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# ESTABLISHMENT OF ENHANCED ANTHOCYANIN PRODUCTION IN *MALVA SYLVESTRIS* L. WITH DIFFERENT INDUCED STRESS

**JAYALAKSHMI N.R[1*], THARA SARASWATHI K.J.[1], VIJAYA B.[1], RAMAN D.N.S.[2] AND SURESH R[3]**

*[1]Department of Microbiology and Biotechnology, J.B. Campus, Bangalore University, Bangalore-560056.*
*[2]Associate Professor, Department of Botany, P.G. Centre, St. Josephs College, Bangalore.*
*[3]Research Scholar, Department of Statistics, J.B. Campus, Bangalore University, Bangalore-56.*

## ABSTRACT

A comparative study in *Malva sylvestris* L. plants was carried out for change in growth pattern and their anthocyanin production with various induced stress. Exogenous administration of 0.1mM concentration of abscisic acid, silver nitrate as foliar spray for five successive days and supplemental thirty minutes exposure dose of UV-B for continuous three days to the plants significantly induced variation in the growth pattern and anthocyanin content. The abscisic acid and silver nitrate treatment induced increased anthocyanin content up to five fold and three fold with induced morphological variations by increasing height, leaf mass, leaf number, fresh and dry weight in comparison to untreated plants whereas the plants with UV-B treatment showed reduced growth with two fold increase in anthocyanin content. The result of the study using biological (abscisic acid), chemical (silver nitrate) and physical (UV-B) stress evidenced both positive and negative responses with induction of high anthocyanin production and varied growth pattern.

**KEYWORDS :Malvidin, delphinidin, induced stress, HPLC, C-18 Sep-Pak column.**

**JAYALAKSHMI N.R**
**Department of Microbiology and Biotechnology, J.B. Campus, Bangalore University, Bangalore-560056.**

**Corresponding author*

# INTRODUCTION

The ability for adapting to variations is essential for plant's survival with production of various metabolites. During the growth and development, plant has to survive with different internal and external stresses like metabolic and environmental changes which often lead to the development of oxidative stress. Hence in recent years, considerable attention has been devoted to the way stress affect plants and how the plants actually respond to stressful conditions[1]. As a defense mechanism the plants show different responses for oxidative stress by producing antioxidants such as anthocyanins, ascorbate and glutathione. The enzymes such as PAL, *ugft*, 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[2]. The plants exposed to different stresses such as hormonal variations, metal toxins, UV-B irradiation, and infection by pathogens etc. has resulted in increased oxidants that affect plant growth and development, trigger wide range of response, from altered gene expression and modification in cellular metabolism to change in growth rate and product yield [3,4]. The application of abscisic acid (ABA) (phytohormone), silver nitrate and UV-B radiation to plant mimics the effect of a stress condition. The presence of ABA triggers heterophyllous switch during the adult vegetative phase resulting in stimulation of growth and development, acts as main triggering signal for the onset of ripening in plants. An increase in the concentration of ABA just before veraison is recognized as an important clue for the aging process by accelerating sugar accumulation[5,6]. The accumulation of sugar beyond the immediate need of a tissue often favors the synthesis of secondary metabolites such as anthocyanins and other flavorants[7]. Silver nitrate (phytotoxicant) is traditionally been considered as an anti-ethylene agent, which suppresses the aging process[8,9,10]. The reaction of plants to silver nitrate exposure is both antagonistic and synergistic[11,12,13]. With treatment some plants showed reduced growth with decreased foliar number and some showed increased cellular proliferation with high multiple shoot formation[1,14,15,16] and enhanced anthocyanin biosynthesis by induction of PAL activity[17]. 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.

The interaction between UV-B radiation and plants are various like additive, synergistic or antagonistic and differs among species[18]. The negative effect results in deformed morphological parameters and positive effect in enhanced acceleration in biosynthesis of phenolics[19]. The phenolics present in the epidermal layers of leaves and tissues are susceptible to UV damage, they activate the enzymes chalcone synthase (CHS) or chalcone isomerase (CHI) involved in the synthesis of anthocyanins to protect the plants from stress[18]. *Malva sylvestris* L. (mallow) is a perennial herb of Malvaceae. The plant harbors polysaccharides, flavonoids with anthocyanins as main components. The secondary metabolites from alcoholic extract of leaves and flowers are widely used as a mild relief for cough and inflammatory diseases of mucus membrane[20]. Further they are also utilized as medicines, food flavors, nutritive food, UV protecting agents (lotions and creams) etc. in pharma industries and in health care[21]. The prime aim of the present investigation was to study the effect of various stresses like biological, chemical and physical on plant growth pattern and accumulation of anthocyanins in *Malva sylvestris*.

# 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).

# METHODS

### *Stress induction*

*Malva sylvestris* plants were grown from seeds sown using top soil with mixture of compost to maintain moisture at room temperature. The flowering plants (ten numbers in three sets) were taken for elicitation. 0.1mM concentration of ABA and silver nitrate was sprayed onto the plants and exposed to the treatment for five successive days. The UV-B radiation was provided for thirty minutes for three consecutive days by Philips sunlamps (Philips TL 20W/12). The sunlamps were wrapped by 0.13mm cellulose diacetate filters for removing UV-C radiation. The plants were observed for their morphological changes. The ABA treated flowers were picked and dried under shade, stored at 4$^{o}$C till further use.

### *Extraction and purification of anthocyanins*

Different methods were adopted for maximum recovery of anthocyanins. As per the percentage yield, extraction and purification were done by the method as explained by Anderson and Markham[22].

Methanol:Acetic acid:Water in the ratio 49:1:50 was added to the powdered flower sample and incubated at 4$^{o}$C for 20-24 hrs. The extract was filtered with Whatmann no.1 filter paper and the residual extract was rotary evaporated under vacuum at 30$^{o}$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 a solvent.

### *Quantitative and qualitative analysis:*

The total monomeric anthocyanin content was determined by pH differential spectrophotometric analysis with cyanidin as standard[23]. The purified sample was qualitatively analyzed by TLC on silica gel 60 F254 with the solvent system butanol:acetic acid:water in the ratio 4:1:5. Pink and blue colored bands were obtained and their *Rf* values were calculated and compared with the standard. 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 out on ODS column. The sample was eluted at a flow rate of 1.5ml/min with two solvent system, solvent A with 15% acetic acid and 85% water (v/v), solvent B with acetonitrile. Gradient separation at room temperature was done with detection at 520nm.

### *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 ANOVA and Student's t-test. The results presented are averaged over the independent experiments with ten quantifications within each sample. Mean values are expressed as ± S.E at 5% level of significance.

# RESULTS

### *Morphological variations*

After five days of ABA and silver nitrate treatments used as foliar spray, and three days of UV-B radiation, the *Malva sylvestris* plants showed morphological changes in plant height; leaf texture, number, area; fresh and dry weight; flower size and color (Table 1 and Box-plot 1).

**Table 1**
***Multiple Comparisons between different stress treatment and morphological characters by Dunnett t-test (2-sided)***

| Morphological characters | (I) Treatment | (J)Treatment | Mean Difference (I-J) | Std. Error Sig. |
|---|---|---|---|---|
| Plant height(cm) | UV | Control | -31.960* | 1.971.000 |
| | Silver nitrate | Control | -1.970 | 1.971.629 |
| | Abscisic acid | Control | 13.460* | 1.971.000 |
| Leaf number | UV | Control | -90.600* | 3.290.000 |
| | Silver nitrate | Control | -13.800* | 3.290.000 |
| | Abscisic acid | Control | -28.400* | 3.290.000 |
| Total leaf area($dm^2$) | UV | Control | -70.46600* | 3.04375.000 |
| | Silver nitrate | Control | 1.25100 | 3.04375.955 |
| | Abscisic acid | Control | 11.83800* | 3.04375.001 |
| Fresh weight(gm/plant) | UV | Control | -18.21860* | 1.12639.000 |
| | Silver nitrate | Control | .96640 | 1.12639.726 |
| | Abscisic acid | Control | 5.84640* | 1.12639.000 |
| Dry weight(gm/plant) | UV | Control | -3.35800* | .23358.000 |
| | Silver nitrate | Control | .64800* | .23358.023 |
| | Abscisic acid | Control | 1.36300* | .23358.000 |

***Dunnett t-tests treat one group as a control, and compare all other groups against it.***
****The mean difference is significant at the 0.05 level.***

The plants leaves with ABA treatment showed smooth appearance with less curling, with silver nitrate treatment showed rugged appearance with change in color from green to yellow and with UV-B treatment the leaf size decreased, color changed to yellow, showed roughness with curling (Figure 1). The flowers obtained from all the treatments expressed deep coloration from pinkish-purple to purplish blue. There was a noted increase in flower size and number with ABA, silver nitrate treatments and decrease in UV-B treatment. The flowers from ABA treatment were normal in appearance, whereas the silver nitrate and UV-B treatment ones showed demarcation with curling (Figure 2).

**Figure 1**
***Variations in leaf morphology with stress induction***

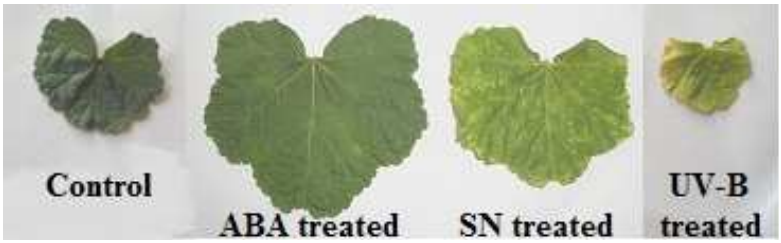
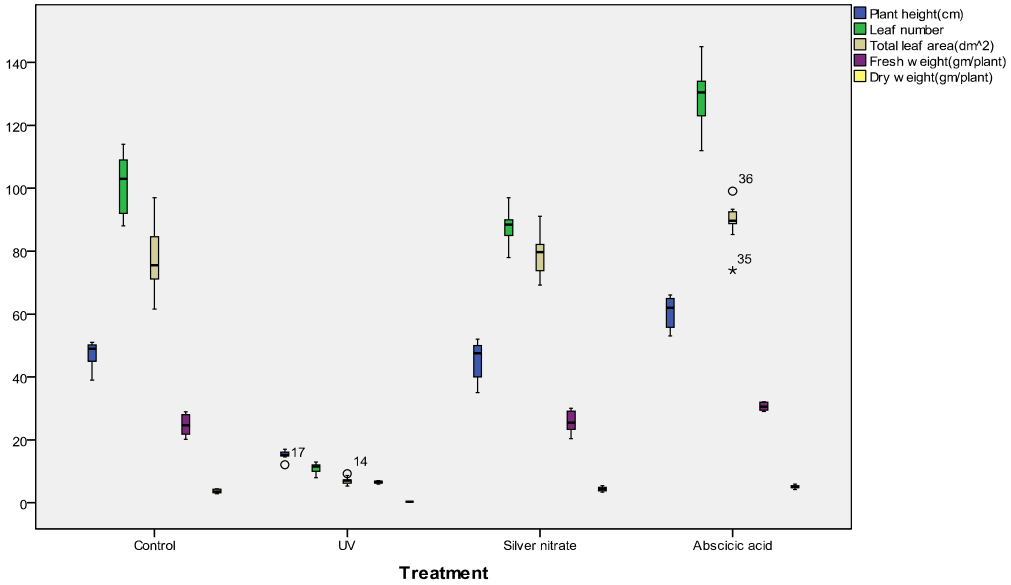


**Figure 2**
***Variations in flower morphology with stress induction***


**Box-plot 1**
***Morphological variations due to stress induction in M. sylvestris***




The ABA treated plants shoot tip showed elongation with increased number of axillary buds resulting in luxuriant plant growth (after 15-20 days) and subsequently showed the formation of lateral branches (after 25-35 days). After 10-15 days of silver nitrate treatment the shoot tips and axillary buds got blackened, with apical shoot tips drying up completely without revival. The plant grew horizontally with lateral branching (after 30-45 days) having bushy appearance. The UV-B treated plants had decreased axillary buds and lateral branches with decreased growth (Figure 3).

**Figure 3**
***Variations in plant growth with stress induction***

### *Variation in HPLC analysis*

An elevation in anthocyanin level was measured in all the treated plants. The total anthocyanin concentration was significantly increased from 49.7µg/g (control) to 220.4µg/g, 161.9µg/g and 89.5µg/g (ABA, silver nitrate and UV-B treated sample) amounting to 443.46%, 325.75% and 180.08% increase respectively (Table 2 and Box-plot 2). The HPLC chromatogram of the dried flower sample extract obtained in the visible spectral region (520nm) revealed two anthocyanidins, malvidin and delphinidin for control and all treated plants. According to area of the corresponding peaks for the two anthocyanindins, malvidin and delphinidin there was an increase in their concentrations in ABA, silver nitrate and UV-B treated extracts when compared to control. The malvidin content got increased to 103.9µg/g, 75.3µg/g and 49.3µg/g from 27.9µg/g and delphinidin increased to 81.6µg/g, 61µg/g and 27.4µg/g from 14.2µg/g respectively (Graph 1).

**Table 2**

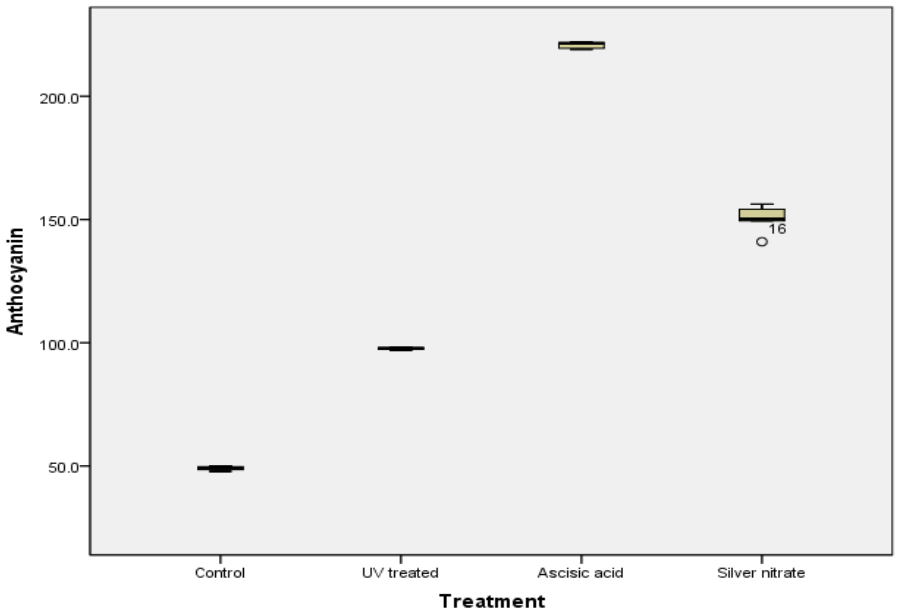
***Group statistics for variations in anthocyanin content due to different stress***

| Treatment | N | Mean± S.D | S.E Mean |
|---|---|---|---|
| Control | 5 | 49.060* ± 0.9236 | 0.4130 |
| Abscisic acid | 5 | 220.756* ± 1.4345 | 0.6415 |
| Silver nitrate | 5 | 160.200* ± 5.8587 | 2.6201 |
| UV treated | 5 | 89.660* ± 0.5320 | 0.2379 |

***Variations among different treatments at 5% level of significance***

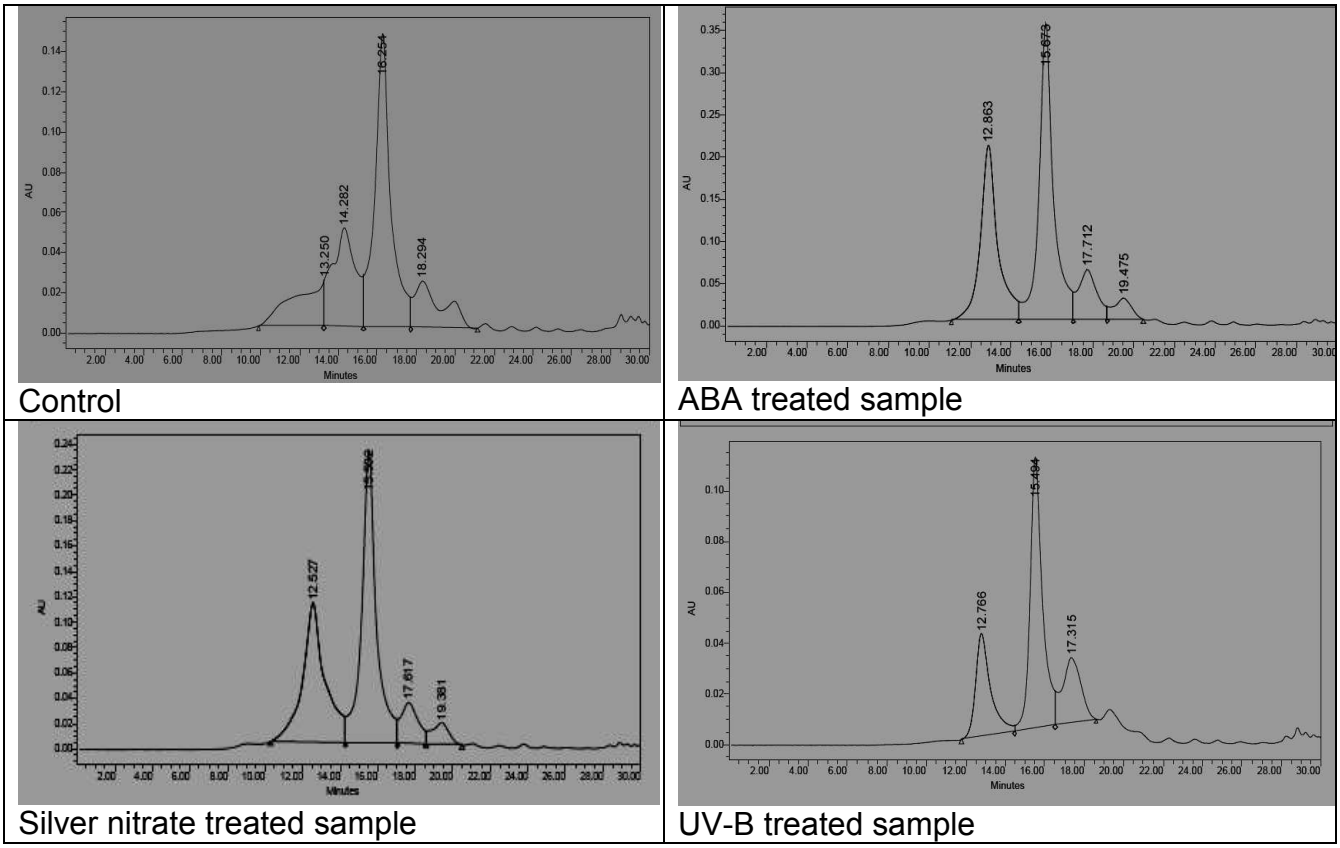
**Box-plot 2**

***Variations in anthocyanin content due to various stress in M. sylvestris***



**Graph 1**

***Variations in malvidin and delphinidin content by various stress induction***




Control | ABA treated sample

Silver nitrate treated sample | UV-B treated sample

## DISCUSSION

In the present study, stress induction from ABA, silver nitrate and UV-B treatment to *Malva sylvestris* plants was additive in the sense of anthocyanin production but non synergistic in terms of plant growth. During the study the ABA treated plants showed changes in shoot morphology with enhanced growth of shoot tips favoring increase in number of lateral branches, resulting in luxurious growth of the plant. The application of ABA at the beginning of the ripening process enhanced fresh and dry weight in the plant biomass[24]. ABA effect was predominantly seen on newly developing leaves, showing increase in leaf number and leaf area. The leaf showed softened texture with less curling when compared to controlled leaves and those leaves fully grown prior to ABA treatment remained unaffected[24-26]. In the study, the flowers showed deepened color from pinkish-purple to purplish-blue with increase in their size[27]. These effects of ABA for inducing morphological variations are substantiated by earlier studies[28-32]. The silver ions classically perform 'triple' response viz., vertical growth retardation, stem swelling and horizontal growth induction[12,33,34]. During the present study, the silver nitrate treated plants showed increased activity in the nodal region expressing numerous lateral branches with bushy appearance thereby enhancing dry and wet mass. The enhanced growth of internode in the treated plants infers that cell elongation in plants gets drastically affected than cell division thus resulting in stimulation of growth and development of lateral branches to increase plant height and weight[17]. The morphological changes seen in UV-B treated plant referred typically as a hallmark of stress and sensitivity of plants to radiations. The

reduced plant growth after treatment might be due to the synthesis of free radicals, which changes the membrane integrity of cells by disrupting the synthesis and transport of plant hormones[35,36]. The decrease in fresh and dry mass is mainly due to deficiency in photosynthetic activity with decreased enzyme activities[35]. The lesser leaf area and leaf number could be an adaptive mechanism to minimize the exposure to radiation[37] with reduced cell division, photosynthetic rate and calmodulin content, a key factor required for leaf growth[38,39]. During the study, the enhanced production of anthocyanins with ABA; silver nitrate; UV-B treatments can be related to, acceleration of ripening by endogenous ethylene synthesis; relative immobility of silver nitrate in plant tissues; photoinduction activating photoreceptors and phytochromes respectively[40]. Foliar applications with ABA and silver nitrate inhibited the ripening process which in turn inhibited the endogenous ethylene synthesis leading to an amazing induction of the enzymes involved in anthocyanin accumulation[41,17]. Phenolics in the epidermal layers of leaves and tissues are susceptible to UV damage and activate the enzymes chalcone synthase (CHS) or chalcone isomerase (CHI) in flavonoid biosynthetic pathway. The expression of flavonoid synthesis and associated genes could be enhanced by the presence of stress inducers[42]. Suttle et al[17] and Navabpour et al[43] provided evidence for anthocyanin accumulation with stress induction is genotype-dependent and reported enhanced mRNA accumulation of PAL gene-a key regulatory enzyme in anthocyanin biosynthesis, *Ufgt* gene-enzyme directing anthocyanin glycosilation and LSC54 gene-a senescence inducing gene[1,44].

## CONCLUSION

The extract of *Malva sylvestris* has been traditionally used since ages as an herb medicine in folk remedies to treat cough, pain, inflammation, and cancer. In the present study, ABA, silver nitrate and UV-B radiations were used as a stress “inducers” acted as “enhancers for anthocyanin production”. Although in nature, ABA a veraison molecule, silver nitrate a toxic substance and UV-B radiation a lethal component, when applied exogenously to plants at specific dosages induced morphological variations in plant growth and enhanced anthocyanin production. These anthocyanins can be intended to be employed as food colorants and antioxidant agents in food, pharmaceutical, and cosmetic industries.

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