Effects of Silver Nitrate and Silver Thiosulfate on Growth Characteristics of Micropropagated Potato (*Solanum tuberosum* L.) Plantlets and Regeneration Efficiency of the Explants

A Dissertation

submitted in partial fulfillment of the
requirement for the award of the degree of

Masters of Science

in

Biotechnology

Submitted

By

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July 2014

DECLARATION

I hereby declare that the work which is being presented in this thesis **"Effects of silver nitrate and silver thiosulfate on growth characteristics of micropropagated potato (*Solanum tuberosum* L.) plantlets and regeneration efficiency of the explants"** 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,** Professor, Department of Biotechnology, 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: July 18, 2014

Place: Patiala

Gupta

Geetika Gupta

CERTIFICATE

This is to certify that the thesis entitled "**Effects of silver nitrate and silver thiosulfate on growth characteristics of micropropagated potato (*Solanum tuberosum* L.) plantlets and regeneration efficiency of the explants**" submitted by Ms. Geetika Gupta (Roll no: 301201003) 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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Geetika Gupta

DEDICATED TO MY PARENTS

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
IAA	Indole-3- acetic acid
BA	6-benzylaminopurine
GA	Gibberellic acid
CW	Coconut Water
BAP	Benzylaminopurine
NAA	Naphthalene-acetic acid
STS	Silver thiosulfate
$AgNO_3$	Silver nitrate
YEM	Yeast Extract Mannitol

ABSTRACT

Potato (*Solanum tuberosum* L.) is an important food crop and ranks only after wheat and rice in terms of productivity. Current areas of research focused to improve the potato cultivars for various desirable traits such as disease resistance, improved yield and processing attributes etc. Due to heterozygosity, it is difficult to improve this crop through conventional breeding methods. Molecular breeding is one of the methods for potato crop improvement, through which the desirable traits can be introduced to potato cultivars. The success of plant genetic engineering depends on various factors such as an efficient tissue culture system for regeneration of plants using different explants. The growth and development of cultured-cells or tissues highly depend upon *in vitro* conditions that include the type of media, concentrations of various phytohormones and growth conditions. In closed-culture vessels, ethylene is produced by plant tissues that may accumulate in large quantities which is likely to influence growth and development. For maximizing the desired *de novo* morphogenic pathway, it is important to modify the accumulation of ethylene in the head-space of culture vessels and altering its biological action. There are various methods which can regulate ethylene biosynthesis and action. The addition of silver nitrate and silver thiosulphate in tissue culture medium are known to inhibit ethylene action by inhibiting its receptors. Ethylene is detected by a family of five membrane-bound receptors in most of plants. Ethylene signal transduction pathway is blocked by the silver ions by replacing the copper ions at ethylene receptors. In this study, we investigated the effect of silver ions on morpholological features of commercially important potato cultivars, Kufri Chipsona-1and Kufri Jyoti along with exotic cv. Desiree. For this purpose, we used different concentrations of silver nitrate (0.5, 1.0, 2.0, 3.0 mg L^{-1}) and silver thiosulfate (1.5, 3, 6 and 9 µM L^{-1}). Addition of silver nitrate resulted in stunted shoot length, increased leaf area and root length, thickening of shoots, shoots and roots became dark green. The effect on these growth features increased with increase in the concentration of silver ions. In case of silver thiosulfate, these growth features were not much affected at lower concentrations. This study helps us to compare the overall effects of silver nitrate and silver thiosulfate on growth characteristics of the potato plantlets. Moreover, *Agrobacterium*-mediated transformation was only initiated by introducing silver ions in the tissue culture media to see the effects during regeneration.

Chapter-1

INTRODUCTION

Potato is a member of the *Solanaceae* family consisted of more than 3,000 species. Some of the other economically important members of this family are tomato, eggplant, petunia, tobacco and pepper. Over 230 wild potato species are known (including *Solanum tuberosum* L., *S. ajanhuiri, S. curtilobum, S. caucha, S. goniocalyx, S. phureja, S. juzepczukii, and S. stenotomum*) (Harris 1992; Struik and Wiersema 1999). In developing countries it is a part of the diet of billion consumers by providing roughly half of the world's annual production (Ghislain et al, 1999). Potato tubers contain significant concentrations of starch, protein and vitamins. The essential amino acids found in potato protein are tyrosine, lysine and sulfur containing amino acid cysteine and methionine. Potatoes are used as animal feed and as raw material for manufacture of starch, alcohol and other food products such as chips and french fries. It is also important crop in terms of energy (216 MJ $ha^{-1}day^{-1}$), dry matter production (2.2 t ha^{-1}) and nutrition (Beukema and Vander Zaag, 1990). Potatoes were first popularized outside the Andes region, four centuries ago with more than 4,000 varieties of native potatoes and then it was shipped to Europe as an essential crop. From Europe it was introduced to North America and rapidly expands over the world. Since then has become an elemental part of much of the world's cuisine. China is the largest potato producing country in the world. Potato ranks third among the world, after wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.). The worldwide production of potato is around 374 million tons in 2011. In terms of production potato is most important tuber crop which accounts for about 45% of the total world production of all tuber crops i.e sweet potato, cassava and yams accounting for about 90 % of total world production. Today, in India Potato is grown over 1.3 million hectares with 23.6 million tones annual production (Directorate of Economics and Statistics, Ministry of Agriculture, Govt. of India, 2004-05). Now, India ranks 3rd in production and 4th in area of potato in the world with the current update of modern potato technologies. The productivity of potato in India is better than the world average (166.3 quintals/hectare). The key objectives of global potato breeding programs include optimization of production levels and resistance to biotic and abiotic stresses. About 190 wild tuber-bearing species being recognized in the section *Petota* of the genus *Solanum*, the potato has one of the richest genetic resources of any cultivated plant (Spooner and Hijmans 2001). In the coming decades root and tuber crops will play an important role in feeding the developing world.

1.1. Morphology of potato plant

Potato is a cool season crop grown in temperate climates. It is a short day C3 plant with a low light saturation point (Demagante and Zaag, 1988). Potato tuber is a modified stem. It is a herbaceous, dicotyledonous plant with alternate leaves on the stem above ground and alternate stolons underground. Usually the root length of potato is around 20-25 cm but in nutrient rich soils roots of some varieties of potato may reach upto 90-100 cm.

Fig. 1. Potato plant with tuber

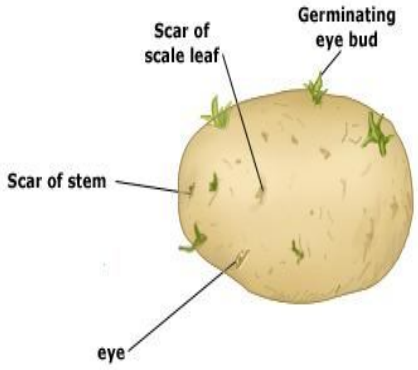


Fig. 2. Potato tuber

1.2. Taxonomy of potato

Including tuber bearing species, the genus *Solanum* consists of approximately 2000 members. Potato consists of 12 haploid no. of chromosomes. These potato species differ in their ploidy status, ranging from diploid ($2n = 2x = 24$) to hexaploid ($2n = 6x = 72$) in addition to triploids, tetraploids, and pentaploids (Spooner et al. 2005). The *tuberosum* (a 48 chromosomes tetraploid) is the most common cultivated species of potato which is a hybrid within the diploid weed *S. sparsipilum* and the diploid species S. *stentotomum* with successive chromosome doubling (Ramanna and Hermsen, 1979).

1.3. Different growth stages of potato plant under field condition

Growth of a potato plant takes place in several stages including sprout development, plant establishment, tuber initiation, tuber bulking, and maturation. Depending upon environmental factors, timing of these growth stages varies such as temperature, availability of moisture, geographic location, soil type and cultivar selected.

Growth stage I (sprout development) – After dormancy if environmental conditions are favorable sprouts are developed from the eyes on seed tubers and grow upward to emerge from the soil. Roots started to develop at the base of emerging sprouts.

Growth stage II (plant establishment) – Plant establishment includes development of both roots and shoots from early sprouting until beginning of new tubers occurs.

Growth stage III (tuber initiation) – Under proper growth conditions, the stolons tips will curve and begin to swell, resulting in the formation of new tubers. In most cultivars this stage ends with early flowering.

Growth stage IV (tuber bulking) - This is the most critical growth phase for both tuber yield and quality. Under ideal growth conditions, tuber growth rates remain almost constant during this period, which is often specified as the linear tuber growth phase. The accumulation of water, inorganic nutrients and carbohydrates is started during this phase.

Growth stage V (maturation) – During maturation phase, dry matter of the tubers increases, which improves quality for both processing and fresh market consumption. In addition, free sugars are converted to starch, which is required for better quality of chips and fries. In this stage, leaves become yellow, decrease photosynthetic rate, tuber growth slows and vines eventually die. Tuber dry matter content reaches a maximum, and tuber skins set.

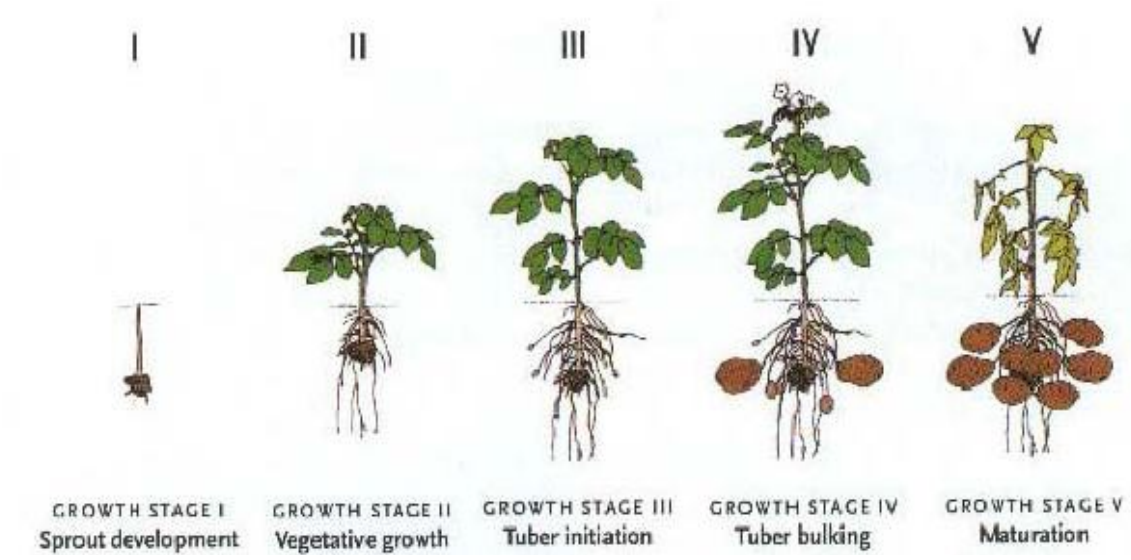


Fig. 3. Different growth stages of potato plant (Dwelle and Love, 1993).

1.4. Mode of propagation of potato germplasm

Vegetative propagation: Potato plant is propagated via tubers by means of vegetative or asexual propagation. The "eyes" on a potato tuber are nodes from where new plants, both stems and roots, can germinate. Most horticulturist sow "seed potatoes" which are pieces of potato (or small whole potatoes) having atleast two eyes. As the plant flourishes, it uses the food reserves in the "seed". The benefit of vegetative propagation is that it guarantees uniformity of the crop in terms of growth and yield. The limitation of this method is that viral infected plants will also propagate from season after season.

Sexual propagation: There is alternative method of potato propagation via botanical seeds of potato generally known as "True Potato Seed" (TPS). TPS can be grown directly in the field or in nursery beds. True potato seeds are used to produce disease free potato plants. 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. 4. Seed Potato

Fig. 5. True Potato Seed

1.5. Improvement of potato crop

The demand of potato crop is high due to the large population and its high nutrition's. It has a good future as a crop that is set to replace rice as a staple in Asian rice-consuming countries. The amount of water content required by potato is less as compared to other basic food products, without compromising the nutrition value. Potato, therefore, is highly 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 accepted potato as the food for the future in order to fight global poverty and hunger. Therefore it is need to boost the potato crop in terms of nutritional quality, disease resistance and productivity. 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 prolong time.

1.5.1. Conventional breeding

Conventional breeding includes cross pollination of the crop plants so that advantageous characteristics from different parent plants could also be assembled in the offspring. It includes natural processes, like sexual and asexual reproduction. For conventional breeding a new plant variety of two closely related plants are 'crossed'. The objective is to combine the desirable traits from both parent plants and exclude their undesirable traits in a singular new and better plant variety. Nonetheless, the progeny of this first cross inherit a mix of genes from both parent plants and so both positive and negative traits may be rooted. Breeders have to watch at all the progeny and choose the ones with the most selective and desirable traits and least negative traits. They then

cross this selected progeny back to one of the authentic parent plants to attempt and transfer more of its positive traits into the following generation.

This process called 'back-crossing' takes place over many generations, which commonly means many years, as far as the progeny have all the desirable traits and none of the negative ones of the authentic two parent plants. Previously the main attention of conventional potato breeding programmes was to hike yield production but in latest years it has transferred to disease resistance (Huisman et al. 1992). Nonetheless, as potato is tetraploid, its advancement by breeding and selection is very tedious and challenging and it is a time engrossing process.

1.5.2. Aspects of molecular breeding

The introduction of desirable genes into plants by genetic engineering has become popular method in plant breeding. Genes of agricultural importance, for example viral, fungal or insect resistance genes have been introduced into the genomes of several crop plants (Schaff, 1991). The plant having the foreign gene is called as a transgenic plant. These improvements have been because of the development of effective gene vector systems for techniques of plant transformation and regeneration. Production of transgenic plants has been anticipated as a probable approach for the control of crop pests, weeds, insects, and plant diseases. Potato is highly heterozygous; therefore, improvement through conventional breeding is more difficult. Moreover it is time consuming and laborious task. This makes potato an outstanding selection for applied molecular genetics which has become adaptive tool to obtain disease and pest resistance in potatoes (Mullins *et al.* 2006). Molecular breeding methods have an asset over conventional breeding in that it allows integration of a specific gene into a plant genome. Molecular techniques can be categorized into two different sets. Firstly the molecular breeding methods that are needed for the identification, isolation, modification and introduction of specific genes coding for traits of interest and secondly, the cellular methods such as transformation and regeneration from completely changed cells into plants (Huisman et al. 1992).

Potato is a dicotyledonous plant and is an outstanding recipient for *Agrobacterium* mediated gene transfer (Vain, 2007). *Agrobacterium*-mediated transformation has been widely accepted in potato modification and potato was one of the first plant species that was changed completely using *Agrobacterium tumefaciens* (An et al. 1986; Shahin and Simpson, 1986). Over the years different potato cultivators have been changed and many transformation methods have been published and developed that rely either on *Agrobacterium*-mediated transformation or biolistics. *Agrobacterium* has been auspiciously used for the transformation of leaves, petioles, stems (Trujillo et al. 2001; Andersson *et al.* 2003), micro tubers (Ishida et al. 1989; Kumar *et al.* 1995) and way of dipping the

flower bolts (combination of flowers on a stem) in an *Agrobacterium* solution (Petti et al. 2005). There are less number of reports on potato transformation using the biolistic method. Romano et al. (2001) reported, potato transformation of leaves, internodal explants and micro tubers with the help of particle bombardment. Particle bombardment can assist the progress of the integration and expression of several genes. Biolistic transformation can also be employed when large DNA fragments require to be transferred to potato (Ercolano et al. 2004).

1.6. Plant tissue culture

Several factors that affect the success of plant genetic engineering include an efficient tissue culture system. Cell and tissue culture techniques like rooting and shoot generation play an important role in realization of their potential and help in plant improvement. Potato is amenable to a number of tissue culture techniques like *in vitro* propagation via shoot culture, protoplast regeneration under sterile conditions in an appropriate culture media. Meristem culture (virus free) and micropropagation (clonal mass propagation) are the techniques which have most prominently been applied to potatoes. Micropropagation is an asexual method of plant regeneration thereby helping in large scale asexual multiplication of potatoes. The nodal segments containing axillary and terminal buds (meristematic and virus free) are commonly used for shoot multiplication.

Micropropagation involves culturing of disease free explant on a semi solid or a liquid culture media. Media is generally composed of major and minor salts, vitamins, sugar which serves as a chief carbon source, plant growth regulators. Commercially Murashige and Skoog (1962) media have been widely used for media formulations. Various plant tissues (roots, leaves, tuber and stem) and cell types have widely been used for plant regeneration.

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 the process of induction and differentiation of roots/shoots from a cell/ cell mass. It is a developmental process. Morphogenesis either occurs directly via explant or it may occur indirectly by dedifferentiation of callus. The complete plant regeneration with the help of tissue culture has enabled the introduction of foreign genes in plant cells and the development of transgenic plants. Different techniques have been used for plant transformation which include *Agrobacterium* mediated gene transfer, particle gun bombardment, microinjection, PEG treatment,

electroporation of protoplast. However, *Agrobacterium* mediated transfer and particle gun bombardment has been most extensively used methods for gene transfer.

1.6.1 Problems associated with plant tissue culture

The tissue culture bottles should be protected from contamination and harsh conditions in order to protect clonal propagated and regenerated tissue culture. Therefore to close culture tubes and bottles polypropylene caps have been used, but these lead to certain abnormalities in potato micro-plants. The main reason of these abnormalities is the lack of gaseous exchange leading to the accumulation of ethylene in culture tubes and bottles. As tissue culture is mainly performed in closed containers, therefore, the gaseous environment experienced by non enclosed plants is different. *In vitro* studies revealed that ethylene affects callus growth; shoot regeneration and somatic embryogenesis (Purnhauser et al. 1987; Songstad et al. 1988; Roustan et al. 1990; Biddington, 1992; Pua and Chi, 1993). The physiological functions of ethylene in plants are discussed in the following section.

1.7. Brief introduction to phytohormone ethylene

Phytohormone regulates physiological activities in plants including germination, growth and metabolism. These hormones also act as stress hormones which initiate quick response to biotic and abiotic stress conditions. Ethylene was one of the first hormones to be identified and is gaseous in nature (Abeles et al. 1992). Ethylene plays different role in developmental processes in different plant species. 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 fruit ripening
- senescence of leaves and flowers
- stimulates the release of dormancy
- shoot and root growth and differentiation
- stimulates flower opening
- stimulates leaf and fruit abscission

1.8. Ethylene biosynthesis

Plants have tendency to generate ethylene from necessarily all parts of higher plants. Their actions involved in biosynthesis of ethylene are well illustrated in the Yang cycle (Yang et al. 1984) displayed in Fig. 6. The two important steps in ethylene biosynthesis are transformation of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and the oxidative

cleavage of ACC to form ethylene. These both steps occur through the activity of ACC synthase and ACC oxidase (ACO), respectively. Regulation of ethylene production can be optimized by harmonizing the levels and activity of both ACC synthase and ACO. The conversion of SAM to ACC by ACC synthase is considered as the rate-limiting step of the ethylene biosynthesis pathway (Liu et al. 2004). The amount of ethylene generated, rely on the tissue-type along with the abiotic and biotic cues. In particular, ethylene levels hike during germination and positively regulate seed germination.

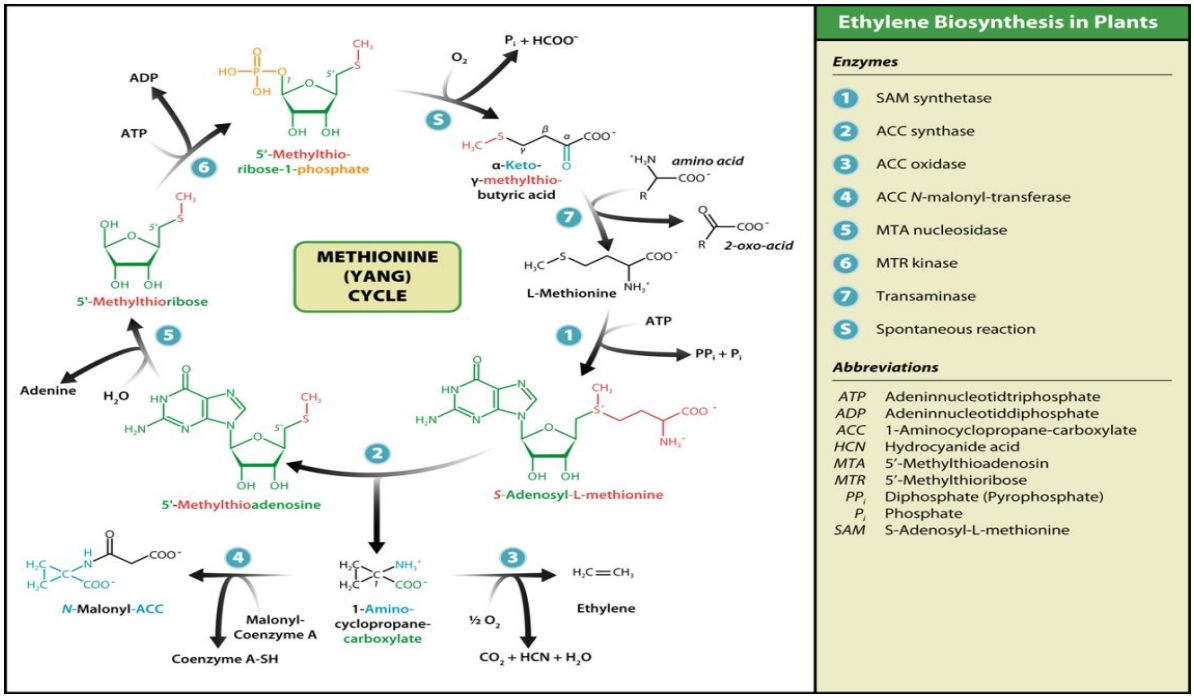


Fig. 6. Schematic view of Ethylene biosynthesis (Wang KC-L,Li H, Ecker JR (2002),Plant cell S131- S151).

1.9. Ethylene signal transduction pathway - an overview

In ethylene signaling pathway, the receptors plays key role. Plant ethylene receptors are associated to bacterial two-component regulators. They are kind of endoplasmic reticulum (ER)-related integral membrane proteins (Chen et al. 2002) with protein kinase activities (Gamble et al. 1998; Moussatche and Klee, 2004). The receptors are dimers of disulfide-linked. In Arabidopsis, a family of five disulfide-linked homodimer receptors situated 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). On account of gene and protein structures, these five ethylene receptors have been categorized into two subfamilies, the subfamily I receptors, ETR1

and ERS1, are similar to histidine kinases. The subfamily II members, ETR2, EIN4 and ERS2, were lacking most of the amino acids critical for histidine kinase activity rather than possess serine kinase activity (Moussatche and Klee, 2004). They incorporate an extra potential membrane-spanning domain at the amino terminus. All receptor isoforms have an N-terminal ethylene-binding domain along with three transmembrane α-helices, a GAF domain exists to mediate interaction between receptors and a kinase domain is also exist. Ethylene binding takes palce at the N-terminal transmembrane domain of the receptors. Histidine kinase behaves as the sensor that autophosphorylates an internal histidine residue in consequence to environmental signals, and a response regulator that triggers the downstream components upon acquiring a phosphate from the histidine residue of the sensor on its aspartate residue (Wurgler et al. 1997). A metal co-factor, copper ion binds to the binding domain found at the interface of the two monomers is needed for ethylene to bind to the receptor (Rodriguez FI et al. 1999; Schaller et al. 1995). Copper is transferred to its receptor by a metal transporter RAN1 (Hirayama et al. 1999; Woeste et al. 2000). Genetic analysis in tomato and Arabidopsis has displayed that the receptors behave as negative regulators for the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al. 2000). Due to lackness of the hormone, receptors strongly restrained ethylene responses. Upon ethylene binding, the suppression is removed and response occurs. Experimentally, it has been depicted that reduction of receptor content develops ethylene sensitivity (Cancel and Larsen, 2002; Hall and Bleecker, 2003; Hua and Meyerowitz, 1998; Tieman et al. 2000), while high receptor content has the opposite effect (Ciardi et al. 2000).

1.10. Inhibition of ethylene signaling by silver ions

Plausible mechanisms of ethylene inhibition by silver ions are described by many researchers. $AgNO_3$ restricts ethylene action by means of silver ions by inhibiting the receptor capacity to bind with ethylene (Yang, 1985), which would result in higher titers of ethylene in the tissues, thus retarding the earlier steps of its own pathway, Miyazaki and Yang (1987) reported the impact of putrescine and $AgNO_3$ on the competitive utilization of SAM. Bais et al. (2000b) also reported that the utilization of SAM by putrescine for its conversion to spermidine would probably yield a lesser possibility of SAM for ethylene biosynthesis (Fig. 7). The ethylene receptor, ETR1, contains one ethylene-binding site per homo dimer and binding is mediated by a single copper ion (Cu^{++}) present in the ethylene-binding site.

The substitution of the copper co-factor by silver also serves to lock the receptor into a conformation such that it endlessly hinders ethylene response (Zhao et al. 2002). The induction of ethylene antagonists into the culture media influences the level of ACC, there by altering ethylene

levels (Gong et al. 2005). Silver ions are 70% larger than copper ions and the bulkiness of the silver ion obstructs the crucial conformational change in response to ethylene (Binder BM et al. 2007). Or another possibility is that the silver-receptor complex is not as stable as the copper-receptor complex, because of which dissociation of ethylene becomes faster.

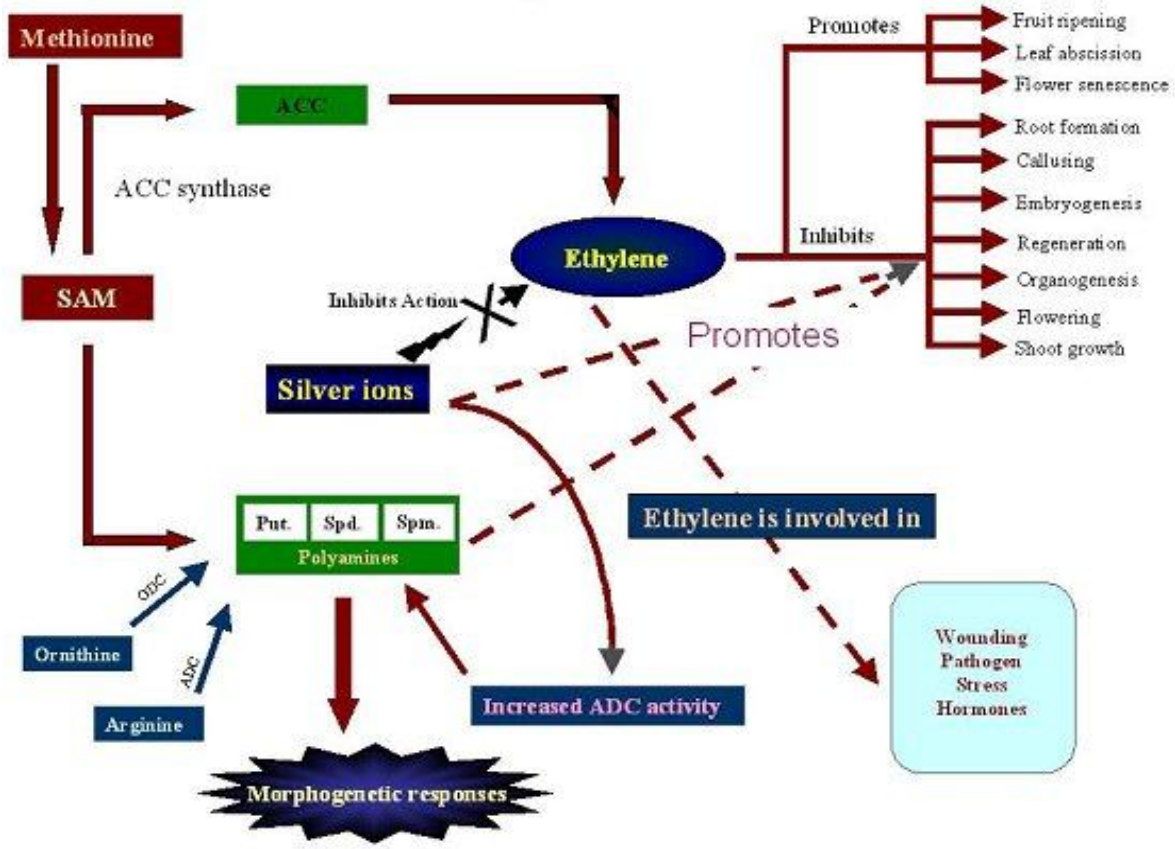


Fig. 7. Schematic view of inhibtion of ethylene action by silver ions Kumar V et al., 2009)

1.11. *In vitro* regeneration

In vitro regeneration of plants or micropropagation achieved by tissue culture is the fundamental tool for crop improvement through genetic transformation. The development of a reliable, rapid and efficient system of tissue culture for plant regeneration has been a foremost prerequisite. Plant tissue culture technique comprises of selection and isolation of plant tissue, maintenance of aseptic conditions during and after manipulation, and *in vitro* maintenance of the cultured tissues / cells in controlled environment. Three different approaches are usually adopted for regenerating plants through culturing of tissues *viz.* 1) use of apical meristem or shoot primordia, 2) direct

organogenesis from explant or through callus, and 3) somatic embryogenesis. Plant regeneration is controlled by many factors mainly including cultivar, explant source and culture medium along with growth hormones. The regeneration of plants through tissue culture is mainly divided into three steps: 1) shoot induction and multiplication, 2) shoot elongation and 3) *in vitro* rooting from the shoots to from stably growing plantlets. Tissue culture techniques for plant regeneration has numerous advantages like simple and rapid propagation of wide range of species, development of true to type plants, a small explant can be grown into a complete plant, controlled physical, chemical and environmental factors etc. Moreover, it can be employed in gene manipulation and plant transformation systems.

1.12. *Agrobacterium*-mediated plant transformation

Agrobacterium tumefaciens is a gram-negative soil bacterium that infects the plant at the site of wounds and causes crown gall disease in various plant species. *A. tumefaciens* possesses a tumor inducing (Ti) plasmid which stimulates tumor formation by transferring a fragment of its DNA, known as transfer DNA (T-DNA) to the host cell after infection and incorporate into the genome of plant (Zupan and Zambryski, 1995). The bacterial genes in T-DNA are expressed in the plant cell and activate the production of phytohormones like auxins and cytokinins that initiate the growth of plant cells in an uncontrolled manner resulting in the formation and proliferation of tumors. Arginine derivatives called opines usually octopine or nopaline are synthesized in these tumors and serve as a source of energy for *Agrobacterium.* Tumor forming genes are removed and the foreign gene of interest may be introduced into the T-DNA of Ti plasmid for its transformation into the plant DNA (Sheng and Citovsky, 1996). *A. tumefaciens* strain carrying such Ti plasmid without tumor inducing oncogenes is called disarmed strain (Klee et al. 1987). The size of Ti plasmid is about 23 kb and T-DNA is a small segment of this plasmid flanked by direct repeats of 25 bp called as T-DNA borders. The endonucleases expressed by *vir* genes recognize these borders of T-DNA for excision (Webb and Morris, 1992). A 35 kb region called virulence (*vir*) region is also a part of Ti plasmid which comprises of 7 loci including *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*G, and *vir*H. These genes synthesize proteins called virulence proteins in response to chemical signals produced at the site of wound and mediate the transmission of T-DNA into infected cell. *Vir* genes assist the movement of T-DNA into the host plant cell. Helper plasmids carrying *vir* genes have been develop to maintain their function in plant transformation with T-DNA carrying gene of interest and use disarmed *Agrobacterium* strains (Hood et al. 1993). Simple vectors with gene of interest expressed under promoter from plant, bacteria or virus are used for plant transformation. Promoters may be used for constitutive expression in the plants or a tissue specific promoter for expression in desired

tissues is coupled with foreign gene (Walden and Wingender, 1995). Reporter genes like β-glucuronidase gene (gus) are used for the analysis of gene expression while, antibiotic resistance genes e.g. neomycin phosphotransferase II (nptII) gene is used for selecting the transgenic cells (McElroy and Brettel, 1994).

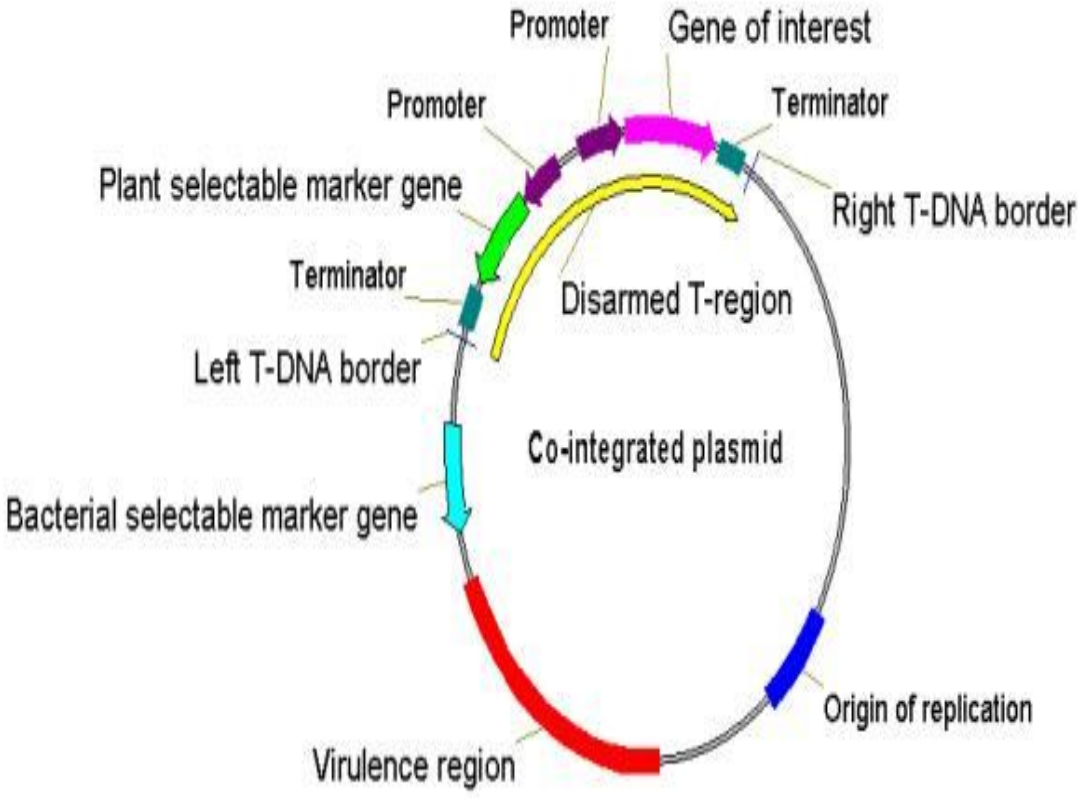


Fig. 8. Schematic view of Ti plasmid

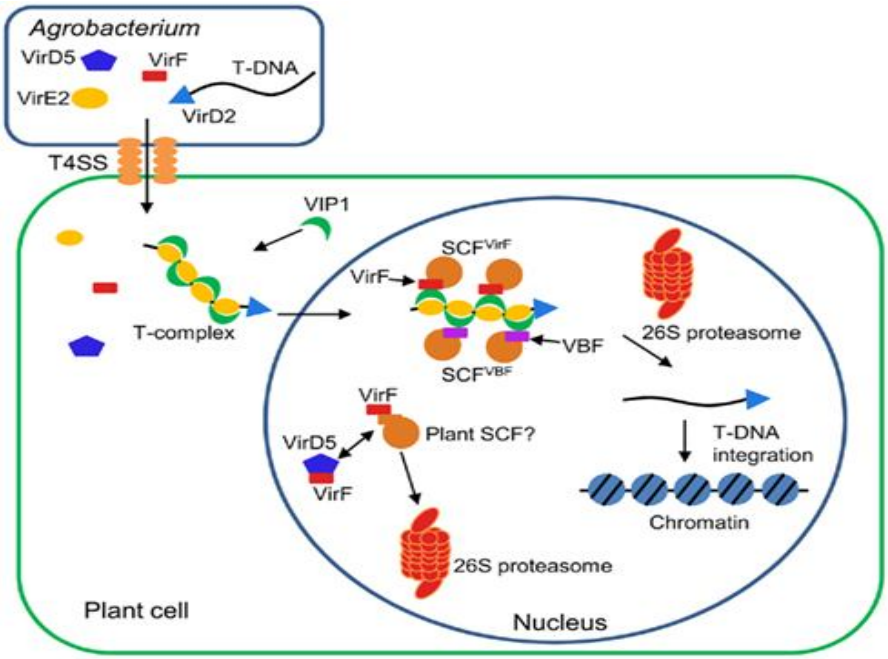


Fig. 9. Mechanism of action of Ti plasmid

Chapter-2

LITERATURE REVIEW

Many researchers have been studied the effect of ethylene on many plant species from time to time. *In vitro* plant cells, tissues and organs produced ethylene. During subculturing of the plant cells *in vitro* major production of this phytohormone occurs as a result of wounding. Plant tissues grown *in vitro* may accumulate large quantities of ethylene from culture vessels, suspension cultures or particularly from rapidly growing non-differentiated callus and hence it influence development and growth in such systems (Biddington, 1992). Gaseous nature of ethylene allows diffusing through intercellular spaces, where it can act as a signal of stress, physical contact or damage leading to appropriate coordinated cellular response. There has been increasing documentation that differentiation and growth of plant cells and tissues *in vitro* can be influenced by ethylene (Chi et al. 1990). Inhibition of ethylene biosynthesis has been shown to stimulate plant regeneration from callus cultures of *Triticum aestivum* (Purnhauser et al. 1987) and *Zea mays* (Vain et al. 1989), stimulate shoot regeneration from cotyledon and hypocotyl cultures of *Brassica*. In case of potato, the tissue cultures are highly sensitive to ethylene, and the growth of potato shoots is decreased in closed culture vessels (Hussey et al. 1981).

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. For long time, many researchers used silver nitrate and silver thiosulfate as silver ions source to the media .Addition of $AgNO_3$ to the culture media enhance the regeneration of both dicot and monocot plant tissue cultures (Beyer, 1976b; 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 different variety of plants for example potato leaf explants produced shoot-forming callus only on medium with $AgNO_3$ (De Block, 1988). Likewise, it stimulated shoot production in cotyledon and seedling explants of several *Brassica* genotypes (Chi and Pua, 1989; Chi et al. 1990). $AgNO_3$ also stimulated shoot formation in *Brassica oleracea* (Sethi et al. 1990). It also improved shoot production in maize callus (Songstad et al. 1988).The use of $AgNO_3$ stimulated 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*) reported that endogenous ethylene may inhibit callus growth: $AgNO_3$ was found to be essential for improving regeneration as well as maintaining callus cultures (J Williams, Biddington NL, 1990).

Studies revealed that although ethylene may enhance 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 et al. 1990). $AgNO_3$ improved shoot regeneration in wheat callus culture, and reversed the inhibitory effect of ethylene and 2, 4-D on morphogenesis. In carrot cell cultures, $AgNO_3$ enhanced embryogenesis and inhibited ethylene production suggesting that embryo production was stimulated by reduced ethylene biosynthesis (Roustan et al. 1990). In *Hevea brasiliensis* the production of embryogenic callus 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 et al. 1990).

In Brussels sprouts (*B. oleracea,* var. *gemmifera*) ethephon, anther culture, and ACC inhibited embryo production and $AgNO_3$, promoted it, particularly with non- or poorly-responsive cultivars (N. L. Biddington et al. 1991). On some species anther culture, studies indicate that ethylene affects microspore embryogenesis (Babbar and Gupta, 1986; Cho and Kasha, 1989; Reynolds, 1987; Dunwell, 1979; Biddington et al. 1991). Biddington et al. (1991) have reported silver nitrate to promote embryogenesis in anther cultures of Brussel sprouts by blocking the inhibitory effect of ethylene.

Beyer (1976a) 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 (*Piscum sativum*cv. Alaska); enhance 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 anti-ethylene properties of Ag (I) are its persistence, specificity, and its lack of phyto-toxicity at effective concentrations.

Hyde and Phillips (1996) disclosed that silver nitrate improves shoot development and plant regeneration in chile pepper (*capsicum annuum* L.) through organogenesis. The explants used were cotyledon. Medium contained-MS salts, vitamins, 2.5 % sucrose, 0.8% agar, 0.5mg/L IAA, 2mg/l BA, 2mg/l GA, 5mg/l $AgNO_3$, 5.7 pH. For multiple shoot production and elongation to occur in the culture, treatment with silver nitrate was necessary and most effective when compared with media without $AgNO_3$. The overall rooting efficiency was increased to 70-72% when shoots transferred to rooting medium developed roots. When grown in the greenhouse, most rooted shoots grew well and produced viable seeds. For plant regeneration cytokinins tested were zeatin and thidiazuron. Out of which thidiazuron induced multiple shoots whereas zeatin induced few shoots.

Eapen S and George L, (1997) reported influence of silver nitrate and silver thiosulfate on plant regeneration from peduncle segments of oil seeds *Brassica* species. Various peduncle segments of B. *juncea*, B. *campestris*, B. *napus*, B.*nigra* and B. *carinata* on Murashige and Skoog medium enriched with benzyladenine and 1-naphthalene acetic acid produced shoot buds. It was observed that enrichment 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.

Naik and Chand, (2003) revealed silver nitrate and aminoethoxyvinylglycine promote *in vitro* adventitious shoot regeneration of pomegranate (Punica granatum L.). MS medium supplemented with 8.9 µmol L^{-1} BA, 5.4 µmol/L NAA and 10% CW promoted adventitious shoot bud differentiation in axenic seedling-derived cotyledons as well as hypocotyl segments. The cotyledons response more than the hypocotyls. When ethylene inhibitors such as $AgNO_3$ (10-40 µmol L^{-1}) and aminoethoxyvinylglycine (AVG) (5-15 µmol L^{-1}) was added to the medium it enhanced number of shoots obtained per explant as well as regeneration frequency.

Turhan H, (2004) showed effect of silver nitrate on potato (*Solanum tuberosum L.*). The results showed an inhibitory effect on ethylene gas produced by potato plantlets when MS basal medium supplemented with $AgNO_3$. Different $AgNO_3$ concentrations used in the cultivars showed genotypic dependence. All cultivars showed best response on concentration 5 or 10 µM $AgNO_3$. For cultivar Nicola and Desiree that initially had branching and abnormal plantlet growth the higher $AgNO_3$ concentrations (25 and 50 µM) can be used.

Buyukalaca S et al. (2004) presented effects of silver nitrate and donor plant growing conditions on production of pepper (*Capsicum annuum* L.) haploid embryos via anther culture. Plant materials used were genotypes U-247 and U-238. From the plants grown either in greenhouse or in open field conditions, flower buds were collected. Different concentrations of silver nitrate (5, 10, 15 and 20 mg L^{-1}) were tested. From all the concentrations tested, haploid embryos were obtained, but with

different production rates. The genotype U-247 in the medium containing 15 mg L^{-1} silver nitrate obtained the highest embryo formation (45.7 embryos per 100 anthers). It was observed that plant growing conditions were also important for embryo formation as the anthers taken from the plants grown in the green-house produced large number of embryos than that of the open field conditions.

Y Gong et al. (2005) observed the effect of silver nitrate in the regulation of direct root and shoot regeneration in sweet potato (*Ipomoea batatas* L.). They developed a high frequency shoot regeneration procedure using stem and lamina explants. According to different explants, silver nitrate showed different result but adding silver nitrate enhanced shoot regeneration. Infect addition of 8.0 mg L^{-1} $AgNO_3$ in MS media increased regeneration of shoots from lamina explants 73.3%. Using silver nitrate for other explants also showed regeneration. It made possible to produce a large number of plantlets from explant in short time. This can be used for conventional breeding programmed gene.

Wu et al. (2006) showed the effects of silver nitrate on the tissue culture of immature wheat embryos. The explants used were the immature embryos of four common wheat (*Triticum aestivum L.*) genotypes. Genotypes were evaluated for their tissue culture response to silver nitrate, ethylene antagonist, added to callus-inductive and subculture media at six concentrations.

Supplementation of $AgNO_3$ significantly enhanced embryogenic callus frequency and callus growth, but reduced the necrosis and relatively did not affect callus induction frequencies. Out of the six concentrations, 10 mg L^{-1} concentration may be considered most favorable for embryogenesis and prevention of callus necrosis, being not harmful for callus induction and stimulatory for callus growth.

Park et al. (2012) improved shoot organogenesis and plant regeneration of gloxinia (*Sinningia speciosa*) by using silver nitrate and putrescine treatment. The explants used were leaves. Culturing of the explants was done on the MS medium combined with different combinations of BAP and NAA for shoot induction. The highest efficiency in shoot regeneration was recorded on MS media containing BAP (2 mg L^{-1}) and NAA (0.1 mg L^{-1}). Addition of $AgNO_3$ and the polyamine putrescine in the regeneration medium improved the shoot induction and enhanced the shoot number. Hardening of the rooted plants was done and it was then transferred to the soil with a survival rate of 90%.

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

• *Somatic embryogenesis* - 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 et al. 1989; Songstad et al. 1991).

• *Multiple shoot induction and shoot regeneration* – In different plants the use of silver nitrate is known to promote multiple shoot formation. *In vitro* shoot formation was enhanced 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 improved the rate of sprouting. At low concentration, $AgNO_3$ was found to cause delayed senescence resulting in increased growth of the proliferated shoots in *Coffea canephora* (Fuentes et al. 2000). *In vitro* shoot growth of C. *Arabica* and C. *canephora* (Giridhar et al. 2003) was greatly improved by $AgNO_3$. Shoot regeneration of Chinese radish *Cv* Red coat was enhanced when cultured in media combined 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 belong 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* - In *Decalepis hamiltonii* addition of 40 µM $AgNO_3$ resulted in root initiation and elongation (Bais et al. 2000a; Reddy et al. 2001). In *Vanilla planifolia* the effect of $AgNO_3$ on rooting and shooting was elucidated (Giridhar et al. 2001). On medium containing 20 µM $AgNO_3$ maximum numbers of shoots and highest shoot length was recorded. Not only $AgNO_3$ induced shoot multiplication but also influenced rooting of vanilla explants. 100% survival was obtained on plantlets medium supplementing 40 µM $AgNO_3$. Silver nitrate also proliferated rooting and flowering *in vitro* on the rare, rhoeophytic woody medicinal plant, *Rotula aquatica* Lour. Dipping of the basal end of shoots in silver nitrate (11.7µM) solution and NAA (2.69 µM) improved rooting efficiency (Sunandakumari et al. 2004).

• *Fruit ripening* - Ethylene plays a critical role in initiating and accelerating the ripening-related process. Treatment of tomato with silver ions has been shown to inhibit fruit ripening and ethylene action (Hobson et al. 1984). In fact, 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; Sisler et al. 1996).

• *Leaf abscission* - Ethylene that enhanced leaf abscission in cotton is blocked by the silver ion (Beyer, 1976b). All the leaves had abscised on the 7th day in ethylene without $AgNO_3$. Progressive less leaf abscission has been showed by plants treated with increasing concentrations of $AgNO_3$ and placed in ethylene. The time required to reach 100% leaf abscission in 2 days were decreased by treatment with 25 mg L^{-1} of $AgNO_3$. Growth retarding effects of ethylene has been reduced by silver nitrate. Other similar experiments have demonstrated a similar ability of $AgNO_3$ to prevent young fruit and flower abscission with mature cotton plants (Beyer, 1976b).

2.3. Role of Silver Thiosulfate in Plant Tissue Culture

T.M. Sridhar et al. (2011) studied the effect of silver thiosulphate (Ethylene inhibitor) on shoot regeneration using axillary bud explants of *Solanum nigrum.* Ethylene inhibitor silver thiosulphate increased the shoot morphogenesis. MS medium supplementing with 40 µM L^{-1} STS resulted in highest frequency of regeneration (95%), increased number of shoots. The optimum range of STS concentration observed was between 10-40 µM L^{-1}. Adventitious root formation was observed and successful field establishment was also achieved at higher concentration. Ethylene hindered 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 helpful as a media supplement to develop efficient protocols for *in vitro* propagation of *Solanum nigrum* as it enhanced the shoot and root formation.

Steinitz et al. (2011) showed thiosulfate enhanced growth and alleviates silver and copper toxicity in root cultures of tomato (*Solanum lycoperiscum*). MS medium containing $Na_2S_2O_3$ with 10–300 µM stimulated root elongation and proliferation of lateral roots. Silver nitrate was showing positive results but in media at 5 µM it caused growth inhibition. This happens because of dissolved Ag^+ and by silver in silver precipitate formation. $CuSO_4$ also showed growth inhibition at 50 µM. Root cultures continued to elongate and proliferate. Tomato roots responded to thiosulfate, adding sodium thiosulphate also increased root growth. Thiosulfate detoxifies effect of dissolved Ag^+ by preventing formation of toxic silver particle precipitate.

Chapter-3

ORIGIN OF PROBLEM

There are more than 40 potato varieties in India which are 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 respect to different attributes such as yield, maturation, disease resistance, texture, soluble sugar content, starch and extent of low temperature sweetening and others. With increase in 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. This can be achieved genetically by inserting desirable trait through transgenic in cultivars. Potato is highly heterozygous; therefore, improvement through conventional breeding is more difficult. Moreover it is time consuming and laborious task. Transgenic approach is more feasible to introduce these desirable traits in potato cultivars. The success of generating transgenic plants depends upon the *Agrobacterium*-mediated transformation and efficient protocols for regeneration. Many reports showed that ethylene produced by tissue-cultured plants or plant tissues affect their morphological features. It is speculated that addition of silver ions may improve regeneration efficiency of the explants. Here, the aim of the study was to see the effects of varying concentrations of silver nitrate and silver thiosulfate on growth characteristics of three potato cultivars, Kufri Chipsona-1, Kufri Jyoti and Desiree under *in vitro* conditions: and also to make a comparison on their effects. Moreover, in order to see the regeneration efficiency during Agrobacterium-mediated transformation, the respective media would be supplemented by silver ions.

Objectives

- Effect of silver nitrate and silver thiosulfate on various growth characteristics of micropropagated potato plantlets of some Indian potato cultivars

- Making a preliminary comparison between the overall effects of silver nitrate and silver thiosulfate on the potato plantlets

- To see the effect of silver nitrate on regeneration efficiency during *Agrobacterium*-mediated genetic transformation

Chapter-4

MATERIALS AND METHODS

4.1. Materials

4.1.1. Procurement of potato germplasm and other materials:

The germplasm of three Indian potato cultivars such as Kufri Chipsona-1 (CS-1), KufriJyoti (KJ) 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 Jyoti	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
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 HiMedia Labs 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 (gL^1)	Use of stock for 1L 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 1L 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 5.6 mgL^{-1} .

4.1.4. Silver thiosulfate - Silver thiosulfate was dissolved in water to make a stock of 20 mM.

4.2. Methodology

4.2.1. Maintenance of potato germplasm (routine micropropagation)

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

4.2.2. Micropropagation of potato plants in varying silver nitrate and silver thiosulfate 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 and STS i.e. 1.5 µm, 3 µm, 6 µm and 9 µm. For each potato cultivar, nodal stem segments were transferred to the above media along with control (without silver nitrate and silver thiosulfate). 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 recorded.

4.2.3. Bacterial strains

Agrobacterium tumefaciens (LBA4404) strain: LBA4404 (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404 (Hoekema et al. 1983). LBA4404 strain was maintained on YEM medium containing rifampicin (15 µg mL^{-1}) and streptomycin (50 µg mL^{-1}).

4.2.4. *Agrobacterium*-mediated co-cultivation: The well characterized transformed *Agrobacterium* strain (corresponding to individual genetic constructs) was used for co-cultivation. The single colony of transformant strain was grown in YEM broth for 24 h up to 0.4-0.5 O.D and then 1 mL of culture was diluted in 10 mL of MS basal medium. Inter-nodal stem segments of five to six weeks old potato plantlets (Kufri Chipsona-1), grown in MS medium, were co-cultivated with diluted culture for 10 min, blot the internodal stem segments on sterile filter paper, placed horizontally on MS basal medium and incubated in dark for 48 hrs in growth room. Internodal stem segments were washed in cefotaxime (250 mg L^{-1}) and shifted to the selective shoot regeneration medium (MS medium containing Zeatin 2 mg L^{-1}, GA_3 3.0 mg L^{-1}, IAA 0.01 mg L^{-1} supplemented with kanamycin 70 mg L^{-1} and cefotaxime 250 mg L^{-1}) for regeneration and primary selection of the transgenic potato lines. The initial shoots were further transferred to the rooting media i.e., MS medium supplemented with IAA (0.5 mg L^{-1}), kanamycin (70 mg L^{-1}) and cefotaxime (250 mg L^{-1}), to obtain complete potato plantlets.

Chapter-5

RESULTS AND DISCUSSIONS

The studies on the effect of silver nitrate on different potato cultivars were initiated last year in our laboratory. The aim of the present study is to know the effects of different concentrations of silver nitrate and silver thiosulfate on growth characteristics of micropropagated potato plantlets of different cultivars. The relevant data was noted after one month of culturing. The various growth characteristics such as shoot length, number of nodes, number of leaves, root length, number of roots, their morphology on various concentrations of silver nitrate (0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1} and 3.0 mg L^{-1}) and silver thiosulfate (1.5 µM L^{-1} ,3.0 µM L^{-1} ,6.0 µM L^{-1} ,9.0 µM L^{-1}) were compared with that of untreated plantlets of same potato cultivar (grown without silver nitrate and silver thiosulfate). At the same time cultivar-wise variation was also reported in response to silver ions. All these results are presented and discussed in the following sections.

5.1. Effect of different concentrations of silver nitrate and silver thiosulfate on growth characteristics of micropropagated potato cultivars

Kufri Chipsona-1 and Kufri Jyoti as well as one exotic cultivar Desiree, are different from each other in many attributes such as quality, crop yield, adaptability, maturation period and tolerance for various stresses. Micropropagated plantlets of these cultivars show some variations in their growth patterns when grown on MS-basal medium. In this study, around one month old nodal stem segments from each cultivar were shifted to normal MS-basal medium supplemented with different concentrations of silver nitrate. Interestingly, the different silver nitrate concentrations were found to influence the overall growth characteristics of the potato plantlets as shown in Fig. 10. In most of the cases, silver ions influenced mainly the following imputes: (i) increase size of leaf (ii) decrease of shoot length (iii) increase in root length (iv) intense pigmentation in leaves.
All these data are provided in the following sections.

Effect of different concentrations of silver nitrate on potato cultivars

Silver nitrate concentrations (mg L^{-1})

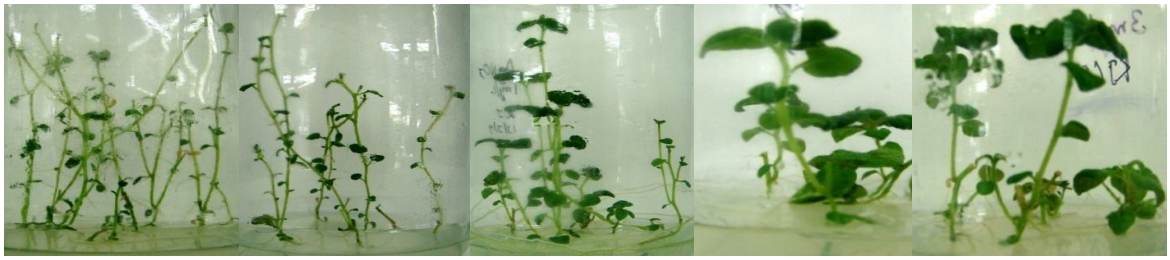
Control 0.5 1.0 2.0 3.0

A. Kufri Chipsona-1

a **b** **c** **d** **e**

B. Kufri Jyoti

a **b** **c** **d** **e**



C.Desiree

a **b** **c** **d** **e**

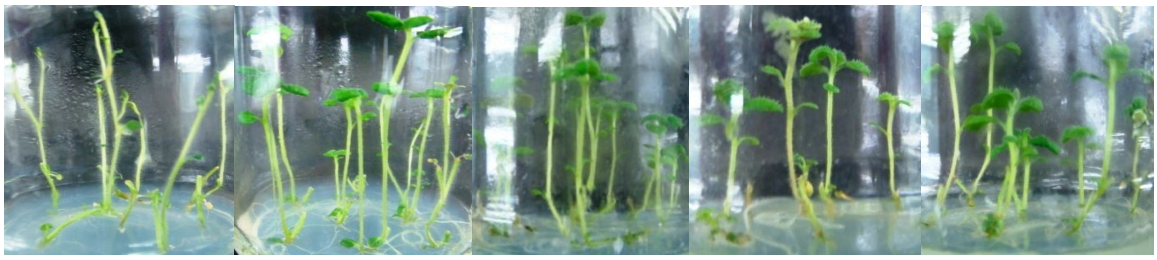


Fig. 10. Effects of silver nitrate during micropropagation of different potato cultivars. **A** Kufri Chipsona-1, **B** Kufri Jyoti, **C** Desiree. Each row refers to particular potato cultivar : **a** control (no $AgNO_3$), **b** 0.5 mg L^{-1}, **c** 1.0 mg L^{-1}, **d** 2.0 mg L^{-1}, **e** 3.0 mg L^{-1} $AgNO_3$ in MS media.

Effect of different concentrations of silver thiosulfate on potato cultivars

Silver thiosulfate Concentrations (µM L^{-1})

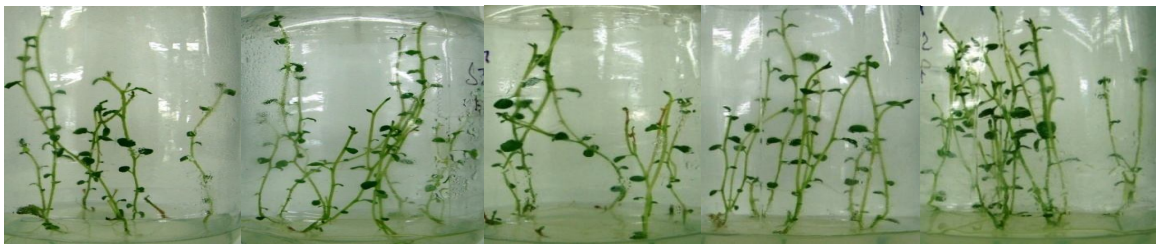
Control 1.5 3.0 6.0 9.0

A. Kufri Chipsona-1

a **b** **c** **d** **e**

B. Kufri Jyoti

a **b** **c** **d** **e**



C. Desiree

a **b** **c** **d** **e**

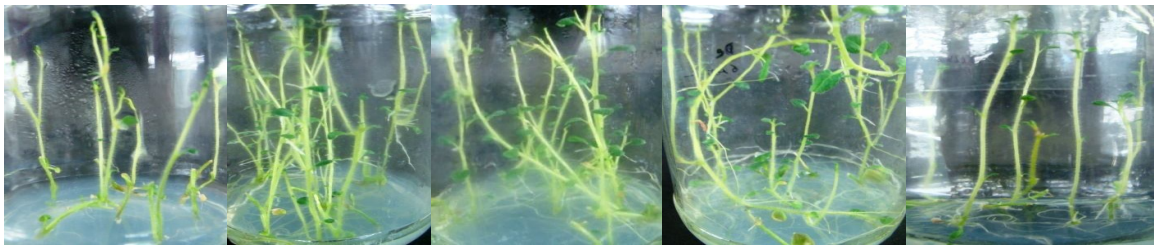


Fig. 11. Effect of silver thiosulfate during micropropagation of different potato cultivars. **A** Kufri Chipsona-1, **B** Kufri Jyoti, **C** Desiree. Each row refers to particular potato cultivar : **a** control (without silver thiosulfate), **b** 1.5 µM L^{-1}, **c** 3.0 µM L^{-1}, **d** 6.0 µM L^{-1}, **e** 9.0 µM L^{-1} silver thiosulfate in MS media.

a. Shoots

Usually, tissue cultured potato plantlets are thin and long on MS-basal medium. In all the cultivars shoot length decreased with respect to control when silver nitrate was added to MS- basal medium. Moreover, the shoot length decreased slowly with increase in silver ion concentration of the medium (0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1} and 3.0 mg L^{-1} $AgNO_3$) and (1.5 µM L^{-1}, 3.0 µM L^{-1} , 6.0 µM L^{-1}, 9.0 µM L^{-1}silver thiosulfate) in case of all the cultivars as shown in Table. 1.1 and 1.2. Kufri Chipsona-1 showed the shortest shoots among all the cultivars. The least effect was observed in case of Desiree. The cultivar Kufri Jyoti showed intermediate effects. Shoot length was not much affected at lower concentrations of silver thiosulfate.

Another observation was the thickening of the shoots along with decrease in shoot length. The shoots colour also became dark green with increasing silver nitrate concentrations in medium as compare to control one. Table 1.1 and 1.2 showing the data obtained from shoot length of all three cultivars.

Table 1.1 Effect of different concentrations of silver nitrate (mg L^{-1}) on shoot length (cm)

Potato Cultivars	**Control**	**0.5**	**1**	**2**	**3**
Kufri Chipsona-1	11.3 ± 0.16	11.0 ± 0.18	10.2 ± 0.30	7.5 ± 0.20	6.9 ± 0.11
Kufri Jyoti	11.7 ± 0.15	11.5 ± 0.30	10.8 ± 0.10	9.1 ± 0.15	8.7 ± 0.25
Desiree	10.6 ± 0.22	10.5 ± 0.12	9.6 ± 0.16	8.8 ± 0.18	7.9 ± 0.20

Values are mean ± SD, n=3

Table 1.2 Effect of different concentrations of silver thiosulfate (µM L^{-1}) on shoot length (cm)

Potato Cultivars	**Control**	**1.5**	**3**	**6**	**9**
Kufri Chipsona-1	11.3 ± 0.16	11.5 ± 0.18	11.1 ± 0.20	10.9 ± 0.11	10.5 ± 0.25
Kufri Jyoti	11.7 ± 0.15	12.2 ± 0.10	11.6 ± 0.25	10.3 ± 0.22	11.1 ± 0.10
Desiree	10.6 ± 0.22	10.5 ± 0.21	11.3 ± 0.17	9.8 ± 0.15	10.9 ± 0.10

Values are mean ± SD, n=3

b. Leaves

Leaf area of all the three potato cultivars was increased near about two to three folds with increase in concentration of silver nitrate in medium as compared to the control as shown in Fig. 10. The effect of silver thiosulfate (Fig. 11.) was observed at higher concentration. The effect was more prominent in leaf area of cultivar Kufri Chipsona-1.

The number of leaves also significantly decreased in all the three cultivars. Conversely, the control potato plantlets consist of more number of leaves than the silver nitrate treated plantlets as shown in Table 2.1**.** The effect of silver thiosulfate (Fig. 11.) was reported at higher concentrations. Kufri Chipsona-1 showed least number of leaves whereas the number of leaves was not much reduced in case of Desiree.

Table 2.1 Effect of silver nitrate concentrations (mg L^{-1}) on number of leaves

Potato Cultivars	**Control**	**0.5**	**1**	**2**	**3**
Kufri Chipsona-1	20 ± 1.0	17.3 ± 0.50	14.3 ± 0.61	13.3 ± 0.86	12 ± 1.0
Kufri Jyoti	20 ± 0.90	19.3 ± 0.81	17.6 ± 0.77	10.6 ± 0.75	11 ± 0.70
Desiree	16.3 ± 0.77	12 ± 0.87	13.3 ± 0.57	9.8 ± 0.57	8.2 ± 0.57

Values are mean ± SD, n=3

Table 2.2 Effect of silver thiosulfate concentrations (µM L^{-1}) on number of leaves

Potato Cultivars	**Control**	**1.5**	**3**	**6**	**9**
Kufri Chipsona-1	20 ± 1.0	18.3 ±0.68	18 ± 0.55	16.6 ±0.98	15 ±0.86
Kufri Jyoti	20 ± 0.90	19.6 ±0.66	19.3 ±0.78	16.3 ±0.99	17.6 ±0.50
Desiree	16.3 ± 0.77	17.3±	17±0.50	15.3±0.85	16±0.90

Values are mean ± SD, n=3

The difference in leaf colour was also observed in all the three cultivars. The plantlets grown on different silver nitrate concentrations showed dark green leaves as compared to the plantlets grown on control MS medium. The addition of silver salts in the medium showed progressively less leaf abscission and senescence even after long time period.

c) Roots

Two main parameters observed in case of roots - root length and number of roots.

Root length: Root length of all the three potato cultivars was found to be increased with increasing concentrations of silver nitrate and silver thiosulfate in the medium. Silver nitrate showed supplementing effect on root elongation as shown in Table. 3.1. In case silver thiosulfate, the effect was only observed at higher concentrations.

Table 3.1 Effect of silver nitrate concentrations (mg L^{-1}) on root length (cm)

Potato Cultivars	**Control**	**0.5**	**1**	**2**	**3**
Kufri Chipsona-1	7.2±0.20	8.5±0.35	9.7±0.25	10.2±0.30	11.6±0.15
Kufri Jyoti	6.8±0.28	7.8±0.20	8.0±0.30	9.2±0.18	10.5±0.22
Desiree	6.9±0.24	7.5±0.36	7.9±25	8.6±0,25	10.7±0.33

Values are mean ± SD, n=3

Table 3.2 Effect of silver thiosulfate concentrations (µM L^{-1}) on root length (cm)

Potato Cultivars	**Control**	**1.5**	**3**	**6**	**9**
Kufri Chipsona-1	7.2±0.20	7.0±0.25	6.8±0.18	7.5±0.25	8.4±0.20
Kufri Jyoti	6.8±0.28	7.2±0.30	6.5±0.26	7.5±0.30	7.2±0.24
Desiree	6.9±0.24	6.7±0.40	7.2±0.35	7.8±0.22	7.6±0.30

Values are mean ± SD, n=3

Longest roots were observed in Kufri Chipsona-1 at higher concentration of silver nitrate and silver thiosulfate. Almost similar effects were observed in the cases of Kufri Jyoti and Desiree.

Number of roots: Although silver nitrate enhanced the root length of all the three potato cultivars but the number of roots decreased. With the increase in concentration of silver ion in the medium the number of roots dropped gradually. It is shown in the Table 4.1

Table 4.1 Effect of silver nitrate concentrations (mg L^{-1}) on number of roots

Potato Cultivars	**Control**	**0.5**	**1**	**2**	**3**
Kufri Chipsona-1	8.2±0.45	8.0±0.25	7.1±0.50	6.3±0.30	4.6±0.56
Kufri Jyoti	8.5±0.35	7.8±0.46	7.2±0.35	6.5±0.48	5.0±0.20
Desiree	8.3±0.38	7.3±0.68	6.8±0.74	5.7±0.23	4.9±0.18

Values are mean ± SD, n=3

Table 4.2 Effect of silver thiosulfate concentrations (µM L^{-1}) on number of roots

Potato Cultivars	**Control**	**1.5**	**3**	**6**	**9**
Kufri Chipsona-1	8.2±0.45	7.8±0.35	8.1±0.56	7.8±0.43	7.3±0.34
Kufri Jyoti	8.5±0.35	8.8±0.45	8.2±0.50	7.9±0.26	7.8±0.40
Desiree	8.3±0.38	8.0±0.24	7.6±0.25	7.4±0.65	7.7±0.52

Values are mean ± SD, n=3

Apart from silver nitrate effects on root length and root number, there was also another effect on root colour was observed. The roots developed green pigment in the presence of silver nitrate in the medium at higher concentrations. This effect was not observed in case of silver thiosulfate Table 4.2. Thickening of roots along with decrease in number of roots was also reported at higher concentrations of both silver nitrate and silver thiosulfate.

Kufri Chipsona-1 showed least number of roots whereas Kufri Jyoti and Desiree showed almost same effect of silver nitrate and silver thiosulfate.

d) Nodes

Number of nodes of all the three potato cultivars was decreased with increasing silver nitrate concentration in the medium (0.5 mg L^{-1} to 3 mg L^{-1}), it may be due to shortened shoot length. Kufri Jyoti showed least number of nodes among all cultivars. The effect of silver nitrate on number of nodes per plantlet is shown in Table 5.1. Silver thiosulfate showed lesser effect as compared to silver nitrate. Moreover, its effect was observed at higher concentrations shown in Table 5.2.

Table 5.1 Effect of silver nitrate concentrations (mg L^{-1}) on number of nodes

Potato Cultivars	**Control**	**0.5**	**1**	**2**	**3**
Kufri Chipsona-1	12.0±0.50	11.6±0.37	10.2±0.30	8.3±0.45	7.1±0.25
Kufri Jyoti	11.3±0.46	11.2±0.45	9.8±0.25	7.8±0.36	6.8±0.50
Desiree	10.8±0.35	11.1±0.25	10.2±.55	8.4±0.18	6.9±0.30

Values are mean ± SD, n=3

Table 5.2 Effect of silver thiosulfate concentrations (µM L^{-1}) on number of nodes

Potato Cultivars	**Control**	**1.5**	**3**	**6**	**9**
Kufri Chipsona-1	12.0±0.50	11.8±0.56	11.2±0.24	11.0±0.35	10.1±0.30
Kufri Jyoti	11.3±0.46	11.5±0.24	10.9±0.30	9.8±0.50	10.4±0.40
Desiree	10.8±0.35	10.4±0.47	10.0±0.18	10.4±0.30	9.7±0.18

Values are mean ± SD, n=3

5.2. Comparative analyses of silver nitrate and silver thiosulfate treated potato plantlets

Addition of silver nitrate resulted in stunted shoot length, increased leaf area and root length, thickening of shoots, shoots and roots became dark green. The effect on these growth features increased with increase in the concentration of silver ions. In case of silver thiosulfate, these growth features were not much affected at lower concentrations. Comparison of control and silver nitrate and silver thiosulfate treated plantlets are shown in Fig. 12. It is noteworthy that the effects of silver nitrate and silver thiosulfate were mostly distinguishable after two months. In other words the effects of silver thiosulfate on growth of potato plantlets appeared to be slow as compared with silver nitrate.

I. Kufri Chipsona-1

a **b** **c**

II. Kufri Jyoti

a **b** **c**

III. Desiree

a **b** **c**

Fig. 12. Effect of silver nitrate and silver thiosulfate treated plantlets during micropropagation of different potato cultivars as compared to control. Top most panel refer to control, different concentrations of silver nitrate and silver thiosulfate. Each row refers to particular potato cultivar: **a** control (no $AgNO_3$ and no STS), **b** $AgNO_3$ concentrations, **c** STS concentrations in MS media. **I.** Kufri Chipsona-1, **a** control, **b** 3mg L^{-1} $AgNO_3$, **c** 9 µM L^{-1} STS, **II.** Kufri Jyoti, **a** control, **b** 3mg L^{-1} $AgNO_3$, **c** 9 µM L^{-1} STS, **III.** Desiree, **a** control, **b** 2mg L^{-1} $AgNO_3$, **c** 6 µM L^{-1} STS.

5.4. *Agrobacterium*-mediated co-cultivation

Inter medium, were co-cultivated with *Agrobacterium* transformant i.e, LBA4404 (pBI121). After co-cultivation, the intermodal stem segments were duly transferred to the selective shoot induction medium. Only single attempt was made in this direction. No significant result was obtained till the submission of this report. Further efforts are still required to see the results and consolidate research base in this area.-nodal stem segments of five to six weeks old potato plantlets from Kufri Chipsona-1, grown in MS.

CONCLUDING REMARKS

With regard to the Indian potato cultivars there was no significant progress with respect to the effect of silver nitrate and silver thiosulfate on micropropagation and regeneration aspects of the potato plantlets. In this context, this work could be considered as a good initiative. The aim of the present study was mainly to see the effects of different concentrations of silver nitrate (0.5, 1.0, 2.0 and 3.0 mg L^{-1}) and silver thiosulfate (1.5 µM L^{-1}, 3.0 µM L^{-1}, 6.0 µM L^{-1}, 9.0 µM L^{-1}) during micropropagations of Indian potato cultivars in MS basal medium. Interestingly all the three cultivars showed significant changes in the growth characteristics even in the low concentration of silver nitrate (i.e. 0.5 mgL^{-1}). Moreover, the effect of silver thiosulfate appeared to be slow as compared to silver nitrate. The effect of the former was prominent only at higher concentrations. In terms of growth characteristics, the observable changes included, decrease in shoot length, number of leaves, nodes and roots but leaf area and increase of root length for all the three potato cultivars. In addition, cultivar-wise variations were also observed and presented in this report.

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