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Enhanced Vigor in Growth and Accumulation of Anthocyanins with Abscisic Acid Treatment in *Malva sylvestris* L.

[1]N.R. Jayalakshmi, [1]K.J. Thara Saraswathi, [1]B. Vijaya, [2]D.N.S. Raman and [3]R. Suresh

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

Abstract: Plants of *Malva sylvestris* L. were studied for change in growth pattern and their anthocyanin production, malvidin and delphinidin with abscisic acid (ABA) treatment. Exogenous administration of ABA as foliar spray for five successive days significantly increased anthocyanin content up to five fold with induced morphological variations by increasing height, leaf mass, leaf number, fresh and dry weight in comparison to untreated plants. By using traditional chilled acidified methanol the total anthocyanins were extracted and estimated by pH differential spectroscopic method. The anthocyanins were purified by C18 Sep-Pak column and further analyzed by thin layer chromatographic (TLC) and high performance liquid chromatographic (HPLC) methods. The result of the study using ABA as stress inducer evidenced a positive response with induction of high anthocyanin production showing vigorous growth of the plants.

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 the plants actually respond to stressful conditions. Kacperska [1] defined stress as "potentially adverse environmental conditions (stressors) that affect plant growth and development, trigger a wide range of response, from altered gene expression and modification in cellular metabolism to change in growth rate and yield".

ABA is an important phytohormone and plays a critical role in response to various stress signals. The application of ABA to plant mimics the effect of a stress condition. The presence of ABA triggers heterophyllous switch during the adult vegetative phase, which in most tissues, result in the stimulation of growth and development. In plants, all three organs of vegetative phase (root, stem and leaf) respond to ABA [2]. ABA induces the formation of aerial type of morphology and enhances the differentiation of mesophyll, aerenchymal, stomatal cells with formation of epicuticular layer [2-4].

Abscisic acid is considered as the main triggering signal for the onset of ripening in plants, an increase in ABA concentration just before veraison is recognized as an important clue for the aging process [5, 6]. The precise conditions that initiate anthocyanin synthesis during veraison are unknown. One hypothesis suggests that sugar accumulation provide the substrate needed for anthocyanin synthesis. The accumulation of sugars beyond the immediate need of a tissue often favors the synthesis of secondary metabolites such as anthocyanins and other flavorants. However, as abscisic acid content is associated with sugar accumulation, it may be the abscisic acid that is the initial trigger for anthocyanin synthesis [7].

Malva sylvestris L. (mallow) is a perennial herb of Malvaceae. The plant harbours 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 [8]. 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 [9].

Corresponding Author: N.R. Jayalakshmi, Department of Microbiology and Biotechnology,
J.B. Campus, Bangalore University, Bangalore-560056, India.

The prime aim of the present investigation was to study the effect of ABA on plant growth pattern and stress induced accumulation of anthocyanins in *Malva sylvestris*.

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).

Methods

Abscisic Acid Treatment: 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) were taken for elicitation. 0.1mM ABA was sprayed onto the plants and exposed to the treatment for five successive days. The plants were observed for their morphological changes. The ABA treated flowers were picked and dried under shade, stored at 4°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 explained by Wrolstad [10]. Methanol:Acetic acid:Water in the ratio 49:1:50 was added to the powdered flower sample and incubated at 4°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°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 [11].

The sample was qualitatively purified with standard malvidin and delphinidin and were separated 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 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 1% level of significance.

RESULTS

Morphological Variations: After five days of ABA 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 133, 147 and 122.22% increase in plant height, leaf number and leaf area and 128% and 154.66% increase in fresh and dry weight biomass were recorded (Table 1 and Fig. 1a). The leaves of the ABA treated plants showed smooth appearance with less curling (Fig. 2). The flowers expressed deep coloration from pinkish-purple to purplish blue and showed an increase in flower size and number (Fig. 3). The shoot tip showed elongation with increase in number of axillary buds. This initially resulted in luxuriant plant growth (after 15-20 days) and subsequently showed the formation of lateral branches (after 25-35 days) (Fig. 4 and 4a).

Extraction and Analysis: In the present study, the 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 ABA. The total anthocyanin concentration was significantly increased from 49.7µg/g (untreated sample) to 220.4µg/g (ABA treated sample) amounting to 443.46% increase (Fig. 1b). The HPLC chromatogram of the dried flower sample extract obtained in the visible spectral region (520nm) revealed two anthocyanidins, malvidin and delphinidin for untreated and ABA treated plants (Fig. 5 and Fig. 6). According to area of the corresponding peaks there was an increase in concentration of anthocyanidins, malvidin (103.9µg/g),

Table 1: Effect of ABA on plant morphology and anthocyanin production

t-test for Equality of Means (for physical characters and anthocyanin content)					
Characters	t	df	N	Sig. (2-tailed)	Mean Difference± S.E
Plant height(cm)	-6.70	18	10	0.000	-13.4±2.0
Leaf number	-6.60	18	10	0.000	-28.4±4.3
Total leaf area(dm^2)	-3.10	18	10	0.006	-11.8±3.8
Fresh weight(gm/plant)	-5.10	18	10	0.000	-5.9±1.1
Dry weight(gm/plant)	-5.60	18	10	0.000	-1.3±0.2
Anthocyanin content (µg/g)	-225.02	8	05	0.000	-171.7±0.8

At 1% level of significance

Table 2: Recovery of anthocyanins by different extraction conditions

Extraction number	Extraction method applied	Solvent used	Temp (°C)	Acidification	Yeild (%)	Loss (%)	Anthocyanins
1	Soxhlet	Methanol	80°C-90°C	5% acetic acid	62	38	Only malvidin, no delphinidin
				1% Hcl	71	29	
				5% formic acid	60	40	
2	Partition	Acetone, chloroform	35°C-40°C	0.1- 0.01% Hcl	50-62	35-40	Malvidin and delphinidin (very less)
3	Cold incubation	Methanol	4°C	1% Hcl	95	05	Malvidin and delphinidin

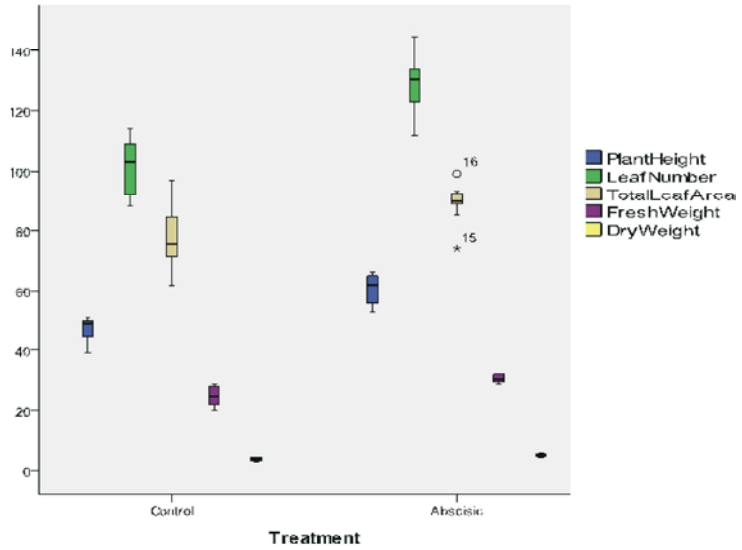

Fig. 1a: Statistical depiction in box plots for morphological variation

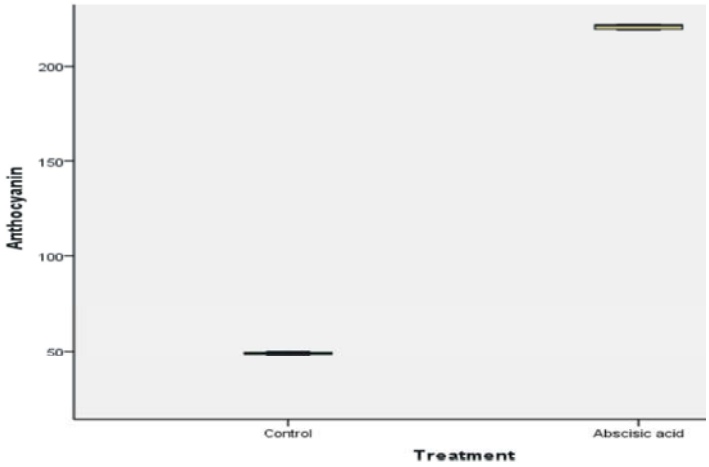

Fig. 1b: Statistical depiction in box plots for increased anthocyanin production

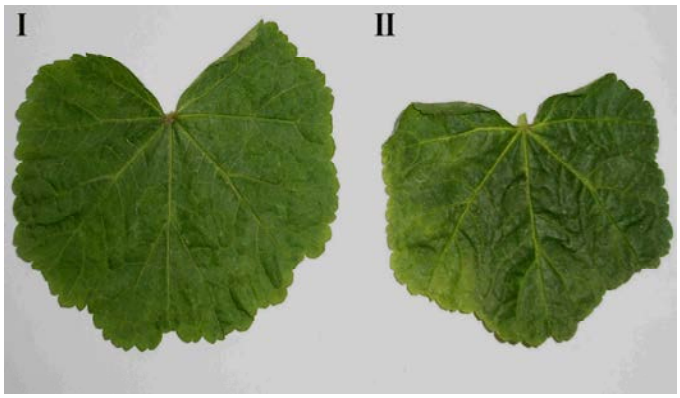

Fig. 2: Morphological variation in the leaves
I- ABA treated: big sized, less crumpled
II- Untreated: normal sized, more crumpled

Fig. 3: Morphological variation in flowers
I- Untreated: Pinkish-purple color, normal size
II- ABA treated: Purplish-blue color, big size

Fig. 4: Height difference with ABA treatment (after15 days)

Fig. 4a: ABA induced morphological variations Untreated plant:
I- Normal growth, without lateral branching
ABA treated plant:
IIa- Vigorous growth (incresein leaf size and no.) (after 15-20 days)
IIb- Profuse lateral branching (after 25-30 days)

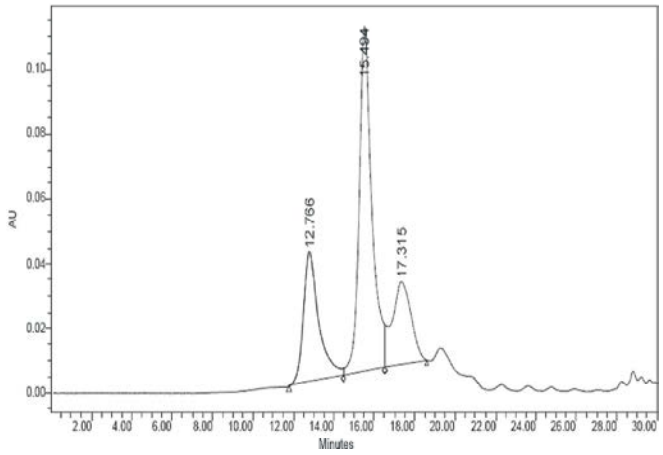

Fig. 5: HPLC chromatogram for untreated sample

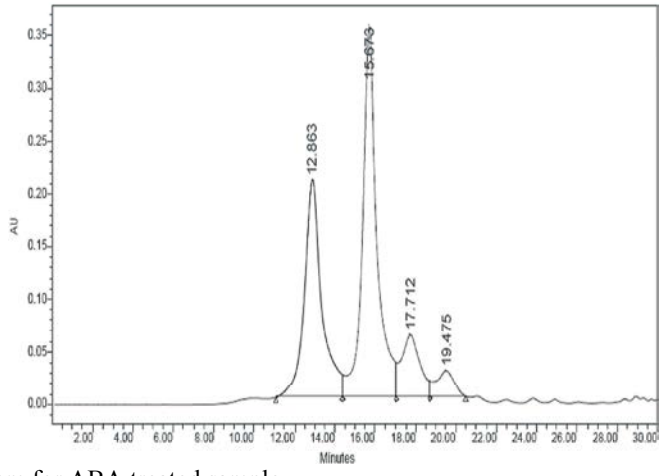

Fig. 6: HPLC chromatogram for ABA treated sample

delphinidin (81.6µg/g) in comparison to the untreated sample showing malvidin (27.9µg/g) and delphinidin (14.2µg/g) respectively.

DISCUSSION

In the present study, foliar application of ABA to *Malva sylvestris* plants was additive and synergistic with induction of high cellular proliferation and plant growth [12]. Morphological characters like plant height, leaf area and number, wet and dry mass of plant, flower size and color etc., were increased with ABA treatment [13].

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. This dramatic increase can be an integral part of the ABA induced morphogenesis [2]. 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 untreated leaves and those leaves fully grown prior to ABA treatment remained unaffected [2, 4, 14]. In the study, the flowers showed deepened color from pinkish-purple to purplish-blue with increase in their size [13]. The effects of ABA for inducing morphological variations have also been substantiated by earlier studies in various plants [15-19].

The solvent extraction of anthocyanin is initial step to determine total and individual anthocyanins prior to quantification, purification, separation and characterization [20]. The polar character of anthocyanins make them soluble in several types of solvents such as methanol, ethanol, acetone, water etc., generally involving 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 [21].

During the study, the enhanced production of anthocyanins with ABA treatment can be related to intermittent activation of few genes involved in anthocyanin biosynthesis. Studies have shown that, administration of ABA to plants mediated the acceleration of ripening by endogenous ethylene synthesis and induction of enzymes involved in anthocyanin accumulation [22]. Expression of flavonoid synthesis gene and those associated with anthocyanin synthesis were enhanced in the presence of ABA [23]. Ithal and Reddy [24] provided evidence to show that anthocyanin accumulation with ABA treatment is genotype-dependent and the ABA treatment also enhanced the mRNA accumulation of seven anthocyanin biosynthetic enzyme genes [7]. Several genes are involved in anthocyanin synthesis of which, the PAL gene (a key regulatory enzyme) and *Ufgt* gene (UDP glucose-flavonoid 3-o-glycosyl transferase- directs anthocyanin glycosilation) are critical and expected to be increased by ABA [25]. The increase in anthocyanin content as studied during the present investigation is justified by similar work carried out by earlier researchers done in various plants [7, 13, 23, 24, 25-29].

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, ABA used as a stress “inducer” acted as “plant growth enhancer” and “activator for anthocanin production”. Although ABA is a veraison molecule in nature, when applied on leaves exogenously induced morphological variations with accelerated plant growth and enhanced anthocyanin production in the present study. These anthocyanins can be intended to be employed as food colorants and antioxidant agents in food, pharmaceutical and cosmetic industries.

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