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Biochemical evaluation of lucerne (*Medicago sativa* L.) cultivars under water stress condition

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Abstract
The present investigation was conducted to find out water stress tolerance in lucerne cultivars using biochemical markers. The lucerne cultivars were grown in pots for 45 days, thereafter water stress was imposed by -0.25 MPa PEG-6000 solution. The leaves of control and stressed plants were analyzed for osmolytes accumulation and activities of antioxidant enzymes. The stressed leaves showed decline in relative water and total chlorophyll content than unstressed. The cultivar Anand-2 recorded the highest RLWC (76%), followed by RL-10-01 (75%). Under the stressed condition, the highest total chlorophyll content was observed in the cultivar RL-10-01, followed by RL-10-02 with 1.10 mg g^{-1} FW and 1.05 mg g^{-1} FW, respectively. Overall the osmolytes, proline and glycine betaine were increased under water stress condition. Water stressed leaves of cultivars, Anand-2 and RL-10-01 accumulated higher proline content of 14.72 and 13.88 µmoles g^{-1} FW, respectively, whereas glycine betaine accumulated higher in Anand-2, followed by RL-10-01 with 24.98 and 22.82 µmoles g^{-1} FW, respectively. The activities of anti-oxidative enzymes, ascorbate peroxidase and superoxide dismutase were higher under water stress. The higher ascorbate peroxidase activity of 651.35 and 612.67 ηmoles ascorbate oxidized mg^{-1} protein min^{-1} and superoxide dismutase activity of 95.0 and 92.0 units mg^{-1} protein was recorded by Anand-2 and RL-10-01, respectively under water stress condition. The lowest lipid peroxidation rate was observed in Anand-2 and RL-10-01, whereas the highest nitrate reductase activity was recorded in Anand-2, followed by RL-10-01 with 386.6 and 358.3 ηmoles NO_2^- formed g^{-1} FW h^{-1}, respectively under water stress condition. The biochemical study revealed that cultivars, Anand-2 and RL-10-01 could be used as promising donors for inducing drought tolerance characters in breeding programme.

Keywords: Anti-oxidative enzymes, lucerne, osmolytes, water stress

Introduction
Lucerne (*Medicago sativa* L.) is a fodder crop belongs to family the Leguminoceae. Lucerne grown globally over an area of 35 million ha, while 1 million ha in India with productivity of 60-130 tons ha^{-1}. The major lucern growing states are Punjab, Maharashtra, Uttar Pradesh, Gujarat, and Tamil Nadu (Anonymus, 2013) [2]. Lucerne is high quality green feed, having high energy, digestibility around 65-72% and high protein content of 12-24%. Benefit of this crop is combination of per hectare higher yield with high nutritional quality (Abid *et al*., 2015) [1]. It has deep rooted system, grown as annual or perennial legume. Drought and irregular rain fall limits plant growth and production mostly in arid and semi-arid regions. Every year 40% of cultivated land is affected by drought. Such water stress induces a disruption of many morphological, physiological and metabolic processes which affects photosynthetic rate, protein biosynthesis and accumulation of solutes etc. The major plant responses to water stress includes changes in stomatal conductance, osmolytes accumulation, and specific gene expression. The abscisic acid is major stress hormone accumulate in severe stress conditions, it participate in physiological and biochemical processes for survival of plant (Huang *et al.*, 2000). The consequence of exposure to water stress is the generation of reactive oxygen species (ROS), which in turn have a negative oxidative stress effects on cellular structures and metabolism. As water and salt stresses occur frequently, plants have developed several strategies to cope with these challenges. One of the stress defense mechanisms is activation of antioxidant defense system, which includes production of antioxidative enzymes and low-molecular antioxidants. The enzyme superoxide dismutase (SOD) converts superoxide radicals (O_2) into hydrogen peroxide (H_2O_2), POD reduces H_2O_2 into water using various substrates as electron donor, Ascorbate peroxidase (APX) uses ascorbate as an electron donor to reduce H_2O_2 to water, and CAT dismutase's H_2O_2 into water and oxygen. Rapid detoxification of both O_2 and H_2O_2 is therefore essential to prevent oxidative damage. Numerous studies indicated that the activity of antioxidant enzymes is correlated with plant tolerance to abiotic stresses, including drought and other stresses. There is also increase in concentration of osmolytes such as glycine betaine and proline in response to abiotic stresses for osmotic adjustment.

After establishment, lucerne has good drought tolerance ability, thus well suited under irregular rainfall; it appears to go dormant for extended dry periods. Analysis of lucerne growth under drought stress has shown that it respond to drought by reduction in shoot and root length, no of basal bud and shoot also found to be reduced (Safarnejad *et al.*, 2008) [18]. Due to perennial nature of lucerne crop, plant suffers from water stress condition many times, which reduces plant population and altimetry forage yield. Thus, in the present investigation drought stress tolerance mechanism in lucern cultivars was studied by evaluating chlorophyll, osmolytes, activities of antioxidative enzymes and nitrate reductase under water stress condition.

Material and methods

Seven lucerne cultivars, *viz*., Anand-2, Anand-3, CO-1, CO-2, RL-10-01, RL-10-02 and RL-88 were obtained from different locations *viz.*, AAU, Anand, TNAU, Coimbatore and MPKV, Rahuri,. The seeds were grown in pots for 45 days containing equal quantity of black cotton soil. Thereafter, pots were divided into two groups i.e. control and stress. The water stress was created by -0.25 MPa PEG-6000 solution for 2 days. The use of -0.25 MPa PEG-6000 solution had optimized before starting the experiment. The leaves of control and stressed plants were analyzed two days after the imposing water stress. The leaves of control and stressed plants were assayed for biochemical parameters *viz.*, osmolytes, proline (Bates *et al.* 1973) [4] and glycine betaine (GB) (Stumf, 1984) [19], activities of antioxidative enzymes such as superoxide dismutase (SOD) (Dhindsa *et al*., 1981) [8] and ascorbate peroxidase (APX) (Nakano and Asada, 1981) [6]. The relative leaf water content (RLWC) (Henderson and Davies, 1990) [12], total chlorophyll content (Aron, 1949) [3], lipid peroxidation rate (Heath and Packer, 1968) [13] and nitrate reductase activity (NR) (Hageman and Hucklesby, 1971) [10]. The data obtained was analyzed for statistical significance using Factorial Randomized Block Design (Panse and Sukhatme, 1985) [17].

Results and Discussion

The data presented in Table 1 showed the effect of -0.5 MPa PEG–6000 -induced water stress on RLWC and total chlorophyll in the leaves of lucern cultivars.

Relative leaf water content

The stressed leaves of lucerne showed a considerable decline in RLWC over unstressed control in all cultivars. The RLWC was ranged from 48.67 to 76 per cent under stressed condition. After imposition of water stress, the highest RLWC of 76 per cent was recorded by cultivars Anand-2, followed by RL-10-01 and RL-88 with 75 and 60 per cent, respectively, also the lowest per cent decrease was observed in these cultivars over control. Luo *et al.* (2019) [14] reported decline in relative water content (RWC) in the range of 79 to 85 per cent, more in roots, followed by stem and leaves of lucerne from unstressed to severe water stress. Reduction in RLWC is an effect of water deficit in soil. Water deficit negatively affect the RWC in leaves of lucerne (Farissi *et al.*, 1913).

Table 1: Effect of PEG-6000 -induced water stress on relative water and total chlorophyll content

Cultivars	Relative leaf water content (%)		Per cent decrease over control	Total chlorophyll (mg g^{-1} FW)		Per cent decrease over control
	Control	Stress		Control	Stress	
Anand-2	90.00	76.00	15.56	1.22	1.01	20.60
Anand-3	79.92	55.82	30.16	1.45	0.92	57.39
CO-1	91.49	58.00	36.60	1.42	0.97	46.87
CO-2	84.18	57.00	32.29	1.25	0.73	70.95
RL-10-01	91.41	75.00	17.95	1.44	1.10	30.22
RL-10-02	67.72	48.67	28.14	1.53	1.05	45.87
RL-88	71.88	60.00	16.53	1.30	0.98	32.35
Mean	81.90	43.84	25.32	1.37	0.967	43.46
Range	67.72-91.49	48.67-76.00	15.56-36.60	1.22-1.53	0.73-1.10	20.60-70.95
	Condition	Variety	C X V	Condition	Variety	C X V
SE±	0.972	0.743	2.572	0.011	0.010	0.035
CD at 5%	2.812	2.148	7.441	0.031	0.022	0.010

Total chlorophyll

The total chlorophyll content was decreased under PEG -induced water stress over the control. Total chlorophyll content was ranged from 0.73 to 1.10 mg g^{-1} FW under stress condition. The stressed leaves of RL-10-01 recorded the highest total chlorophyll content of 1.10, followed by RL-10-02 and Anand-2 with 1.05 and 1.01 mg g^{-1} FW, respectively. The lowest decrease in total chlorophyll content was 20.6 and 30.22 per cent observed in Anand-2 and RL-10-01, respectively. Similar results reported by Moharramnejad *et al*. (2015) [15] that PEG induced stress significantly decreased chlorophyll a, b and total in maize. Damame (2013) [6] reported significant decline in Chlorophyll content under moisture stress created by PEG or withholding water under field condition in sorghum genotypes.

The data depicted in Table 2 showed the effect of -0.5 MPa PEG–6000 -induced water stress on proline and GB accumulation and activities of enzymes in the leaves of lucern cultivars.

Proline

Considerably higher proline content was accumulated under stressed than the unstressed condition in all lucerne cultivars. The proline content under stressed condition was ranged from 6.83 to 14.72 µmoles g^{-1} FW in which cultivar Anand-2 recorded the highest proline content of 14.72, followed by RL-10-01 with 13.88 µmoles g^{-1} FW, while the cultivar RL-10-02 recorded lowest proline content. The Anand-2 and RL-10-01 also recorded the higher per cent increase of 143.57 and 129.42, respectively over unstressed condition. Luo *et al* (2019) [14] marked that significant increase in proline concentration in lucerne leaves from 2.04 to 4.28 µg g^{-1} FW under full water to severe water stress treatment and correlated with biomass. Damame *et al.* (2014) [7] reported PEG –induced osmotic stress significantly increased proline content in stressed than unstressed sorghum genotypes. Moharramnejad *et al*. (2015) [15] also reported PEG stress significantly increased proline content in maize.

Table 2: Effect of PEG-6000 -induced water stress on osmolytes and enzyme activities

Cultivars	Proline (µmoles g^{-1} FW)		Per cent increase over control	Glycine betaine (µmoles g^{-1} FW)		Per cent increase over control	APX (ηmoles of ascorbate oxidized $min^{-1}mg^{-1}$ protein)		Per cent increase over control
	Control	Stress		Control	Stress		Control	Stress	
Anand-2	6.04	14.72	143.57	8.62	24.98	189.75	343.54	651.35	89.98
Anand-3	5.57	8.27	48.47	7.50	10.04	33.91	389.57	485.61	18.75
CO-1	6.66	12.04	80.80	7.52	16.50	119.36	349.70	543.54	33.33
CO-2	5.67	10.96	93.29	6.13	13.76	124.47	260.09	418.55	60.92
RL-10-01	6.05	13.88	129.42	7.98	22.82	185.96	341.67	612.67	79.47
RL-10-02	4.29	6.83	59.21	8.39	13.52	61.14	288.49	514.91	56.25
RL-88	5.78	11.98	111.49	8.37	22.76	171.95	333.95	568.94	70.36
Mean	5.58	10.78	92.05	8.07	17.769	118.47	260.09	418.55	45.85
Range	4.29- 6.66	6.83-14.72	48.47-143.57	6.13- 8.62	10.04-24.98	33.91-189.75	260.09-389.57	418.55-651.35	18.75-89.98
	Condition	Variety	C X V	Condition	Variety	C X V	Condition	Variety	C X V
SE±	0.008	0.006	0.022	0.190	0.145	0.504	0.236	0.180	0.625
CD at 5%	0.025	0.019	0.065	0.550	0.420	1.456	0.684	0.522	1.808

Cultivars	Superoxide dismutase activity (Units mg^{-1} protein)		Per cent increase over control	MDA (ηmoles g^{-1} FW)		Per cent increase over control	*In vivo* NR activity (ηmoles NO_2^- formed $hr^{-1}g^{-1}$ FW)		Per cent increase over control
	Control	Stress		Control	Stress		Control	Stress	
Anand-2	71.44	95.00	32.98	18.00	25.16	39.78	619.68	386.62	37.61
Anand-3	77.04	85.00	10.34	19.03	33.29	74.92	498.79	176.43	64.63
CO-1	86.12	90.23	4.78	17.31	32.00	73.31	585.92	194.94	66.73
CO-2	73.25	82.25	12.29	25.10	40.25	60.38	499.88	246.13	50.76
RL-10-01	78.20	92.00	17.65	20.15	29.15	44.67	646.91	358.30	44.61
RL-10-02	84.00	88.89	5.82	23.16	39.35	69.92	437.80	223.26	49.00
RL-88	76.00	91.21	20.01	21.11	31.15	47.56	510.00	270.09	47.04
Mean	78.01	89.226	14.84	20.55	32.90	58.64	542.71	265.11	51.48
Range	71.44-86.12	82.25-95.00	4.78-32.98	17.31-25.10	25.16-40.25	39.78-74.92	437.80-646.91	176.23-386.62	37.61-66.73
	Condition	Variety	C X V	Condition	Variety	C X V	Condition	Variety	C X V
SE±	0.256	0.196	0.678	0.403	0.307	1.065	7.915	6.045	20.942
CD at 5%	0.741	0.566	1.961	1.164	0.889	3.080	22.894	17.485	60.571

Glycine betaine

In present study, glycine betaine accumulation in stressed lucerne leaves was more than the unstressed control. Under stressed condition, GB was ranged from 10.04 to 24.98 µmoles g^{-1} FW. The highest GB content under stress was recorded in Anand-2 with 24.98, followed by RL-10-01 and RL-88 with 22.82 and 22.76 µmoles g^{-1} FW, respectively. The highest per cent increase of 189.75 and 185.96 GB was also recorded by Anand-2 and RL-10-01 cultivars, respectively over control. Similar results were reported by Damame *et al.* (2014) [7] that PEG induced osmotic stress significantly increased glycine betaine content in stressed sorghum genotypes than unstressed one. Moharramnejad *et al.* (2015) [15] showed increase in glycine betaine content significantly under PEG stress in maize.

Ascorbate peroxidase

Significantly higher APX activity was observed under water stress condition in all lucerne cultivars. The cultivar Anand-2 recorded the highest APX activity under stressed condition with 651.35, followed by RL-10-01 and RL-88 with 612.67 and 568.94 ηmoles ascorbate oxidized mg^{-1} protein min^{-1}, respectively. The highest per cent increase over control was also recorded in Anand-2 and RL-10-01. The results reported by Babkhani *et al.* (2011) and Wang *et al.* (2011) [21, 22] indicated APX activity was higher under stressed than unstressed lucerne cultivars, it was further higher in tolerant than susceptible cultivar. Wang *et al.* (2009) [20] reported significantly higher APX activity in tolerant than susceptible genotype under PEG and salt stress.

Superoxide dismutase

The superoxide dismutase activity in stressed lucerne leaves was higher than the unstressed control. Under stressed condition, SOD activity was ranged from 82.25 to 95.00 units mg^{-1} protein. The cultivar, Anand-2 recorded the highest SOD activity of 95.00, followed by RL-10-01 and RL-88 with 92.00 and 91.21 units mg^{-1} protein, respectively under stressed condition. The highest per cent increase of 32.98 was recorded in Anand-2. Similar results were reported by Babkhani *et al.* (2011) that under NaCl salt stress, SOD activity found much higher in stressed than unstressed lucerne cultivar. Wang *et al.* (2009) [20] reported higher activity of SOD in tolerant than susceptible genotype under PEG and salt stress in lucerne genotypes.

Lipid peroxidation

The lipid peroxidation rate was measured by i.e. malondialdehyde (MAD) formed in lucerne leaves. Overall it was increased under stress condition. Under stress, MDA content was ranged from 25.16 to 40.25 ηmoles MDA g^{-1} FW. The cultivar Anand-2 recorded the lowest MDA content of 25.16, followed by RL-10-01 and RL-88 with 29.15 and 31.15 ηmoles MDA g^{-1} FW, respectively under stress condition. The lowest per cent increase in MDA was recorded in Anand-2 and RL-10-01 cultivars over control. Similar result was reported by Farissi *et al.* (1913) that under water stress at 25% field capacity recorded higher values for MDA in roots, shoots and leaves of lucerne. Babkhani *et al.* (2011) reported that MDA content was higher under salt stress than control and it was increased more than 6 fold. Tolerant

genotypes exhibited lowest percent increase ion MDA than susceptible.

In vivo nitrate reductase

In vivo nitrate reductase activity was found decreased under stress condition. Under stressed condition, *in vivo* NR activity was ranged from 176.23 to 386.62 ηmoles NO_2^- formed g^{-1} FW h^{-1}. The lucerne cultivar Anand-2 recorded the highest activity of 386.62, followed by RL-10-01 with 358.30 ηmoles NO_2^- formed g^{-1} FW h^{-1}, while cultivar Anand-3 recorded the lowest NR activity of 176.23 ηmoles NO_2- formed g^{-1} FW h^{-1} under stressed condition. The lowest per cent decrease of 37.61 in NR activity was recorded in Anand-2. Similar results were reported by Yang *et al*. (2011) [22] that NR activity in fresh leaves of irrigated lucerne in the range of 193 to 1293 ηmoles NO_2^- formed g^{-1} FW h^{-1}. Farissi *et al.* (1913) reported water stress caused great reduction in NRA as well as nitrate content in leaves of lucerne but maintaining adequate level of NR activity under stress is an indicator of tolerance nature of genotype.

Conclusion

Under water stress condition, the lucerne cultivar RL-10-01 recorded the highest total chlorophyll content, while Anand-2 and RL-10-01 recorded highest RLWC. The cultivars, Anand-2 and RL-10-01 accumulated higher proline and GB under stressed condition. These cultivars also exhibited higher antioxidative enzyme activity, lowest lipid peroxidation and higher *in vivo* NR activity under stressed condition. Thus, the cultivars, Anand-2 and RL-10-01 appear to have water stress tolerant character.

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