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Alleviation of Cold Stress Effects on Alfalfa (*Medicago sativa* L.) by Stigmasterol and Ascorbic acid under Tabouk Governorate, Saudi Arabia Kingdom Conditions

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ABSTRACT

Changes in plant growth parameters and the total yield of Alfalfa plant (*Medicago sativa* L.) were registed and chemical constituents were determined under the effect of brassinoteroides (BR_S) as stigmasterol (Stig.) and/or Ascorbic acid (*Vitamin C*; A.SA) according the different concentrations, i.e. 40 and 80 mg/l Stig. and/or 30 and 60 mg/l A.S.A. as foliar spraying. The treatments were applied during cold stress period. The results observed that, combined treatments of both 80 mg/l Stig. + 60 mg/l was the most favorable treatments in increasing plant height, fresh and dry weight and total yield of alfalfa plants. On the other hand, no significant changes was found in growth characters was found at the treatments with 30 and/or 60 mg/l ASA, whereas single application with 40 and/or 80 mg/l of Stig. recorded moderate effects of different parameters of growth characters. Moreover, photosynthetic pigments especially chlorophyll (a and b) showed significant promotion due to various treatments under study compared with control treatments. Protein content of green forage was positively increased with foliar spraying with the eight concentration treatments under study in the two cutting except the differences between the control treatments and 40 mg/l Stig. + 30 mg/l ASA in the 2nd cutting failed to reach the significant level at 5% level. On the contrary, our results indicate that all treatments exhibited negative response for ash content. In general, fiber % did not affect so much at the two cuttings. In additions, SOD enzyme was quantitatively increased according to Stig. or ASA treatments, whereas the increase compared with control treatments was significant in the first cutting and not significant in the second cutting.

Key words: Alfalfa (*Medicago sativa* L.), plant growth parameters, Ascorbic acid, Saudi Arabia

Introduction

Medicago sativa L. is a highly valuated legume forage. It has been heralded as having the highest feeding value of all commonly growth producing highly crops more protein per/ ha than any other crop for livestock. Alfalfa is used for soil improvement and no nitrogen fertilizer required through being the host plant for the nitrogen.

In northendimater, alfalfa is very sensitive to winter injury. Winter hardness (McKersie *et al*., 1999 and Melis, 1999) which resulting in the formation of activated forms of oxygen that altimately results in various types in injury might be reduced in crop plants if there tolerance to activated stress were increased towards that objective. Alfalfa plants were engineered to over express many forms of superoxide dismutase (SOD), as well as, enzyme and the eliminate oxidative radicals (Foyer and Harbinson, 1994 and Osmond and Grace, 1995).

Chilling stress (<20^{o}C) is a direct results of low temperature effects on cellular macromoluler which lead to slowing metabolism, solidification of cell membranes and loss of membrane functions. Chilling stress effects include reduced leaf expansion and growth (Sowinski *et al*., 2005), wilting (Bagnall *et al*., 1983) and chlorosis and may lead to necrosis and resulted seed and pod development in sensitive plant species (Kaur *et al*, 2008, Ohnishi *et al*., 2010 and Kaurly *et al*., 2011).

Brassinosteroids (BR_S) are a group of naturally occuning plant steroidal compounds with ranging biological activity that play pivotal role in the regulating of various plant growth and development processes; BR_S biosynthetic or signaling mutants clearly indicate that these plant steroids area essential for regulating a variety of physiological processes including cellular expansion and proliferation, vascular differentiation, limiting senescence and leaf development. BR_S regulate the expression of hundreds of genes, affect the activity of numerous metapolic pathways and help to control overall developmental programs leading to morphogenesis. On the other hand, the potential application of BR_S in agriculture to improve growth and yield under various stress conditions (Fariduddin *et al*., 2014).

In plants ascorbic (ASA) is essential for photosynthetic activity via the detoxification of superoxide and hydrogen peroxide in chloroplasts. In addition, to its general antioxide function, ASA has been implicated in cell division, cell expansion and cell wall metabolism (Sminoff, 1996 and Noctor and Foyer, 1998). Moreover,

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Ascorbic acid is one of the most extensively studied antioxidants and has been detected in the majority of plant cell types. Ascorbic is a major metabolite in plants, it is antioxidant and; with association with other components of the antioxidant system, protects plants against oxidative damage resulting from chilling stress, ascorbic metabolism, photosynthesis and range of pollutions (Smirnoff, 1996 and Noctorand Foyer, 1998). Antioxidants in plant cells mainly include ascorbate. It can protect plant cells from oxidative damage by scavenging reactive oxygen species (ROS); (Hong *et al*., 2008).

From this point of view, this study were carried out to investigate the effect of foliar application of stigmasterol and ascorbic acid and their combinations of their concentrations on plant response and tolerance to cold stress, productivity and chemical constituents of alfalfa (*Medicago sativa* L.).

Material and Methods

Two field experiments were carried out in two successive following Autumns seasons at Astra Farm, Tabouk Governorate, Saudi Arabia Kingdom. Alfalfa (*Medicago sativa* L. var. Cuf. 101) was provided from the same farm to study the effect of foliar application with stigmasterol (i.e. Chemical Co., St. Louis Mo. BR_S related compound), and ascorbic acid (ASA) (i.e. BDH Chemical Ltd. Poole England) and their interactions of its concentrations on plant tolerance to cold stress that respective by vegetative growth, yield and chemical constituents of alfalfa plants. Soil temperature of the experimental sites are presented in (Table 1).

Table 1: Temperature month/year during first and second seasons (Metarological of Astra Farms, Tabouk Governorate, Saudi Arabia Kingdom)

Average of the soil temp. 20cm depth	Average of the months temp./years		Month/year
	Mini.	Maxi.	
20.6	9.5	22.9	10/2010
17.5	4.6	18.2	11/2010
11.5	1.3	13.8	12/2010
10.0	6.0	14.7	1/2011
12.5	4.0	15.9	2/2011
18.7	7.1	22.0	3/2011
23.5	12.6	27.7	10/2011
16.9	6.0	20.4	11/2011
17.5	8.3	23.8	12/2011
11.1	1.7	13.4	1/2012
14.0	4.7	19.2	2/2012
18.3	6.8	21.0	3/2012

The plot size was 42 m^2, six meters long and seven meters width. The seeds were sown in the 15th of October at the two seasons by means of seed drilling. The experimental design was complete randomized blocks with five replicates. Before sowing, phosphorus fertilizer at 100 kg/fed. In the form of supper phosphate 15.5 P_2O_5 was added, whereas, nitrogen fertilizer was applied in the form of ammonium sulphate 33% N at 100kg/ha was added and after one month from planting date potassium fertilizer as K_2O 48% was added at 50 kg/fed. rate. Other cultural practices of growing alfalfa were conducted as recommended. Foliar spray treatments were carried out using manual atomizer and liquid soap as a wetting agent was added to spraying solution at a rate of 0.1 %, while, control plants were sprayed with water only.

Each experiment consisted of nine treatments which were:

1-Control (tap water)
2-Foliar spraying with 40 mg/l Stig.
3-Foliar spraying with 80 mg/l Stig.
4-Foliar spraying with 30 mg/l V.C. (ASA)
5-Foliar spraying with 60 mg/l V.C. (ASA)
6-Treatment 2 + treatemt 4
7-Treatments 2 + treatments 5
8-Treatments 3+ treatments 4
9-Treatments 3 + treatments 5

Foliar spraying was carried out late afternoon after one month from seedling and replicate 10 days later from the first one. All foliar treatments were applied also two weeks after the first cutting. Plant samples were collected from all replicates for the 1st cutting. Although, plant samples for the 2nd cutting were taken one month after foliar spraying treated plants. The growth characters for both cutting were recorded including plant height "cm", fresh weight of vegetative parts "kg/1m^2", then these green forage were dried in an electrical oven with fan at 75^{o}C till contest dry weight and recorded as kg/m^2. Moreover, dried samples were crushed and kept in desiccators for chemical analysis. In addition, freshly samples for every treatments were quickly freezing under -180^{o}C by using liquid nitrogen then immediately kept at -20^{o}C in deep freezer for super oxide dismutase (SOD) enzyme determination, meanwhile, the chemical analysis for vegetative parts samples, i.e. photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined in fresh leaf discs using the method

adopted by Mertzener *et al*. (1965), and crude protein content was determined using procedure of A.O.A.C (1970) and micro-kjekdahl according to Park (1946). on the other hand, ash % and crude fiber % were determined using the method according (A.O.A.C. 1995), whereas, super oxide dismutase (SOD) activity (unit/mg protein) for freezing tissue crude extract was determined using the method described by Ohindsa *et al*. (1981) by Digital Spectrophotometer, Bechman DU-640.

All the obtained data were subjected to statistical analysis using analysis of variance for complete randomized blocks design according to Snedeer and Cochran (1990). For comparison between means L.S.D. test was used

Results and Discussion

Growth characters:

Effect of stigmasterol (stig.) and/or ascorbic acid (ASA) on plant growth:

Through the 1st cut; results presented in Table (2) revealed that different treatment of stig. and/or V.C. resulted positively effects in increasing plant height "cm" comparing with control plants (untreated plants), meanwhile, the best treatment concerning to this parameter of growth was 80 mg/l stig. + 60 mg/l V.C., on the other hand, plant treated with 30 mg/l A.S.A. (V.C.) recorded the lowest value of plant height. Table (2) showed clearly that an increasing in fresh weight of alfalfa plant as a result of almost all applied treatments with exception of 30 mg/l. A.S.A. which revealed insignificant increasing in plant fresh weight.

Table 2: Effect of stigmasterol (Stig.) and/or ascorbic acid (ASA) on vegetative growth of alfalfa (*Medicagostiv*L.) plants. (Average of 2010/2011 and 2011/2012 season)

Vegetative growth characters / Treatments	First cut.						Second cut.					
	Plant height "cm"	Plant height % of control	Fresh weight "ton/ha"	Fresh weight % of control	Dry weight "ton/ha"	Dry weight % of control	Plant height "cm"	Plant height % of control	Fresh weight "ton/ha"	Fresh weight % of control	Dry weight "ton/ha"	Dry weight % of control
Control (tap water)	42.20	--	2.609	--	0.413	--	39.85	--	3.855	--	0.414	--
Stig. 40 mg/l	45.51	107.84	2.867	109.36	0.467	113.23	41.33	103.71	4.517	117.7	0.487	117.71
Stig 80 mg/l	46.01	109.04	3.068	117.59	0.483	116.93	41.68	104.58	4.657	120.80	0.494	119.38
Ascorbic acid 30 mg/l	42.91	101.67	2.620	100.41	0.442	107.07	39.53	99.20	4.133	107.23	0.461	111.39
Ascorbic acid 60 mg/l	43.00	101.90	2.707	103.73	0.464	112.33	40.03	100.43	4.470	115.96	0.470	113.57
Stig. 40 mg/l + Ascorbic acid 30 mg/l	46.70	110.66	3.108	119.12	0.507	122.97	42.85	107.53	4.693	121.74	0.499	120.39
Stig. 40 mg/l + Ascorbic acid 60 mg/l	46.52	110.24	3.178	121.66	0.557	134.87	43.38	108.75	4.752	123.28	0.506	122.13
Stig. 80 mg/l + Ascorbic acid 30 mg/l	47.07	111.55	3.257	124.83	0.565	137.02	43.45	109.03	4.790	124.28	0.514	124.23
Stig. 80 mg/l + Ascorbic acid 60 mg/l	47.13	111.69	3.441	131.88	0.567	137.46	43.99	110.37	4.820	125.05	0.525	126.74
L.S.D. at 5% level	1.51	--	0.037	--	0.036	--	1.10	--	0.039	--	0.028	--

At the same time, 80 mg/l stig. combined with 60 mg/l A.S.A realized the highest plant fresh weight compared with control treatment, whereas, plants sprayed with 60 mg/l A.S.A alone recorded the lowest significant increasing of plant fresh weight and they gave 131.88% and 103.73% as percentage comparing with untreated plants, respectively. The results in the same table observed that all treatments under study significantly increased

plant dry weight except for the lowest concentration of A.S.A (i.e. 30 mg/l A.S.A) which revealed insignificant increasing in plant dry weight. It could be noted that foliar spraying with 80 mg/l stig. + 60 mg/l A.S.A followed by 80 + 30 mg/l of the same later compounds realized the highest effecting in increasing dry matter of alfalfa plants. Concerning to data illustrated in Table (2), it could be noted that the 2nd cutting almost to all treatment was given the same trend as the 1st cutting, while ststig. at low level had lowest significant value of plant height, also, treatment with 60 mg/l A.S.A. insignificant increasing in plant height, also. At the same time 30 mg/l recorded insignificant increasing in this parameter of growth herease applying bilateral treatments. The highest increasing of plant height was recorded especially under 80 mg/l stig. + 60 mg/l A.S.A. which reached to 110.37% comparing with control plants. Moreover, all plants that have been treated with stig. and/or A.S.A recorded increments in fresh and dry weight of alfalfa plants, and it could be elected that there was a positive correlation between fresh and dry weight at the 2nd cutting, and increasing the concentrations for each of stig. and/or A.S.A on fresh and dry weight recorded 4.82 and 0.83 tan/ha, respectively as a result of 80 mg/l stig. + 60 mg/l A.S.A comparing with control plants that recorded 3.85 ton/ha of fresh forage weight and 0.40 ton/ha for forage dry weight. It could be concluded that almost all characters so far affected by foliar spraying application with stig. and/or ABA at various treatments, while the highest positive effects always realized with incorporated treatments followed by single treatments of both stig. or A.S.A treated plants. The above mentioned results are agree with that of Balbaa *et al*. (2008) concluded that plant height, number of branches, herb fresh and dry weight of *Tagetserecta*L. were significantly affected by stig. application at 50 and 100 mg/l , also. Fariduddin*et al*., (2014); stated that BR_S characteriscally avoka both cell elongation and cell division resulting in elongation in stem and roots, photo-morphogenesis, leaf senescence and also in stress responded by playing pivotal roles in wide range of developmental phenomena. Bin Huang *et al*. (2006) reported that the exogenous treatment with BR_S could allow the plant to recover from the inhibited growth caused by chilling. It is note worthy to mention that similar results on the effect of stig. were obtained by Ahmed *et al.* (2011) on wheat, Ahmed and Shalaby (2014) on wheat and Shalaby *et al*. (2014) on barley.

Furthermore, Zonouri *et al*. (2014) stated that under environmental stress A.S.A play important roles in membrane stability, soluble protein and lipid peroxidation. Ascrobic acid with oxgen free radical scavenging in reduce damage to fatty acid, protein and thus reduced structure effects of various stresses (Akhila *et al*., 2008). It is worthy that ascorbate is a major metabolite in plants and are in association with other components of antioxidant system, protect plant cells, metabolism and photosynthesis from oxidative damage (Samiroff, 1996). It is worthy to mention that similar results on the effect of A.S.A were obtained by Magda Shalaby *et al.* (2013) on barley and Ebtesam El-Housini *et al*. (2014) on Stevia.

Effect of stegmasterol and/or Ascorbic acid on chemical contituents of alfalfa plants:

Data illustrated in table (3) shows clearly that the different concentration of both stig. and/or ABA (i.e. V.C.) caused significant increase in photosynthetic pigments content in leaves of alfalfa treated plants except for 30 mg/l ASA that realized insignificant increment. Moreover, the results also indicate that the effect of the combination between Stig. and ABA substances caused the best and favorable results especially the foliar spraying with 80 mg/l stig. + 60 mg/l ASA, meanwhile, foliar application with 60 mg/l ASA caused the lowest significant increase of the total chorophyll. With respect of carotenoids content, all treatments under study were more effective in increasing carotenoids content, except the two treatments 30 mg/l and/or 60 mg/l ASA which showed insignificant increases in carotenoids content. It could be concluded that the highest significant increase in carotenoids content was harvested under 80 mg/l stig. + 60 mg/l ASA, meanwhile the lowest value caused by 40 mg/l stig. treatment. Generally, data in table (3) observed that all the pigments estimating results in the 2nd cutting that have the same trend of the 1stsutting and caused significant increase in total chorophyll and carotenoids content especially with the treatment by combination between stig. + ASA, whereas, the single application showed the lowest increases in photosyhthetic pigments content. It could be mention that 60 mg/l ASA recorded insignificant decrement in carotenoids content, however, other treatments under study realized significant increase but treatment 80 mg/l Stig. + 60 mg/l ASA was the most positive impact treatment, whereas, the highest percentages of the total pigments were realized with the highest concentrations of stig. and/or ASA and amounted between 133.61 – 168.77 compared with control plants. In addition, these results are agreement with Janeezko *et al*. (2007) which found that strongly reduced of leaf content from chlorophyll a and chlorophyll b and carotenoids at 2^OC but less markedly at 20^OC. In rape seedlings when exposed to 2^OC pigments was significantly higher in BR_{27} treated leaves as compared to water/ethanol control and there were no differences between pigment contents of leaves injected with BR_{27} solution or only with water/ethanol at 20^OC.

Table 3: Effect of stigmasterol (Stig.) and/or ascorbic acid (ASA) on photosynthetic pigments content and the activity of superoxide dismutase (SOD) in alfalfa (*Medicagostiv*L.) plants. (Average of 2010/2011 and 2011/2012 season)

Characters / Treatments	First cut.							
	Chlorophyll a "mg/g"	Chlorophyll b "mg/g"	Total chlorophyll "mg/g"	Carotenoids "mg/g"	Total photosynthetic pigments "mg/g"	Total photosynthetic pigments %	SOD conc. u/mg protein	SOD %
Control (tap water)	0.357	0.262	0.619	0.137	0.756	--	115.92	--
Stig. 40 mg/l	0.437	0.300	0.737	0.146	0.882	116.71	150.018	129.35
Stig 80 mg/l	0.445	0.365	0.811	0.157	0.968	128.04	163.958	141.37
Ascorbic acid 30 mg/l	0.359	0.265	0.624	0.135	0.759	100.40	149.491	128.89
Ascorbic acid 60 mg/l	0.415	0.274	0.689	0.134	0.823	108.91	161.30	139.07
Stig. 40 mg/l + Ascorbic acid 30 mg/l	0.474	0.394	0.868	0.166	1.034	136.73	169.677	146.30
Stig. 40 mg/l + Ascorbic acid 60 mg/l	0.479	0.407	0.886	0.184	1.070	141.53	173.59	149.67
Stig. 80 mg/l + Ascorbic acid 30 mg/l	0.533	0.426	0.958	0.177	1.135	150.13	187.273	161.47
Stig. 80 mg/l + Ascorbic acid 60 mg/l	0.662	0.420	1.041	0.195	1.237	163.58	199.922	172.37
L.S.D. at 5% level	0.045	0.151	0.059	0.004	0.059	--	4.84	--
Characters / Treatments	Second cut.							
	Chlorophyll a "mg/g"	Chlorophyll b "mg/g"	Total chlorophyll "mg/g"	Carotenoids "mg/g"	Total photosynthetic pigments "mg/g"	Total photosynthetic pigments %	SOD conc. u/mg protein	SOD %
Control (tap water)	0.349	0.290	0.639	0.137	0.776	--	116.744	--
Stig. 40mg/l	0.491	0.384	0.875	0.146	1.021	131.53	143.659	123.05
Stig 80mg/l	0.493	0.382	0.875	0.153	1.028	132.52	144.007	124.11
Ascorbic acid 30mg/l	0.469	0.269	0.738	0.144	0.882	113.66	139.711	119.67
Ascorbic acid 60mg/l	0.482	0.285	0.767	0.132	0.859	115.89	144.007	123.35
Stig. 40mg/l + Ascorbic acid 30mg/l	0.578	0.412	0.990	0.176	1.166	150.30	148.594	127.28
Stig. 40mg/l + Ascorbic acid 60mg/l	0.574	0.417	0.991	0.166	1.157	149.13	154.504	138.34
Stig. 80mg/l + Ascorbic acid 30mg/l	0.644	0.442	1.086	0.185	1.271	163.83	154.997	132.77
Stig. 80mg/l + Ascorbic acid 60mg/l	0.642	0.477	1.119	0.191	1.310	168.77	159.73	136.22
L.S.D. at 5% level	0.042	0.024	0.035	0.006	0.036	--	--	--

Protein content

Table (4) indicated clearly that almost treatments significantly increased protein content as "mg/g" and total protein % with exception for ASA at 30 and 60 mg/l treated plants which revealed little increases in protein %, but the increases failed to reach the significant levels at 0.05 throughout the 1st and 2nd cuttings. At the same time; compound treatments of 80 mg/l Stig. + 60 mg/l ASA was the most favorable treatments in increasing protein content as mg/g and/or protein % which amounted to 115.96 % and 119.77 % at 1st and 2nd cutting,

sequently, followed by 80 mg/l Stig. + 30 mg/l ASA treated plants and realized 112.65 % and 117.00 % at the 1st and 2nd cutting, respectively.

As a results, it could be stated that BR_S in the form of stigmasterol may have an important role at RNA building and protein produce. Balbaa *et al.* (2008) as 50 and/or 100 mg/l of Stig. increased protein % significantly in *Tageteserecta* L. plant.

Ash and fiber contents:

Table (4) show the effect of Stig. and/or ASA at the different concentrations under study on ash and fiber contents through winter cold stress at Tabouk Governorate. Data observed that all treatments decreased ash contents and obvious decrease was realized by Stig. 80 mg/l + ASA at 60 mg/l which recorded 10.84% comparing with untreated plants where showed 14.27%, while plants treated with 60 mg/l ASA gave the highest value comparing with other treatments and recorded about 13.9% of ash content. Data illustrated in the same table indicate that the same trend of the 1st cut was showed in the 2nd cut as a result of plants that had been treated with foliar spray concentrations from stig. And/or ASA. It is worthy that, all treatments under study cause significant decrement of ash content compared with control treatments (untreated plants) which recorded 13.10%, meanwhile, ASA at low concentration (30 mg/l) recorded about 12.8% as lowest decrease of plant ash %.

With respect of the fiber content; data reported in table (4) observed that, in constrast of ash, fiber % increased as a result of different foliar application treatments, since the treatments with addition of Stig. and/or ASA significantly increased fiber content especially at 80 mg/l stig. + 30 mg/l ASA which recorded the highest value of fiber content. On the other hand, single treatments did not affect significantly fiber content of alfalfa plants. Table (4) showed that as the 1st cut, double treatments released in the highest value of fiber percentage especially under 80 mg/l Stig. + 60 mg/l A.S.A which recorded 14.13% of the fiber content. In the opposite single treatments for each compounds especially 60 mg/l ASA revealed insignificant increase, valued with 12.8% comparing with 12.5% of fiber recorded by untreated plants. It can be concluded from the above mentioned results that the increases in fiber content comparing to the reduction in ashes content indicates that both Stig. and/or A.S.A play an important roles in the tissues building and protecting various organs of plants. These role of both compounds were illustrated by many investigators (Yokote and Takahashi, 1986), Yuxine *et al.* (2001), Kriska (2003), Sminoff (1996) and Noctor and Foyer (1998), those observed that BR_S have a biological activity in increasing crop yield through both changing plant metabolism and protecting plants from environmental stress. Also, ASA has been implicated in cell division, cell expansion and cell metabolism.

Super oxid dismutase (SOD) activity (units/mg protein):

Table (3) revealed that foliar spraying plants with Stig. and/or ASA resulted in an obvious increasing (SOD) enzyme activity in comparing with untreated plants through the two cutting of the 1st and 2nd season. At the same time, incorporated treatments of the various concentrations were the superiors especially with80 mg/l Stig. + 60 mg/l A.S.A. sequentially, meanwhile, 30 mg/l of ASA reflected in the lowest increase of (SOD) activity as an unit comparing with other treatments. It can be mentioned that the values of enzyme activity ranged from 150.00 to 199.90 u/mg protein according to various treatments compared to untreated plants which recorded 116.00 u/mg protein. Thus, the same trend of results were observed with 2nd cut and the same table (3) showed that the percentage of enzyme positively affected with the highest concentrations of the two substances compound used on alfalfa plants especially when applied with each other the single substances from Stig. and/or A.S.A.

According to the results of SOD, it can be suggested that, various treatments under study had a positive effects against cold stress which revealed the increase in antioxidant enzyme activity and SOD which reflected an improvement in growth and development of alfalfa plant. Thus, there are many researches agreement with this proposal is that BR_S as (Stig.) have an important role in increasing enzyme activity. Reactive oxygen species (ROS) such as CO_2 and CO_2, H_2O_2 and OH are extremely reactive in nature because they can interact with a number of cellular molecules and metabolites, thereby leading to irreparable metabolic dysfunction and death. Plants have well developed enzymatic scavenging pathways or detoxification systems to counter the deleterious effects of (ROS) that include SOD enzyme. SOD m RNA levels have been observed to increase during recovery from naturally established winter stress (Karpinski *et al.,* 1993 and 1994). In alfalfa plants, Camp *et al*. (1994), demonstrated that Fe-SOD and Mn-SOD have different protective properties in response to shilling treatments, whereas, Morello *et al*. (2005) stated that; the concentration of superoxide and hydrogen peroxide reduces and the resk of hydroxyle radical decreases as affected by ASA.

Table 4: Effect of stigmasterol (Stig.) and/or ascorbic acid (ASA) on chemical constituents for forage yield of alfalfa (*Medicagostiv* L.) plants. (Average of 2010/2011 and 2011/2012 season)

Characters / Treatments	First cut.						Second cut.					
	Ash. Mg/g D.W.	Ash. % D.W.	Fiber % D.W.	Protein "mg/g" D.W.	Total protein % D.W.	Total protein in related to control %	Ash. Mg/g D.W.	Ash. % D.W.	Fiber % D.W.	Protein "mg/g" D.W.	Total protein % D.W.	Total protein in related to control %
Control (tap water)	142.72	14.27	13.61	221.33	22.13	--	131.037	13.1	12.50	205.667	20.57	106.73
Stig. 40 mg/l	130.105	13.01	13.86	238.66	23.87	107.83	122.642	12.26	13.27	219.50	21.95	107.78
Stig 80 mg/l	133.498	13.35	13.90	236.33	23.63	106.78	118.141	11.81	13.71	221.667	22.17	104.54
Ascorbic acid 30 mg/l	137.360	13.74	13.95	228.33	22.83	103.16	127.551	12.76	12.97	215.00	21.50	103.57
Ascorbic acid 60 mg/l	139.773	13.98	13.94	229.33	22.93	103.61	123.354	12.34	12.80	213.00	21.30	103.57
Stig. 40 mg/l + Ascorbic acid 30 mg/l	129.271	12.93	14.09	239.33	23.93	108.13	111.686	11.17	13.78	228.967	22.90	111.33
Stig. 40 mg/l + Ascorbic acid 60 mg/l	122.310	12.23	14.03	240.66	24.07	108.73	108.291	10.83	13.92	231.933	23.19	112.77
Stig. 80 mg/l + Ascorbic acid 30 mg/l	114.023	11.4	14.81	249.33	24.93	112.65	99.972	10.00	14.29	240.633	24.06	117.00
Stig. 80 mg/l + Ascorbic acid 60 mg/l	108.426	10.84	14.45	256.66	25.67	115.96	103.929	10.39	14.37	246.333	24.63	119.77
L.S.D. at 5% level	2.526	0.30	0.45	7.929	0.82	--	2.938	0.30	0.60	9.648	0.97	--

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