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Full Research Article

Evaluation of Growth and Biochemical Markers for Salt Stress in Fenugreek (*Trigonella foenum-graecum* L.)

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Abstract

Fenugreek (*Trigonella foenum-graecum*) also known as methi, is used both as herb (leaves) and as spice (seeds), also known for its medicinal values. The effects of salinity on plant growth are associated with low osmotic potential of soil solution (water stress), nutritional imbalance, specific ion effect (salt stress), or a combination of these factors. All of these cause adverse pleiotropic effects on plant growth and development at physiological, biochemical and molecular levels. A number of enzymes regulate intracellular H_2O_2 levels but catalase (CAT), total peroxidase (POD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOX) are known to play an important role. In the present study, an effort has been made to study the effect of salt stress on levels of different biochemical stress markers in seedlings of fenugreek cultivar (RMt-1), subjected to solution of -0.5, -0.7, -1.2, -1.7 Mega Pascal (MPa) osmotic potential. A decrease in seedling vigor and moisture content was observed when osmotic potential was increased from -0.5 Mpa to -1.7 MPa whereas Proline, H_2O_2, MDA, Total Chlorophyll, Chl a, Chl b and total phenolic content increased with the duration of stress. H_2O_2 metabolizing enzymes viz., APX, GR and GPOX showed a moderate increase in activity in comparison to SOD and POD, which showed a far greater increase in the activity, CAT activity declined with time. The results of these studies on fenugreek cultivar may be useful in elucidating tolerance mechanisms at the cellular level and may thus facilitate development of salt tolerant fenugreek cultivars.

1. Introduction

Salinity affects plant growth and development in two ways. First it imposes osmotic stress by reducing the soil water potential leading to limiting the water uptake, second it causes excessive uptake of ions particularly of Na^+ and Cl^- thus ultimately interferes with metabolic processes. Salt stress like other abiotic stress, can also leads to oxidative stress through the increase in reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) etc., which may hamper normal cellular functioning through oxidative damage to lipids, proteins and nucleic acids (Neill et al., 2002). To overcome these effect plant make use of a complex antioxidative system which is composed of antioxidant like glutathione, ascorbate and carotenoids as well as ROS scavenging enzyme like ascorbate peroxidase, superoxide dismutase, guaicol peroxidase and glutathione reductase. Though plants have a potential antioxidant defence system, still plant suffer extensive damage on exposure to salinity stress. There is an evidence to suggest that the alleviation of oxidative damage and increased resistance to salinity stress is correlated with an efficient antioxidant system. In plants, a number of enzymes regulate intracellular H_2O_2 levels but catalase (CAT), total peroxidase (POD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOX) are known to play an important role. Despite this the mechanism of salinity/drought tolerance in fenugreek is not fully understood. Fenugreek is one of the most important medicinal and spice plant in the world. The properties of Fenugreek such as hypocholesterolemic and hypolipidemic have also been reported (Puri et al., 2002). Fenugreek galactomannans results in reduced glucose absorption within the digestive tract.

2. Materials and Methods

2.1. Germination of seeds

Seeds of Fenugreek cultivar RMt-1 were procured from Department of Genetics and Plant Breeding, Rajasthan Agriculture University, Bikaner Rajasthan. Seeds were first washed with distilled water, followed by surface sterilization with 0.1% $HgCl_2$ for 1 min. Seeds were then germinated on germination papers moistened with autoclaved distilled water to ensure adequate moisture to seeds, in Petri-plates. After 7 days of germination seedlings were irrigated with Hoagland's solution for further 7 days.

2.2. Seedling physiology and of NaCl stress

Different morphological characters like seedling vigour index, moisture content of early seedlings irrigated with Hoagland solution having different conc. of NaCl having osmotic potentials (0 (control), -0.5 MPa, -0.7 MPa, -1.2 MPa and -1.7 MPa) at 1st, 3rd , 5th and 7th day from the day of irrigation were determined by the following formulas

Moisture content=(fresh weight-dry weight)/fresh weight×100

Vigour index= (mean root length+mean shoot length)×percent germination.

2.3. Evaluation of biochemical stress markers

Free proline was determined by the method of Bates et al. (1973). Hydrogen peroxide content was measured spectrophotometrically after reaction with potassium iodide (KI) through Alexieva et al. (2001) method. Total chlorophyll content was estimated with the method given by Arnon (1949). The procedure of Swains and Hillis, (1959) was used to measure the total phenolics content.

2.3. Anti-oxidative enzyme analysis

Ascorbate peroxidase activity was determined according to the method of Nakano and Asada (1981). Guaiacol peroxidase activity was determined as described by Urbanek et al. (1991). The enzyme activity expressed as µmol min^{-1} mg^{-1} protein. Specific Catalase activity was measured according to Beers and Sizer (1952). Peroxidase activity was determined according to Tatiana et al. (1999) with some modifications. Superoxide dismutase (SOD) activity was assayed spectrophotometrically as the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm Beauchamp and Fridovich (1971). Glutathione reductase (GR) activity was estimated by recording NADPH oxidation at 340 nm, according to Foyer and Halliwel (1976).

3. Results and Discussion

3.1. Effect of salt stress on morphological characteristics during early seedling stage

3.1.1. Seedling vigour

A decrease in vigour index of seedlings was recorded under increasing osmotic potential of NaCl containing media, at different time intervals. In -1.7MPa osmotic potential a 19.62% decrease in seedling vigour was observed on 7th day, while decrease of 28.97%, in -1.2 MPa and no substantial change was observed in -0.7 and -0.5 Mpa over their respective controls. There was a decrease of 27.11% and 31.53% seedling vigour in -1.7, -1.2 Mpa osmotic potential and further no substantial change was observed when osmotic potential was -0.7 and -0.5 MPa respectively, from 1st to 7th day (Figure 1). Joshi et al. (2011) reported reduction of vigour index in all the varieties of Brassica juncea tested under increasing salt concentration.

3.1.2. Moisture content

Moisture content of seedlings was significantly lowered under increasing osmotic potential of NaCl containing media, at different time intervals. In -1.7 MPa 16.91% decrease in moisture content was observed on 7th day (Table 1.)

3.2. Effect of salt stress on biochemical stress markers

3.2.1. Proline content

An increase in Proline content in seedlings was observed under increasing osmotic potential of NaCl in the media, at different time intervals. There was an increase of Proline from 1st to 7th day from their respective control. Free Proline has been proposed to act as osmoprotectant, a protein stabilizer, a metal chelator. Proline serves as a sink of energy to regulate redox potentials, a hydroxyl radical scavenger (Sharma and Dietz, 2006).

3.2.2. Hydrogen peroxide (H_2O_2) content

An increase in hydrogen peroxide (H_2O_2) level in seedlings was observed under increasing osmotic potential of NaCl containing media, at different time intervals. As shown figure 3, in -1.7 MPa H_2O_2 level was increased by approximately 2.9 fold on 7th day, while an increase of 2.1 folds, 2.5 folds and 2.4 folds was observed when osmotic potential was increased from -0.5 MPa to -1.2 Mpa over their respective controls (Figure 2). Higher oxidative damage,

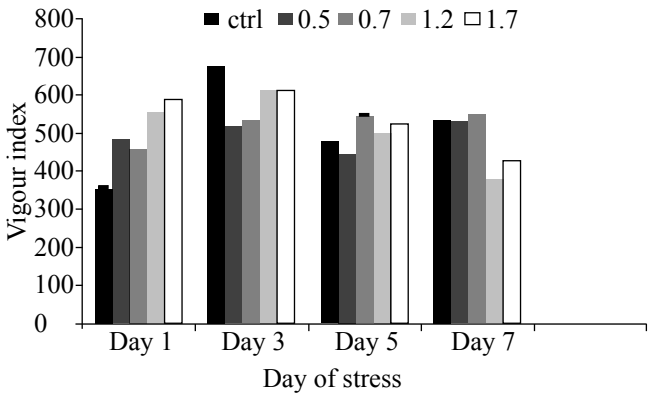

Figure 1: Effect of salt stress on seedling vigour (%)

Table 1: Effect of salt stress on Moisture content (%) in early seedling stage. Data shown below are mean value±standard error (n=3)

Osmotic potential NaCl (-MPa)	Days of stress			
	1st day	3rd day	5th day	7th day
	Moisture content (%) in early seedling stage			
control	6.28±0.57	8.41±0.86	10.47±0.59	10.17±0.61
-0.5	11.35±0.57	11.09±0.61	12.63±0.58	11.35±0.59
-0.7	7.89±0.60	9.75±0.62	10.17±0.61	12.82±0.63
-1.2	8.64±0.60	7.36±0.61	9.15±0.62	10.94±0.69
-1.7	9.48±0.61	11.05±0.59	11.61±0.58	8.45±0.61

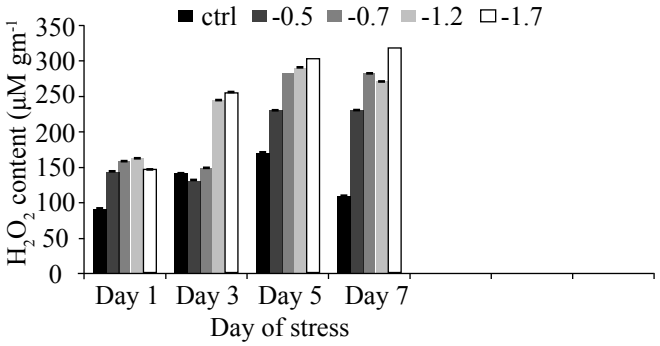

Figure 2: Effect of salt stress on H_2O_2 Content (µmol gm^{-1})

leading to increased H_2O_2 accumulation, could be used as an important parameter for screening of crop varieties for oxidative stress tolerance (Khan and Panda, 2008).

3.2.3. Total phenolics content

An increase in total phenolics content in seedlings was observed under increasing osmotic potential of NaCl containing media, at different time intervals. In -1.7 MPa osmotic potential total phenolics content was increased by 1.4 fold on 7th day, while an increase of 1.4, 1.3 and 1.05 folds was observed when osmotic potential was -1.2, -0.7 and -0.5 MPa respectively, as compared to control (Figure 3).

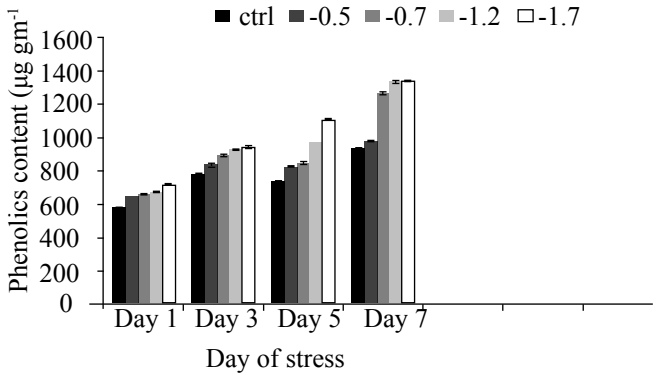

Figure 3: Effect of salt stress on Phenol Content (µg gm^{-1})

3.2.4. Chlorophyll content

Chlorophyll content was analyzed in seedlings at different time intervals, under increasing osmotic potential of NaCl containing media. There was increase of approx 4.8, 6.0 folds in -1.7,-1.2, respectively, from 1st to 7th day while no substantial change was observed in other. Total chlorophyll content increased by 1.8 folds, 1.5 folds and 1.3 folds in -1.7, -0.7 and -0.5 MPa respectively, as compared to control on 7th day. The increase in pigments may be due to an increase in the number of chloroplasts in stressed leaves. The results regarding the increase in chlorophyll content with the corresponding increase in salt concentration agree with that of Misra et al. (1997).

3.3. Effect of salt stress on antioxidative enzymes activity and total protein

3.3.1. Guaiacol peroxidase (GPOX) activity

An increase in Guaiacol Peroxidase (GPOX) activity of seedlings was observed under increasing osmotic potential of NaCl containing media, at different time intervals. Approx 1.2 to 1.3 fold increase in Guaiacol Peroxidase activity was observed when osmotic potential was increased by -0.5 Mpa to -1.7 Mpa. At lower potential there was decrease in GPOX activity but it increased by 30% to 60% more from 1st to 7th day when osmotic potential was increased to -1.2 and -1.7 MPa respectively. Under sub lethal salinity, level of peroxidases has been used as potential biomarker to evaluate the intensity of stress (Mittal and Dubey, 1991).

3.3.2. Ascorbate peroxidase (APX) activity

The activity of Ascorbate Peroxidase (APX) showed a concomitant increase in seedlings with increasing osmotic potential of NaCl containing media, at different time intervals. In comparison to control an increase of approximately 4 times increase in APX activity was observed on 7th day when osmotic potential was increased from control to -1.7 Mpa. The APX activity was also observed to higher acting at 7th day as compared to 1st day of stress which indicates the tolerance of the plant against stress. It has been shown that over expression of APX gene in plants increases protection against oxidative stress (Wang et al., 1999).

3.3.3. Peroxidases (POD) activity

Total Peroxidase activity increased remarkably with increasing osmotic potential of NaCl containing media, at different time intervals. In comparison to control an increase of approximately 1.5 folds was observed in -0.5 Mpa to, -1.7 Mpa, respectively, on 7th day. An increase of 1.6 folds in -0.5 to -1.7 MPa respectively, was observed from 1st to 7th day (Table 2). POD activity increased to high levels because they are known to play a significant role in various processes such as lignin biosynthesis and formation of isodityrosine bridges that are

Table 2: Effect of salt stress on Peroxidase activity in early seedlings. It is expressed in µmol min^{-1} mg^{-1} protein. Data shown below are mean value±standard error (n=3)

Osmotic potential NaCl (-MPa)	Days of stress			
	1st day	3rd day	5th day	7th day
	Peroxidase activity in early seedlings (µmol min^{-1} mg^{-1} protein)			
control	3840.11± 9.77	2823.86± 12.83	8190.87± 17.91	5024.56± 45.30
-0.5	3929.82± 12.67	3980.35± 24.24	5541.05± 23.19	5159.29± 19.30
-0.7	5608.42± 2.41	10065.9± 9.45	4861.75± 14.96	6894.03± 12.72
-1.2	3216.84± 34.17	6461.75± 1.02	5630.87± 75.45	4839.29± 12.36
-1.7	3211.22± 12.35	7404.91± 1.25	4917.89± 53.99	6046.31± 12.36

believed to crosslink between structural protein molecules, in addition to antioxidative activity. POD scavenges H_2O_2 in chloroplasts which are produced through dismutation of superoxide (O_2^{-}) catalysed by SOD. Increased POD activity has also been reported in salt tolerant and sensitive species of tomato (Shalata and Tal, 1998).

3.3.4. Catalase (CAT) activity

Catalases are indispensable for ROS detoxification during stressed condition. Catalase has one of the highest turnover number enzyme. A uniform increase in POD, APX and GPX activities paralleled with gradual decrease in catalase (CAT) activity in seedlings. In the present investigation the activity decreased by apporx 2 folds in -0.5 MPa to -1.7 Mpa from 1st to 7th day respectively, in comparision to control whereas no effect was observed of salt stress on catalase activity on 7th day (Figure 4). It may be due to production of H_2O_2 when salt stress was increased. Since catalase have highest turnover number and may be useful to remove H_2O_2 produced during salt stress (Figure 4). Though a decrease in CAT activity poses an oxidative threat a corresponding increase in total peroxidase (POD) activity may play a role in H_2O_2 detoxification (Peixoto et al., 1999). A reduction in CAT activity under stressful conditions has been attributed to the inactivation of enzyme protein due to ROS (Engel et al., 2006), or decrease in enzyme synthesis or change in assembly of enzyme subunits (Hertwig et al., 1992). They reported decrease in CAT activity in soyabean root nodules with increasing salt concentration.

3.3.5. Superoxide dismutase (SOD) activity

SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all sub

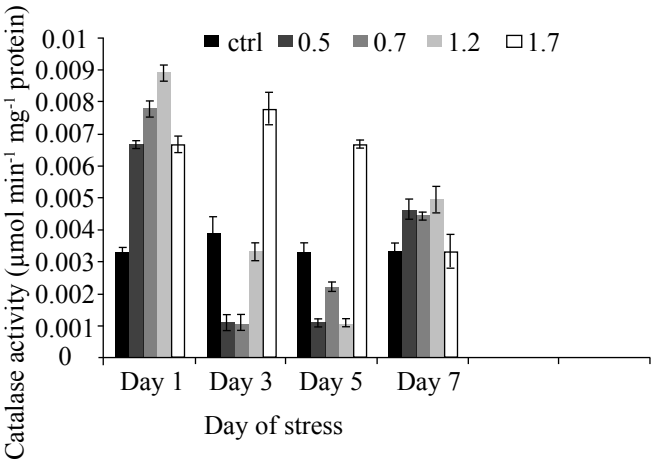

Figure 4: Effect of salt stress on catalase content (µmol min^{-1} mg^{-1} protein)

cellular compartment prone to ROS mediated oxidative stress. SOD has been proposed to be important in plant stress tolerance and provide the first line of defence against the toxic effect of elevated level of ROS. Superoxide dismutase (SOD) activity in seedling subjected to NaCl stress increased in comparison to their respective controls. On 7th day SOD activity increased by 3.2 folds, 3.6 folds, 4.4 folds and 5.3 folds when osmotic potential was increased from 0.5 Mpa to -1.7 Mpa (Table 3). SOD activity was observed to increase 3 to 4 times from 1st to 7th day in all treatments. The increase in SOD activity with respect to salt stress may be correlated to sensitive and tolerant variety as there have been many aspect of production of abiotic stress tolerance transgenic plant. Panda et al. (2003) reported increased SOD activity under salt stress.

Table 3: Effect of salt stress on Superoxide dismutase activity in early seedling stage. It is expressed in µmol min^{-1} mg^{-1} protein. Data shown below are mean value±standard error (n=3)

Osmotic potential NaCl (-MPa)	Days of stress			
	1st day	3rd day	5th day	7th day
	Superoxide dismutase activity in early seedling stage (µmol min^{-1} mg^{-1} protein)			
Control	4.14± 0.075	7.03± 0.189	11.04± 0.216	15.11± 0.166
-0.5	15.37± 0.37	13.49± 0.326	16.34± 0.251	48.67± 0.257
-0.7	13.09± 0.019	12.83± 0.167	28.02± 0.173	54.44± 0.288
-1.2	22.50± 0.41	12.77± 0.117	28.79± 0.149	67.25± 0.276
-1.7	20.84± 0.61	22.74± 0.97	30.12± 0.189	81.30± 0.331

3.3.6. Glutathione reductase (GR) activity

GR is a flavoprotein oxidoreductase potential enzyme of ASH-GSH cycle and plays an essential role in defence system against ROS by sustaining the reduced status of GSH. Glutathione reductase (GR) activity in seedling subjected to NaCl stress, increased in comparison to their respective controls. It was observed that there is an increase in GR activity from 1st to 7th day in all seedling stage. As the seedling grows GR activity increased in control as well as in all treatment from 1st to 7th day. In stress condition it was 1.5 times higher than control on 7th day (Table 4).

Table 4: Effect of salt stress on Glutathione reductase activity in early seedling stage. It is expressed in µmol min^{-1} mg^{-1} protein. Data shown below are mean value±standard error (n=3)

Osmotic potential NaCl (-MPa)	Days of stress			
	1st day	3rd day	5th day	7th day
	Glutathione reductase activity in early seedling stage (µmol min^{-1} mg^{-1} protein)			
Control	0.048± 0.002	0.048± 0.009	0.078± 0.012	0.099± 0.017
-0.5	0.045± 0.001	0.047± 0.004	0.084± 0.011	0.096± 0.019
-0.7	0.031± 0.004	0.015± 0.003	0.107± 0.007	0.109± 0.018
-1.2	0.044± 0.006	0.078± 0.007	0.111± 0.009	0.129± 0.016
-1.7	0.061± 0.011	0.092± 0.013	0.126± 0.015	0.179± 0.022

4. Conclusion

With increasing concentration of NaCl, a moderate increase in APX, GR and GPOX activity was observed while a greater increase in POD and SOD activity was observed, whereas CAT activity declined with time. Pyramiding of ROS scavenging enzymes may also be used to obtain abiotic stress tolerance plants. Therefore, plants with the ability to scavenge and/or control the level of cellular ROS may be useful in future to withstand harsh environmental conditions.

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