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BIOTECHNOLOGY

Expression of MYB Transcription Factor Genes in Response to Methyl Jasmonate, Salicylic Acid and Sodium Nitropruside in *Selaginella bryopteris* (L.) Baker

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ABSTRACT

The effects of elicitors [methyl jasmonate (MeJ), salicylic acid (SA) and sodium nitropruside (SNP)] on expression of three MYB transcription factor genes (*SbMYB1*, *SbMYB2* and *SbMYB3*) and flavonoid content was studied in *Selaginella bryopteris*. Gene expression analysis showed that *SbMYB2* was responsive to MeJ as its expression increased (1.6-2.36 fold) as compared to control between 3 to 6h. The effect of SA was most prominent on *SbMYB1* and *SbMYB2* as their expression level increased (11-7.9 fold for *SbMYB1* and 8.35 fold for *SbMYB2*) as compared to control between 3 to 6h. While the effect of SNP on expression *SbMYB2* and *SbMYB3* was prominent as compared to their respective control. The expression level increased (2.6-4 folds for *SbMYB2* and 1.15-3.16 folds for *SbMYB3*) between 3 to 6h. The effect of elicitors on the flavonoid production was also evident in the present study. The content of flavonoid in methanolic extract of MeJ treated sample was found to be increased (1.2 fold) after 3h but declined at 6h and 9h as compared to control. Similarly, the content of flavonoid in methanolic extract of SA treated sample was found to be higher (1.85 fold) than control at 3h and later declined at 6h and 9h.The flavonoid content in methanolic extract of SNP treated sample was higher (1.84 fold) at 3h, 6h (2.13 fold) and at 9h (1.42 fold) as compared to control. The correlation was established between the gene expression and flavonoid content in response to elicitors. Out of three MYB genes studied only *SbMYB2* was found to be most responsive to elicitors.

Highlights

- Out of three MYB genes (*SbMYB1*, *SbMYB2* and *SbMYB3*) studied only *SbMYB2* was found to be most responsive to elicitors (MeJ, SA, and SNP)
- The correlation was established between the gene expression and flavonoid content in response to elicitors

Keywords: *Selaginella bryopteris*, elicitors, MYB transcription factor, flavonoid content

Selaginella bryopteris (L.) Baker, commonly considered as "Sanjeevani-like plant", has traditionally been used as a remedy for several human health complications for centuries in India. The immense medicinal value of *Selaginella* species is largely due to the presence of large number of bioactive compounds, the most important being biflavonoids (Setyawan 2011). Amentoflavone, the most common biflavonoid of *Selaginella*, has various biological and pharmacological effects, including antioxidant (Shi *et al.* 2008), anti-cancer (Guruvayoorappan and Kuttan 2007), anti-inflammatory (Woo *et al.* 2005), antimicrobial (Jung *et al.* 2007), antivirus (Flavin *et al.* 2002), vaso-relaxation (Kang *et al.*

2004), anti-stomachic-ache (Kim *et al.* 1998), and anti-depressant (Baureithel *et al.* 1997). Ginkgetin is the second most studied biflavonoid of *Selaginella* beside amentoflavone. Water extract of *S. bryopteris* increases endurance to oxidative stress; and assists cell growth and protects from free radical stress caused by H_2O_2 (Sah *et al.* 2005). Flavonoids are synthesized via the phenylpropanoid (PP) pathway and flavonoid (FL) pathway using phenylalanine as precursor. MYB genes play important roles in the regulation of these biosynthetic pathways at the transcriptional level besides controlling other biological processes in plant (Stracke *et al.* 2007, Jung *et al.* 2008, Nakatsuka *et al.* 2012, Huang *et al.* 2013). The MYB family of proteins is large, functionally diverse and represented in all eukaryotes. Most MYB proteins function as transcription factors with varying numbers of MYB domain repeats conferring their ability to bind DNA.

Despite immense potential for commercial exploitation of this plant for bioactive molecules particularly flavonoids, it has been least studied on aspect of their biosynthesis. There was no report on molecular studies in this plant, prior to RNA-Seq based transcriptome analysis of *S. bryopteris* in our lab. Molecular aspect of biosynthesis of flavonoids involving transcription factors, transporters and biosynthetic pathway genes is very important for overall understanding of regulation of biosynthesis of flavonoids. In this direction, the present research work on the effects of elicitors [methyl jasmonate (MeJ), salicylic acid (SA) and sodium nitropruside (SNP)] on expression of MYB transcription factor genes and flavonoid content was studied.

MATERIALS AND METHODS

Plant Material

The plants of *S. bryopteris* were collected from Girihinda Hills (25°08′29′′N; 85°52′08′′E) near Sheikhpura town in Bihar, and maintained in pots containing soil, sand, compost (1:1:1) ratio at the Bihar Agriculture College, Sabour (25°14′12′′N; 87°02′57′′E). The present work was conducted at the Department of Plant Breeding and Genetics, Bihar Agricultural College, Sabour.

Elicitor treatment

To see the elicitor's response on gene expression and flavonoid content, fronds were treated with elicitors. The plant of *S. bryopteris* uprooted from pots and washed thoroughly and put into liquid KNOP medium (Reski and Abel 1865). Methyl Jasmonate (MeJ), salicylic acid (SA) and sodium nitropruside (SNP as donor of nitric oxide) were purchased from Sigma-Aldrich, USA. Stock solution (20 ml) of salicylic acid (1 mM) was prepared adding 2.762 mg SA in 1% ethanol. As surfactant Triton X-100 (Sigma-Aldrich, USA) 0.1% added in diluted spray solution of SA (100μM). Similarly, 10 ml of MeJ (100 μM) solution was prepared adding 229.3 μl MeJ from 4.362 M stock in 1% ethanol and 0.1% Triton X-10. While 100 ml stock of SNP (100 mM) was prepared adding 2.979 g SNP in 1% ethanol. SNP solution was diluted to 100 μM and 0.1% Triton X-10 added to it prior to spray on fronds. Experiments were carried out in triplicate.

RNA isolation

For the purpose of RNA isolation, the fronds (leaves), stem and roots of plants were harvested at different time point (3, 6, 9 h) post treatment along with their respective control and instantly preserved into tmsRNA Stabilizer reagent (Xcelris Genomics, Ahmedabad, India). Total RNA was extracted following protocol with slight modification as described earlier (Ghawana *et al.* 2011, Singh and Kumar 2012) and using RaFlex™ total RNA isolation kit (Banglore Genei, Banglore, India). Absorbance of RNA was measured using a spectrophotometer; the purity of RNA was determined by calculating the ratio of absorbance at 260 and 280 nm. A value for the ratio between 1.8-2.0 was considered ideal for finding the purity of RNA. The integrity of RNA was checked on a 1.2% agarose gel containing formaldehyde. For this, 1.0-5.0 μg of RNA was mixed with RNA loading dye, incubated at 65 °C for 10 min and electrophoresed at 72 volts in 1X MOPS buffer. Quality and quantity of RNA was assessed by monitoring absorbance using a Spectrophotometer (Genova Plus, JENWAY, U.K.). High quality RNA [with $A_{260/280}$ ratio of 1.98, $A_{260/230}$ ratio of 1.94] was used in the present study.

Reverse transcription-PCR (RT-PCR) analysis

The following components were added to a nuclease-free micro-centrifuge tube RNA Sample (2.0 μg), 10X DNase I Reaction Buffer (2.0 μl), DNase

Table 1: List of primers and PCR condition used for gene expression analysis

Name of primers	Sequence (5′→3′; Forward primer, F and Reverse primer, R)	PCR Condition
SbMYB1	F-GAGCTCCTTCAGCGTTTTGT	30 cycles: 94°C, 30 sec; 55°C, 40 sec; 72 °C, 1 min; Final extension at 72°C, 7 min
	R-GATGTTCTTGCCGGTTTCTT	
SbMYB2	F-AACCTCTGGTGGGCATAGTG	30 cycles: 94°C, 30 sec; 55°C, 40 sec; 72 °C, 1 min; Final extension at 72°C, 7 min
	R-GTCACCACCAACTGACAACG	
SbMYB3	F-TTCATCTCCACAACCATCCA	30 cycles: 94°C, 30 sec; 55°C, 40 sec; 72 °C, 1 min; Final extension at 72°C, 7 min
	R-GGACATGCTCTTCGTTCTCC	
26S rRNA	F-CACAATGATAGGAAGAGCCGAC	30 cycles: 94°C, 30 sec; 52°C, 40 sec; 72 °C, 1 min; Final extension at 72°C, 7 min
	R-CAAGGGAACGGGCTTGGCAGAATC	

I Amp grade (1.0 U /µl) (2.0 µl), DEPC-treated autoclaved distilled water added to make volume up to 20 µl. The reaction mixture was incubated for 15 min at 25 °C followed by addition of 2.0 µl of 25.0 mM EDTA to the reaction mixture. The reaction mixture was heated for 10 min at 65 °C and chilled on ice.

These components were added to a nuclease-free micro-centrifuge tube: 2.0 µg DNase I digested RNA, 1 µl Oligo (dT) (10 µM), 1 µl dNTP Mix (10.0 mM). The reaction mixture was heated at 65 °C for 10 min, placed on ice for 2 min followed by addition of the 4.0 µl 5X First Strand Buffer, 2.0 µl 100 mM DTT, 1 µl MMLV Reverse Transcriptase (200U/µl; Xcelris Genomics, Ahmedabad, India). These contents were mixed and centrifuged for a while to settle any component sticking to the walls of the tube. The tubes were incubated at 42 °C for 60 min and the reaction was terminated by heating the sample at 70 °C for 15 min.

PCR-based Gene expression analysis

SbMYB1, *SbMYB2* and *SbMYB3* sequences from NGS data of *S. bryopteris* available in our laboratory were used for primer designing by Primer 3 Input (Primer3_www.cgi v.0.2; http://frodo.wi.mit.edu/). PCR was carried out on a thermal cycler (Veriti®#9902, ABI, Singapore) as follows:, 10X PCR Buffer 2.5 µl, dNTPs (10 mM) 0.5 µl, Forward Primer (10.0 µM) 0.5 µl, Reverse Primer (10.0 µM) 0.5 µl, cDNA1.0 µl, *Taq* DNA Polymerase (5.0 U /µl; Xcelris Genomics, Ahmedabad, India) 0.25 µl and autoclaved distilled water 19.75 µl to make total reaction volume 25 µl. PCR was carried out using MYB specific primers, and the expression evaluated at exponential phase of amplification (Table 1). The cycles of PCR amplification was standardized initially, and the amplification in exponential phase was taken for analysis. PCR was carried out and the expression was evaluated at exponential phase of amplification. The cycles of PCR amplification was standardized initially, and the amplification in exponential phase was taken for analysis. Expression of *26S rRNA* was used as internal control to equalize cDNA quantity in various reactions (Singh *et al.* 2004). Gel was viewed on a UV trans-illuminator and captured on gel documentation system (UVITEC, Cambridge, U.K.). Intensity value of amplicons was calculated by Fire Reader software (UVITEC Cambridge, United Kingdom). The data was used to calculate the relative change in gene expression. The correlation analysis was done between gene expression and flavonoid content in tissues, and further to test the significance of correlation value, the p-value was calculated using a t -distribution with n−2 degrees of freedom.

Phytochemical analysis

Preparation of extracts

Plant parts of *S. bryopteris* (fronds, roots and stem) were dried in oven (Tanco, PLT12A) for eight hour at 55°C, and subsequently powdered using a mixer and grinder (Philips HL 3294/c, India). The powdered material of fronds, roots and stem, each of 2.5 g mixed with 100 ml of methanol and distilled water, separately and kept overnight at room temperature. Thereafter, these samples were sonicated at 33 KHz for 30 min using Ultra-sound Sonicator (Qsonica, USA) and filtered by filter paper (Whatman® No. 1). These filtrates were evaporated under reduced pressure using rotary

evaporator (Parmultico, India). The residue thus obtained were dissolved in an appropriate volume of distilled water and methanol, and stored at 4°C till further use.

Total flavonoid estimation

Flavanoid content in the plant extract of *S. bryopteris* was determined by spectrophotometric method (Quettier *et al.* 2000). The total flavonoid content was quantified using aluminium chloride method. In brief, 1 mL extract was taken in 10 ml volumetric flask containing 4 ml of distilled water, 0.3 mL 10% $AlCl_3$. $6H_2O$. The mixture was incubated for 6 min at room temperature. Thereafter, 2 mL of 1M NaOH were added and the solution was diluted to 10 mL with distilled water. The mixture was mixed well by vortex for 2 min. The absorbance was taken immediately at 510 nm through UV-VIS spectrophotometer. The concentration of the total flavonoid contents was expressed as mg/100g dry weight rutin equivalent. The estimation of flavonoids in the fraction was carried out in triplicate and the results were averaged.

RESULTS AND DISCUSSION

Transcriptional regulation of gene is achieved through binding of transcription factors to *cis*-acting regulatory elements. These are usually located in the promoter regions, which are located upstream of the coding sequences (Udvardi *et al.* 2007). MYB proteins are transcription factors, and understanding their regulation is important, as they play key roles in the regulation of biosynthesis of secondary metabolites including flavonoids at the transcriptional level besides controlling various biological processes in plant (Stracke *et al.* 2007, Jung *et al.* 2008, Nakatsuka *et al.* 2012, Huang *et al.* 2013). Biosynthesis and regulation of secondary metabolites are influenced by several environmental cues (Yazaki *et al.* 2002, Touno *et al.* 2005) including signaling network involving H_2O_2, NO, Ca^{2+}, cAMP, cGMP, MAPK cascades, SA, MJ, ethylene and ABA signalling (Li and Xue, 2010, Zhao *et al.* 2005). A number of flavonoid-related MYB transcription factors were identified in model plants, such as *Arabidopsis thaliana* and *Zea mays*, but few have been identified in woody plants (Dubos *et al.* 2010, Li 2014, Liu *et al.* 2015). Specific R2R3-MYB and bHLH transcription factors interact with WDR proteins to form MBW complexes that contribute to the tight regulation of expression of late flavonoid biosynthetic pathway genes (Xu *et al.* 2015).

Effect of elicitors on expression of MYB transcription factor genes

The gene expression analysis was conducted in fronds in response to three elicitors MeJ, SA and SNP. From the gene expression it can be visualized that *SbMYB2* was most responsive to MeJ as its expression increased between 3 to 6h (1.6-2.36 fold) as compared to control (Fig.1 a, d). In contrary, the expression of *SbMYB1* didn't show any response while *SbMYB3* expression declined. The exogenous MeJ elicited massive accumulation of caffeoylputrescine in tomato leaves by up-regulating genes of phenylpropanoid and polyamine pathways (Chen *et al.* 2006). MeJ was reported to stimulate the synthesis of shikonins by up-regulating the expression of PGT (Yazaki *et al.* 1997, Matsuno *et al.* 2002, Yazaki *et al.* 2002). There were reports that the genes involved in the biosynthesis of the triterpene aglycone of saponin are up-regulated by MeJ, including squalene synthase (SS), squalene epoxidase (SE) and b-amyrin synthase (b-AS) (Suzuki *et al.* 2002, Hayashi *et al.* 2003). Yi *et al.* (2016) reported that in response to MeJ and SA treatment on various MYB genes in broccoli and Kale leaves it was found that MYB28 gene (Bol036743) was up-regulated in broccoli leaves under MeJ treatment, whereas MYB28 genes (Bol007795) in broccoli and Bol036286 in kale leaves were down-regulated under both MeJ and SA treatment. Similarly, MYB51 genes (Bol013207 and Bol030761) were up-regulated in kale under SA treatment and MYB122 gene Bol026204 was up-regulated in broccoli leaves under MeJ treatment *In Panx ginseng* out of four *PgMYBs* studied, *PgMYB3* was up-regulated and other three were down-regulated in response to MeJ and SA, suggesting a role for all these genes in stress response (Choi *et al.* 2017).

The effect of SA was prominent on *SbMYB1* and *SbMYB2* as their expression level increased between 3 to 6h (11-7.9 fold for *SbMYB1* and 8.35 fold for *SbMYB2*) as compared to control, while *SbMYB3* expression declined (Fig. 1 b, c). SA reported to inhibit the activity of PAL, a key enzyme in the synthesis of phenolic compounds and stimulates activity of chalcone synthase a key

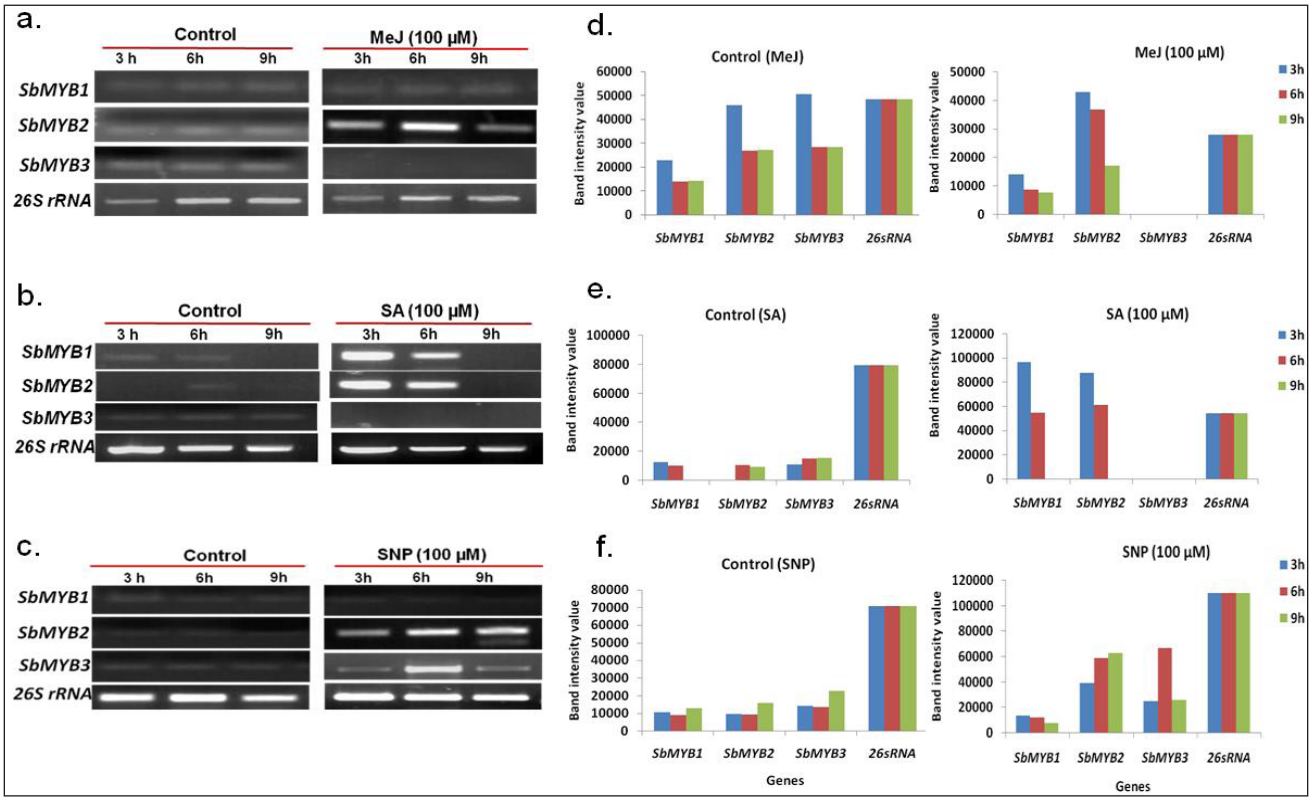

Fig. 1: Expression of *SbMYB1*, *SbMYB2* and *SbMYB3* in frond of *S. bryopteris* in response to (a) MeJ (100 µM), (b) SA (100 µM) and (c) SNP (100 µM) at 3, 6 and 9h after the treatment. *26S rRNA* was used as an internal control as shown previously (Singh *et al.* 2004, Singh *et al.* 2010). Graphical representation of respective gene expression after normalization of band intensity value based upon the amplicons for *26S rRNA* (d-f)

enzyme in the synthesis of flavonoids (Nicholson and Hammerschmidt 1992). Specifically, regarding SA exogenous application, it may also induce the expression of many defense genes which encode particular enzymes of secondary metabolic pathway to form bioactive compounds such as phenolics (Ali *et al.* 2007). The higher SA (250 mM) dose in *M. chamomilla* resulted in the rise of the activity of the enzyme PAL, followed by an increase in the accumulation of soluble phenolic compounds and lignin (Kováčik *et al.* 2009). SA is known to induce gene expression related to biosynthesis and production of some classes of secondary metabolites in plants, which function as phytoalexins (Taguchi *et al.* 2001). Saha *et al.* (2016) reported five differentially up-regulated *BrMYBs* (*BrMYB55*, 118, 147, 217 and 222) against *Fusarium* treatment in Chinese cabbage (*Brassica rapa ssp. pekinensis*), which were also found to be induced against JA and SA treatments. Particularly, *BrMYB55*, 147 and 217 showed up-regulation (2-4 folds) against exogenous JA and SA treatments.

The effect of SNP on gene expression was prominent on *SbMYB2* and *SbMYB3* as compared to their respective control. The expression level increased between 3 to 6h (2.6-4 fold for *SbMYB2* and 1.15-3.16 fold for *SbMYB3*) (Fig. 1 c, e). In contrast, *SbMYB1* didn't show any response of SNP. NO (released by SNP) is a signal molecule that was reported to stimulate the regulation of secondary metabolites in plants like potato, soybean and *Taxus* (Wu *et al.* 2009). NO affects the activities of a variety of nuclear regulatory proteins and the formation of S-nitrosylated proteins seems to be an especially important mechanism in the regulation of the function/activity of transcription factors (Grun *et al.* 2006). We could not find any study related to effect of NO on MYB gene expression in plants. But its effect have been studied in several pathogen-induced genes (e.g. NBS-LRRs,NDR1), genes coding for disease resistance proteins and several plant defense response modulating transcription factors, like WRKYs, EREBPs (ethylene responsive element binding proteins) several zinc finger proteins, and dehydration responsive element binding proteins (DREB1and DREB2), were induced by the NO donor

SNP (Grun *et al.* 2006).While its effect on genes of biosynthetic pathways were reported by Wu *et al.* (2009), in which they found that the expression level of genes of three key enzymes (*PGT*, *PAL*, and *HMGR*) involved in shikonin biosynthesis could be stimulated by SNP (40 µM) in *Onsoma paniculatum* cells.

Effect of elicitors on flavonoid content

MeJ treatment showed initially positive effect on flavonoid content in fronds of *S. bryopteris*. The content of flavonoid in methanolic extract was found to be increased (1.2 fold) after 3h but declined at 6h and 9h as compared to control (Fig. 2 a).

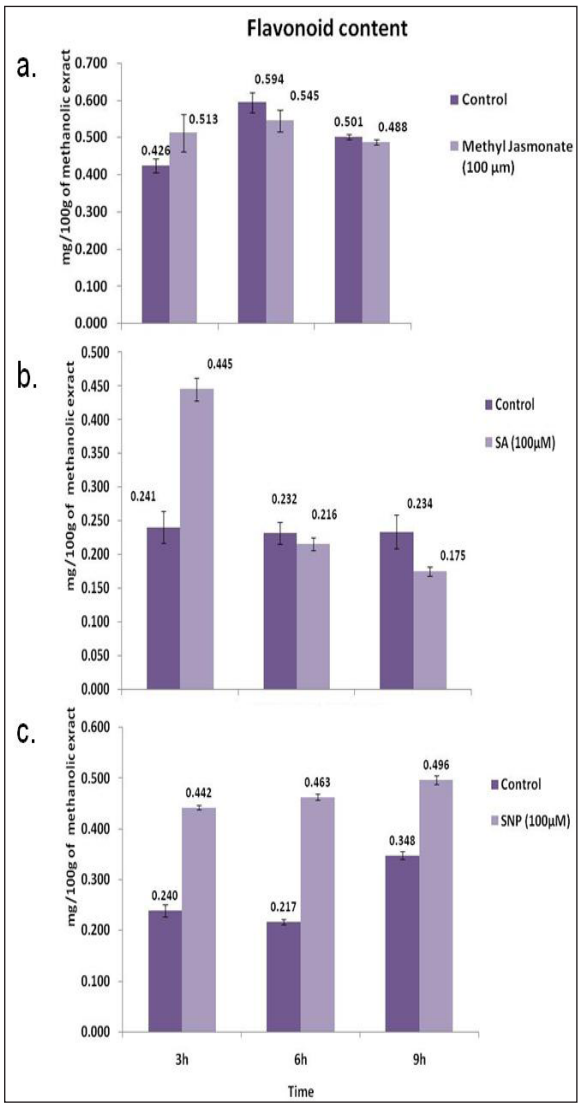

Fig. 2: Effect of MeJ (100 µM), SA (100 µM) and SNP (100 µM) on flavonoid content in fronds of *S. bryopteris*. The methanolic extract of fronds was prepared and flavonoid content estimated at 3, 6 and 9 h after the treatment. Similarly, one control was also set up i.e. with water. All the values for flavonoid content represents mean of three replicates ± standard deviation (SD).

MeJ induced biosynthesis of volatile organic compounds was reported in grape berries of *Vitis vinifera* cv. Lemberger, in which the emission of linalool rapidly surged within 2-6 hours after application of the elicitor (May and Wust 2015). Enhanced production of flavonoids by MeJ elicitation in cell suspension culture of *Hypericum perforatum* was reported in which the treatment of the cell cultures with 100 µM MeJ on day 15 resulted in the highest flavonoid production (280 mg/L) and 2.7 times of control cultures and the activities of *PAL* increased, which led to the enhancement of flavonoid production (Wang *et al.* 2015). Parthenolide content in feverfew (*Tanacetum parthenium*) leaves was quantified by high-performance liquid chromatography after foliar application of MeJ (100 µM) on in time course experiment (3-6-9 h), the results showed that exogenous application of MeJ or SA activated parthenolide biosynthesis as the parthenolide content reached its highest amount at 24 h after the treatments and were 3.1- and 1.96 fold higher than control plants, respectively (Majdi *et al.* 2015).

The SA also had positive effect at 3 h on flavonoid content in fronds of *S. bryopteris* (Fig. 2 b). The content of flavonoid in methanolic extract was found to be 1.85 fold higher than control at 3h and later declined at 6h and 9h. Our result is in agreement with the previous report that the parthenolide content in feverfew plant (*Tanacetum parthenium)* increased after foliar application of salicylic acid (1.0 mM) (Majdi *et al.* 2015). SA also reported to induce the expression of genes related to biosynthesis and production of phytoalexins, these are secondary metabolites involved in defense response of the plant against pathogens (Taguchi *et al.* 2001). The induction mechanism of defense is generally thought to be related to the elevation of ROS including H_2O_2, which could serve as secondary messengers in defense signaling pathway (Ebel and Mithofer 1998, Qian *et al.* 2006, Jannat *et al.* 2011).

Like SA and MeJ, SNP (100 µM) also elicited the flavonoid production in fronds of *S. bryopteris.* The effect of SNP on flavonoid content was higher (2.4 fold) at 3h, and declined at 6h (1.14 fold) and again high at 9h (1.42 fold) as compared to control (Fig. 2 c). Previous studies were reported that NO is being involved in elicited production of secondary

metabolites such as ginseng saponin (Hu *et al.* 2003), hypericin (Xu *et al.* 2005), puerarin (Xu *et al.* 2006), catharanthine (Xu and Dong 2005), artemisinin (Zheng *et al.* 2008), and taxanes (Wang *et al.* 2006) in plant cell and tissue cultures. NO elicited the synthesis of phenolics, flavonoids, and caffeic acid derivatives in the adventitious roots of *Echinacea purpurea, w*hen roots were treated with 100 μM SNP (Wu *et al.* 2007). Addition of SNP at 10–160 μM significantly increased shikonin production by 30.1–78.1% at the end of the culture period compared with the control, showing the maximum effect at 40 μM (Wu *et al.* 2009).

The correlation analysis was done between gene expression and flavonoid content in tissues used in the present study (Table 2). The p-value was calculated using a t -distribution with n–2 degrees of freedom. It was found that *SbMYB1* gene expression was positively correlated (0.89) with flavonoid content in response to SA and negatively correlated with SNP (-0.98). While *SbMYB2* showed positive correlation with content of flavonoid in response to MeJ (0.68), SA (0.82) and SNP (0.88). In case of *SbMYB3* a negative correlation was found with SNP (-0.11).

Table 2: Correlation between expressions of MYB genes with flavonoid content in fronds of *S. bryopteris*

Gene expression	Flavonoid content		
	MeJ	SA	SNP
SbMYB1	0.058*	0.89*	-0.98*
SbMYB2	0.68*	0.82*	0.88*
SbMYB3	—	—	-0.11*

**Significant at P < 0.05 and P < 0.01*

Gene expression analysis suggested that enhanced flavonoid content by elicitors in fronds of *S. bryopteris* could be the result of up-regulation of flavonoid biosynthetic pathway genes. In contrary, for the decline in content of flavonoid a feed forward or feedback inhibition could be the reason. For the similar case in *A. euchroma*, possibility of the substrate/product mediated feed-forward and feed-back inhibition of PGT and HMGR and other enzymes of shikonins biosynthesis pathway were envisaged for decrease in shikonins accumulation in response to mevinolin (Singh *et al.* 2010). Feed-back and feed-forward regulation of biosynthetic pathways is well known mechanism in plants and animals (Goldstein *et al.* 2006, Oulmouden and Karst 1991).

CONCLUSION

In the present study, the expression pattern of three genes of MYB transcription factor (*SbMYB1*, *SbMYB2* and *SbMYB3*) was studied in response to elicitors (MeJ, SA, and SNP) in *S. bryopteris*. The response was evident on the expression of MYB genes and flavonoid production. Out of three MYB genes studied, *SbMYB2* was found to be highly responsive to known elicitors (MeJ, SA, and SNP) of FL pathway. This could be helpful in future study related to modulation of flavonoid biosynthesis.

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