Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 7 (1): 23-31, 2012
ISSN 1816-4951 / DOI: 10.3923/jps.2012.23.31

Morphological Variations with Enhanced Accumulation of Anthocyanins in *Malva sylvestris* L. With Accumulation of Silver Nitrate Treatment

[1]N.R. Jayalakshmi, [1]K.J.T. Saraswathi, [1]B. Vijaya, [2]D.P.H.S.S. Prasad and [3]R. Suresh
[1]Department of Biotechnology, JB Campus, Bangalore University, Bangalore-56, India
[2]Lotus Labs Pvt Ltd., Bangalore-51, India
[3]Department of Statistics, Jnana Bharathi campus, Bangalore University, Bangalore-56, India

Corresponding Author: N.R. Jayalakshmi, Department of Microbiology and Biotechnology, Jnana Bharathi Campus, Bangalore University, Karnataka, INDIA, Bangalore-560056, India

ABSTRACT

Plants of *Malva sylvestris* L., were studied for morphological variations and anthocyanin production, malvidin and delphinidin with silver nitrate treatment. Effects of exogenous silver nitrate on anthocyanin accumulation have been examined. Foliar spray of 0.1 M silver nitrate for five consecutive days significantly increased the anthocyanin content showing distinct morphological variations with respect to plant height, plant biomass, leaf number and leaf mass. By traditional chilled acidified methanol the total anthocyanins were extracted and estimated by pH differential spectroscopic method. The anthocyanins were purified by C-18 Sep-pak column and further analyzed for different anthocyanins by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) methods respectively. The evidence presented in this paper indicates that the enhanced anthocyanin production is related to different levels of oxidative stress induced by silver nitrate to the plant tissues.

Key words: Malvidin, delphinidin, induced stress, HPLC, C-18 Sep-pak column

INTRODUCTION

In recent years, considerable attention has been devoted to the way stress affect plants and how plants actually respond to stressful condition. During the growth and development, plant has to survive with different internal and external stresses like metabolic and environmental changes often leading to oxidative stress. Hence the ability to adapt to variations is essential for plant's survival (Ozudogru *et al*., 2005).

As a defense mechanism the plant shows different responses for oxidative stress by producing antioxidants such as anthocyanins, ascorbate and glutathione. The enzymes such as PAL enzyme, ugft enzyme, catalase, superoxide dismutase and ascorbate peroxidase are involved in the production of antioxidants. When these defenses fail, cell-death occurs with premature senescence and necrosis. The plants exposed to different stresses such as UV-B irradiation, infection by pathogens, metal toxins, increased hormonal level etc. has resulted in increased oxidants and responded by showing symptoms of premature senescence followed by necrotic cell death (Pell *et al*., 1997; Jansen *et al*., 1998; Attri *et al*., 2008).

Silver nitrate is an important phytotoxicant (Mazumdar and Ahmed, 2011; Namasivayam and Chitrakala, 2011) playing a critical role in the induction of various stress signals (Musante and

White, 2010). Silver nitrate is traditionally been considered as an anti-ethylene agent. The foliar application of silver nitrate effectively blocks ethylene-induced abscission in leaves, flowers and fruits (Beyer, 1976, 1979). When treated to plants suppression in aging process occurs (Sahandi *et al*., 2011).

The application of silver nitrate to the plant mimics the effect of stressed condition. The plants react to silver nitrate in both antagonistic and synergistic manner where some plants showed reduction in growth with decreased foliar number and some others showed increased cellular proliferation with high multiple shoot formation (Ozudogru *et al*., 2005; Giridhar *et al*., 2003; Pestana *et al*., 1999; Mafla *et al*., 2004). Subsequent studies made on morphology of the plant revealed that, the silver nitrate treatment induced variation in plant height, leaf area, leaf number, wet and dry mass, auxiliary branching etc. and also enhanced the acceleration of flavonoid biosynthesis especially anthocyanins which occur by the induction of enhanced PAL activity (Suttle and Schreiner, 1982). Silver nitrate though being an anti-aging agent, still induces the production of anthocyanins where these are specifically synthesized during cell senescence or aging process.

Malva sylvestris L., (mallow) is a perennial herb belonging to Malvaceae family. The plant harbors polysaccharides, flavonoids with anthocyanins as the main components. The secondary metabolites from alcoholic extract of leaves and flowers have been widely used as a mild relief for cough and inflammatory diseases of the mucus membrane (Farina *et al*., 1995). They are also utilized as medicine, food flavor, nutritive food, UV protecting agent (lotions and creams) etc. in pharma industries and health care (Andersen and Markham, 2006).

The present study investigates the effect of supplementation of silver nitrate to *Malva sylvestris* plants for their morphological variations and enhanced production of anthocyanins.

MATERIALS AND METHODS

Materials: Malvidin-3-glucoside and Delphinidin chloride were purchased from Sigma Aldrich (Germany), Solid phase extraction columns C-18 Sep-pak column from Agilent (USA) and all other HPLC graded chemicals from Himedia (India).

Method

Abscisic acid treatment: Malva sylvestris plants were grown from seeds sown by using top soil with a mixture of compost to maintain the moisture at room temperature. The flowering plants (ten numbers) were taken for elicitation and 0.1 mM silver nitrate was sprayed onto the plants and exposed to same treatment for five consecutive days. After five days of treatment the plants were observed for morphological changes and the flowers were collected, dried under shade stored at 4°C till further analysis of anthocyanins.

Extraction and purification of anthocyanins: Different extraction methods were adopted for the maximum recovery of anthocyanins. As per the percentage of yield, extraction and purification were done by the method explained earlier by Wrolstad *et al*. (2005). Accordingly Methanol: Acetic acid: Water in the ratio of 49:1:50 was added to the powdered flower sample and was incubated at 4°C for 20-24 h. Filtered with Whatmann No. 1 filter paper, the residual extract was rotary evaporated under vacuum at 30°C. The anthocyanins were separated by solid phase extraction using Accu Bond C-18 cartridge (Sep-pak column) with acidified (0.1% HCl) methanol as the solvent.

Quantitative and qualitative analysis: The total monomeric anthocyanin content was determined by pH differential spectrophotometric analysis with cyanidin as the standard (Mazza *et al*., 2004). Qualitatively the purified sample of anthocyanin was tested with standard malvidin and delphinidin. The compounds were separated on silica gel 60 F254 TLC with the solvent system butanol;acetic acid; water in the ratio 4:1:5. Reverse phase HPLC analysis was carried out on Waters separation module (Waters Corp., Milford, Mass., USA) equipped with an auto injector and separation was carried on ODS C-18 column. The sample was eluted at a flow rate of 1.5 mL min^{-1} with two solvent systems, solvent A with 15% acetic acid and 85% water (v/v) and solvent B with acetonitrile. Gradient separation at room temperature was done with detection at 520 nm.

Statistical methods: Calculations and statistical analysis were performed using SPSS 11.5 windows software. Based on the experimental design adopted in the study, data were analyzed using Student's t-test. The results presented are averaged over the independent experiments with ten quantifications within each sample. Mean values are expressed as ±SE at 1% level of significance.

RESULT

Morphological variation: After five days of silver nitrate treatment as foliar spray the plant showed morphological changes in plant height; leaf texture, number, area; fresh and dry weight; flower size and color. About 5% increase in plant height, 15% decrease in leaf number, 3% decrease in leaf area, 1% increase in fresh weight biomass and 19% increase in dry weight biomass were recorded (Table 1 and Fig. 1a). The leaves of the silver nitrate treated plants showed rugged appearance with change in color from green to yellow (Fig. 2). The flowers expressed deepening in color from pinkish-purple to purplish blue with demarcation in appearance with curling (Fig. 3). The shoot tips and axillary buds got blackened, with apical shoot tips drying up completely without revival and growth in axillary buds after 20-25 days of treatment giving the plant bushy appearance (Fig. 4).

Extraction and analysis: In the present study, anthocyanin extraction was maximum (95%) with 1% acidified methanol by cold temperature incubation method compared to soxhlet (60-70%) and partition extraction (50-60%) methods (Table 2). An elevation in the anthocyanin level was measured in plants treated with silver nitrate. The total anthocyanin concentration was significantly increased to 161.9 μg g^{-1}, compared to untreated sample with 49.7 μg g^{-1} with 325.75% increase (Fig. 1b). The HPLC chromatogram of the dried flower sample extract obtained in the visible spectral region (520 nm) revealed two anthocyanidins, malvidin and delphinidin for

Table 1: Statistical analysis with t-test for equality of means (for physical characters and anthocyanin content)

Characters	c	df	N	Sig. (2-tailed)	M D±SE
Plant height (cm)	0.86	18	10	0.398	1.97±2.3
Leaf number	0.33	18	10	0.001*	13.8±3.41
Total leaf area (dm^2)	0.33	18	10	0.743	1.25±3.76
Fresh weight (g/plant)	0.62	18	10	0.538	0.96±1.54
Dry weight (g/plant)	2.23	18	10	0.038*	0.64±0.29
Anthocyanin content (μg g^{-1})	38.13	18	10	0.000*	101.1±2.7

*At 1% level of significance

Table 2: Recovery of anthocyanins by different extraction conditions

No.	Method	Solvent	Temp (°C)	Acidification (%)	Yeild (%)	Loss (%)	Anthocyanins
1	Soxhelt	Methanol	80-90	5 acetic acid	62	38	+mal,- del
				1 HCl	71	29	
				5 formic acid	0	40	
2	Partition	Acetone	35-40	0.1- 0.01 HCl	50-62	35-40	+ mal, + del
3	Cold	Methanol	4	1 HCl	95	05	+ mal, + del

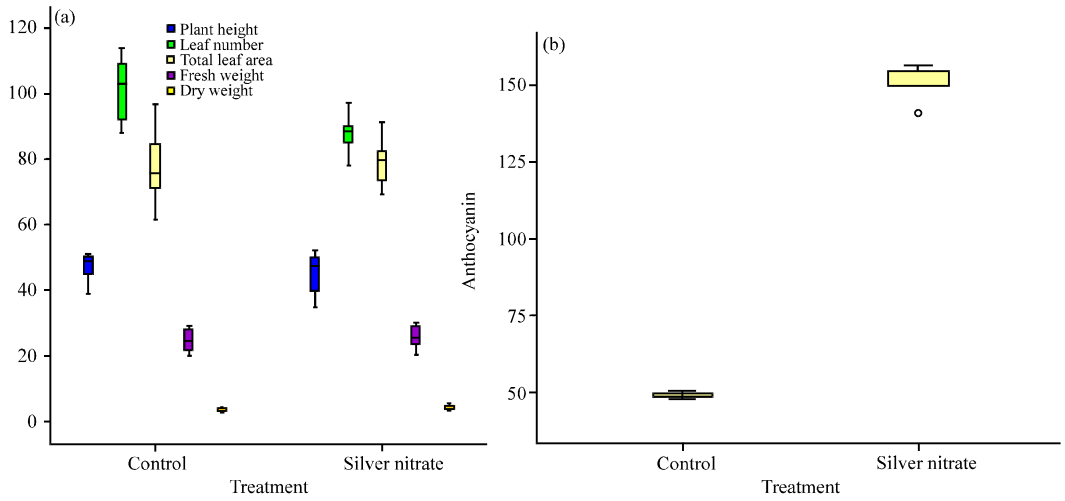

Fig. 1(a-b): (a) Statistical depiction in box plots for morphological variations and (b) statistical depiction in box plots for increased anthocyanins

Fig. 2: Morphological variations in leaves by silver nitrate treatment, (a): Treated-Roughed, small sized yellow colored leaf and (b): Control-smooth, normal sized green colored leaf

untreated and silver nitrate treated plants (Fig. 5 and 6). According to the area of the corresponding peaks there was an increase in concentrations 75.3 μg g^{-1} (malvidin), 61 μg g^{-1} (delphinidin) of anthocyanidins in comparison to the untreated sample where it showed 27.9 μg g^{-1} (malvidin) and 14.2 μg g^{-1} (delphinidin), respectively.

DISCUSSION

In the present study there was marginal increase in height of the treated plants due to elongation of internodal region. Such increased activity in the nodal region gave rise to the

Fig. 3: Morphological variations in flowers by silver nitrate treatment, (a): Treated-purplish-blue colored, curled and demarked flower and (b): Control-pinkish-purple colored normal flower

Fig. 4: Growth variations, (a): control with apical growth and (b): Treated with lateral growth giving bushy appearance

expression of numerous lateral branches giving a bushy appearance to the plant with enhanced dry and wet mass. It could be inferred that cell elongation in plants were drastically affected than the cell division, as silver nitrate is well known for its involvement in enhancing the growth of internode. The resulted stimulation in the growth and development of lateral branches contributed to an increase in plant height and weight. In many plants, the organs of vegetative phase (root, stem, leaf) and senescence phase (flowers) responds to silver ions with growth induction. The silver ions applied as silver nitrate classically performs 'triple' response viz., vertical growth retardation, stem swelling and horizontal growth induction (Beyer, 1976; Zavattieri *et al*., 2010; Sharma *et al*., 2008). The young leaves decolorized from green to yellow resulting in abscission of leaves in 8-10 days of treatment. This reduced the number of leaves in the plants and the leaves which survived expressed variation with respect to size, shape and area. After 25-30 days of treatment the newly emerged leaves remained small and deformed. Silver ions are well known for induction of enhanced flowering and change in flower color (Bais *et al*., 2001; Reddy *et al*., 2001).

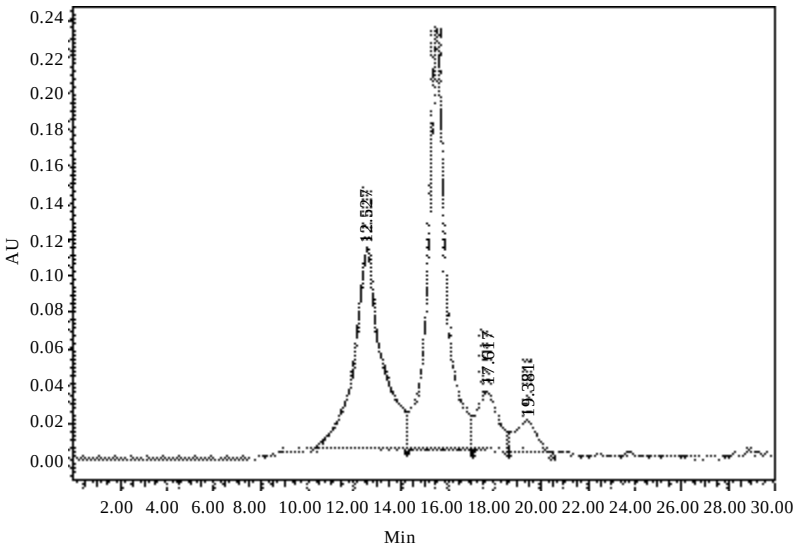

Fig. 5: Chromatogram for silver nitrate treated sample

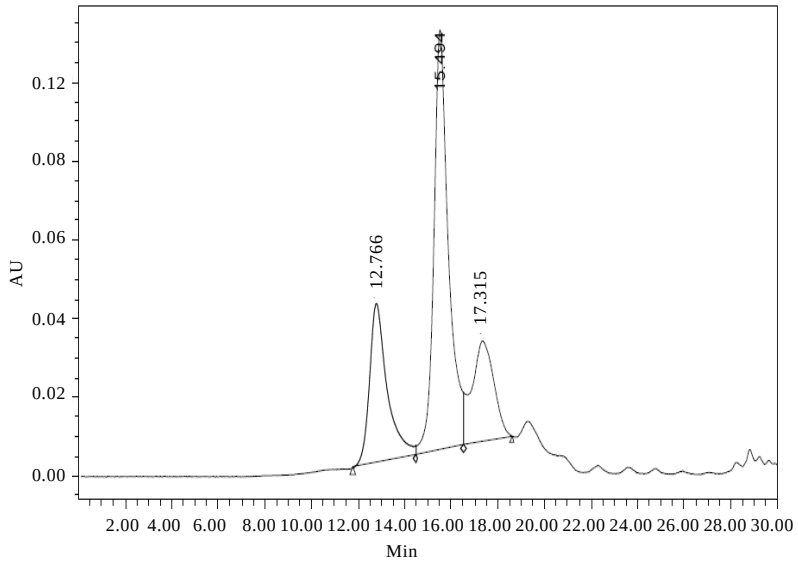

Fig. 6: Chromatogram for untreated sample

The blue color of the flower in the treated plant might be due to the production of polyamines and phenolic compounds induced by silver ions where in normal or untreated plants it is ethylene induced (Bais *et al*., 2001). Flowering is one of the processes inducing senescence, though silver nitrate is an ethylene inhibitor or ethylene action inhibitor, its participation in the induction of flowering is still a mystery. The present investigation is substantiated by earlier work done on etiolated soybean hypocotyls (Suttle and Schreiner, 1982), *Cannabis sativa* (Sarath and Mohan Ram, 1979; Mohan Ram and Sett, 1982) *Ricinis communis* (Mohan Ram and Rina, 1980), *Pelargonium hortorum, Calceolaria herbeohybrida and Bougainvillea glabra* (Arthur and Michael, 1983).

The solvent extraction of anthocyanins is the initial step in the determination of total and individual anthocyanins prior to quantification, purification, separation and characterization (Durst and Wrolstad, 2001). The polar character of anthocyanins makes them soluble in several types of solvents such as methanol, ethanol, acetone, water etc. and generally involves the use of acidified methanol or ethanol. The use of acid stabilizes anthocyanins in the flavylium cation form which is red at low pH. Even though methanol is toxic, it is best preferred for complete extraction because ethanol is less efficient and more difficult to eliminate later during purification (Corrales *et al*., 2009).

During the study, the enhanced production of anthocyanins with silver nitrate treatment can be related to intermittent activation of few genes in anthocyanin biosynthesis. Studies have revealed that the relative immobility of silver nitrate within the plant tissue could be the reason for induction of the stress leading to high accumulation of anthocyanins (Baird *et al*., 1984). The foliar application with silver nitrate inhibited the ripening process which in turn inhibited the endogenous ethylene synthesis leading to an amazing induction of those enzymes involved in anthocyanin accumulation (Bais *et al*., 2001; Suttle and Schreiner, 1982). The expression of flavonoid synthesis gene and those associated with anthocyanin synthesis could be enhanced by the presence of stress inducers Jeongi *et al*. (2008), Suttle and Schreiner (1982) and Navabpour *et al*. (2003) provided evidence for anthocyanin accumulation with silver nitrate treatment is genotype-dependent and reported enhanced mRNA accumulation of PAL gene-a key regulatory enzyme in anthocyanin biosynthesis and LSC54 gene-a senescence inducing gene. Expression of PAL gene and LSC54 gene encoding a metallothionein protein increases during leaf senescence and cell death and the extent of its expression is related to the levels of oxidative stress in the tissue (Ozudogru *et al*., 2005; Butt *et al*., 1998). Several other genes were expressed under stressed condition, like LSC94 which encodes the pathogenesis related protein PR1a, LSC222 which encodes chitinase (Hanfrey *et al*., 1996) and LSC760, LSC790 and LSC803 which encodes aspartic protease, cysteine protease, lipid hydroperoxide dependent glutathione peroxidase res pectively (Page *et al*., 2001). Similar studies for expression of anthocyanin genes for induced oxidative stress has been reported in various plants like *Cannabis sativa* (Mohan Ram and Rina, 1980), *Capsicum frutersens* (Sharma *et al*., 2008), Date Palm (Al-Khayri and Al-Bahrany, 2001), Virginia-type peanut plants (Ozudogru *et al*., 2005), Coffee (Giridhar *et al*., 2003) and *Arachis hypogaea* (Pestana *et al*., 1999).

CONCLUSION

The extracts of *Malva sylvestris* have traditionally been used since ages as an herb medicine in folk remedies to treat cough, pain, inflammation and cancer. In the present study, silver nitrate used as an stress "inducer" acted as an "enhancer". Although silver ions or silver nitrate as such are toxic in nature, when applied on leaves exogenously morphological variations occurred with accelerated growth and proliferation thereby enhancing the anthocyanin production. These anthocyanins can be intended to be employed as food colorants and antioxidant agents in food, pharmaceutical and cosmetic industries.

ACKNOWLEDGMENT

I am greatly indebted to Prof. Jagadish Chandra (retd), department of Botany, Mysore University for his review and correction of this paper.

REFERENCES

Al-Khayri, J.M. and A.M. Al-Bahrany, 2001. Silver nitrate and 2-isopentyladenine promote somatic embryogenesis in date palm (*Phoenix dactylifera* L.). Sci. Hortic., 89: 291-298.

Andersen, O.M. and K.R. Markham, 2006. Flavonoids: Chemistry, Biochemistry and Applications. CRC Press, Taylor and Francis Group, Boca Raton, FL., USA., ISBN-13: 9780849320217, Pages: 1237.

Arthur, C.C. and S.R. Michael, 1983. Use of silver thiosulfate to prevent flower abscission from potted plants. Scientia Hoticulturae, 19: 373-378.

Attri, L.K., H. Nayyar, R.K. Bhanwra and A. Pehwal, 2008. Pollination-induced oxidative stress in floral organs of *Cymbidium pendulum* (Roxb.) Sw. and *Cymbidium aloifolium* (L.) Sw. (Orchidaceae): A biochemical investigation. Sci. Hortic., 11: 311-317.

Baird, L.M., M.S. Reid and B.D. Webstar, 1984. Anatomical and physiological effects of silver thisulphate on ethylene-induced abscission in Coleus. J Plant Growth Regul., 3: 217-225.

Bais, H.P., G. Sudha and G.A. Ravishankar, 2001. Influence of putrescine, silver nitrate and polyamine inhibitors on the morphogenetic response in untransformed and transformed tissues of *Cichorium intybusand* their regenerants. Plant Cell Rep., 20: 547-555.

Beyer E.M., 1976. A potent inhibitor of ethylene action in plants. Plant Physiol., 58: 268-271.

Beyer Jr., E.M., 1979. Effect of silver ion, carbon dioxide and oxygen on ethylene action and metabolism. Plant Physiol., 63: 169-173.

Butt, A., C. Mousley, K. Morris, J. Beynon and C. Can *et al.*, 1998. Differential expression of a senescence-enhanced metallothionein gene in *Arabidopsis* in response to isolated of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J., 16: 209-221.

Corrales, M., A.F. Garcia, P. Butz and B. Tauscher, 2009. Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. J. Food Eng., 90: 415-421.

Durst, R.W. and R. Wrolstad, 2001. Separation and Characterization of Anthocyanins by HPLC. In: Current Protocols in Food Analytical Chemistry, Wrolstad, R.E., T.E. Acree, H. An and E.A. Decker (Eds.). John Wiley and Sons, New York, USA., ISBN-13: 9780471325659, pp: F1.3.1-3.13.

Farina, A., A. Doldo, V. Cotichini, M. Rajevic, M.G. Quaglia, Mulinacci and F.F. Vincieri, 1995. HPTLC and reflectance mode densitometry of anthocyanins in *Malva sylvestris* L.: A comparison with gradient-elution reversed-phase HPLC. J. Pharm. Biomed. Anal., 14: 203-211.

Giridhar, P., E.P. Indu, D.V. Ramu and G.A. Ravishankar, 2003. Effect of silver nitrate on *in vitro* shoot growth of coffee. Trop. Sci., 43: 144-146.

Hanfrey, C., M. Fife and V. Buchanan-Wollaston, 1996. Leaf senescence in *Brassica napus*: Expression of genes encoding pathogenesis-related proteins. Plant Mol. Biol., 30: 597-609.

Jansen, M.A.K., V. Gaba and B.M. Greenberg, 1998. Higher plants and UV-B radiation: balancing damage, repair and acclimation. Trends Plant Sci., 3: 131-135.

Jeongi, S.T., N.G. Yamamoto, K. Hashizume and M. Esaka, 2008. Expression of multi-copy flavonoid pathway genes coincides with anthocyanin, flavonol and flavan-3-ol accumulation of grapevine. Vitis, 47: 135-140.

Mafla, G., J.C. Roa, C.H. Ocampo, G. Gallego, G. Jaramillo and D.G. Debouck, 2004. Efficacy of silver nitrate for slow-growth conservation of cassava (*Manihot esculenta* Crantz). Determination of viability and genetic stability. Proceedings of the 6th International Scientific Meeting of the Cassava Biotechnology Network, March 8-14, 2004, CIAT, Cali, Colombia, pp: 87-110.

Mazumdar, H. and G.U. Ahmed, 2011. Phytotoxicity effect of silver nanoparticles on *Oryza sativa*. Int. J. ChemTech Res., 3: 1494-1500.

Mazza, G., J.E. Cacace and C.D. Kay, 2004. Methods of analysis for anthocyanins in plants and biological fluids. J. AOAC Int., 87: 129-145.

Mohan Ram, H.Y. and R. Sett, 1982. Induction of fertile male flowers in genetically female *Cannabis sativa* plants by silver nitrate and silver thiosulphate anionic complex. Theor. Applied Genet., 62: 369-375.

Mohan Ram, H.Y. and S. Rina, 1980. Induction of male flowers in a pistillate line of *Ricinus communis* L. by silver and cobalt ions. Planta, 149: 413-415.

Musante, C. and J.C. White, 2010. Toxicity of silver and copper to *Cucurbita pepo*: Differential effects of nano and bulk-size particles. Environ. Toxicol., (In Press). 10.1002/tox.20667

Namasivayam, S.K.R. and K. Chitrakala, 2011. Ecotoxicological effect of *Lecanicillium lecanii* (Ascomycota: Hypocreales) based silver nanoparticles on growth parameters of economically important plants. J. Biopest., 4: 97-101.

Navabpour, S., K. Morris, R. Allen, E. Harrison, S.A.H. Mackerness and V. Buchanan-Wollaston, 2003. Expression of senescence-enhanced genes in response to oxidative stress. J. Exp. Bot., 54: 2285-2292.

Ozudogru, E.A., Y. Ozden-Tokatli and A. Akcin, 2005. Effect of silver nitrate on multiple shoot formation of Virginia-type peanut through shot tip culture. *In vitro* Cell. Dev. Biol., 41: 151-156.

Page, T., G. Griffiths and V. Buchanan-Wollaston, 2001. Molecular and biochemical characterisation of post harvest senescence in broccoli. Plant Physiol., 125: 718-727.

Pell, E.J., C.D. Schlagnhaufer and R.N. Arteca, 1997. Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiol. Plant., 100: 264-273.

Pestana, M.C., C. Lacorte, V.G. de Freitas, D.E. de Oliveira and E. Mansur, 1999. *In vitro* regeneration of peanut (*Arachis hypogaea* L.) through organogenesis: Effect of culture temperature and silver nitrate. *In vitro* Cell. Dev. Biol., 35: 214-216.

Reddy, O.B., P. Giridhar and G.A Ravishankar, 2001. *In vitro* rooting of *Decalepis hamiltonii* Wight and Arn., an endangered shrub, by auxins and root-promoting agents. Curr. Sci. India, 81: 1479-1482.

Sahandi, M.S., A. Sorooshzadeh, H. Rezazadeh and H.A. Naghdibadi, 2011. Effect of nano silver and silver nitrate on seed yield of borage. J. Med. Plants Res., 5: 171-175.

Sarath, G. and H.Y. Mohan Ram, 1979. Comparative effect of silver ion and gibberlic acid n the induction of male flowers on female *Cannibis* plants. Cell. Mol. Life Sci., 35: 333-334.

Sharma, A., V. Kumar, P. Giridhar and G.A. Ravishankar, 2008. Induction of *in vitro* flowering in *Capsicum frutescens* under the influence of silver nitrate and cobalt chloride and pollen transformation. Electron. J. Biotechnol., Vol. 11, No. 2. 10.2225/vol11-issue2-fulltext-8

Suttle, J.C. and D.R. Schreiner, 1982. Effects of DPX-4189 (2-chloro-N-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl)benzenesulfonamide) on anthocyanin synthesis, phenylalanine ammonia lyase activity and ethylene production in soybean hypocotyls. Can. J. Bot.,60: 741-745.

Wrolstad, R.E., R.W. Durst and J. Lee, 2005. Tracking color and pigment changes in anthocyanin products. Trends Food Sci. Technol., 16: 423-428.

Zavattieri, M.A., A.M. Frederico, M. Lima, R. Sabino and B. Arnholdt-Schmitt, 2010. Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron. J. Biotechnol., Vol. 13, No. 1. 10.2225/vol13-issue1-fulltext-4.