

THE EFFECT OF SALICYLIC ACID AND POTASSIUM SULPHATE ON THE AMOUNT OF SOLUBLE SUGARS, STARCH, PROLINE AND PROTEIN OF GRAPE BUDS UNDER COLD STRESS

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Abstract. Askari grape is the dominant cultivar of the region which is damaged annually by winter cold. To study the biochemical changes and to reduce the effect of cold stress during the winter, this study was conducted in one of the vineyards of Dana city as a factorial experiment with a randomized complete block design with three replications and two vines in each plot. In this experiment, salicylic acid at concentrations of 0, 150, 300 and 450 mg/l and potassium sulphate at 0, 1, and 2% were used as foliar spray in 2014. Sampling was performed at different periods: two weeks after spraying, the onset of dormancy, deep dormancy stage and before bud growth in the following year, and related characteristics was measured and recorded in each stage. Sampling stages had significant effect on the increase of soluble sugars at 1%, while the interaction of salicylic acid \times stages of sampling, and salicylic acid \times potassium sulphate \times stages of sampling had significant effect at 5%, and other treatments had no significant effect on soluble sugar content. Sampling stages had significant effect on the starch of grape bud at 1%, and other treatments had no significant effect on the starch. The effects of sampling stages and the interaction of potassium sulphate \times stages of sampling on the protein content of grape buds were significant at the levels of 1% and 5%, respectively, and other treatments did not show significant effect on the protein content. The proline content of grape buds was significantly affected by salicylic acid at 5%, as well as the stages of sampling, the interaction of salicylic acid \times potassium sulphate, salicylic acid \times stages of sampling, potassium sulphate \times stages of sampling and salicylic acid \times potassium sulphate \times stages of sampling at the level of 1%, and other treatments had no significant effect on proline content. Increase in the amount of salicylic acid and potassium sulphate and their interaction caused a significant effect on the increase in the amount of soluble sugars, protein and proline of buds, however, their effect on the starch was not significant.

Keywords: Grape, Dormancy, Chilling, Bud, Resistance.

1. INTRODUCTION

The area under grape cultivation in Yasuj province, especially in the city of Dana, is on the rise due to the desire of local farmers as well as revenue of this crop as one of the main sources of income of the people. Winter chilling causes severe damage to the vines of the region, and leads to severe restrictions for vineyards. Salicylic acid and potassium sulphate may increase the amount of soluble sugars, protein, proline or starch during cold stress and reduce cold damage in this region.

When plants have potassium deficiency, protein is not synthesized despite an abundance of nitrogen, and instead, constituent compounds of protein (precursors) such as amino acids, amides and nitrates accumulate. Nitrate reductase enzyme accelerates the reduction of nitrate, and potassium is probably responsible for its activation and synthesis (Better crops). Salicylic acid (SA) plays an important role in the adjustment of apoplastic proteins and antioxidant enzymes associated with resistance to cold stress (Tasgin et al., 2003). Reduction or increase in the production of protein decomposition can be attributed to the increase or decrease in antioxidant activity (El-Tayeb, 2005). However, the accumulated protein as a result of salicylic acid application in the rye had no role in antifreeze activity (Yu, 2001), and role of salicylic acid in resistance to frost is still unknown and further research is needed.

The enzyme responsible for the synthesis of starch (starch synthase) is activated by potassium. So, when the amount of potassium is inadequate, the level of starch reduces while soluble carbohydrate and nitrogen compounds accumulate. The sugar produced in photosynthesis must be transported to other parts of the plant through the phloem for use and storage. Transportation system of plants uses the energy in the form of ATP. If potassium is insufficient, less ATP is available, and material transportation system is disrupted. This issue causes photosynthesis to occur in upper leaves and therefore the amount of photosynthesis reduces. Natural development of energy storage organs such as grain faces trouble. A good amount of potassium helps all the processes and systems of transportation to operate normally (Better crops). Potassium is one of the most important and widely used nutrients in plants, which plays an important role in the growth, metabolism and survival. It has also a role in the activities of enzymes, protein synthesis, photosynthesis, osmotic adjustment, stomatal movements, synthesis and transport of carbohydrates and stress tolerance (Marschner

2012). Large amounts of plants' potassium is removed through the harvested fruits (Fernandez-Escobar, 2004), while potassium plays an important role in regulating water status of olive tree (Arquero et al., 2006). Spraying is a good way of supplying plants' nutritional requirement because nutrients are rapidly absorbed and transported after spraying (Mengel 2002).

It has been reported in tomatoes (Khavarinejad and Ghafarzadeh, 1998), rice (Karimi et al., 2005), wheat and barley (Keles and Oncel 2004) that accumulation of sugar increases by using salicylic acid treatment following oxidative stress. By creating an osmotic gradient in plants, increase of sugars in plants increases the resistance of wheat plant against water loss under stress conditions (Tasgin et al., 2003). Baghbanha et al. (2007) in a study on the seedlings of lemon found that use of salicylic acid at the concentration of 0.5 mM led to the accumulation of soluble carbohydrates in the leaves of lemon seedlings. Sugars such as fructose and glucose are important organic solute with low molecular weight in higher plants which are accumulated under drought stress conditions and changes in the relative water content in plant tissues, and maintain osmotic regulation and turgor inside the plant. The sugars also cause a reduction in tissue freezing point (Kerepesi 1998). Sugars stabilize proteins and membranes, and prevent proteins destruction by creating hydrogen bonds with polar polypeptide sequences and phospholipids groups (Ashraf and Foolad 2007).

Salicylic acid, as a growth regulator, is effective in the induction of flowering, the movement of materials and photosynthesis (Hayat and Ahmad 2007). Different concentrations of salicylic acid and its effective period cause multiple reactions in plant (Hare and Cress, 2004; Senaratna et al., 2003)). It has been reported that treatment with salicylic acid increased proline in walnut (khoramshahi 1391) and wheat (Singh and Usha 2003). Although salicylic acid decreased proline content in okra; the plants treated with salicylic acid produced higher proline content than untreated plants under drought stress conditions (Ashraf and Foolad 2007). Free radical production increases under stress conditions and causes a disruption to electron transport in chloroplasts and mitochondria, and causes destruction and collapse of the membranes under stress conditions (Davies, 1987; Hassibi et al., 2007). Increase in proline content under stress conditions as a result of increase in proline synthesis or decrease in decomposition is to cope with salinity. In general, proline accumulation

under salinity and drought conditions has been reported by many researchers. It has been reported that proline adjusted secretion under osmotic conditions (Meloni et al. 2001). Furthermore, it was reported that sodium chloride increased soluble sugars in barley and tomato (Gao et al., 2004). Fertilization of several varieties of mango was amended by spraying potassium (Oosthuyes, 1993). The main role of potassium is the activation of many enzymes, which are involved in the synthesis of organic materials, increase of photosynthesis and transportation of carbohydrates to storage organs (Marschner, 1986). Overall, potassium is involved in several major physiological actions, resulting in modification of fruit qualitative characteristics such as TSS%, soluble sugars and coloration (El Seginy, et al., 2003). These effects can be attributed to the role of potassium in the increase of stress tolerance and improvement of the formation and accumulation of sugars (Saleh and Abd El-Monem, 2003; Wahdan, et al., 2011). The aim of this study was to evaluate the effect of salicylic acid and potassium sulphate on the rate of changes in protein, proline, soluble sugars and starches in the buds of grape cv. Askari in four sampling stages (two weeks after spraying, the onset of dormancy, deep dormancy and before breaking dormancy) and increase of resistance to cold.

2. MATERIALS AND METHODS

This experiment was conducted in one of the vineyards of Sisakht city to study biochemical changes and cold tolerance. The experiment was performed in a two-factor factorial design with three replications and two plants per plot in 2014. The first factor was salicylic acid at four levels (0, 150, 300 and 450 mg/l), and the second factor was potassium sulphate at three levels (0, 1, 2%). To study soluble sugars, starch, proline and protein, sampling was performed at four steps: (1) two weeks after spraying (buds and leaves), (2) after leaf drop and the start of dormancy, (3) deep dormancy stage, and (4) before the growth of buds in the following year, and the amount of electrolyte leakage was measured at dormancy stage. All the characteristics were measured at each stage.

2.1 Quantitative measurement of protein

For quantitative measurement of proteins, Bradford assay method (1976) was used. The method is based on the connection of Kumasi Brilliant Blue G-250 to protein in an acidic environment and determination of maximum absorption from 465 to 595 nm. Absorbance at 595 nm is directly proportional to the concentration of protein.

2.1.1 Preparation of Bradford solution

1.0 g Kumasi Brilliant Blue G-250 was added to 50 ml ethanol, stirred by using a magnetic stirrer and brought up to the volume of 800 ml by using double distilled water. Then, 100 ml 85% phosphoric acid was added to the obtained solution, and the solution was brought up to the volume of 1000 mL using double distilled water. The resulting solution filtered with Whatman no. 1 filter paper.

2.1.2 Preparation of Bovine Serum Albumine (BSA) protein

The concentrated solution of 10 mg/ml bovine serum albumin was prepared by dissolving 100 mg bovine serum albumin in 10 mM of 0.15 M NaCl. Then concentrations of 0, 5, 10, 15, 20, 25 and 50 mg/ml were prepared by diluting the obtained solution.

2.1.3 Preparation of plant tissue extracts

In order to extract the protein from plant samples (buds), 0.5 g of plant sample weighed and crushed inside a mortar containing 2 ml phosphate buffer over an ice bath. Finally, after homogenization of the sample, the obtained solution was transferred to 2-ml micro-tubes and was centrifuged at 12,000 rpm at 4 ° C for 30 minutes. After centrifugation, the upper phase (supernatant) of the extract was transferred to other micro-tubes. The extracts obtained were used to measure the concentration of soluble protein. In order to measure the concentration of protein, 0.1 ml protein extract and 5 ml biuret were added to the test tubes and vortexed immediately. After 2 minutes, the absorption was measured at 595 nm by using a spectrophotometer.

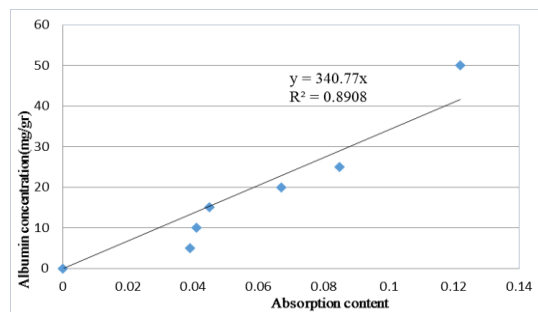


Figure 1. Diagram of bud standard protein (mg /gram of fresh weight of bud)

The linear equation was first fitted between standards adsorption (x axis) and their concentrations (y axis), and the linear relationship

between absorption and concentration was obtained (Figure 1).

Note: The linear equation passes from zero point, therefore intercept (a) is zero in equations.

2.2 Proline Measurement

2.2.1 Preparation of alcoholic extract

To measure the amount of proline, alcoholic extract was first prepared from buds. For this purpose, 0.5 g of fresh or frozen leaf tissue was smashed in a mortar. Then, 5 ml 95% ethanol was added to the obtained sample and the obtained solution was transferred to the test tube and vortexed for 30 seconds (strongly shaken). Next, the supernatant was separated and transferred to a tube with the volume of 20 cc. Then, 10 ml 70% ethanol was added to the remaining solid two times, each time 5 ml, and washed thoroughly. The supernatant was then transferred to a test tube and finally 15 ml of the extract obtained was centrifuged at 3500 rpm at low temperature, and the supernatant was carefully separated and transferred to a refrigerator at 4 ° C (Paquine and Lechasseur, 1979).

2.2.2 Measurement of proline

The amount of light absorption of the samples was measured using a spectrophotometer at 515 nm. Before performing the steps above, standards of proline (L-proline) with different concentrations (0, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 $\mu\text{mol/ml}$) were prepared. The regression curve was then drawn and the amount of free proline of the samples was calculated based on $\mu\text{mol/gram}$ leaf fresh weight (Figure 2).

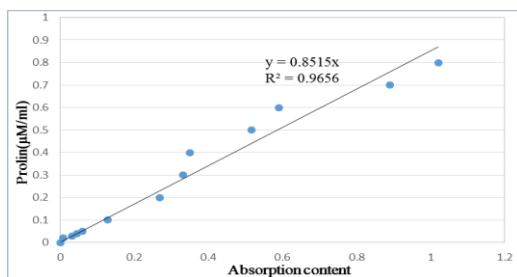


Figure 2- Standard proline diagram ($\mu\text{mol/ml}$)

2.3 Extraction and measurement of soluble sugars

In order to measure soluble sugars, 100 mg of dried and powdered samples were weighed. Then, extraction was performed by using 10 ml of 95% ethanol. Extracts were first shaken for 15 minutes

on a shaker with 360 rpm and were then centrifuged for 15 minutes at 3500 rpm. Then 3 ml anthrone reagent was added to the top phase of centrifuged samples. Finally, after applying boiling water temperature for 10 minutes, the amount of light absorption was recorded at the wavelength of 630 nm.

To prepare the standard solution (Figure 3), pure glucose was used in this experiment (Hedge and Hofreitor, 1962).

The regression equation for data conversion $Y = 1664x$

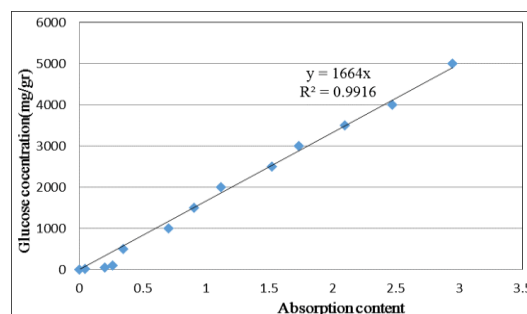


Figure 3. The standard curve of soluble sugars (mg/l)

2.4 Extraction and measurement of starch

The sediment left from the step of soluble sugars measurement (100 mg of dried and powdered buds and leaves) was used to measure starch (Hedge and Hofreitor, 1962). The extracts prepared for measuring starch were first shaken for 15 minutes and then centrifuged for 10 minutes at 5,000 rpm. The supernatant was separated and added to the solution of the previous test tube. The test tube was placed inside the ice for half an hour and then brought up to the volume of 100 cc by using distilled water. 2.5 cc from the solution of the previous step was taken and mixed with 10 cc anthrone solution. The solution was kept in hot water bath at 100 ° C for 7.5 minutes, and then ice water. After the blank of the device, samples were read at the wavelength of 630 nm. The amount of glucose in one sample was calculated by using the standard curve and multiplied by 9.0 to determine the amount of starch.

3. RESULTS AND DISCUSSION

Grape buds were taken at four separate stages (two weeks after spraying, the onset of dormancy, deep dormancy and before breaking bud dormancy), and soluble sugar, starch, protein and proline were measured at each stage. Each trait was analyzed

using split plot statistical design in time. The results of analysis of variance related to the measured traits

Table 1- The results of analysis of variance related to the effect of SA and K₂SO₄ on biochemical characteristics of Askari grape buds

Source of variation	Df	Mean Square of traits			
		Soluble sugars	Starch	Protein	Proline
Block	2	2.054*	0.498**	0.154 ^{ns}	0.137*
SA	3	1.175 ^{ns}	0.084 ^{ns}	0.009 ^{ns}	0.087*
K	2	0.021 ^{ns}	0.143 ^{ns}	0.059 ^{ns}	0.012 ^{ns}
SA×K	6	0.361 ^{ns}	0.091 ^{ns}	0.039 ^{ns}	0.135**
Error	22	0.538	0.046	0.093	0.026
Date	3	23.977 ^{ns}	1.634**	1.049 ^{ns}	7.154**
SA×Date	9	0.978*	0.035 ^{ns}	0.063 ^{ns}	0.150**
K×Date	6	0.142 ^{ns}	0.056 ^{ns}	0.194*	0.083**
SA×K×Date	18	0.836*	0.044 ^{ns}	0.063 ^{ns}	0.083**
Error	72	0.447	0.068	0.067	0.025
Cv(%)		8.66	21.23	30.29	18.21

ns: Difference is not significant, *: Difference is significant at five percent, **: Difference is significant at one percent

3.1 Soluble sugars

The results obtained from analysis of variance showed that sampling stage had significant effect on soluble sugars at 1%, while interaction of salicylic acid × sampling stage and salicylic acid × sulphate potassium × sampling stage had significant effect at 5%, and other treatments had no significant effect on soluble sugar content of grape buds in different periods.

Sampling stage had different effect on the amount of soluble sugars of buds. The average values of soluble sugars in sampling stages were different. The least amount of soluble sugars in the grape buds was observed in the first stage of sampling, while the greatest amount of soluble sugars in the bud was seen in dormancy stage. Cold stress increases along with the onset of dormancy; therefore bud increases its soluble sugars to prevent frost damage and to increase tolerance to cold stress (Figure

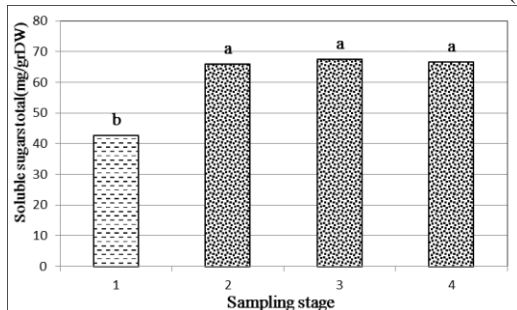


Figure 4. The effect of sampling stage on the amount of soluble sugars (mg/grFW)

Figure 5 shows that soluble sugar content in the grape bud at dormancy stage was higher than the active

in grape buds at four stages are shown in Table 1.

growth stage of the plant. Increase in the concentrations of salicylic acid caused an increase in soluble sugar content in deep dormancy phase compared to other stages. Salicylic acid at concentrations of 300 and 450 mg/l had the greatest effect on soluble sugar content of the grape buds at deep dormancy stage. Increase in soluble sugar content regulates cell osmosis and decreases cell water by increasing materials such as sugars, protein and proline to prevent the cell from freezing. Reserves of soluble sugars and starch in cold period vary greatly (Ranney et al., 1991). Increased cold resistance is related to the amount of soluble carbohydrates, and carbohydrates such as sucrose, sorbitol and raffinose are subunits protecting plant. The amount of carbohydrates affects the plant against low temperatures (Ranney et al., 1991). Salicylic acid stimulates the hydrolysis of carbohydrates and reduces frost damage by increasing compounds such as soluble sugars, which create an osmotic source (Hayat and Ahmad 2007).

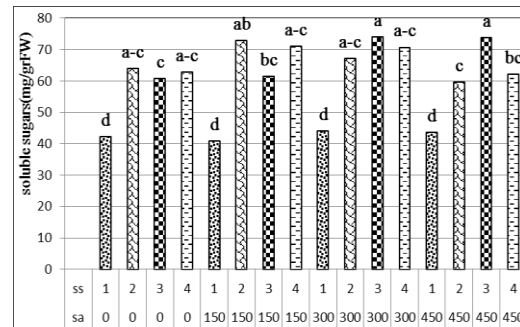


Figure 5. The effect of SA and sampling stage on the soluble sugar content (mg/ grFW)

3.2 Starch

Analysis of variance related to buds starch in various stages of sampling showed that sampling stages had significant effect on starch at 1%, and other treatments had no significant effect.

The amount of starch varied in different sampling stages, so that the lowest amount of starch was observed in the stage of plant growth (Figure 6). In the third stage of sampling, potassium level was less than the other two stages of dormancy, because starch reduces as a result of cold stress and converts into soluble sugars, leading to the reduction of cold stress in the stress period. Photosynthetic activity also affects the rate of sugar formation for production and storage of final starch. Under high levels of potassium, starch effectively transferred from the

place of production to storage organs (Better crops). Spring frost reduces the amount of starch and increases soluble sugars in the plant. Increase of cold resistance is associated with the level of soluble carbohydrates, and carbohydrates such as sucrose, sorbitol and raffinose are the first subunits protecting plants from freezing (Levitt, 1972-1980).

Acclimation period is accompanied with the accumulation of one or more products made in plant cells and mostly in the vacuole. One of these ingredients is sugar (Weiser, 1970). The reserve of soluble sugars and starch in cold period shows high variations (Ranney et al., 1991).

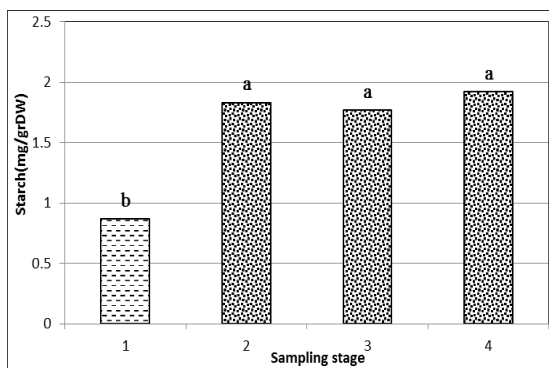


Figure 6- The effect of sampling stages on the amount of starch (mg/grDW)

3.3 Protein

ANOVA of protein showed that different stages of sampling at 1% and the interaction of different percentages of potassium sulphate and sampling stages at 5% caused significant effects on the protein content of bud, and other treatments did not have significant effect on the protein content of grape bud in four stages of sampling.

In this study, the interaction of salicylic acid and potassium sulphate caused the average values of protein to be higher in the growth stage of the plant than the three dormancy stages of bud. The average amount of protein in deep dormancy stage was higher than that of the stages of onset of bud dormancy and before breaking bud dormancy (Figure 7). Protein is necessary for building cell membranes, pigments, and defensive enzymes (anti-oxidant and non-oxidant), and that is why the amount of protein in the growth stage of plant is higher than dormancy stage. When the plant is under cold stress, it increases its protein content. In deep dormancy stage, an increase occurs in genes increasing resistance to cold stress. Potassium is needed for each basic steps of protein synthesis. Reading genetic code in plants cells for

production of proteins and enzymes that regulate all the processes of growth will be impossible without adequate potassium (Better crops).

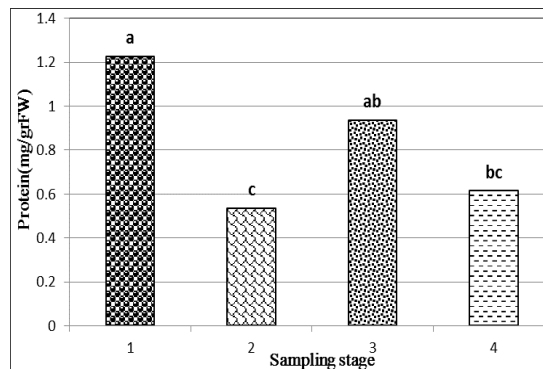


Figure 7. The effect of sampling stages on the amount of protein (mg/grFW)

The comparison of the interaction of potassium sulphate and sampling stage on the amount of protein (Figure 8) showed that potassium sulphate at the concentrations of 0, 1 and 2% in the third stage of sampling had the highest effect on the average values of protein in bud, respectively. In the first sampling stage, potassium sulphate at 2% had the greatest effect on the amount of protein. At two stages of the onset of dormancy and deep dormancy, increase in the concentration of potassium sulphate increases the protein content of the buds. The highest amount of protein in deep dormancy phase was recorded with potassium sulphate at zero percent, followed by potassium sulphate at 1 and 2%, respectively.

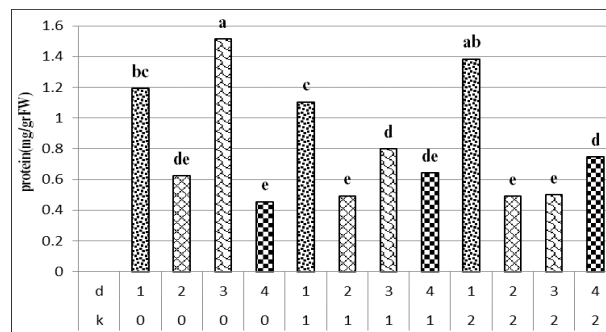


Figure 8. Effect of potassium sulphate and sampling stage on the protein content (mg/grFW)

The activity of defensive enzymes involved in oxidative stress showed an increase during cold stress. After cold stress, plant protein storage increases to get back to normal state (Millard, 1988). The increasing trend of total protein content in the early hours after stress can be interpreted in this way that with the start of stress, plant begins to increase the expression of genes involved in the synthesis of

defensive enzymes to protect cell structures and to maintain normal activities. Therefore, with synthesis of adequate amount of defensive enzymes by cell, there is no need for higher increase in the rate of enzymes which are not subset of total protein, and after passing enough time after the start of stress, the situations will be under the control of plant organization. On the other hand, any damage to the structure of the cell DNA by reactive oxygen species results in a lack of synthesis of suitable protein for crop yield (Mundree, et al., 2002).

3.4 Proline

Results of statistical analysis related to the effect of salicylic acid and potassium sulphate on proline in four sampling stages showed that salicylic acid at 5%, and sampling stages, the interaction of salicylic acid × potassium sulphate, salicylic acid × sampling stages, potassium sulphate × sampling stages, and potassium sulphate × salicylic acid × sampling stages at 1% caused significant effect on proline. Interaction of salicylic acid at 450 mg and potassium sulphate at 2% had the greatest effect on the proline of buds (Figure 9). It has been reported that treatment with salicylic acid increased proline in Walnut (Khoramshahi) and wheat (Singh and Usha 2003). Although use of salicylic acid in okra decreased proline in control plants, the plants treated with salicylic acid produced higher proline under drought stress conditions than untreated plants (Ashraf and Foolad 2007). Under stress conditions, an increase occurs in the production of free radicals, which disrupt the transport of electrons in chloroplasts and mitochondria and can cause the collapse of the membranes under stress conditions (Davies, 1987; Hassibi et al., 2007). Non-structural proline amino acid has antioxidant role and is effective in eliminating free radicals, thereby protecting proteins and membranes against oxidative damage (Zhang et al., 2000).

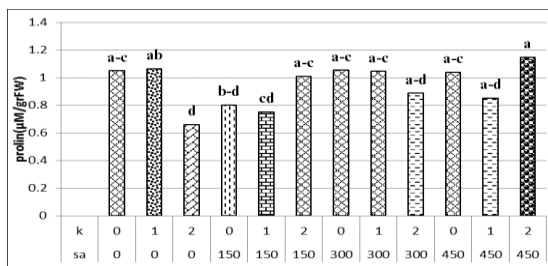


Figure 9- The effect of salicylic acid and potassium sulphate on proline ($\mu\text{M}/\text{grFW}$)

Salicylic acid at 0, 150, 300 and 450 mg/l, respectively, had the greatest effect on proline in sampling stages of 4 and 1. In the fourth and third

sampling stages, salicylic acid at 150 mg had the greatest effect on proline (Figure 10). Furthermore, in deep dormancy stage and before breaking dormancy which buds are exposed to cold stress, using salicylic acid causes an increase in the proline content of bud.

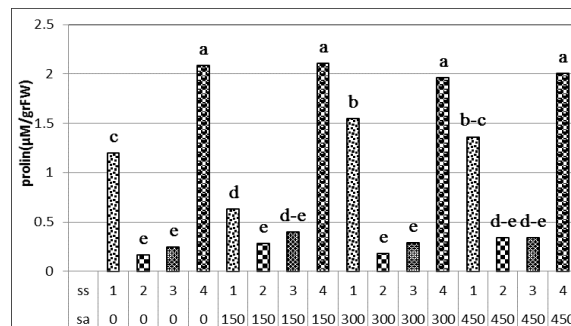


Figure 10. The effect of salicylic acid and sampling stage on proline content ($\mu\text{M}/\text{grFW}$)

Increase in the level of potassium sulphate in the first sampling stage decreased proline (Figure 11), while increase of potassium sulphate in the third sampling stage increased proline. In the fourth stage of sampling, different percentages of potassium sulphate had similar effect on proline. Free proline content in many plants increases greatly in response to environmental stresses such as cold and drought, and stabilizes the membrane during cold stress (Ranney et al., 1991). Free proline accumulation is often associated with plant resistance under high stress conditions, especially low temperature (Ranney et al., 1991). Proline plays an important role in the metabolism of plants under stress, osmotic adjustment of cells and protection of proteins (Yelonsky, 1979). The effects of salicylic acid on the amount of soluble proteins in different plants may be different, and conflicting results are associated to the age, developmental stage of the plant and experimental stage (Janda et al., 2007).

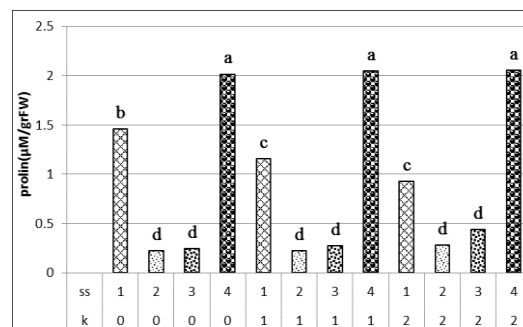


Figure 11- The effect of potassium sulphate and sampling stage on the amount of proline ($\mu\text{M}/\text{grFW}$)

4 CONCLUSION

Increase in the amount of salicylic acid and potassium sulphate and their interaction had a significant effect on the increase of soluble sugars, protein and proline in buds during cold stress or deep dormancy, although no significant effect was observed on the starch. Increase in soluble sugars, protein and proline increases resistance to cold stress and prevents ice formation in cells. Salicylic acid affects physiological and biochemical activities of plants. It also leads to osmotic adjustment, protects cell membranes and reduces free radicals by increasing soluble sugars, proline and protein. Furthermore, salicylic acid increases plant tolerance to biotic and abiotic stresses. Free radicals increase as a result of abiotic stresses, and reduce plant resistance to cold by causing damage to cell membranes. Like salicylic acid, potassium plays key role in the regulation of enzyme activities, protein synthesis, photosynthesis, osmotic adjustment, stomatal movements, synthesis and transport of carbohydrates, and stress tolerance. In general, increasing the amount of salicylic acid and potassium sulphate led to the increase in soluble sugar content, proline and protein in buds and increases resistance to cold stress.

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