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RESEARCH PAPER

Assessing the expression of plant metabolites in different cultivated cotton species at various stages of plant growth and development

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Abstract: Plants synthesize various metabolites in different concentrations at various stages of growth and development to exhibit natural defense mechanism and adapt to diverse stresses. The present study was aimed to evaluate the levels of primary and secondary metabolites in four cultivated *Gossypium* spp. at different stages of growth and development. The total soluble protein content was significantly increased in *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* with the age of the plant with maximal accumulation at 120 DAS (144.32, 111.35, 67.56, 120.54 mg/g fr. tissue), whereas, the carbohydrate content exhibited differential expression with optimal accumulation at 60 DAS (176.43, 178.04, 151.26 and 182.64 mg/g fr. tissue) respectively. Secondary metabolites increased significantly with the growth stage of the plant and at 90 DAS the metabolites like gossypol (0.82, 1.53, 0.92 and 1.03 mg/g fr. tissue), total phenols (15.75, 14.49, 10.24 and 11.24 mg/g fr. tissue), flavonoids (32.11, 30.14, 27.61 and 32.92 mg/g fr. tissue) and tannins (163.04, 161.71, 176.38 and 238.47 mg/g fr. tissue) accumulated to maximum levels in *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum*. The study provides information on distribution of plant metabolites in cotton at various stages of their growth and development.

Keywords: Cotton, Host plant resistance, Plant metabolites, Protein

Introduction

Cotton (*Gossypium* spp.), 'The silver fiber', also known as 'king of natural fibers' and 'white gold' is an important commercial crop of India which plays a significant role in Indian farming and industrial economy of the country by providing raw material to the textile industry. Cotton belongs to Malvaceae family and genus *Gossypium*. About 50 cotton species have been recognized in the world, out of which only four species are cultivated in different regions both in the old world and new world, of which *Gossypium arborerum* and *Gossypium herbaceum* are diploids ((AA) ($2n = 2x = 26$)) and *Gossypium hirsutum* and *Gossypium barbadense* are tetraploids ((AADD) ($2n = 4x = 52$)). In India, all the four cultivable species of cotton and different hybrid combinations are grown commercially (Khadi *et al.,* 2010).

Plants synthesize a large diversity of low molecular weight organic compounds from simple inorganic precursors which play a definite physiological role in their growth and development. Phytochemicals are grouped into two categories based on their role in major metabolic processes, i.e., primary metabolites and secondary metabolites (Krishnaiah *et al.,* 2009). Primary plant metabolites are engaged directly in growth and metabolism that includes amino acids, proteins, carbohydrates, lipid and chlorophylls (Fernie and Pichersky, 2015), whereas, secondary plant metabolites, are products of subsidiary pathways such as the shikimic acid pathway that comprises of alkaloids, steroids, saponins, flavonoids, tannins and phenols (Hartmann, 2007). Secondary metabolites are the by-products of primary metabolism and are generally not involved in metabolic activity but exhibit definite functions, such as attracting pollinators or defence against pathogen and herbivore attack (Cheenickal and Sheela 2016). A large number of secondary metabolites have a direct role in plant defence (Moraes *et al.,* 2008).

Host plant resistance results from the expression of genes responsible for the chemical or physical attributes that interfere with herbivore and pathogen attack (De-Ponti and Garretsen 1980). The plant secondary metabolites together with nutrients and fibers form an integrated part of natural defense system against various biotic and abiotic stress conditions. The variability in the quantity of secondary plant metabolites though depends on the genetic description, biotic and abiotic factors also influence their expression (Villagrasa *et al.,* 2006; Oikawa *et al.,* 2004). Understanding the expression pattern of plant metabolites is therefore necessary for exploitation of host plant resistance to control insect pests for sustainable production of cotton. Although there are several reports on expression of plant metabolites in relation to biotic and abiotic factors, but there is a lack of information on the expression pattern of plant metabolites at different growth stages in cotton. The present work was therefore conducted to quantify the levels of both primary and secondary metabolites in four cultivated species of *Gossypium* at different stages of growth and development.

Material and methods

Cultivated cotton species *viz*. *G. hirsutum* (Sahana, Source: ARS, Dharwad), *G. barbadense* (Suvin, Source: CICR Regional Station Coimbatore), *G. arboreum* (DLSa-17, Source: ARS, Dharwad) and *G. herbaceum* (DDhC-11, Source: ARS, Dharwad) were evaluated for plant metabolites at different stages of plant growth. The sowing of cotton was done in deep black soil field

by dibbling at Agricultural Research Station, Dharwad Farm, Dharwad during 2019-20. The plot size was 3.6 x 6 m^2. Each plot accommodated four rows with 10 plants per row and a total of 40 plants per plot were maintained and the experiment was laid out with three replications following randomized block design. An intra row spacing of 60 cm and inter row spacing of 90 cm was followed. In each plot plant population was maintained with gap filling and thinning after a week of germination. The fertilizer application was at the rate of 100:50:50 kg of NPK/ha in the form of urea, DAP and MoP with two splits of N, at sowing and at 40 DAS. The crop was kept weed free through regular intercultural operations and hand weeding. The crop was protected from insect pests and diseases by following management strategies as provided in package of practices, University of Agricultural Sciences, Dharwad.

Collection of Leaf Samples : Leaf samples from all the four species were collected at 30 day interval *i.e*., from 30 DAS (days after sowing) up to 120 DAS. Fully opened top leaf from five randomly selected plants, one from each, was collected as a representative of entire plot from each of the replication, and brought to laboratory in an ice bouquet. The leaves were ground to fine powder in a mortar with a pestle using liquid nitrogen and preserved at -20°C until further used.

Quantification of Primary Metabolites: Protein and carbohydrate content was assessed in the leaf samples collected at 30, 60, 90 and 120 DAS from *G. hirsutum, G. barbedense, G. herbaceum* and *G. arboreum* cotton plants. For estimation of total soluble proteins, about 100 mg of leaf sample was disrupted in 2 ml of ice-cold extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 5 mM 2-mercaptoethanol, 1% polyvinylpyrrolidone, 1 mM DTT and 0.5 mM EDTA) by using bead-ruptor (Sharma *et al*., 2016). The homogenate was centrifuged at 10,000 rpm for 15 min. and the protein content in the supernatant was estimated according to the Lowry method (Lowry *et al,* 1951) using bovine serum albumin (BSA) as a standard. For estimation of total carbohydrates, about 5 ml of 2.5 N HCl was added to 100 mg of leaf samples and incubated in boiling water bath for 3 h. After the incubation period, the samples were cooled to room temperature, neutralized by adding sodium carbonate until effervescence ceases. The volume was then made up to 10 ml, centrifuged at 10,000 rpm for 10 min. at 4°C and the supernatant was analysed for carbohydrate content by adopting anthrone reagent method with some modifications using glucose as a standard (Sadasivam and Manickam, 2008).

Quantification of Secondary Metabolites : Gossypol was extracted by plunging the leaf sample (100 mg) into boiling 95 % ethanol for 5 min. The extract was filtered and the residue was re-extracted and the filtrates were combined. The extract was diluted to 40% ethanol and pH was adjusted to 3.0 using 1 N HCl. The contents were mixed with 1.5 volumes of diethyl ether at 10°C using a separating funnel. The ether phase was preserved and washed twice with distilled water. The ether extract was evaporated to dryness under vacuum and the residue was re-dissolved in 95% ethanol and to 0.1 ml of extract, 0.5 ml of phlorglucinol reagent and 1 ml of concentrated HCl was added and incubated for 30 min. with occasional shaking. The contents were diluted to 5 ml with 80% ethanol and the absorbance was read at 550 nm in spectrophotometer against reagent blank. Amount of gossypol was quantified using standard curve of gossypol and expressed as mg/g fresh tissue (mg/g fr. tissue) (Sadasivam and Manickam, 2008).

Total phenol content was estimated in cotton leaf samples by following Folin–Ciocalteau reagent method (Bray and Thorpe 1954). About 100 mg of leaf sample was ground in 80% ethanol by using bead ruptor, then the homgenates were centrifuged at 10,000 rpm for 15 min. The supernatant was saved and the residue was re-extracted thrice with ethanol and all the extracts collected after centrifugation was pooled in a test tube. The extracts were evaporated to dryness. Leftover residue was dissolved in 2 ml of distilled water. To 0.1 ml of aqueous extract, 0.5 ml of Folin-Ciocalteau reagent was added and incubated at room temperature for 3 min. After incubation, 2 ml of 20% Na_2CO_3 was added mixed properly and incubated in boiling water bath for exactly 1 minute. Samples were cooled rapidly and the absorbance was read at 650 nm against reagent blank. Amount of total phenols was assessed from a standard curve prepared using catechol and expressed as mg equivalents of catechol/g fr. tissue.

For flavonoid content analysis cotton leaves (100 mg) were ground with sufficient amount of methanol and water mixture (1:1) using bead ruptor and filtered. The pellet was extracted again and both the filtrates were combined. The filtrates were subjected to evaporation of most of the methanol and the volume was reduced to one-third of the original. The concentrated filtrates were washed thrice with hexane by using separating funnel and the aqueous layer was collected for determination of flavonoid content. About 0.1 ml of extract was evaporated to dryness in boiling water bath. To the residue, 4 ml of vanillin-H_2SO_4 reagent was added and incubated in boiling water bath for 15 min. and the absorbance was recorded at 340 nm spectrophotometrically (Egbuna *et al.,* 2019). Flavonoid content was calculated from a standard curve prepared using catechin and expressed as mg equivalents of catechin/g fr. tissue.

Tannin content was estimated according to vanillin hydrochloride method by adopting slight modifications (Bray and Thorpe 1954). About 100 mg of leaf sample was ground in 2 ml of methanol using bead ruptor, and the tubes containing the methanol extract was left at room temperature for 20-28 h. by occasional swirling. The supernatant was collected by centrifugation. To 0.1 ml of methanolic extract, 2.5 ml of vanillin-HCl reagent was added and the absorbance was recorded at 500 nm spectrophotometrically after 20 min. of incubation at room temperature. Tannin content was determined from the standard curve prepared using catechin and expressed as mg equivalents of catechin/g fr. tissue.

Statistical analysis

The data of the experiment was analysed statistically following the procedure described by Gomez and Gomez (1984).

The level of significance used in the 'F' and 't' test was p = 0.05. The critical difference was calculated wherever the 'F' value found to be significant by using RBD.

Results and discussion

Primary metabolites in cultivated species of cotton

Total soluble protein content in *G. hirsutum, G. barbadense*, *G. herbaceum* and *G. arboreum* at 30 DAS was 62.62, 61.62, 38.37 and 44.86 mg/g fr. tissue, which increased significantly at 60 DAS (66.31, 65.62, 52.43, 78.64 mg/g fr. tissue), 90 DAS (90.81, 78.97, 55.67, 98.37 mg/g fr. tissue), and 120 DAS (144.32, 111.35, 67.56, 120.54 mg/g fr. tissue) respectively (Table 1). Whereas, the carbohydrate content in cotton species, *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* at 30 DAS was measured to be 162.18, 158.85, 175.40, and 175.74 mg/g fr. tissue, which increased non-significantly at 60 DAS (176.43, 178.04, 176.26, 182.64 mg/g fr. tissue), but reduced significantly at 90 DAS (89.65, 93.90, 85.40, 100.11 mg/g fr. tissue), which again increased considerably at 120 DAS (126.32, 213.10, 153.10 and 172.75 mg/g fr. tissue) respectively (Table 2). The total soluble proteins and total carbohydrates were differentially expressed among the four species, except that the protein content in *G. herbaceum* was measured to be low throughout the various growth stages when compared to the other species.

Primary metabolites are essentially required for the growth of plants. The growth dependent accumulation of total soluble proteins was recorded in all the four *Gossypium* spp. reaching to a maximum concentration at 120 DAS, whereas the amount of total carbohydrates was maximum at 60 DAS, later reduced drastically at 90 DAS, and again increased at 120 DAS, but the concentration was significantly low when compared to at 30 DAS in all the four species. This is a natural phenomenon in plants where the primary metabolites accumulate progressively during their growth and later they are deviated to various plant parts to meet the requirement for other vital processes which is again depended on the physiology of the species. Cotton plant spends its vegetative growth stage till 35 to 40 DAS, and appearance of the first square can be seen at around 40 DAS, and from 80 DAS onwards it enters boll bearing stage wherein it deviates most of the energy for boll filling. The reduced levels of carbohydrates at 90 DAS in *Gossypium* spp. was therefore due to the deviation of photosynthates to filling of bolls. The total sugars in cucumber reached maximum to 4.26% and 4.10% during the second week after sowing in the first and second season, then gradually decreased at 14th week and again increased to 4.10% and 3.98% at the end of growth in both the seasons, respectively, with a similar trend for the levels of total amino acids, conjugated amino acids and total nitrogen (Kamel and El-Gengaihi 2009).

Secondary metabolites in cultivated species of cotton

Plants have developed diverse strategies for protection against different stressful factors through development of structures like thorns, glandular hairs, foliar trichomes, and production of secondary metabolites (Anaya *et al*., 2003; Granados *et al*., 2008). Secondary metabolites do not have any direct role in the growth and development of plant, but they provide protection from abiotic and biotic stresses. Secondary metabolites in cultivated species of cotton in the present study were observed to be accumulative with the age of the plant and found maximum at 90 DAS which reduced drastically at 120 DAS.

The gossypol content in the leaves of cotton species, *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* at 30 DAS was 0.49, 0.54, 0.86 and 0.70 mg/g fr. tissue, which increased significantly at 60 DAS (0.80, 1.06, 0.88, 0.93 mg/g fr. tissue) and 90 DAS (0.82, 1.53, 0.92 and 1.03 mg/g fr. tissue) but decreased drastically at 120 DAS (0.32, 0.29, 0.25, 0.19 mg/g fr. tissue) respectively (Table 3). The total phenol content in cotton species, *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* at 30 DAS was recorded to be 1.30, 2.28, 1.69, 1.27 mg/g fr. tissue, which increased significantly at 60 DAS (6.13, 3.73, 2.70, 5.22 mg/g fr. tissue), and accumulated to greater levels at 90 DAS (15.75, 14.49, 10.24, 11.24 mg/g fr. tissue), then reduced significantly at 120 DAS (1.66, 2.72, 0.35, 2.73 mg/g fr. tissue) respectively (Table 4).

The flavonoid content in cotton species, *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum* at 30 DAS was corresponding to 14.38, 18.04, 12.19, 14.21 mg/g fr. tissue, which increased significantly at 60 DAS (17.40, 20.42, 15.35, 17.69 mg/g fr.

Table 1. Total soluble protein content in cultivated species of cotton at different plant growth stages of cotton species.

	Protein (mg/g fr.tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	62.62	66.31	90.81	144.32
G. barbedense	61.62	65.62	78.97	111.35
G. herbaceum	38.37	52.43	55.67	67.56
G. arboreum	44.86	78.64	98.37	120.54
SE. m ±	0.74	0.34	0.53	0.59
C.D. @ 1%	2.62	1.22	1.87	2.08

Table 2. Carbohydrate content in cultivated species of cotton at different plant growth stages of cotton species.

	Carbohydrate (mg/g fr.tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	162.18	176.43	89.65	126.32
G. barbedense	158.85	178.04	93.90	213.10
G. herbaceum	175.40	151.26	85.40	153.10
G. arboreum	175.74	182.64	100.11	172.75
SE. m ±	0.31	0.23	0.25	1.64
C.D. @ 1%	1.11	0.83	2.22	1.71

Table 3. Gossypol content in cultivated species of cotton at different plant growth stages of cotton species.

	Gossypol (mg/g fr.tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	0.49	0.80	0.82	0.42
G. barbedense	0.54	1.06	1.53	0.34
G. herbaceum	0.86	0.88	0.92	0.28
G. arboreum	0.70	0.93	1.03	0.15
SE. m ±	0.02	0.023	0.01	0.01
C.D. @ 1%	0.07	0.08	0.06	0.03

Table 4. Phenol content in cultivated species of cotton at different plant growth stages of cotton species.

	Phenol(mg/g fr. tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	1.30	6.13	15.75	1.66
G. barbedense	2.48	3.73	14.49	2.72
G. herbaceum	1.69	2.70	10.24	0.35
G. arboreum	1.27	5.22	11.24	2.73
SE. m ±	0.05	0.10	0.07	0.22
C.D. @ 1%	0.18	0.36	0.25	0.77

Table 5. Flavonoids content in cultivated species of cotton at different plant growth stages of cotton species.

	Flavonoids(mg/g fr. tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	14.38	17.40	32.11	28.19
G. barbedense	18.04	20.42	30.14	22.47
G. herbaceum	12.19	15.35	27.61	23.90
G. arboreum	14.21	17.69	32.92	22.40
SE. m ±	0.23	0.20	0.17	0.34
C.D. @ 1%	0.82	0.73	0.61	0.89

Table 6. Tannins content in cultivated species of cotton at different plant growth stages of cotton species.

	Tannins (mg/g fr. tissue)			
	30 DAS	60 DAS	90 DAS	120 DAS
G. hirsutum	48.00	73.90	163.04	26.28
G. barbedense	51.80	84	161.71	98.85
G. herbaceum	70.85	94.09	176.38	56.38
G. arboreum	82.66	111.80	238.47	76
SE. m ±	0.77	0.70	0.58	0.11
C.D. @ 1%	1.71	1.68	2.05	0.39

tissue) and 90 DAS (32.11, 30.14, 27.61, 32.92 mg/g fr. tissue), but decreased gradually at 120 DAS (28.19, 22.47, 23.90, 22.40 mg/g fr. tissue) (Table 5). Likewise, the tannin content in *G. hirsutum, G. barbadense, G. herbaceum* and *G. arboreum,* at 30 DAS was corresponding to 48.00, 51.80, 70.85, 82.66 mg/g fr. tissue, which increased significantly at 60 DAS (73.90, 84.00, 94.09, 111.80 mg/g fr. tissue) and 90 DAS (163.04, 161.71, 176.38 and 238.47 mg/g fr. tissue), but decreased gradually at 120 DAS (26.28, 98.85, 56.38 and 76 mg/g fr. tissue) (Table 6).

As the flowering in cotton plant progresses to the top, the plant puts all its energy in boll development and ceases further flowering and growth which slows down the other metabolic pathways too, thus resulting into reduced amounts of secondary metabolites at 120 DAS. The composition of plant metabolites is dependent on the genetic makeup of a species. Secondary metabolites vary qualitatively and quantitatively between different species, populations of the same species, individuals of a population and between the organs of a certain individual (del Valle *et al.,* 2015). Quantity of primary and secondary metabolites is usually influenced by the plant developmental stage and environmental conditions (Krischik and Denno 1983). Higher phenol content was observed during vegetative stage which decreased significantly at fruit set stage in *Achillea millefolium* L.*,* a medicinal plant, and a similar pattern was observed for flavonoid content but there was no significant difference in flavonoid content at both the stages (Farhadi *et al.,* 2020). In contrast, the secondary metabolites in *Rumex crispus* L. and *R. obtusifolius* were higher in quantity during flowering and fruiting period (Feduraev *et al.,* 2019). The levels of gossypol, total phenols, flavonoids and tannins were differentially expressed among the four species and comparable to each other at corresponding growth stage.

Cotton is a host for a variety of insect pests which comprises sucking and chewing pests that poses a large economic impact on cotton production. With the increase in concern over development of resistance to Bt toxin in bollworms, there has been renewed attention in improving the host plant resistance by increasing levels of phytochemicals that are toxic to insect pests. Susceptibility or tolerance of a plant to insect pests is usually depended on the composition of phytochemicals. The constitutive expression of defensive phytochemicals in a plant enhances its natural defence mechanism to insect pests. The information on expression pattern of the plant metabolites in the present study is thus useful for evaluation and better exploitation of host plant resistance in cotton against insect pests.

Conclusion

In conclusion, among the primary metabolites, total soluble proteins were accumulated progressively with the growth of the cotton plant reaching to maximum levels at 120 DAS, but total carbohydrates exhibited differential expression at various growth stages with optimal production at 60 DAS, whereas, the defensive secondary metabolites were accumulated to maximum levels at 90 DAS in cultivated *Gossypium* spp. The information generated by the study on distribution of plant metabolites in cultivated species of cotton at various stages of growth can be utilized in screening of cotton cultivars and better exploitation for host plant resistance against insect pests.

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