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Effect of Water Stress on Biochemical Metabolites in Fenugreek (*Trigonella Foenum-Graecum L.)* Genotypes

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Annotation: A field experiment was conducted at Agronomy farm, S.K.N. Collage of Agriculture, Jobner during Rabi season 2014-2015 to investigate "Biochemical changes in fenugreek (Trigonella foenum graecum L.) genotypes under water stress" using Five genotypes of fenugreek (UM-100,UM-112,UM-124,UM-134,UM-140). The significant increment observed in proline content, Glycine betaine content and Peroxidase activity due to water stress at both the stages. A Reduction in the content of total chlorophyll and GSH in stressed plants of all genotypes was recorded at both stages. Significant reductions in total carotenoids content were found in all the genotypes at 65 DAS under water stress conditions. The MDA values in stressed plants were found higher over respective controls at both stages in all the genotypes.

Keywords: Fenugreek, water stress, antioxidants, proline, glycine betaine, Glutathione Reduced, peroxidase activity, chlorophyll, Malondialdehyde.

1. Introduction

Fenugreek popularly known by its vernacular name 'methi' is an important condiment crop, largely grown in Northern during Rabi season. The leaves and shoots are quite rich in protein, minerals and vitamin A and C. Rao and Sharma (1987) reported that fenugreek seeds contain 25.5% protein, 7.9%fat, 20% mucilaginous matter and 4.8% saponins. The seeds also contain cellulose, hemicellulose, and major nutrients such as calcium, iron, sodium and amino acids like leucine, valine, lysine and phenylalanine. Seeds are bitter in taste due to presence of an alkaloid "trigonelline". In recent years the importance of fenugreek has further increased due to presence of a steroid called "diosgenin".

Environmental factors such as water, temperature and nutritional status affect the biochemical responses of plants to stress. Plants have genetically controlled mechanisms that allow them to live and grow under stress (Boyer, 1982). One of the most important environmental factors is the availability of water which is included in all vital activities. Due to insufficient, untimely and erratic rainfall in semi-arid and arid areas, the fenugreek crop often suffers from drought at the end of the cropping season. Therefore, understanding crop response to this stress is very important. It is well known that drought stress brings about numerous metabolic and biochemical changes in

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plants like pigment content and photosynthetic activity (Ekmekc *et al.*, 2005). Drought impacts include pigment content, osmotic adjustment and photosynthetic activity (Benjamin and Nielsen, 2006; Praba *et al*., 2009).

2. Materials and Methods

1. L-Proline content (Bates *et al.* 1973)

Fresh leaf sample (0.2 g) were extracted in 5.0 ml sulphosalicylic acid [SSA 3%, Appendix-III A (a)] using mortar-pestle at room temperature. The homogenate were centrifuged at 8000 rpm for 10 minutes. The clear supernatants were collected in clear test tubes separately. To 1.0 ml of supernatant was added 2 ml of glacial acetic acid and mixed throughly, followed by 2.0 ml acid ninhydrin reagent [Appendix-III A (b)] was added and mixed well. The test tubes containing assay mixtures were incubated in a boiling water bath for an hour and then cooled to room temperature. Four ml of toluene solvent was added to each tube and mix well using vortex mixture. The pink colour of L-Proline as extracted in SSA and taken up by the solvent after incubation was separated using a separating funnel. Toluene fractions were collected and intensity of pink colour read at 520 nm on a spectrophotometer. A standard curve was prepared using LProline (0.1 mg/ ml).

2. Glycine Betaine: (QAC) (Grieve and Grattan,1983)

Leaf sample (1.0 g) was finely grind in 20 ml distilled water and shaken mechanically for 24 hrs at 25 oC. The samples were then filtered. The extract were diluted in the ratio of 1:1 with 2N H_2SO_4 [Appendix-III B (a)]. Aliquots (0.5 ml) were measured into 2 ml eppendorf tubes and cooled in ice water for 1 hr. Cold KI-I2 reagent [Appendix-III B (b)] (0.2 ml) was added to each test tube and tubes were gently stirred on a vortex mixture. The tubes were stored at 4 oC for 16 hrs and then centrifuged at 10,000 rpm for 15 minutes at 0oC. The supernatant were carefully aspirated. The per-iodide crystals were dissolved in 9 ml of 1, 2 dichloromethane, with vigorous mixing required to effect complete solubilization in the developing solvent. After 2-2.5 hrs, the absorbance was measured at 365 nm on a spectrophotometer (UV range). Reference standard of GB (50-200 µg/ml) were prepared in 1N H_2SO_4. Standard curve were prepared and the GB content of sample was calculated using following formula:

Glycine Betaine = sample O.D. x graph factor x dilution factor

Where, graph factor = 38, dilution factor = 4000

3. Glutathione Reduced (GSH) (Bailey, 1998)

Fresh leaf samples (0.25 g) were extracted in mortar-pestle using 5.0 ml of 0.1 M phosphate- buffer, pH 7.8 [Appendix-III C (a)] containing EDTA (1mM- 50 mg EDTA, disodium salt) [Appendix-III C (c)] was dissolved in 10 ml 1.0 M phosphate buffer (pH 7.8) and centrifuged at 8,000 rpm for 10 minutes. Supernatant collected after centrifugation was then used for assay. To 1.0 ml of aliquot, 2.8 ml of 0.1 M phosphate buffer (pH 7.8) was added followed by 0.2 ml 5-5 Dithiobis Nitro Benzoic Acid (DTNB) [Appendix-III C (b)]. After mixing, the reaction mixture was incubated for 30 minutes at room tempature after which 4 ml distilled water was added. The intensity of yellow colour was measured at 410 nm on spectrophotometer (Systronic) against reagent blank. A standard curve was prepared using GSH (0.1 mg/ml).

4. Peroxidase: EC. 1.11.1.7 (Costa *et al.* 2002)

200 mg fresh leaves were homogenized in a pre chilled mortar pestle kept under ice cold condition using 2 ml extraction buffer, containing 0.1M sodium phosphate buffer, pH 7.2 [Appendix-III D (a)] with the addition of 1 mM β-mercaptoethanol and 1% (w/v) polyvinyl pyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes. The supernatant were used for the assay. POX activity was determined in the supernatant of

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centrifuged homogenates by measuring the increase in absorption at 470 nm in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA , 0.05 ml enzyme extract, and 10 mM H_2O_2.

5. **Total chlorophyll content (Hiscox and Israelstam, 1979).**

Total chlorophyll in leaves was determined by DMSO (dimethylsulphoxide) method. Finely chopped 50 mg fenugreek leaves were weighed in graduated test tubes. 10 ml of DMSO was added to each tube and incubated at 65oC for 3 hrs. After incubation the tubes were allowed to cool at room temperature and the volume made up to a total of 10 ml by adding DMSO. The optical density (OD) was recorded at 663 and 645 nm by taking DMSO as blank .The amount of chlorophyll present in the sample was calculated using standard formulae:-

Total chlorophyll (mg/g) = 22.2 (O.D. at 663) + 8.02 (O.D. at 645)

6. **Carotenoid content (Wellburn, 1994).**

The procedure was same as total chlorophyll content and absorbance recorded at 480 nm. Carotenoid content was calculated using the formulae:-

Carotenoid µg /ml = (1000 A 480 - 2.14 Ca- 70.16 Cb / 220)

Ca (mg/litre) = (12.7 A663- 2.69 A645)

Cb (mg/Litre) = (22.9 A645 - 4.68 A663)

7. **Malondialdehyde (Heath and Packer, 1968).**

Fresh leaf samples (0.2 g) were extracted in 5.0 ml of 6% trichloroacetic acid (TCA) [Appendix-III E (a)] solution by centrifugation at 8,000 rpm for 10 minute. Supernatant were collected in separate tubes. To 1 ml of the supernatant taken in a clean, dry test tube, was added 2.0 ml of Thio-Barbituric Acid (TBA) reagent [Appendix-III E (b)], mixed and incubated for half an hour in a boiling water bath. The tubes were than cooled to room temperature. The assay mixture was then centrifuged at 5,000 rpm for 10 min. and clear supernatant bearing yellow to light orange colour was read on spectrophotometer at two wavelengths *viz*. 532 nm (major for MDA) and 600 (minor for interfaring substance) millimolar concentration of MDA was calculated as follows:-

MDA (mM) = (O.D.532 –O.D. 600) x 155 (extinction coefficient)

3. Results and discussion

1. **L-Proline content**

Result of the estimation show that significant increase in proline content due towater stress at both the stages. At both the stages, maximum increase dueto water stress was observed in genotype UM-134 with 37.16% at 40 DASand 56.06% at 65 DAS. However, the increase was more at 65 DAS thanat 40 DAS(Table 3.1).Increase in proline content under stress conditions has been suggested due to enhanced synthesis of proline and/or stress induced decrease in incorporation of proline into proteins (Mishra *et al*., 1995).

Table 3.1: Effect of water stress on proline in fenugreek genotypes at two stages

Genotypes	Proline (mg 100g-1 FW)					
	40DAS			65DAS		
	Control	Stressed	Percent Increase	Control	Stressed	Percent Increase
UM-100	63.81	98.12	34.96	163.83	363.58	54.94
UM-112	56.89	66.31	14.20	154.45	244.89	36.93
UM-124	64.92	100.17	35.19	173.47	370.31	53.16
UM-134	62.23	99.02	37.16	170.71	388.50	56.06

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UM-140	53.33	67.37	20.85	159.95	257.16	37.80
SEm±	1.495		4.667			
CD(p=0.05)	4.19		13.48			
CV	4.09		3.81			

2. **Glycine Betaine**

The present investigation showed significant increase in Glycine betaine content due to water stress at both the stages. The maximum increase due to water stress was observed in genotype UM-100 (44.75%) at 40 DAS. At 65 DAS also, this genotype showed the highest increase (43.25%) on the basis of GB, genotype UM-124, significantly increased under water stress conditions (Table 3.2).Several reports on GB accumulation and drought stress have shown that accumulation of GB under drought stress was found to be high in drought tolerant species than drought susceptible species. (Hitz & Hanson, 1980; Wyn Jones & Storey, 1981; Rhodes *et al*., 1987; Mittal, 2010; Ranganayakulu *et al*., 2015).

Table 3.2: Effect of water stress on glycine betaine (GB) in fenugreek genotypes at two stages

Genotypes	GB(mgg^{-1}FW)					
	40DAS			65DAS		
	Control	Stressed	Percent Increase	Control	Stressed	Percent Increase
UM-100	1.99	3.61	44.78	1.17	2.06	43.25
UM-112	2.14	3.12	31.46	1.29	1.88	31.17
UM-124	1.87	3.21	41.59	1.43	2.49	42.44
UM-134	2.16	3.66	40.96	1.36	2.35	42.33
UM-140	2.52	3.68	31.68	1.22	2.05	40.56
SEm±	0.075		0.044			
CD(p=0.05)	0.216		0.128			
CV	5.36		5.12			

3. **Glutathione Reduced**

A lower accumulation of GSH in stressed plants of all genotypes was recorded at both stages. Minimum decline was observed in genotypes UM-100 with 1.80% at 40 DAS and 2.71% at 65 DAS followed by UM-124 with 4.44% at 40 DAS and at 65 DAS, the significant decrease was found (Table 3.3).In the present study, a lowering of GSH contents during stress, the magnitude being higher at 65 DAS reveals that the fenugreek plants had detoxified ROS intermediates ($O_2^- \rightarrow H_2O_2$) at this stage quickly thus indicating the operation of ASC-GSH cycle during water stress (Asada and Takahashi, 1987).

Table 3.3: Effect of water stress on glutathione reduced (GSH) in fenugreek genotypes at two stages

Genotypes	GSH(mgg^{-1}FW)					
	45 DAS			65 DAS		
	Control	Stressed	Percent Decrease	Control	Stressed	Percent Decrease
UM-100	1.49	1.47	1.80	1.73	1.69	2.71
UM-112	1.94	1.60	17.36	1.81	1.51	16.36
UM-124	1.51	1.45	4.44	2.08	1.90	8.41
UM-134	1.65	1.55	6.10	1.82	1.67	8.49
UM-140	1.83	1.46	20.22	1.78	1.40	21.13
SEm±	0.023			0.04		

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CD(p=0.05)	0.068	0.115
CV	2.94	4.58

4. **Peroxidase**

The present study showed significant increase in POX activity due to water stress at both the stages. The maximum increase due to water stress was observed in genotype UM-124 (37.84%) at 40 DAS. At 65 DAS also, the maximum increase was observed in same genotype (24.77%). However, the increase was more at 40 DAS than at 65 DAS. Our results thus show that a significantly higher POX activity in stress condition may scavenges the ROS (Table 3.4).Karmakar *et al.*, (2014) worked on response of fenugreek (*Trigonella foenum-graecum* L.) seedlings under moisture and heavy metal stress with special reference to antioxidant system. They found increased POD activity, indicating that this enzyme serves as an intrinsic defence; to resist PEG induced oxidative damage. Our results are supported by many other scientists. (Pant *et al*., 2014, Mittal *et al*., 2006 and Mittal, 2010).

Table 3.4: Effect of water stress on Peroxidase (POX) in fenugreek genotypes at two stages

Genotypes	POX(ODunitperminute/100mg)					
	40DAS			65DAS		
	Control	Stressed	Percent Increase	Control	Stressed	Percent Increase
UM-100	0.015	0.021	28.24	0.031	0.039	20.58
UM-112	0.022	0.025	12.24	0.034	0.037	9.43
UM-124	0.006	0.009	37.84	0.012	0.016	24.77
UM-134	0.005	0.007	23.21	0.010	0.013	22.31
UM-140	0.008	0.009	8.70	0.011	0.012	7.37
SEm±	0.0001		0.001			
CD(p=0.05)	0.0001		0.002			
CV	6.79		6.23			

5. **Total chlorophyll content**

Reduction in the content of total chlorophyll was observed in all the genotypes at both the stages under water stress conditions. The decline was relatively lower at 40 DAS than at 65 DAS.Amongst the five genotypes, the lowest reduction was observed in genotypes UM-124 (10.55%) at 40 DAS due to water stress. While the least reduction under stress at 65 DAS was found in genotype UM-100 (5.14%) (Table 3.5). However the total chlorophyll content was more at 40 DAS than of 65 DAS and significant reduction was observed at 40 DAS than 65 DAS (Table 3.5). The results are supported by the findings of Abdouli *et al*., 2012 and Aggrawal *et al*., 2013.

Table 3.5: Effect of water stress on total chlorophyll content in fenugreek genotypes at two stages

Genotypes	Chlorophyll(mgg^{-1}FW)					
	40DAS			65DAS		
	Control	Stressed	Percent Decrease	Control	Stressed	Percent Decrease
UM-100	11.60	8.65	**25.44**	6.84	6.49	**5.14**
UM-112	18.19	6.61	**63.66**	8.50	5.35	**37.03**
UM-124	9.88	8.84	**10.55**	6.42	5.63	**12.19**
UM-134	10.20	7.52	**26.23**	7.61	6.19	**18.67**
UM-140	12.32	6.13	**50.27**	7.68	5.15	**32.86**
SEm±	0.420			0.229		

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CD(p=0.05)	1.213	0.663
CV	8.40	6.97

6. **Carotenoid content**

Significant reductions in total carotenoids content were found in allthe genotypes at 65 DAS under water stress conditions. Minimum decline was observed in genotype UM-124 (23.45%) at 65DAS and in genotype UM-100 (9.99%) at 40 DAS followed by genotypeUM-100 (24.44%) at 65 DAS and genotype UM 124 (14.30%) at 40 DAS (Table 3.6).Total carotenoids showed similar trends as by chlorophyll contents.Our results are also supported by research on mannitol-induced drought stress on calli of *Trigonella foenum-graecum* L. Var. RMt-303 (Pant *et al*., 2014).

Table 3.6: Effect of water stress on Carotenoids content in fenugreek genotypes at two stages

Genotypes	**Carotenoid (mg g^{-1} FW)**					
	40 DAS			**65 DAS**		
	Control	**Stressed**	**Percent Decrease**	**Control**	**Stressed**	**Percent Decrease**
UM-100	1.11	1.00	9.99	2.59	1.96	24.44
UM-112	0.65	0.48	26.61	2.56	1.78	30.44
UM-124	0.89	0.76	14.30	2.08	1.59	23.45
UM-134	0.88	0.71	19.23	2.43	1.82	25.18
UM-140	0.56	0.36	35.07	3.63	2.14	40.97
SEm±	0.057			0.054		
CD(p=0.05)	NS			0.156		
CV	15.37			4.78		

7. **Malondialdehyde**

The present investigation showed the minimum increase in MDA content in genotype UM-100 (8.18%) at 40 DAS, followed by genotype UM-124 (12.17%) at the same stage. Whereas at 65 DAS, the minimumincrease in MDA was found in genotype UM-124 (2.04%) followed by UM-100 (10.16%) to have better stability of membranes. Genotypes UM-140 (39.51 at 40 DAS, 40.58 at 65 DAS) and UM-112 (33.89%, 17.92 at 65 DAS) which have less membrane stability due to higher accumulation of MDA content at both stages (Table 3.7).The results are supported by the earlier findings of Pant *et al*., 2014, Mittal *et al*., 2006, Mittal, 2010, Karmakar *et al*., 2014.

Table 3.7: Effect of water stress on Malondialdehyde (MDA) content in fenugreek genotypes at two stages.

Genotypes	**MDA (m moles g^{-1}FW)**					
	40DAS			**65DAS**		
	Control	**Stressed**	**Percent Increase**	**Control**	**Stressed**	**Percent Increase**
UM-100	28.71	31.27	8.18	20.54	22.86	10.16
UM-112	20.11	30.42	33.89	21.31	25.96	17.92
UM-124	16.78	19.10	12.17	18.60	18.99	2.04
UM-134	27.09	35.50	23.69	18.21	20.93	12.96
UM-140	19.10	31.58	39.51	15.89	26.74	40.58
SEm±	0.197		0.837			
CD(p=0.05)	0.570		2.418			
CV	1.52		7.97			

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4. Conclusion

- The results of present investigations indicate that significant biochemical changes occur under water stress at the two stages 40 DAS and 65 DAS.
- Based on most of the parameters, at both stages genotype UM-134 was highly tolerant to water stress.
- Genotypes UM-100 at 40 DAS and UM-124 at 65 DAS were moderately tolerant. Genotypes UM-112 and UM-140 were sensitive to water stress.
- Based on most of the parameters, 65 DAS stage was moresensitive to water stress than 40 DAS.
- These genotypes contrasting in their response to water stress may be used for breeding programmes for water stress studies or to generate drought tolerant genotypes by exploiting genetic engineering techniques.

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