Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 238-241
Received: 17-03-2020
Accepted: 19-04-2020

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Biochemical constituents in transgenic mulberry expressing barley *HVA1*, *tobacco osmotin* and *BCH1* (β-Carotene Hydroxylase) genes

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Abstract
Mulberry is a perennial tree crop, trait improvement through traditional breeding is cumbersome and time consuming. Recently, mulberry transgenic plants expressing *Arabidopsis* gene, *AtSHN1* to improve post-harvest water loss and impart stress tolerance has been developed. Mulberry is rich sources of protein, carbohydrate, carotenoids, lipids, ascorbic acid, anthocyanins etc. Hence the current investigation was undertaken to study the biochemical constituents in leaves of transgenic mulberry lines. Biochemical analysis revealed that transgenic mulberry lines showed significant difference for chlorophyll a content with maximum being in non-transgenic genotype V1 (0.84 mg/g FW) followed by transgenic lines, ST series (0.87 mg/g FW) and BT series (0.81 mg/g FW). Similarly, Highest chlorophyll b content was observed in non-transgenic mulberry line V1 (0.83 mg/g FW) followed by transgenic lines ST series (0.78 mg/g FW), MT series (0.74 mg/g FW) and BT series (0.70mg/g FW). For the total chlorophyll content, the experiment revealed that all six treatments were significantly different. Among six treatments, V1 was found to be having highest total chlorophyll content (1.66 mg/g FW). The maximum protein content of 14.25 mg/g was noticed in transgenic lines, MT series followed by RD series (14.00 mg/g) wherein non-transgenic type the content was around 11.50 mg/g tissue. The highest carbohydrate content (0.28 mg/g) was recorded in ST series followed by V1 series which recorded 0.27 mg/g of carbohydrate content and RD series with 0.24 mg/g of carbohydrates.

Keywords: Mulberry, chlorophyll, silk worms, protein

Introduction
Mulberry is a perennial tree crop, trait improvement through traditional breeding is cumbersome and time consuming. Combined efforts by conventional breeding and modern biotechnological approaches are needed to improve mulberry leaf yield and quality under stressful conditions. To achieve a quick progress in crop improvement in this perennial tree crop, biotechnological interventions by genetic engineering would be one of the best options, especially when traits are governed by one or a few genes. Similar other major crops for targeted crop improvement, attempts have been made through modern biotechnological approaches, including transgenic in mulberry (Das *et al*., 2011; Checker *et al*., 2012) [2, 1]. The targeted manipulation of elite mulberry genotypes through incorporation of specific genes encoding desired traits using modern biotechnological methods offers a new opportunity for crop improvement. Such an attempt has been possible due to development and standardization of *in vitro* regeneration and transformation protocols in recalcitrant mulberry (Sajeevan *et al*., 2011; Raghunath *et al*., 2013) [4, 3]. There is an option for rapid and precision tree improvement program through targeted genetic manipulation using transgenic technologies.
There are transgenic approaches attempted in mulberry with varied degrees of success (Das *et al*., 2011; Checker *et al*., 2012; Sajeevan *et al*., 2017) [2, 1, 5]. Recently, mulberry transgenic plants expressing *Arabidopsis* gene, *AtSHN1* to improve post-harvest water loss and impart stress tolerance has been developed (Sajeevan *et al*., 2017) [5]. The transgenic plants displayed dark green shiny appearance with increased leaf surface wax content (Sajeevan *et al*., 2017) [5]. In these transgenic plants increased wax content altered leaf surface properties. The transgenic plants showed significant improvement in leaf moisture retention capacity and there was slow degradation of proteins in detached leaves compared to non-transgenic plants. The study demonstrated the possibility of specific trait manipulation using transgenic approach in mulberry.
Mulberry transgenic research carried out at the University of Delhi South Campus, New Delhi, resulted in the development of stable transgenic plants expressing barley *HVA1*, *tobacco Osmotin* and *BCH1* (β-carotene hydroxylase) genes (Lal *et al*., 2008; Das *et al*., 2011; Checker

et al., 2012; Saeed *et al*., 2015) [6, 1, 2, 7]. Controlled laboratory studies indicated that these transgenic plants exhibit superior phenotypes under stressful conditions. The *BCH1* transgenic plants are more tolerant to high-light and high temperature and to UV stress than the non-transgenic plants under growth room conditions. The transgenic lines have also been developed with inducible promoters with the *HVA* gene so as to control the expression of this gene under stress. It is believed that the tobacco *osmotin* gene expressed under a constitutive and an inducible promoter to in mulberry would be better suited to tolerate not only salt, drought and cold stresses but is also capable of providing protection upon fungal pathogenesis. From this context the major goal of the study was to evaluate the select transgenic lines expressing barley *HVA1*, *tobacco Osmotin* and *BCH1* (β-carotene hydroxylase) genes their suitability for silkworm rearing.

Material and Methods

The experiment was conducted in green house/ containment facility of the Department of Crop Physiology, and Department of Sericulture, University of Agricultural Sciences (UAS), GKVK campus, Bengaluru, Karnataka. The site is situated at 12° 58' North latitude, 77° 35' East longitudes and an altitude of 930M above Mean Sea Level (MSL). The plant species used for the study were transgenic plants of *Morus indica* L. and cultivated mulberry genotype V1. All the transgenic materials (VR series- Rd29A::*HVA;* ST series-CaMV35S::*HVA1;* BT series-CaMV35S::*bch1;* MT series-CaMV35S::*Osmotin* and RD series- Rd29A::*Osmotin*) were developed at the University of Delhi, South Campus, New Delhi and transferred to the Department of Crop Physiology, UAS, Bengaluru as per the Department of Biotechnology, Government of India guidelines. These plants were multiplied and grown in the containment facility. The transgenic mulberry lines were raised and maintained in pots, which were initially pruned at once and were regularly irrigated once in two days. Each plant was provided with 5.00 g N, 2.88 g P and 3.00 g K after 15 days of pruning. Leaves were harvested on 45th day for silkworm rearing.

Chemical Constituents of Transgenic Mulberry Varieties

Bio-chemical constituents such as carbohydrates, leaf protein, chlorophyll 'a', chlorophyll 'b', total chlorophyll, nitrogen, phosphorus, potassium, calcium, magnesium, sulphur and zinc in the leaves of different transgenic mulberry varieties were estimated. The biochemical analysis was carried out in Department of Crop physiology and Department of Soil Science and Agricultural Chemistry, UAS, GKVK, Bengaluru.

Estimation of Chlorophyll (mg/g FW)

The leaf discs were collected from wild type and transgenic plants to estimate the chlorophyll content. About 100mg of leaf discs were incubated in acetone (80%, v/v): dimethyl sulfoxide (1:1) solution (10 ml) overnight. The extract was used to determine total chlorophyll content by taking absorbance at 645, 652 and 663 nm, using spectrophotometer. Chlorophyll a, chlorophyll b and total chlorophyll were estimated by substituting the absorbance values in the formulae given below and expressed as mg g^{-1} fresh weight (Arnon, 1949).

Chlorophyll a: (12.7 (A_{663})-2.69(A_{645}) V) / (weight * 1000)

Chlorophyll b: (22.9 (A_{645})-4.68(A_{663}) V)/(weight * 1000)

Total chlorophyll: (Chlorophyll a + Chlorophyll b)

Estimation of Carbohydrates (mg/g)

Carbohydrates content in different mulberry samples was quantitatively measured by the method provided by Dubios *et al*. (1956) [9]. For estimation of carbohydrates content, 2 g leaves of different position of mulberry plant samples were grinded in distilled water with the help of mortar and pestle. Leaf samples were the centrifuged at 5000 rpm for 10 minutes. 0.5ml clear supernatants with 0.5 ml distilled water were collected in different test tubes and 4 ml of Anthrone reagent was added to obtain green colour. The absorbance of green colour was taken to estimate the carbohydrate content at 625 nm wave length by using U-V Spectrophotometer. The carbohydrates content was calculated by standard sugar solutions (Dextrose L) method and was expressed as mg/g.

Estimation of Leaf Protein (mg/g of fresh weight of tissue)

Leaf (3rd, 4th and 5th from top of the plant) samples were collected in liquid N_2. The extract and measurement of soluble proteins was performed according to the method of Bradford (1976), where 50 mg of sample homogenized in 500 µl phosphate buffer (pH-7.4) transferred to eppendorf tube, centrifuged at 10000 rpm for 10 minutes, and the supernatant was transferred to new eppendorf tube and stored at-20^{0}C until further estimation. From the supernatant 10 µl of sample was added to 290µl of Bradford reagent where the sample colour turns to blue. The final absorbance was seen at 595 nm. Protein concentration was calculated through standard curve using bovine serum albumin.

Result and Discussion

The results revealed that these mulberry lines showed significant difference for chlorophyll a content with maximum in transgenic mulberry line (0.87 mg/g FW) followed by V1variety, (0.84 mg/g FW) and BT series (0.81 mg/g FW), while least chlorophyll content was recorded in transgenic lines VR series with 0.63 mg/g FW of chlorophyll a (Table 1and fig.1).

Similarly, chlorophyll b content was found to be significantly different among the six mulberry lines evaluated (Table 1). Highest chlorophyll b content was observed in wild mulberry line V1 (0.83 mg/g FW) followed by transgenic lines ST series (0.78 mg/g FW), MT series (0.74 mg/g FW) and BT series (0.70 mg/g FW), while least chlorophyll b content was observed in VR series (0.50 mg/g FW).

For the total chlorophyll content, the experiment revealed that all six treatments were significantly different. Among six treatment non-transgenic mulberry (V1) was found to be having highest total chlorophyll content (1.66 mg/g FW) followed by ST series with total chlorophyll content of 1.65 mg/g FW and BT series (1.51 mg/g FW). The least total chlorophyll content was observed in VR series with 1.13 mg/g FW. The protein content though non-significant was higher in MT series and ST series. Accumulation of higher levels of chlorophyll, protein and carbohydrates has facilitated higher cocoon weight, shell weight and silk productivity in transgenic lines. Similar results wherein V1 was better for chlorophyll, protein and carbohydrate content has been reported by Ahmed, (2002) [10], Mamatha, (2014) [12] and Manjula and Kumar (2014) [13] when compared to other varieties of mulberry such as M5, DD and S36. Transgenic mulberry expressing *AtSHN1* had higher protein, chlorophyll and sugars compared to untransformed mulberry wild type plant (Pallavi, 2015) [11]. However in the present study V1

was on par with transgenic mulberry line ST series for the above parameters.

Total buffer soluble protein was extracted and quantified in the leaf tissue of transgenic and wild type plants. Total buffer soluble protein was estimated by Bradford's reagent method. The maximum protein content of 14.25 mg/g was noticed in transgenic lines (MT series) followed by ST series (14.00 mg/g), whereas in non-transgenic plants (V1) the content was around 11.50 mg/g tissue (Table 1).

All the experiment material revealed significant difference for the carbohydrate content. The highest carbohydrate content (0.28 mg/g) was recorded in ST series followed by V1 series which recorded 0.27 mg/g of carbohydrate content and RD series with 0.24 mg/g of carbohydrates. The least value observed in VR series (0.19 mg/g). MT, BT and VR series were found to be on par with each other (Table 1).

Table 1: Biochemical constituents in the leaves of transgenic mulberry lines

Transgenic/ Non-transgenic lines[@]	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll (mg/g FW)	Protein (mg/g)	Carbohydrate (mg/g)
VR SERIES	0.63^{a}	0.50^{a}	1.13^{a}	10.25	0.19^{a}
ST SERIES	0.87^{d}	0.78^{bc}	1.65^{c}	14.00	0.28^{e}
BT SERIES	0.81^{abcd}	0.70^{bc}	1.51^{bc}	11.00	0.22^{abc}
MT SERIES	0.68^{abc}	0.74^{bc}	1.43^{abc}	14.25	0.21^{ab}
RD SERIES	0.66^{ab}	0.64^{ab}	1.30^{ab}	12.75	0.24^{bcd}
V1 SERIES	0.84^{bcd}	0.83^{c}	1.66^{c}	11.50	0.27^{de}
F test	*	*	*	NS	*
Sem±	0.06	0.05	0.11	1.79	0.01
CD at 5%	0.18	0.16	0.32	-	0.03

Note: Significant at 5%, NS: Non- Significant, Figure with same super script are statistically on par. [@] VR-Rd29A::*HVA1*, ST-CaMV35S::*HVA1*, BT-CaMV35S::*bch1*, *MT*-CaMV35S::*Osmotin*, RD-Rd29A::*Osmotin*, V1- *Victory1*

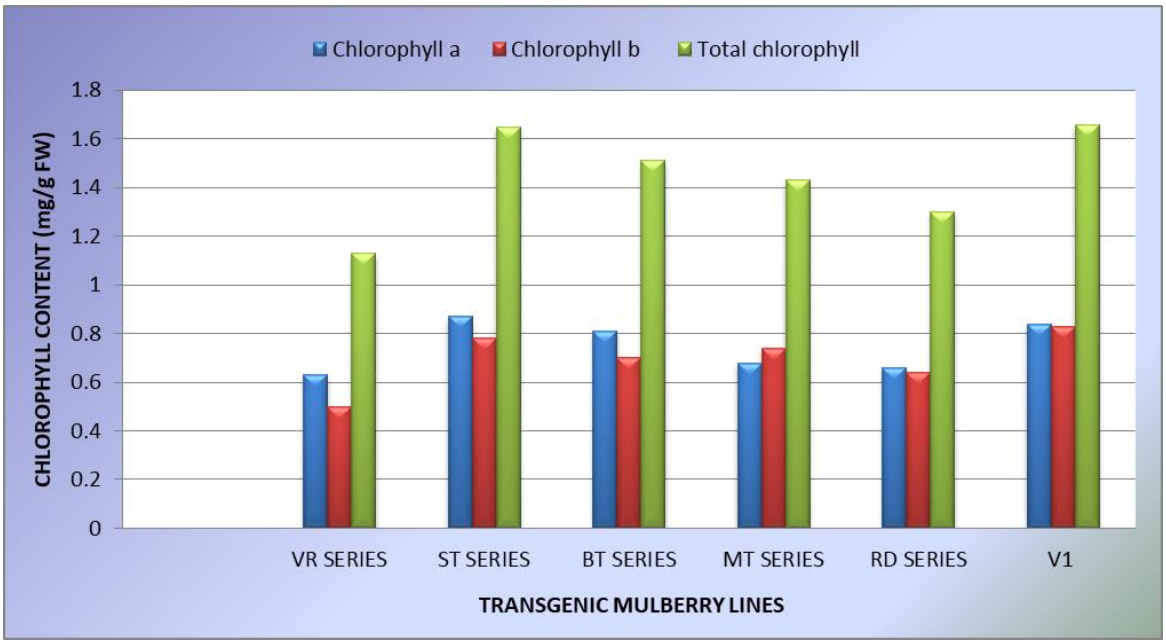

Fig 1: Chlorophyll contents in the leaves of transgenic mulberry lines.

The protein content though non-significant was higher in MT series and ST series. Accumulation of higher levels of chlorophyll, protein and carbohydrates has facilitated higher cocoon weight, shell weight and silk productivity in transgenic lines. Similar results wherein V1 was better for chlorophyll, protein and carbohydrate content has been reported by Ahmed, (2002) [10], Mamatha, (2014) [12] and Manjula and Kumar (2014) [13] when compared to other varieties of mulberry such as M5, DD and S36. Transgenic mulberry expressing *AtSHN1* had higher protein, chlorophyll and sugars compared to untransformed mulberry wild type plant (Pallavi, 2015) [11]. However in the present study V1 was on par with transgenic mulberry line ST series for the above parameters.

Conclusion

Biochemical analysis revealed that transgenic mulberry lines showed significant difference for chlorophyll a content with maximum being in non-transgenic genotype V1 (0.84 mg/g FW) followed by transgenic lines, ST series (0.87 mg/g FW) and BT series (0.81 mg/g FW). Similarly, Highest chlorophyll b content was observed in non-transgenic mulberry line V1 (0.83 mg/g FW) followed by transgenic lines ST series (0.78 mg/g FW), MT series (0.74 mg/g FW) and BT series (0.70mg/g FW). For the total chlorophyll content, the experiment revealed that all six treatments were significantly different. Among six treatments, V1 was found to be having highest total chlorophyll content (1.66 mg/g FW). The maximum protein content of 14.25 mg/g was noticed in transgenic lines, MT series followed by RD series (14.00 mg/g) wherein non-transgenic type the content was around 11.50 mg/g tissue. The highest carbohydrate content (0.28 mg/g) was recorded in ST series followed by V1 series which recorded 0.27 mg/g of carbohydrate content and RD series with 0.24 mg/g of carbohydrates.

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