Original Research Article

Study on Variation of non-polar metabolites in *Gossypium hirsutum* L. under water stress condition using gas chromatography-mass spectroscopy technique

Abstract

Present work was aimed at studying variation of non polar metabolites content in *Gossypium hirsutum* L. under water stress condition using a gas chromatography-mass spectrometry (GC-MS) technique. A total 17 non-polar metabolites were detected in control and water stressed *G. hirsutum* leaf. The major metabolites were quinoline derivative (26.37±0.29%), 2-methylhexadecan-1-ol (7.47±0.07%), phytol (7.71±0.02%), myristic acid (5.94±0.04%), hexadecanol (14.30±0.94%), nonadecane (1.67±0.05%) and palmitic acid (3.20±1.39%). Fourteen metabolites were detected in control and water stressed *G. hirsutum* stem. The major metabolites were dodecene (1.67±0.11%), L-lysine (0.65±0.06%), dibutylphthalate (5.06±1.88%), linoleic acid (10.26±0.07%), campesterol (0.87±0.04%) and stigmasterol (1.13±0.55%). Statistical analysis of GC-MS data was carried out by Mann-Whitney U test without normal distribution using statistical software SYSTAT version 12.0. Significant variation in content of the most of the metabolites were observed between control and water stressed leaf or stem (Mann-Whitney U test, P =0.05). It concludes that the major metabolites played an important role during water stress and can be consider as metabolites responsible for water stress tolerance in *G. hirsutum.*

Keywords: *Gossypium hirsutum***,** water stress, metabolites, gas chromatography-mass spectrometry.

Introduction

Cotton is one of the most important industrial crops under the genus “Gossypium” in the Malvaceae family and popularly known as “white gold” [1]. Globally, the Gossypium genus includes about 50 species [2]. There are four main species in the genus Gossypium, namely *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L. domesticated independently as sources of textile fibre. *Gossypium hirsutum* L. was named due to its hairiness (hirsute), it is commonly known as upland cotton, American cotton or Mexican cotton [3]. Globally, about 90% of all cotton production is of cultivars derived from this species. It is native to Mexico, the West Indies, northern South America, Central America

and possibly tropical Florida. *Gossypium hirsutum* includes a number of varieties or cultivars with varying quality. Cotton requires a minimum temperature of 16 °C during germination, 21 °C to 27 °C for proper crop growth and during the fruiting phase, the temperature ranging from 27 °C to 32 °C. It is cultivated largely under rain fed or dry land conditions and its harvesting period begins from mid-September to November [4]. It can successfully grow on all soils except sandy, saline or water logged types. It is moderately tolerant to salinity but sensitive to water logging as well as frost and extreme cold temperature [5]. Cotton has been utilized as fibre material since ancient times [6]. It is harvested as seed cotton which then ginned in order to separate the seed and linter. Processed cotton (linter) can be used in a variety of products including foods. The linters which have a longer fibre length can be used in the production of mattresses, furniture upholstery and mops. Linters have a much shorter fibre length and are the major source of cellulose for both food and other applications. It is also used in a variety of products including edible vegetable oils and margarine, soap and plastics. Its seeds and flour or hulls are also used in food products for animal feed [7].

Water stress is one of the most important environmental factor which affects crop productivity and adversely affects fruit production, square and boll shedding and fiber quality in cotton [8]. Moreover, water stress is considered as the single most devastating environmental factor [9]. It severely affects plant development with substantial reductions in crop growth rate and biomass accumulation by reduction in the cell division, root proliferation, plant water and nutrient relations [10, 11]. Previous studies revealed that 2 to 4 °C increase in temperature and the expected 30% decrease in precipitation may adversely affect crop productivity and water availability by the year 2050 [12]. Thus, screening cotton varieties for resistance to water stress conditions and improving cotton tolerance to this stress conditions will mitigate negative consequences of this adversity. Cotton is normally not classified under water stress tolerant crop as some other plants species like sorghum [13]. Nevertheless, cotton has mechanisms that make it well adapted to semi-arid regions [14]. An understanding of the response of cultivars to water deficits is also important to model cotton growth and estimate irrigation needs [15]. The alteration of metabolites due to water stress was previously reported for plant species and considered to be responsible for water stress tolerance [16, 17].

Lv *et al.*, evaluated five homozygous transgenic *G. hirsutum* L. plants under water stress condition and the result suggested that glycine betaine may be involved in osmotic

adjustment in the plant [18]. Rodriguez-Uribe et al., used microarray analysis to identify water deficit-responsive genes in the *G. hirsutum* under water stress conditions [19]. Yoo and Wendel, conducted comparative transcriptome profiling of developing *G. hirsutum* fibres using RNA-Seq by Illumina sequencing [20]. Although some other aspect of the changes in *G. hirsutum* under water stress conditions have been reported, there has been no reports on a thorough study on non polar metabolites content and their variation in *G. hirsutum* under water stress condition by gas chromatography-mass spectrometry (GC-MS) method. This can be an important study for identifying the metabolites responsible for water stress tolerance in *G. hirsutum* under water stress condition. Therefore, it was imperative to study the variation of non-polar metabolites in *G. hirsutum* plants under water stressed condition since identifying the metabolites responsible for water stress tolerance may be helpful for agriculture researchers in understanding metabolic pathways during water stress.

Material and Methods

Cotton seeds were purchased from Central Institute for Cotton research, Regional station, Coimbatore, Tamil Nadu, India. These seeds were sown in trays (52 cm x 27 cm) placed in a cultivation chamber. The seedlings were transplanted into pots. After four months, the best plants of approximately the same height and with the same number of leaves were selected for the study (Figure.1). Further, these selected plants were divided into two groups. First group of plants were irrigated in every 12 hour interval at room temperature and considered as control plant. While second group plants were maintained in the same environment as the control plants but without addition of water to the container for 4 days. This would allow the pots to dry out and plants were then considered as water stressed. Finally leaf and stem samples were collected from each group of plants for further study.

Figure 1. Selected plant of *G. hirusitum*

Dried samples of 3g of each leaves and stems were taken for extraction with hexane (1:10 w/v). The solvent portion was collected by filtration and repeated five times until the hexane layer became almost colourless. The separated solvent layer was concentrated under reduced pressure by using evaporator. The resulting sticky mass was stored at -5 ºC. Volatile trimethylsilyl (TMS) derivatives of the samples were prepared by using 3.6 mg of the sample, 40 µl of methoxylamine hydrochloride in GC grade pyridine (20 mg/ml). The mixture was shaken for 2 hours at 37 °C in a temperature controlled vortex, followed by the addition of 70 µl of the N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). Thereafter, the mixture was further continuously shaken for 30 min at the same condition. After completion of TMS derivatization, 1µl of derivatized mixture was taken for GCMS analysis. The GC-MS analysis was performed using a GCs-Agilent 7890 A coupled with a 5975 C MS: MS detector and Electron Impact Ionization to generate mass spectra. The scan mass range was 30m/z-600m/z and the total run time in minutes was 54 min. The split was 1:90, with helium as the carrier gas at a flow rate of 1 ml/min, while the damping gas flow was 0.3 ml/min. The GC oven temperature program was as follows: 40 °C-220 °C, by ramping at 3 °C, and held at 220 °C for 20 min. The injector temperature was maintained at 220 °C and the transfer line was held at 220 °C. The resulting GC-MS profile was analyzed using the NIST mass spectral library and by matching the chromatogram with appropriate standards. The estimation of the metabolites was done using the percentage peak area that appeared at the total ion

chromatogram in the GC-MS analysis. The molecular weights and fragmentation patterns were ascertained by use of the NIST library and the Duke phytochemical data base.

The Mann-Whitney U test, a nonparametric test of the null hypothesis was used to compare differences in metabolites content between two independent groups i.e., control and water stressed leaf or stem. Statistical analysis of metabolites was carried out by Mann-Whitney U test without normal distribution using statistical software SYSTAT version 12.0 (Microsoft Corp. SYSTAT Software, Inc., USA).

Results and Discussion

Different non-polar metabolites were identified from non-polar extracts of leaf and stem of *G. hirsutum* (Table 1). Plotted GCMS chromatogram of the control and water stressed leaf of *G. hirsutum* are shown in figure 2 and 3.

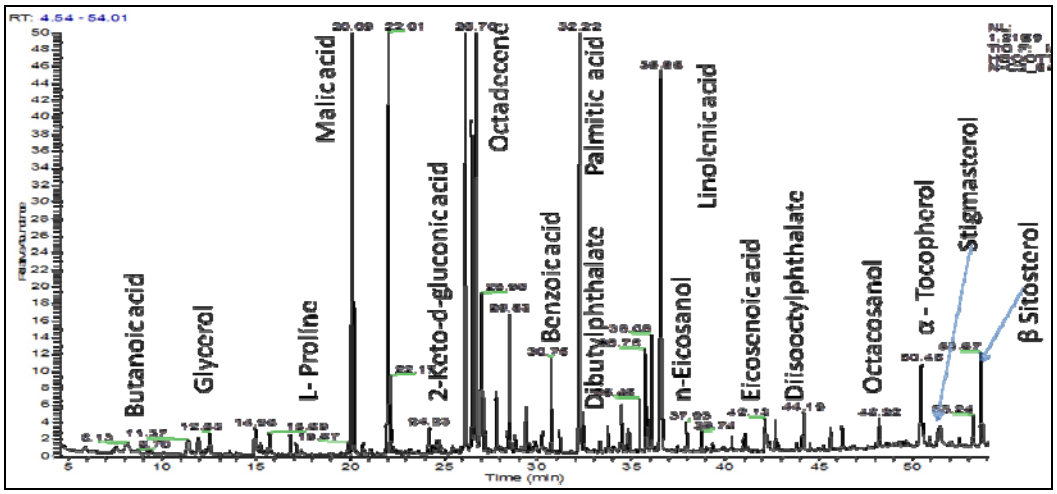
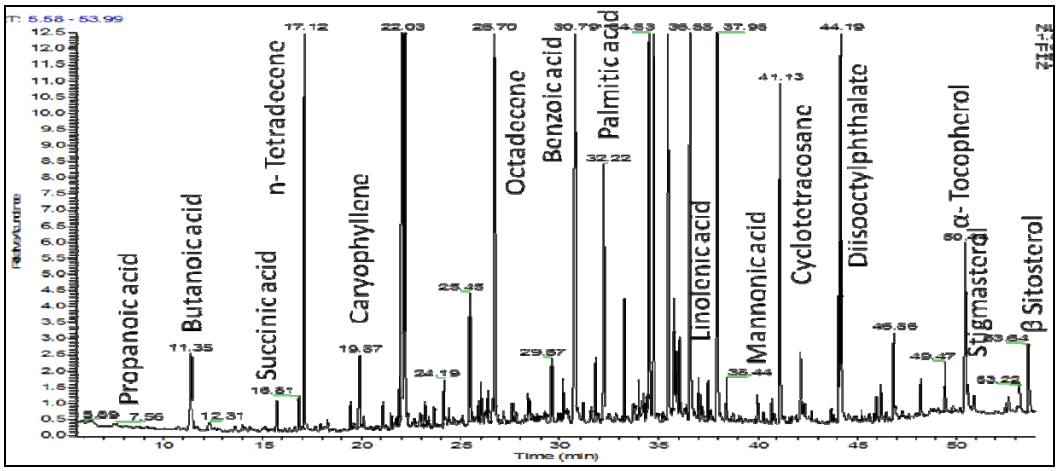


Figure 2. Major non polar metabolites in control *G. hirsutum* leaf



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130 **Figure 3.** Major non polar metabolites in water stressed *G. hirsutum* leaf

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132 **Table1**: Mass data of major metabolites from control and water-stressed *G. hirsutum* leaf and

133 stem**.**

Serial Number	tR (min)	Compound	Molecular Weight	Mass Data *(m/z)*
1.	11.66	Dodecene	168	*m/z* 168 (M^+) (6%), 97 (24%), 84 (28%), 83 (30%), 70 (48%), 56 (62%), 55 (72%), 43 (100%)
2.	17.12	Tetradecene	196	*m/z* 196 (M^+) (2%), 111 (34%), 97 (70%), 70 (82%), 69 (100%), 55 (78%),
3.	17.45	Nonanoic acid	230	*m/z* 230 (M^+) (2%), 215 (70%), 129 (22%), 117 (52%), 97 (62%), 73 (100%), 75 (80%)
4.	19.75	L-Lysine	450	*m/z* 450 (M^+) (2%), 258 (12%), 232 (34%), 172 (30%), 102 (88%), 77 (48%), 73 (100%)
5.	19.87	Caryophyllene	204	*m/z* 204 (M^+) (2%), 189 (24%), 147 (34%), 133 (84%), 105 (58%), 93 (74%), 69 (100%)
6.	22.36	Quinoline derivative	278	*m/z* 278 (M^+) (16%), 264 (20%), 263 (100%), 73 (26%)
7.	24.23	2-Keto-d-gluconic acid	554	*m/z* 554 (M^+) (2%), 437 (22%), 217 (30%), 204 (72%), 73 (100%)
8.	24.56	Cinnamic acid	220	*m/z* 220 (M^+), (98%), 215 (72%), 132 (26%), 75 (94%), 73 (100%)
9.	25.86	Maleic acid dibutylester	228	*m/z* 228 (M^+) (2%), 173 (10%), 155 (16%), 117 (42%), 57 (48%), 41 (38%), 99 (100%)
10.	26.15	Butanal	467	*m/z* 467 (M^+) (2%), 307 (28%), 217 (20%), 160 (10%), 147 (18%), 103 (64%), 73 (100%),
11.	26.39	2- Methylhexadecan-1-ol	256	*m/z* 256 (M^+) (2%), 111 (22%), 97 (38%), 71 (52%), 69 (58%), 57 (100%)
12.	26.72	Octadecene	252	*m/z* 252 (M^+) (2%), 111 (44%), 97 (89%), 83 (92%), 69 (76%), 57 (100%),
13.	27.78	Phytol	296	*m/z* 296 (M^+) (2%), 123 (28%), 95(32%), 82 (38%), 81 (46%), 71 (100%), 57 (64%)
14.	28.53	Myristic acid	300	*m/z* 300 (M^+) (4%), 285 (86%), 145 (34%), (80%)
15.	29.61	Tridecanedial	212	*m/z* 212 (M^+) (2%), 150 (18%), 109 (42%), 95 (96%), 81 (78%), 67 (84%), 55 (100%)
16.	29.94	Hexadecanol	314	*m/z* 314 (M^+) (2%), 300 (22%), 299 (100%), 103 (18%), 75 (50%), 73 (22%)

17.	31.12	Nonadecane	266	*m/z* 266 (M^+) (2%), 111 (32%), 97 (62%) 83 (64%), 57 (80%), 55 (92%), 43 (98%), 41 (100%)
18.	32.16	Quinoline Acetamide derivative	366	*m/z* 366 (M^+) (28%), 351 (26%), 235 (68%), 219 (58%), 75 (38%), 73 (100%)
19.	32.22	Palmitic acid	328	*m/z* 328 (M^+) (4%), 314 (6%), 313 (34%), 145 (26%), 132 (38%), 117 (72%), 75 (82%)
20.	35.87	Dibutylphthalate	278	*m/z* 278 (M^+) (2%), 149 (100%), 150 (10%), 104 (6%), 41 (8%)
21.	36.05	Linoleic acid	352	*m/z* 352 (M^+) (6%), 337 (70%), 129 (44%), 95 (40%), 73 (100%), 54 (52%)
22.	36.14	Stearic acid	284	*m/z* 284 (M^+) (4%), 145 (24%), 132 (38%), 129 (64%), 117 (72%), 75 (72%), 73 (100%)
23.	38.32	Docosene	308	*m/z* 308 (M^+) (2%), 125 (12%), 111 (28%), 97 (62%) ,69 (68%), 55 (100%)
24.	41.50	n-Eicosanol	298	*m/z* 298 (M^+) (2%), 125 (12%), 111 (30%), 97 (52%) 53 (60%)
25.	44.60	Dioctylphthalate	390	*m/z* 390 (M^+) (2%), 279 (20%), 167 (40%), 149 (100%), 113 (14%), 71 (26%), 57 (38%)
26.	47.25	Nonacosanol	424	*m/z* 424 (M^+) (2%), 139 (10%), 125 (22%), 111 (38%), 97 (90%) ,69 (68%), 57 (100%)
27.	48.22	Octacosanol	482	*m/z* 482 (M^+) (2%), 467 (76%), 111 (18%), 103 (44%), 83 (34%), 75 (100%), 57 (58%)
28.	52.56	Campesterol	472	*m/z* 472 (M^+) (4%), 343 (28%), 257 (20%), 147 (24%), 137 (44%), 69 (74%), 73 (100%), 57 (72%)
29.	53.77	Stigmasterol	486	*m/z* 486 (M^+) (38%), 255 (98%), 217 (34%), 147 (36%), 129 (18%), 95 (34%), 73 (100%)

tR =Retention time

Metabolites in leaf

A total of 17 non-polar metabolites were detected from leaves of water stressed *G. hirsutum*. The higher amounts were of quinoline derivative (26.37%), 2- methylhexadecan-1-ol (7.47%), phytol (7.71%), myristic acid (5.94%), hexadecanol (14.30%), nonadecane(1.67%) and palmitic acid (3.20%) detected in water stressed leaves as compared to the control. Moreover two metabolites i.e. caryophyllene and phytol were detected only in stressed leaves.

Table 2: Variation of non-polar metabolites in control and water stressed *G. hirsutum* leaf.

Serial Number	Compound Name	Control Leaf (Area %)	Stress Leaf (Area %)
1.	Caryophyllene	ND	0.58 ± 0.02[a]
2.	Quinoline derivative	7.70±0.11[a]	26.37±0.29[a]
3.	2-Keto-d-gluconic acid	7.13± 0.17[a]	ND
4.	Cinnamic acid	23.93± 0.49[a]	9.18 ± 0.11[a]
5.	Maleic acid dibutylester	1.16± 0.07[a]	ND
6.	Butanal	2.92± 0.24[a]	ND
7.	2- Methylhexadecan-1-ol	1.05± 0.01[a]	7.47 ±0.07[a]
8.	Octadecene	6.74± 0.38[a]	1.64 ± 0.17[a]

9.	Phytol	ND	7.71 ± 0.02[a]
10.	Myristic acid	0.63± 0.01[a]	5.94 ±0.04[a]
11.	Tridecanedial	1.63± 0.03[a]	ND
12.	Hexadecanol	6.14± 0.24[a]	14.30±0.94[a]
13.	Nonadecane	0.49± 0.05[a]	1.67 ± 0.05[a]
14.	QuinolineAcetamide derivative	1.03± 0.06[a]	0.79 ± 0.12[a]
15.	Palmitic acid	0.81± 0.21[a]	3.20 ± 1.39[a]
16.	Dibutylphthalate	1.43± 1.05	0.88 ± 0.57
17.	Stearic acid	2.06± 0.03[a]	0.43 ± 0.21[a]

Mean values ± SD (standard deviation) values of mg/gm of fresh weight. ND = Not Detected; **a** denotes statistical significance $P = .05$ between groups (control vs stress).

The higher amount of metabolites: cinnamic acid (23.93%), octadecene (6.74%), quninoline acetamide derivative (1.03%) and stearic acid (2.06%) were present in control leaf as compared to stressed leaf. While the higher amount of quinoline derivative (26.37%), myristic acid (5.94%), hexadecanol (14.30%), nonadecane (1.67%) and palmitic acid (3.20%) were detected in stressed leaf as compared to control leaf. The other non-polar metabolites such as 2-keto-d-gluconic acid (7.13%), maleic acid dibutylester (1.16%), butanal (2.92%) and tridecanedial (1.63%) were detected only in control leaf. The caryophyllene (0.58%) and phytol (7.71%) were present only in stressed leaf (Table 2 and Figure 4).

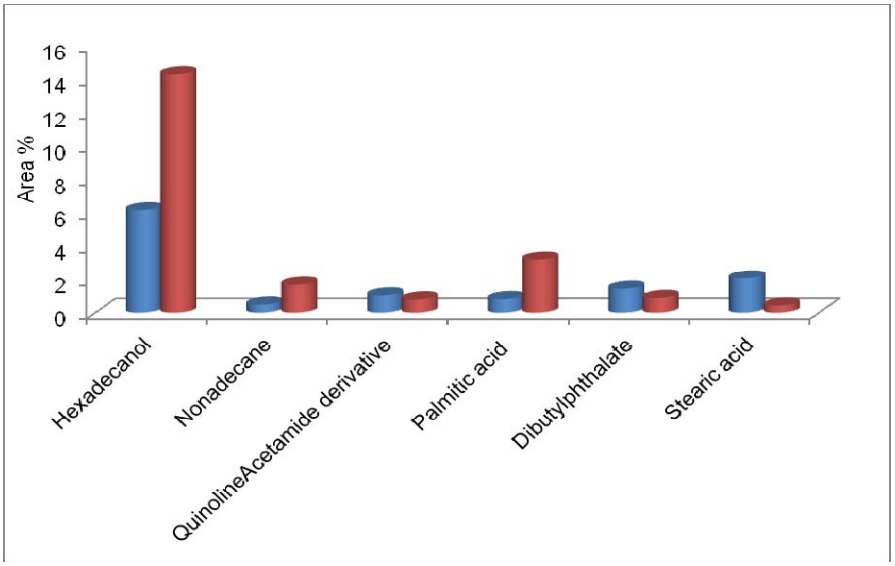

Figure 4. Variation of major non polar metabolites in control vs water stressed *G. hirsutum* leaf

Metabolites in stem

Total 14 non-polar metabolites were detected from water stressed *G. hirsutum* stem (Table 3). Higher amount of L-lysine (0.65%), linoleic acid (10.26%) and campesterol (0.87%) were

detected in water stressed stem than in the control. The rest of the other metabolites slightly decreased in the control as compared to the stressed stem.

Table 3: Variation of non-polar metabolites in control and water stressed *G. hirsutum* stem.

Serial Number	Compound Name	Control Stem (Area %)	Stress Stem (Area %)
1.	Dodecene	1.04 ± 0.04[a]	1.67 ± 0.11[a]
2.	Nonanoic acid	5.36 ± 0.24	5.24 ± 0.05
3.	L-Lysine	0.43± 0.11[a]	0.65 ± 0.06[a]
4.	Quinoline derivative	28.01 ±0.17	25.87± 1.16
5.	Maleic acid dibutylester	0.72± 0.11[a]	0.51 ± 0.03[sa]
6.	2- Methylhexadecan-1-ol	0.73± 0.03[a]	ND
7.	Dibutylphthalate	4.85± 0.21	5.06 ± 1.88
8.	Linoleic acid	3.63± 0.49[a]	10.26±0.07[a]
9.	Docosene	3.47± 0.23	3.05 ± 0.28
10.	n-Eicosanol	2.20± 0.08	2.06 ± 0.25
11.	Dioctylphthalate	4.56± 0.07[a]	3.77 ± 0.09[a]
12.	Nonacosanol	0.50± 0.06	0.46 ± 0.05
13.	Campesterol	0.31± 0.04[a]	0.87 ± 0.04[a]
14.	Stigmasterol	0.44± 0.26	1.13 ± 0.55

Mean values ± SD (standard deviation) values of mg/gm of fresh weight. ND= Not Detected;
a denotes statistical significance $P = .05$ between groups (control vs stress).

Maleic acid dibutylester (0.72%) and dioctylphthalate (4.56%) were more detected in control than in the stress stem while dodecene (1.67%), L-lysine (0.65%), linoleic acid (10.26%) and campesterol (0.87%) were more found in the stressed stem than in control (dodecene (1.04%), L-lysine (0.43%), linoleic acid (3.63%) and campesterol (0.31%)). 2-Methylhexadecan-1-ol (0.73%) was present only in control stem. Statistically significant variation ($P = 0.05$) in few metabolites content was found between control and water stressed *G. hirsutum* stem (Table 3, Figure 5).

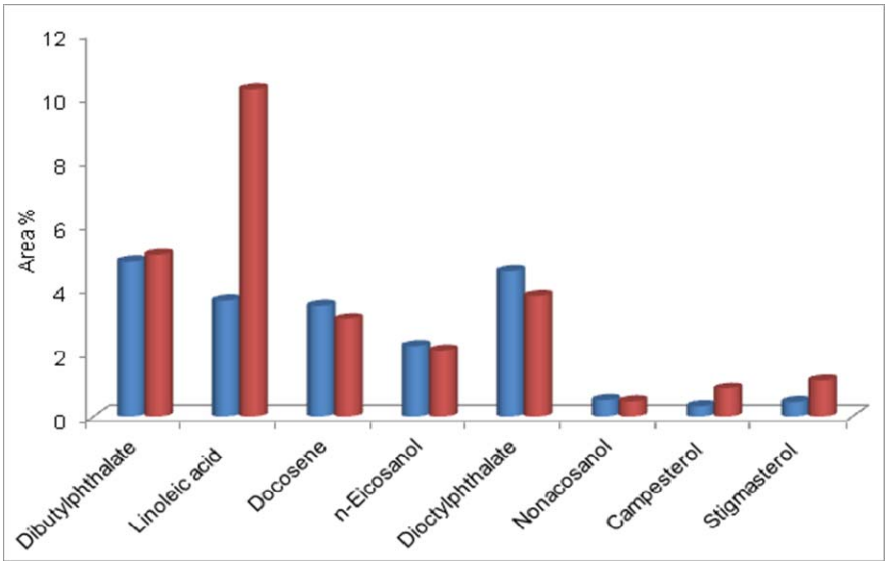

Figure 5. Variation of major non-polar metabolites in control vs water stressed *G. hirsutum* stem

Generally 2- methylhexadecan-1-ol, hexadecanol and palmitic acid in the leaf and linoleic acid in the stem were found to be accumulating under water stress condition. Some metabolites such as cinnamic acid, octadecene, stearic acid in leaf and quinoline derivative, docosene, dioctylphthalate decreased in the stem. These observations suggest that the selective accumulation and consumption of the metabolites occurred during the water stress in *G. hirsutum* leaf and stem. This would further indicate that the above metabolites played a crucial role during the water stress and thus would be considered as metabolites responsible for water stress tolerance in *G. hirsutum*. The accumulation of the above metabolites (2-methylhexadecan-1-ol, hexadecanol, palmitic acid, linoleic acid) was previously reported for other plant species. These metabolites were observed to be responsible for water stress tolerance [16, 17]. Moreover, plant sterol i.e. campesterol was found in high amount in the stress stem. Plant sterols regulate fluidity and permeability of phospholipid bilayer [21], cell division and plant growth [22]. Sterols are also essential for synthesis of prostaglandins and leukotrienes, important component for immune system [23].

Conclusion

Accumulations of metabolites such as quinoline derivative, 2- methylhexadecan-1-ol, phytol, myristic acid, hexadecanol and palmitic acid were more observed in the stressed leaf compared to control leaf. Metabolites like L-lysine, linoleic acid, campesterol and stigmasterol also accumulated more in the stressed stem as compared to control stem. While

reduction in the amount of the metabolites i.e., cinnamic acid, octadecene, stearic acid in leaf and quinoline derivative, docosene, dioctylphthalate in stem was noticed. These observations indicate that the selective accumulation and consumption of the metabolites occurred during the water stress in *G. hirsutum* leaf and stem. In conclusion, the above metabolites could have played a crucial role during the water stress and could in this case be considered as metabolites responsible for water stress tolerance in *G. hirsutum*. However the effect of chemical variation within *G. hirsutum* species, entails that further studies of individual species be carried out to investigate variation in their non-polar metabolites content and component under water stress conditions. Further still, salt stress experiments and the impacts of osmotic potential study on cotton species would widen the knowledge of this research.

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