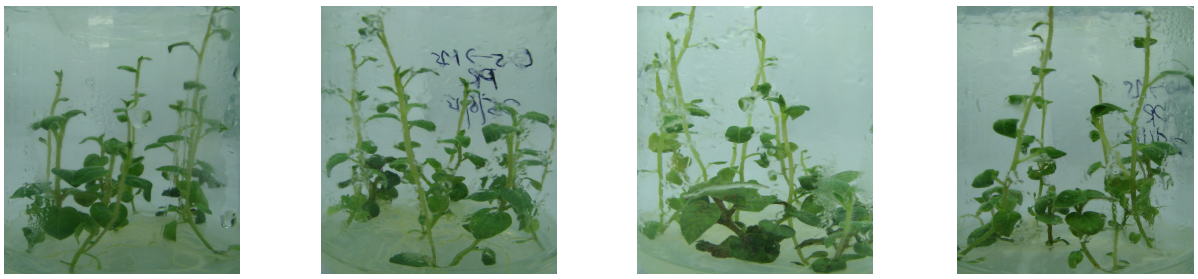


**Fig 8.** Growth characteristics of silver nitrate treated microplants on MS-basal medium. Here figures shows the resuming of original growth characteristics of few cultivars in first observation after four weeks. The changes in the growth characteristics of silver nitrate-treated microplants of different potato cultivars are shown here. **A** Kufri Chipsona-1, **a** 1 mg $L^{-1}$ and **b** 2 mg $L^{-1}$ , **B** Kufri Chipsona-2, **c** 0.5 mg $L^{-1}$ **d** 1 mg $L^{-1}$ and **e** 2 mg $L^{-1}$, **C** Kufri Pukhraj, **f** 0.5 mg $L^{-1}$, **g** 1 mg $L^{-1}$, **h** 2 mg $L^{-1}$ and **i** 3 mg $L^{-1}$

### 5.2.2. Growth characteristics of silver nitrate treated microplants on MS medium after prolong time

After a long time of transferring of silver nitrate treated cultivars on MS-basal medium, few cultivars showed total disappearance of silver nitrate effect in their growth behaviors. There top-most shoot parts showed growth characteristics like original one. It means they resumed there growth behaviors (shown in Fig. 9) Here in the following figures the growth of Kufri Ashoka, Kufri Jyoti and Kufri Pukhraj is showed. Other cultivars behaviors are under-progress.

**A. Kufri Ashoka**

**a**

**b**

**B. Kufri Jyoti**

**c**

**d**

**e**

**C. Kufri Pukhraj**

**f** **g**

**Fig 9.** Silver nitrate (3 mg $L^{-1}$) treated microplants resuming their characteristics on MS basal approximately after 2 months. **A** Kufri Ashoka, **a** and **b**; **B** Kufri Jyoti **c-e**; **C** Kufri Pukhraj **f** and **g**

## Concluding Remarks

*In vitro* studies indicated that ethylene can affect the morphology of micropropagated plants, callus growth, shoot regeneration and somatic embryogenesis. Silver nitrate has been known to inhibit ethylene action. These observations will be very helpful particularly during regular plant tissue culture and regeneration from the explants. With regard to the Indian potato cultivars there was no considerable progress with respect to the effect of silver nitrate on micropropagation and regeneration aspects of the potato plantlets. In this context, this work could be regarded as a good initiative. The aim of the present study was mainly to see the effects of varying concentrations of silver nitrate (0.5, 1.0, 2.0 and 3.0 mg $L^{-1}$) during micropropagations of on Indian potato cultivars in MS basal medium. Interestingly all the cultivars showed significant changes in the growth characteristics even in the low silver ion concentration (i.e. 0.5 $mgL^{-1}$). Noticeable changes include decrease in shoot length, number of leaves, nodes and roots but leaf area and root length were found to be increased for all the cultivars. However, cultivar-wise variations could also be observed. Another important aspect of the study is that if silver nitrate treated plantlets are allowed to grow in MS medium, slowly and gradually resume their original characteristics. Efforts are to be made to see the effects of silver nitrate during regeneration of potato plantlets from the explants. Since any sort of promotive effects of silver ions could help in undertaking potato transgenics.

# Chapter 6

## Summary

Ethylene produced by plant tissues grown *in vitro*, can influence growth and development in such systems. Silver nitrate has been known to inhibit ethylene action as it blocks the signal transduction of ethylene. This application of silver nitrate can used for improving the growth characteristics of micropropagated plantlets and regeneration of different explants. The present study focused to see the effects of varying concentrations of silver nitrate (0.5, 1.0, 2.0 and 3.0 mg $L^{-1}$) on micropropagated microplants of six Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti, Kufri Chandramukhi, Kufri Ashoka, Kufri Pukhraj and one exotic cultivar Desiree. Approximately, 7 to 8 nodal segments from each cultivar transfered on all the concentrations of silver nitrate. After four weeks, number of growth parameters were found to be affected such as decrease in shoot length, number of leaves, node and roots whereas the promoting effect was observed on leaf area and root length in all the cultivars. These morphological parameters were proportionately effected with increase in silver nitrate concentrations. Among the all cultivars, the effect of silver nitrate was observed predominantly on Kufri Ashoka and Kufri Chandramukhi. As compared to other cultivars, Kufri Pukhraj was the least effected cultivar, which showed very little effect on leaf size and on shoot length with respect to control.

An important aspect of this study was to see the behavior of silver nitrate treated microplants on basal MS medium. It was observed that up to certain time period these microplants retain their silver nitrate effect, but after two to three month they started resuming their original growth characteristics from the apical portion of the microplants grown on MS medium. All these findings will be helpful to improve the tissue culture aspects of agriculturly and industrially important Indian potato cultivars.

# Chapter 7

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