Original Research Article

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

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Abstract

Present work was aimed at the study on variation of non polar metabolites content in *Gossypium hirsutum* L. under water stress condition. The variation of non-polar metabolites was observed by gas chromatography-mass spectrometry (GC-MS) technique. 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%). Total 14 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%). Significant variation (P = .05) in most of the metabolites content in leaf and stem was observed during water stress. The above major metabolites played an important role during water stress and can be consider as metabolites responsible for water stress tolerance in *G. hirsutum* under water stress condition. Further, this study will be valuable for the better understanding of overall water stress tolerance mechanism in *G. hirsutum.*

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

Introduction

Cotton is one of the most important industrial crop comes under the genus “Gossypium” in the Malvaceae family and popularly known as “white gold” [1]. Globally, the Gossypium genus includes about 50 species [2]. Mainly four species in the genus Gossypium, namely *G.*

hirsutum L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L. were 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 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. While the linters which have a much shorter fibre length are a 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].

India is the second largest producer of cotton worldwide and one of the largest producers as well as exporters of cotton yarn. The Indian textile industry contributes about 11 % to industrial production, 14 % to the manufacturing sector, 4% per cent to the GDP and 12 % to the country's total export earnings [8]. In year 2014-15, cotton cultivation in India stands at 12.25 million hectares while in the year 2013-14 was about 11.5 million hectares. Major Cotton producing states in India are Gujarat, Maharashtra, Andhra Pradesh (AP), Haryana, Punjab, Madhya Pradesh (MP), Rajasthan, Karnataka and Tamil Nadu (TN). India was an importer of cotton in the range of 8.00 to 9.00 lakh bales per annum till 1970s. Since launch of "Technology Mission on Cotton" by Government of India in February 2000; significant achievements have been made in increasing yield and production. This increase in yield due to development of high yielding varieties, appropriate transfer of technology, better farm management practices, increased area under cultivation etc. All above these developments have contributed to a turnaround in cotton production in India. The changes that are taking

place in cotton cultivation in India have the potential to take the current productivity level near to the world's average cotton production per hectare in the near future. 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 [9]. Moreover, water stress is considered as the single most devastating environmental factor [10]. 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 [11, 12].

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 [13]. 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 [14]. Nevertheless, cotton has mechanisms that make it well adapted to semi-arid regions [15]. An understanding of the response of cultivars to water deficits is also important to model cotton growth and estimate irrigation needs [16]. The alteration of metabolites due to water stress was previously reported for plant species and considered to be responsible for water stress tolerance [17, 18].

Lv et al. evaluated five homozygous transgenic *Gossypium hirsutum* L. plants under water stress condition and the result suggested that glycine betaine may be involved in osmotic adjustment in the plant [19]. Rodriguez-Uribe et al. used microarray analysis to identify water deficit-responsive genes in the *G. hirsutum* under water stress conditions [20]. Yoo and Wendel, conducted comparative transcriptome profiling of developing *G. hirsutum* fibres using RNA-Seq by Illumina sequencing [21]. Although some other aspect of the changes in *G. hirsutum* under water stresses conditions have been reported. But still there was need to study the non polar metabolites changes in *G. hirsutum* under water stress condition, so that the metabolites responsible for water stress tolerance can be investigated. Therefore, it was imperative to study the variation of non-polar metabolites in *G. hirsutum* L. plants under water stressed condition. Further, the finding of this study may helpful for agriculture researchers in better understanding of metabolic pathways during water stress. To the best of our knowledge, this was the first study which deals with the variations of non-polar metabolites content in *G. hirsutum* L. plants under water stressed condition by gas chromatography-mass spectrometry (GC-MS) method.

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 will allow the pots to dry out and plants were 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 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 rota vapour. 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 h 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 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 GC-MS data 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 class of non-polar metabolites were identified from non-polar extracts of leaf and stem of *G. hirsutum* (Table 1). Plottted GCMS chromatogram of the control and water stressed leaf of *G. hirsutum* are shwon in figure 2 and 3.

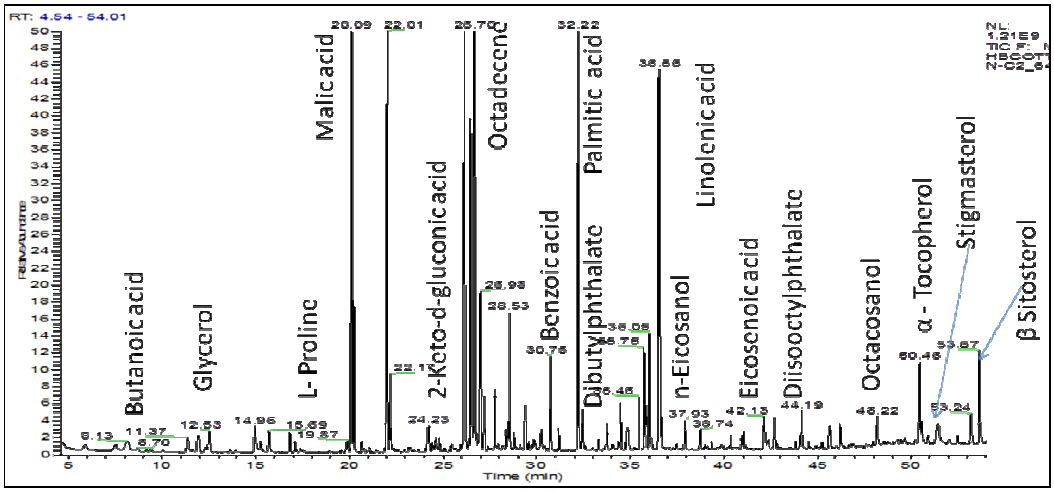

Figure 2. Major non polar metabolites in control *G. hirsutum* leaf

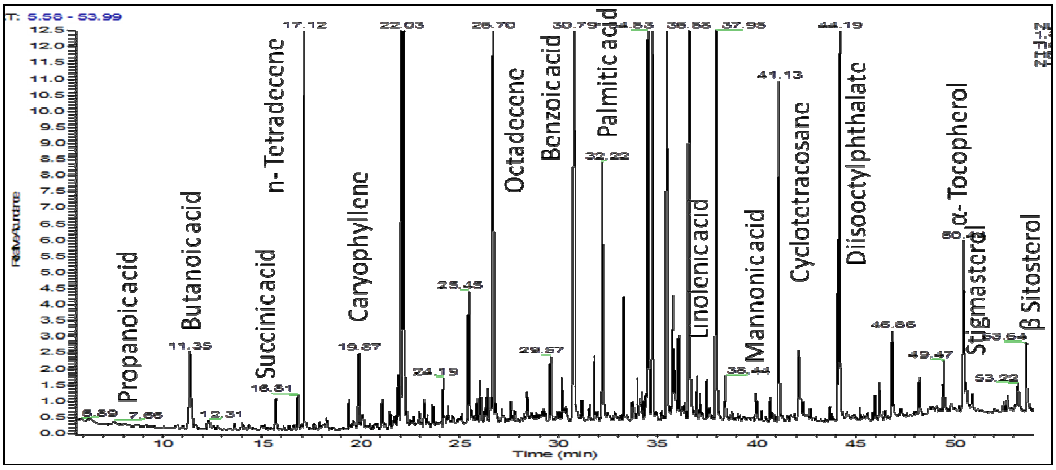

Figure 3. Major non polar metabolites in water stressed *G. hirsutum* leaf

Table1: Mass data of GC-MS identified metabolites from control and water-stressed *G. hirsutum* leaf and stem**.**

Serial Number	tR (min)	Compound	Molecular Formula	Molecular Weight	Mass Data *(m/z)*
1.	11.66	Dodecene	$C_{12}H_{24}$	168	*m/z* 168 (M^+) (6%), 97 (24%), 84 (28%), 83 (30%), 70 (48%), 56 (62%), 55 (72%), 43 (100%)
2.	17.12	Tetradecene	$C_{14}H_{28}$	196	*m/z* 196 (M^+) (2%), 125 (8%), 111 (34%), 97 (70%), 70 (82%), 69 (100%), 55 (78%),
3.	17.45	Nonanoic acid	$C_{12}H_{26}O_2Si$	230	*m/z* 230 (M^+) (2%), 215 (70%), 129 (22%), 117 (52%), 97 (62%), 73 (100%), 75 (80%)
4.	19.75	L-Lysine	$C_{18}H_{46}N_2O_3Si_4$	450	*m/z* 450 (M^+) (2%), 360 (4%), 258 (12%), 232 (34%), 172 (30%),102 (88%), 77 (48%), 73 (100%)
5.	19.87	Caryophyllene	$C_{15}H_{24}$	204	*m/z* 204 (M^+) (2%), 189 (24%), 147 (34%), 133 (84%), 105 (58%), 93 (74%), 69 (100%)
6.	22.36	Quinoline derivative	$C_{18}H_{18}N_2O$	278	*m/z* 278 (M^+) (16%), 264 (20%), 263 (100%), 73 (26%)

7.	24.23	2-Keto-d-gluconic acid	$C_{21}H_{50}O_7Si_5$	554	*m/z* 554 (M^+) (2%), 437 (22%), 292 (10%), 217 (30%), 204 (72%), 73 (100%) (Me_3Si)
8.	24.56	Cinnamic acid	$C_{12}H_6O_2Si$	220	*m/z* 220 (M^+), (98%), 215 (72%), 132 (26%), 75 (94%), 73 (100%)
9.	25.86	Maleic acid dibutylester	$C_{12}H_{20}O_4$	228	*m/z* 228 (M^+) (2%), 173 (10%), 155 (16%), 117 (42%), 57 (48%), 41 (38%), 99 (100%)
10.	26.15	Butanal	$C_{18}H_{45}NO_5Si_4$	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	$C_{17}H_{36}O$	256	*m/z* 256 (M^+) (2%), 125 (10%), 111 (22%), 97 (38%), 71 (52%), 69 (58%), 57 (100%)
12.	26.72	Octadecene	$C_{18}H_{36}$	252	*m/z* 252 (M^+) (2%), 139 (10%), 111 (44%), 97 (89%), 83 (92%), 69 (76%), 57 (100%),
13.	27.78	Phytol	$C_{20}H_{40}O$	296	*m/z* 296 (M^+) (2%), 123 (28%), 95(32%), 82 (38%), 81 (46%), 71 (100%), 57 (64%)
14.	28.53	Myristic acid	$C_{14}H_{28}O_2$	300	*m/z* 300 (M^+) (4%), 285 (34%), 132 (18%), 75 (100%), 73 (8
15.	29.61	Tridecanedial	$C_{13}H_{24}O_2$	212	*m/z* 212 (M^+) (2%), 150 (18%), 109 (42%), 95 (96%), 81 (78%), 67 (84%), 55 (100%)
16.	29.94	Hexadecanol	$C_{19}H_{42}OSi$	314	*m/z* 314 (M^+) (2%), 300 (22%), 299 (100%), 103 (18%), 75 (50%), 73 (22%)
17.	31.12	Nonadecane	$C_{18}H_{38}$	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	$C_{20}H_{18}N_2O_5$	366	*m/z* 366 (M^+) (28%), 351 (26%), 235 (68%), 219 (58%), 75 (38%), 73 (100%)
19.	32.22	Palmitic acid	$C_{19}H_{40}O_2Si$	328	*m/z* 328 (M^+) (4%), 314 (6%), 313 (34%), 201 (2%), 145 (26%), 132 (38%), 117 (72%), 75 (82%)

20.	35.87	Dibutylphthalate	$C_{16}H_{22}O_4$	278	*m/z* 278 (M^+) (2%), 149 (100%), 150 (10%), 104 (6%), 41 (8%)
21.	36.05	Linoleic acid	$C_{21}H_{40}O_2Si$	352	*m/z* 352 (M^+) (6%), 337 (70%), 129 (44%), 95 (40%), 73 (100%), 54 (52%)
22.	36.14	Stearic acid	$C_{18}H_{36}O_4$	284	*m/z* 284 (M^+) (4%), 145 (24%), 132 (38%), 129 (64%), 117 (72%), 75 (72%), 73 (100%)
23.	38.32	Docosene	$C_{22}H_{44}$	308	*m/z* 308 (M^+) (2%), 139 (6%), 125 (12%), 111 (28%), 97 (62%) ,69 (68%), 55 (100%)
24.	41.50	n-Eicosanol	$C_{20}H_{42}O$	298	*m/z* 298 (M^+) (2%), 153 (4%), 139 (6%), 125 (12%), 111 (30%), 97 (52%) 53 (60%)
25.	44.60	Dioctylphthalate	$C_{24}H_{38}O_4$	390	*m/z* 390 (M^+) (2%), 280 (4%), 279 (20%), 167 (40%), 149 (100%), 113 (14%), 71 (26%), 57 (38%)
26.	47.25	Nonacosanol	$C_{29}H_{60}O$	424	*m/z* 424 (M^+) (2%), 139 (10%), 125 (22%), 111 (38%), 97 (90%) ,69 (68%), 57 (100%)
27.	48.22	Octacosanol	$C_{31}H_{66}OSi$	482	*m/z* 482 (M^+) (2%), 468 (12%), 467 (76%), 111 (18%), 103 (44%), 83 (34%), 75 (100%), 57 (58%)
28.	52.56	Campesterol	$C_{31}H_{56}OSi$	472	*m/z* 472 (M^+) (4%), 343 (28%), 257 (20%), 147 (24%), 137 (44%), 69 (74%), 73 (100%), 57 (72%)
29.	53.77	Stigmasterol	$C_{32}H_{58}OSi$	486	*m/z* 486 (M^+) (38%), 398 (6% (98%), 217 (34%), 147 (36%), 129 (18%), 95 (3 (100%)

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153 **Metabolites in leaf**

154 Total 17 non-polar metabolites were detected from leaves of water stressed *G. hirsutum*. The
155 higher amount 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%) were detected in water stressed leaves in compare to 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 in compare 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 in compare 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).

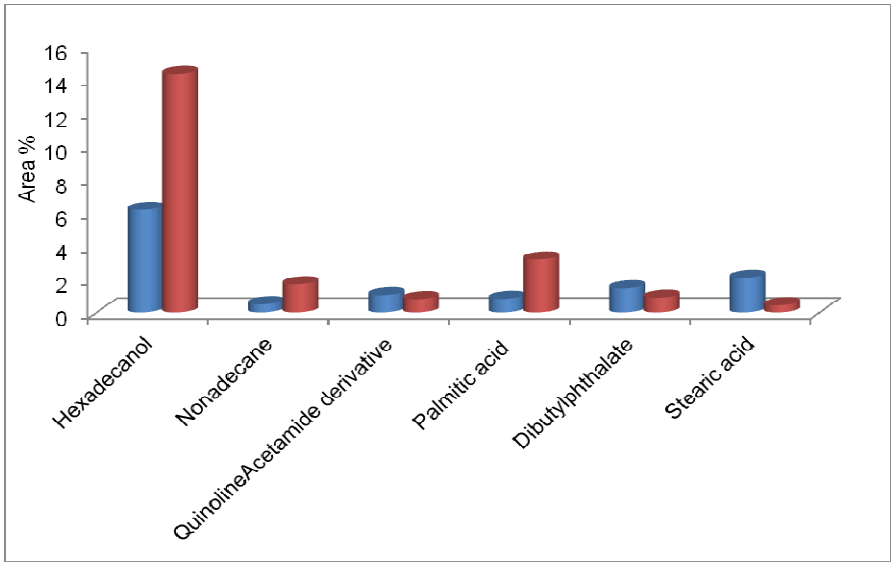

Figure 4. Variation of major non polar metabolites in control vs water stressed *G. hirsutum* leaf

Metabolites in stem

Total 14 non-polar metabolites were detected from water stressed *G. hirsutum* stem (Table 3). The higher amount of L-lysine (0.65%), linoleic acid (10.26%) and campesterol (0.87%) were detected in water stressed stem in compare to control. While the other metabolites were slightly decreased than control in compare to stress 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).

The higher average amount of maleic acid dibutylester (0.72%) and dioctylphthalate (4.56%) were detected in control stem compare to stress stem. The higher average amount of dodecene (1.67%), L-lysine (0.65%), linoleic acid (10.26%) and campesterol (0.87%) were found in stress stem compare to control. 2- Methylhexadecan-1-ol (0.73%) was present only in control stem. Statistically significant variation (P = .05) in few metabolites content was found between control and water stressed *G. hirsutum* stem (Table 3, Figure 5).

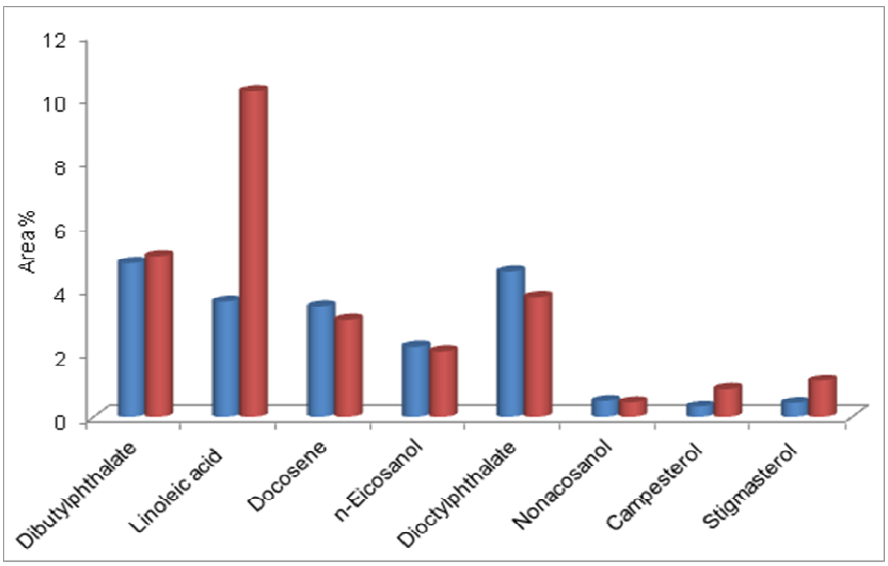

Figure 5. Variation of major non-polar metabolites in control vs water stressed *G. hirsutum* stem

Mainly 2- methylhexadecan-1-ol, hexadecanol and palmitic acid in leaf while linoleic acid in stem was found to be accumulating under water stress condition. The accumulation of these metabolites was previously reported for other plant species. These metabolites were observed to be responsible for water stress tolerance [17, 18]. Moreover, plant sterol i.e. campesterol was found in high amount in stress stem. Plant sterols regulate fluidity and permeability of phospholipid bilayer [22], cell division and plant growth [23]. Sterols are also essential for synthesis of prostaglandins and leukotrienes, important component for immune system [24].

Conclusion

The higher content of the metabolites such as quinoline derivative, 2- methylhexadecan-1-ol, phytol, myristic acid, hexadecanol and palmitic acid was observed in the stressed leaf compare to control leaf. The high content of the metabolites such as L-lysine, linoleic acid, campesterol and stigmasterol was detected in stressed stem compare to control stem.

Similarly, consumption of the metabolites i.e., cinnamic acid, octadecene, stearic acid in leaf and quinoline derivative, docosene, dioctylphthalate in stem was observed. These observations indicate that the selective accumulation and consumption of the metabolites were occurred during the water stress in *G. hirsutum* leaf and stem. It concludes that above metabolites played a crucial role during the water stress and can be considered as metabolites responsible for water stress tolerance in *G. hirsutum.*

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