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The Effects of Spermine and Salicylic Acid on Pistachio (*Pistacia vera* L.) Cultivars (Badami and Qazvini) under Copper Stress

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ABSTRACT

The aim of this study was to identify the effect of Spermine and Salicylic acid on several growth parameters, such as shoot length, shoot fresh and dry weights, amount of protein and carbohydrate, of Badami and Qazvini pistachio cultivated variety under copper stress. The experiment design was completely randomized with three replicates per treatment and four levels of copper (o.5, 30, 45, 60 µM), one level of SA (o.5mM) and one level of Sp (o.5 mM). The excess copper significantly reduced the fresh and dry weight of the shoot and amount protein in pistachio plants. The carbohydrate was also increased as a response to the increase of copper. However, the damage was higher in Qazvini pistachios compared to Badami pistachios. The SPM and SA treatments increased the shoot length and root and shoot fresh weights as well as the amount of protein and moderated the carbohydrate in the pistachio plants under copper stress. The carbohydrate in plant was also increased. It was concluded that SA and SPM could be used as a potential growth regulators to improve copper stress in pistachio plants.

Keywords: Copper stress, Growth regulators, Pistachio, Potential growth.

Abbreviations: SPM: spermine, SPD: spermidine; 2, 4-D: 2 4-dichlorophenoxyacetic acid, SA: Salicylic acid

Introduction

Over the past years, pistachio has become one of the most important and most commercial horticultural products in Iran. Pistachio is mostly produced in the Middle East, especially Iran. In 2010, Kerman and Rafsanjan had 45.5% and 20.6% of the total world pistachio production, respectively, and Damghan were 3% of the production (Afrousheh *et al*., 2010). In the same year, Iran and USA shares of world exports were 69% and 8.9%, respectively (Razavi, 2005). Sources of copper contamination include mining and smelting, urban, industrial and agricultural wastes and the use of agrochemicals (Sheldon *et al*., 2005). Although copper is an essential element, the increase of copper has a harmful effect on plant growth (Jain *et al*., 2009). The increase of copper concentration generates oxidative stress by causing increase in reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and the hydroxyl radical. ROS could damage lipids, proteins, carbohydrates and nucleic acids (Mittler *et al*., 2004). Polyamines are aliphatic amines with low molecular weight and exist in all living cells. Putrescine (PUT), spermidine (SPD) and spermine (SPM) are the most common polyamines expressed in plants (Roychouldhury *et al.*, 2011).

Polyamines are important modulators in different biological processes, including membrane stabilization, enzyme activity, cell division, elongation, root

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initiation, shoot formation, flower initiation, development, fruit ripening, senescence and embryonic formation in tissue cultures (Galston and Kaur-Sawhney, 1995; Kuznetsov and Shevyakova, 2007).

Polyamine participates in the regulation of plant responses to different environmental stresses such as drought, salinity, heat, chilling and heavy metal, scavenging of free radicals, binding to membrane phospholipids, and supporting a cation-anion balance (Kakkar and Sawhney, 2002; Alcazar *et al*., 2006, Tang and Newton, 2005). Salicylic acid (SA) or ortho-hydroxy benzoic acid and other salicylates participate in regulating plant growth and productivity (Hayat *et al*., 2005). It has been reported that salicylic acid is an endogenous growth regulator of phenolic nature, which enhanced the leaf area and dry mass production in corn and soybean (Khan, 2003). Fariduddin (2003) showed that *Brassica juncea* plants sprayed with low concentrations of SA produced larger amounts of dry matter and had higher photosynthetic rate in comparison with control plants. In addition, Hayat (2005) reported that soaking wheat grains in low concentrations of SA significantly increased the growth of wheat seedlings. SA could protect the serpentine plant from the detrimental effects of salt stress by improving physiological parameters such as water content (%), photosynthetic pigments and protein contents (Misra, 2012). SA could be used as a potential growth regulator for improving plant growth under water stress (El Tayeb and Ahmed, 2010). Exogenous SA has an important impact on different processes in plants including seed germination (Cutt and Klessig, 1992), stomatal closure (Larquer- Saaveda, 1979), ion uptake and transport (Harper and Balke, 1981), plant growth and development, evaporation, ion transmission and absorption, (Sakhabutdinova *et al*., 2003), membrane permeability (Barkosky and Einhellig 1993). SA protects plants against a number of abiotic stresses such as heat stress in mustard seedlings (Dat *et al*., 1998), chilling stress in wheat plants (Tasgin *et al.*,2003), heavy metal stress in barley (Metwally *et al*., 2003) and water stress in wheat plants (Singh and Usha, 2003). In this study, we examined the effects of SA and SP on several of growth parameters, such as shoot length, shoot fresh and dry weights, amount of protein and carbohydrates, in pistachio cultivars (Badami and Qazvini) under copper stress.

Materials and Methods

Plant material

Seeds of pistachio cultivars (Qazvini and Badami) were taken from the Pistachio Research Institute of Rafsanjan. After separating their hard skin, they were sterilized by hypochlorite of soduim 10% for 10-12 minutes, and then they were washed with distilled water for 30 minutes. The seeds were soaked in distilled water for 24 hours, and then they were placed in sterile fabrics and moisted for germination. Five germinated seeds were planted at the depth of 2cm in pots containing cocopeat to perlite 1/4 ratio. The pots were irrigated every other day by distilled water and Hoagland solution. We used ½ concentration of Hoagland solution in the first week, and then it was used completely. The number of seedlings was reduced to three in the fourth week (Hokmabadi *et al.*, 2005).

Spermine, Salicylic acid and copper treatments

Seven-week old plants were treated by the concentrations of spermine (0.5 mM) and salicylic acid (0.5mM) every other day for ten days. Then, concentrations of copper (0.5, 30, 45, 60 µM) that supplied $CuSO_4$ was added to the pots every other day and irrigated by Hoagland solution. After the treatments, the growth and biochemical parameters were measured. This experiment was carried out based on factorial and randomized complete block design with three replications (Hokmabadi *et al.*, 2005)

Shoot and Root fresh weight, Shoot length

Roots and stems were separated and then, they were weighted with digital scale grams. Stem length was measured in centimeters using a ruler.

Protein

A certain amount of fresh leaf was macerated in phosphate-buffered saline (PBS), and the homogenate was centrifuged for 15 minutes. The supernatant (100 µl) was mixed well with 5 ml of diluted dye binding solution. After at least five minutes, the absorbency was checked at 595 nm (Bradford, 1976).

Carbohydrate

Reducing carbohydrate content was measured by adapting Somogyi-Nelson's method (Somogyi, 1952). Approximately 0.04 g of the fresh leaves was extracted with 10 ml distilled water. The mixture was boiled in a boiling water bath, cooled and filtered. Then, 2 ml of the extract was mixed with 2 ml of alkaline copper tartarate, and the reaction mixture was heated for 20 minutes. Two ml of phosphomolibdate solution was added to the mixture, and the intensity of its blue color was measured at 600 nm using spectrophotometer. The sugar content was expressed as mg/g FW.

Results

After exposure of plants to different concentrations of copper (0.5, 30, 45 and 60 µM), morphological changes were observed and the growth rate of both cultivars gradually decreased by increasing copper concentration. The results of the shoot length and root fresh weight are shown in Fig. 1. The copper treatment significantly ($p \leq 0.05$) reduced shoot length, shoot and Root fresh weight in the pistachio plants compared to the control plant (0.5 µM copper). Copper in level of 30µM in Badami cultivars was not significant compared to the control plant (0.5 µM copper). However, copper damage was higher in Qazvini pistachio compared to Badami pistachio. SPM and SA reduced copper stress and increased growth parameter in pistachio plants under copper stress. The effects of interaction of SPM and SA on reduce of copper stress was more than compare to separately in pistachio cultivars under stress. Analysis of variance (ANOVA) about the effects of Spermine and Salicylic acid on pistachio *(Pistacia vera* L.)Cultivar (Badami and Qazvini) under copper stress are shown in Tables 1 and 2.

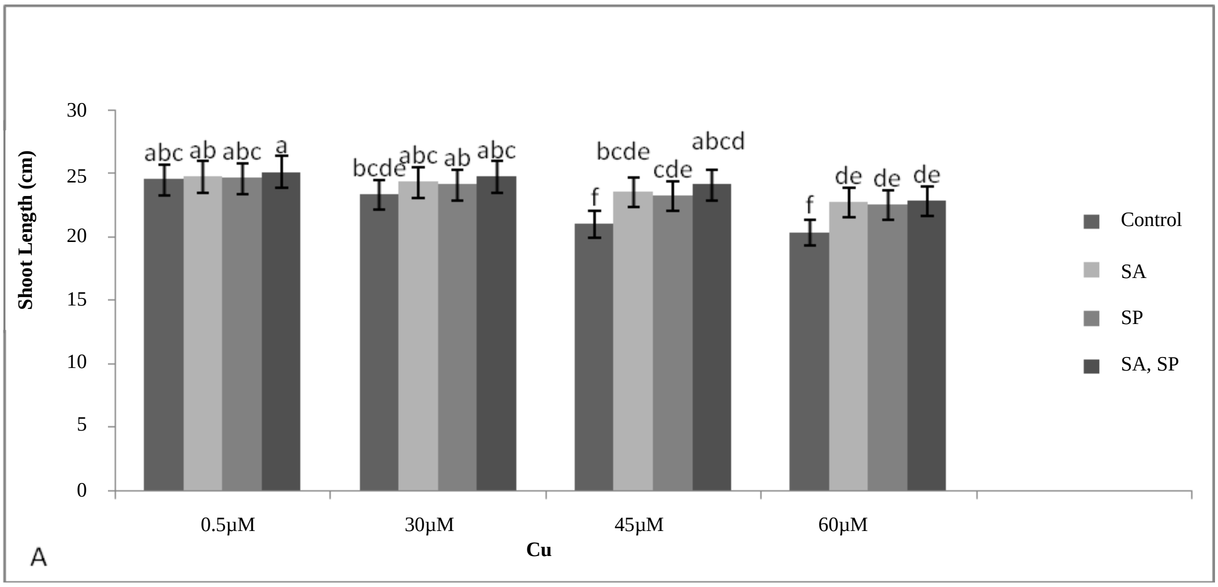
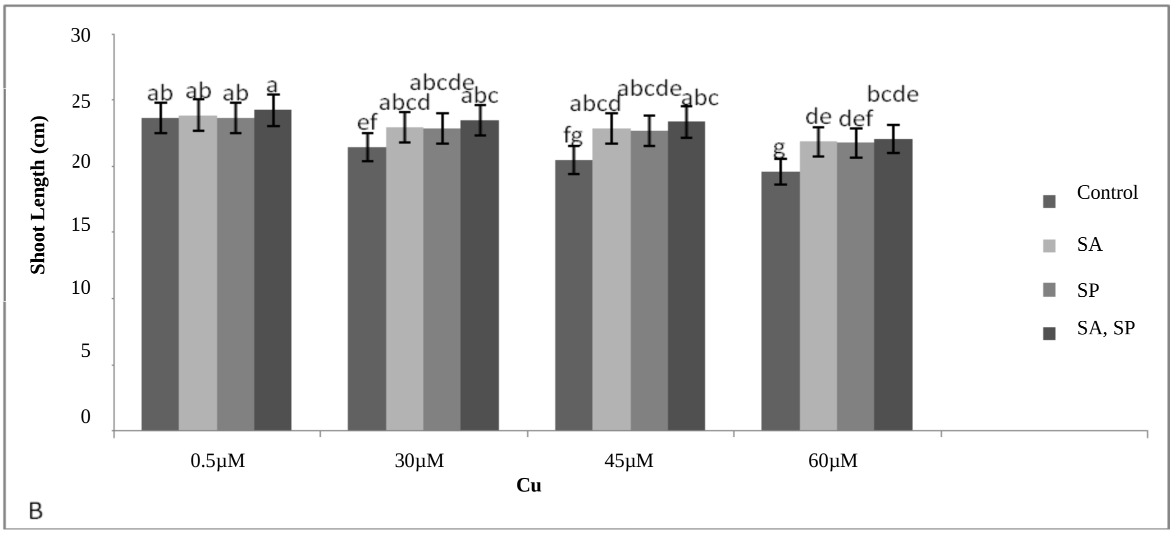
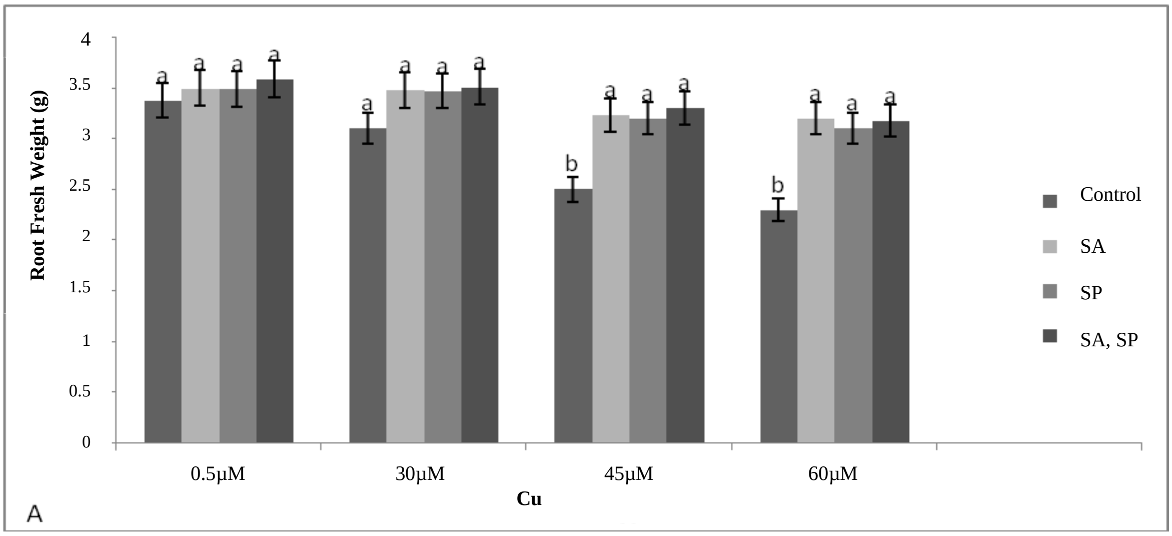
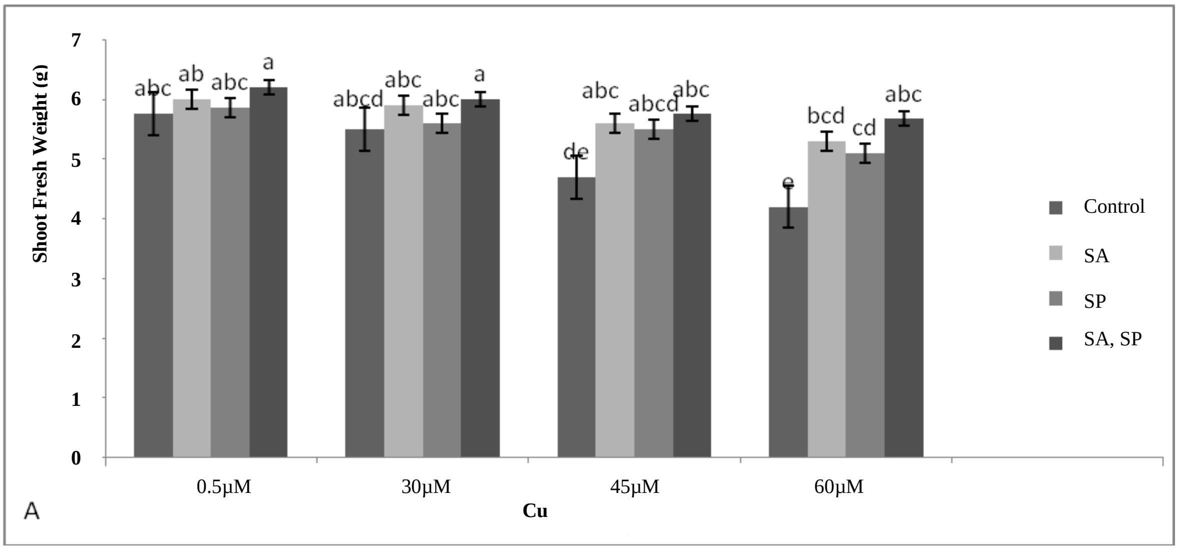


Fig. 1. Effects of SA and SPM on shoot length shoot and Root fresh weight of Badami (A) ording to LSD.







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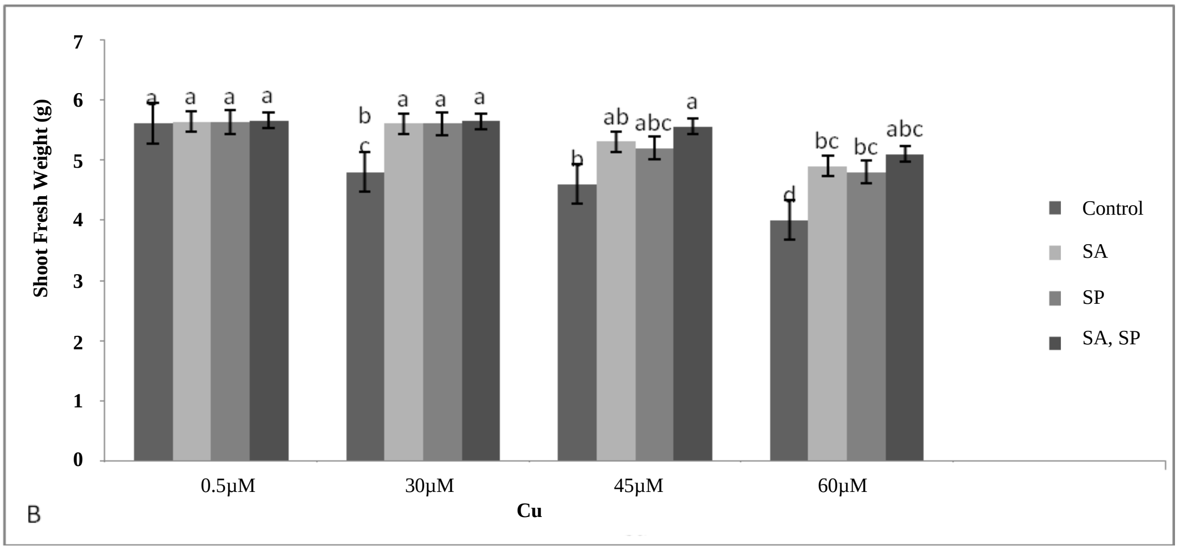

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Copper treatment significantly ($p \leq 0.05$) reduced the amount of protein in the pistachio plants compared to the control plants (0.5μM copper), more so in Qazvini pistachios compared to Badami pistachios. SPM and SA increased protein in the pistachio plants under copper stress. The results in Figs. 2 and 3 showed that copper treatments decreased the amount of proteins and increased the amount of carbohydrates in the pistachio plants compared to the control plants. The increase of carbohydrate was higher in Badami pistachios compared to Qazvini pistachios. SPM and SA increased proteins and moderated the carbohydrate in the pistachio plants under copper stress. The effects of interaction of SPM and SA on the reduction of copper stress was more in combination compared to separately in pistachio cultivars under stress. Analysis of variance (ANOVA) about the effects of spermine and salicylic acid on pistachio *(Pistacia vera* L.) cultivar (Badami and Qazvini) under copper stress are shown in Tables 1 and 2.

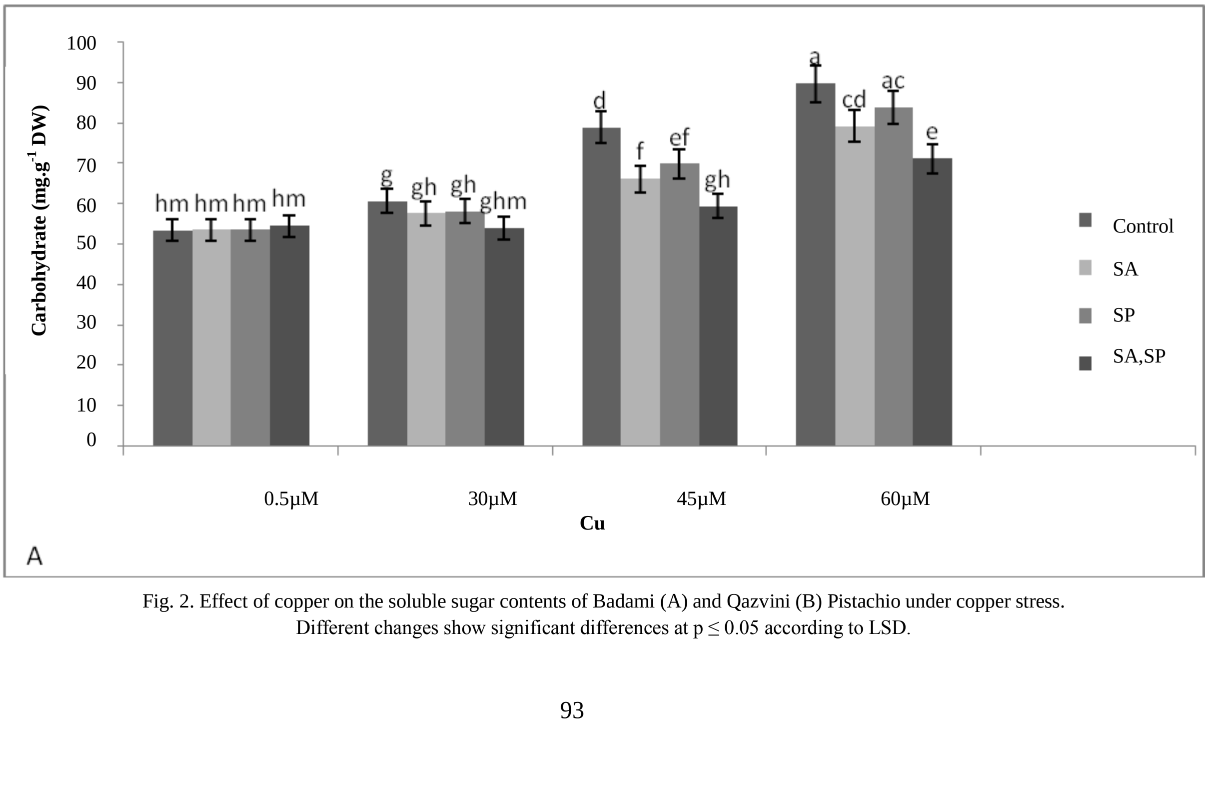
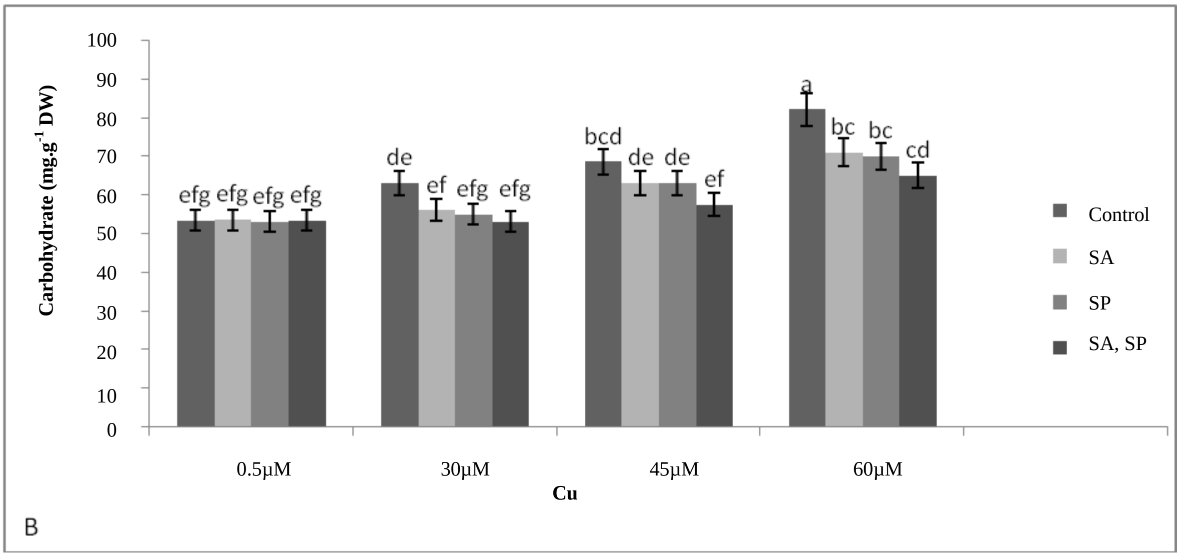


Fig. 2. Effect of copper on the soluble sugar contents of Badami (A) and Qazvini (B) Pistachio under copper stress. Different changes show significant differences at $p \leq 0.05$ according to LSD.



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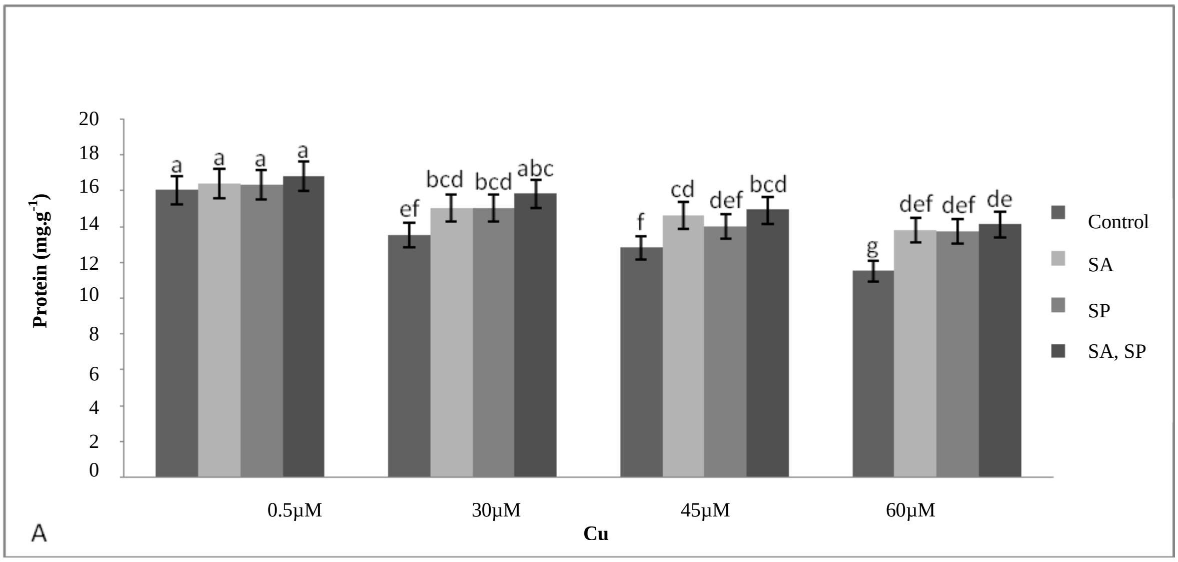

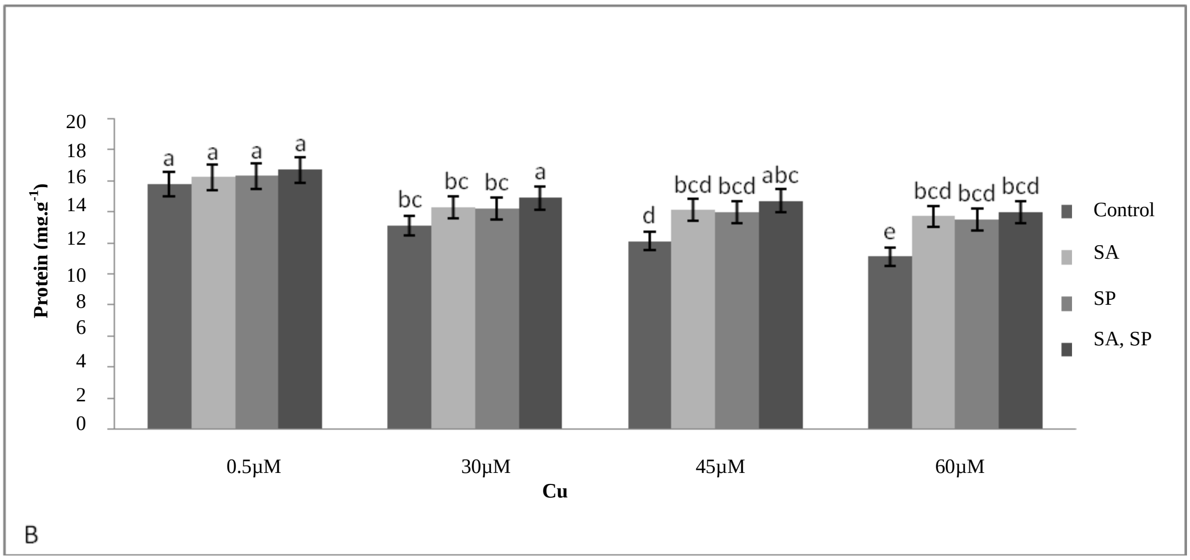

Fig. 3. Effect of copper on the protein contents of Badami (A) and Qazvini (B) pistachio under copper stress.

Different changes show significant differences at $p \leq 0.05$ according to LSD.

Table 1. Analysis of variance (ANOVA) about the effects of Spermine and Salicylic acid on pistachio *(Pistacia vera* L.) Cultivar (Badami) under copper stress.

Badami cultivar	Shoot Length	Shoot weight	Root weight	Carbohydrate	Protein
0.5 µM Cu	24.5±0.4	5.76±0.7	3.38±0.3	53.4±1.5 ns	16±1.9 ns
0.5µM Cu *SA	24.7±0.8 ns	6±0.34 ns	3.5±0.65 ns	53.49±1 ns	16.4±1.2 ns
0.5µM Cu *SP	24.6±0.33 ns	5.59±0.89 ns	3.49±0.7 ns	53.46±1.33 ns	16.3±1.8 ns
0.5µM Cu *SA*SP	25.1±0.9 ns	6.2±0.12 ns	3.59±0.43 ns	54.48±1.66 ns	16.8±1.1 ns
30 µM Cu	23.3±0.2 ns	5.5±0.44 ns	3.1±0.65 ns	60.58±98*	13.5±0.99*
30µM Cu *SA	24.3±0.3 ns	5.9±0.72 ns	3.48±0.32 ns	57.57±0.66*	15±1.45*
30µM Cu *SP	24.1±0.44 ns	5.59±0.66 ns	3.47±0.5 ns	58.1±1.2*	15±1.88*
30µM Cu *SA*SP	24.5±0.7 ns	6.2±0.21 ns	3.51±0.4 ns	53.9±1.67*	15.8±1.39*
45 µM Cu	21±0.53*	4.7±0.8*	2.5±0.2 *	78.8±0.87*	12.8±1.27*
45µM Cu *SA	23.5±1.2*	5.6±0.4*	3.23±0.5*	66.1±0.99*	14.6±1.1*
45µM Cu *SP	23.2±0.66*	5.5±0.45*	3.2±0.71*	69.8±0.67*	14±0.96*
45µM Cu *SA*SP	24.1±0.32*	5.75±0.71*	3.3±0.31*	59.3±0.88*	14.9±0.95*
60µM Cu	20.3±0.2*	4.2±0.3*	2.3±0.34*	89.6±9*	11.5±0.56*
60µM Cu *SA	22.7±1.3*	5.3±0.5*	3.2±0.6*	79.1±1.76*	13.8±0.49*
60µM Cu *SP	22.5±0.9*	5.1±0.61*	3.1±0.77*	83.7±1.34*	13.7±0.78*
60µM Cu *SA*SP	22.8±0.6*	5.67±0.7*	3.18±0.6*	71.1±0.87*	14.1±1.45*

Results significantly different from control at (P< 0.05)*, ns: non-significant.

Table 2. Analysis of variance (ANOVA) about the effects of Spermine and Salicylic acid on pistachio *(Pistacia vera* L.) Cultivar (Qazvini) under copper stress.

Qazvini cultivar	Shoot Length	Shoot weight	Root weight	Carbohydrate	Protein
0.5 µM Cu	23.7±0.8	5.61±0.5	3.4±0.4	53.34±0.86	15.8±0.8
0.5µM Cu *SA	23.9±0.2 ns	5.63±0.8 ns	3.5±0.7 ns	53.55±0.94v	16.2±0.94
0.5µM Cu *SP	23.7±0.55 ns	5.53±0.7 ns	3.4±0.44 ns	53.1±1.65 ns	16.3±1.7
0.5µM Cu *SA*SP	24.3±0.3 ns	5.65±0.76 ns	3.5±0.66 ns	53.4±1.81 ns	16.7±1.1
30 µM Cu	21.5±0.67*	4.8±0.5*	2.8±0.7*	58.58±1.3*	13.1±1.9*
30µM Cu *SA	23±0.78*	5.6±0.66*	3.33±0.7*	56.47±0.91*	14.3±1.5*
30µM Cu *SP	22.9±0.4*	5.6±0.34*	3.31±0.3*	56.99±0.93*	14.2±1.94*
30µM Cu *SA*SP	23.5±0.6*	5.64±0.65*	3.34±0.8*	53.8±1.5*	14.9±1.65*
45 µM Cu	20.5±0.44*	4.6±0.45*	2.5±0.88*	69.8±1.15**	12.1±1.34*
45µM Cu *SA	22.9±0.3*	5.3±0.7*	3.1±0.55*	64.4±0.99*	14.1±1.77*
45µM Cu *SP	22.7±0.7*	5.2±0.9*	3±0.44*	65. ±0.66*	14±0.99*
45µM Cu *SA*SP	23.4±0.76*	5.55±0.54*	3.2±0.6*	57.5±0.68*	14.7±0.63*
60µM Cu	19.6±0.32*	4±0.32*	2.4±0.4*	85.6±0.96*	11.1±0.78*
60µM Cu *SA	21.9±0.5*	4.9±0.56*	2.99±0.78*	78.4±1.14*	13.7±1.88*
60µM Cu *SP	21.8±0.7*	4.8±0.6*	2.93±0.41*	81.3±1.8*	13.5±1.11*
60µM Cu *SA*SP	22.1±0.61*	5.1±0.3*	3.05±0.45*	76.1±1*	14±1.76*

Results significantly different from control at (P< 0.05)*, ns: non-significant.

Discussion

The results of this study showed that copper stress decreased shoot length, shoot and root fresh weight in pistachio plants. However SA and SPM reduced copper stress in pistachio plants. Copper stress decreased the growth parameters in maize (Aly a *et al.*, 2012), Eleusine coracana (Duraipandian *et al.*, 2015), and wheat (Eleftheriou and Karataglis, 1989). SA treatment enhanced the growth of wheat plants under water stress (Singh and Usha 2003), maize (Khodary, 2004), Barley (El-Tayeb, 2005) and Stevia (EL-Housin *et al*., 2014) under salinity stress, SPM treatment in wheat (Abdel-Fattah *et al*., 2013) and SPD treatment in rice (Roychoudhury *et al.*, 2011) . Prasad (1995) reported that the copper stress decreased the growth parameters by increasing ethylene and decreasing cytokinin and polyamine. The inhibition of root growth is one of the major responses by toxic copper levels (Eleftheriou and Karataglis, 1989). Excess copper decreased growth, which as a result, led to the reduction of cell wall elasticity by binding to the cell wall pectin (Aidid and Okamato, 1993). Salicylic acid is able to block the ACC oxidase and thus, inhibit ethylene biosynthesis (Raskin, 1992). Salicylic acid is an endogenous growth regulator of phenolic nature that enhanced the leaf area and dry mass production in corn and soybean (Khan *et al*., 2003). Salicylic acid is a plant growth regulator and also an antioxidant (Raskin *et al*., 1992). SA decreased the effect of salinity in plant growth and development (EL-Tayeb, 2005). Polyamines may play a role as a nitrogen source and stimulates growth in plants (Smith, 1992). In this study, the excess copper reduced the protein in the pistachio plant. SA and SP decreased the effects of copper stress. This finding is consistent with studies performed by Guzel (2013), Wang (2006) and Zhang (2009), SA treatment in barely (Pancheva, 1996) and SA treatment in wheat under Ni stress (H. Siddiqui *et al*., 2013).

Heavy metals produce reactive oxygen species (ROS), which can damage lipids, proteins, carbohydrates, and nucleic acids (Mittler *et al*., 2004). Mouratao *et al*. (2009) reported that copper ions can oxidize the thiol bonds existing in the proteins and cause a disruption in their structure and functions. Salicylic acid is effective on defensive proteins, proteinkinase and Robisco synthesis (Popova, 1997; Raskin, 1992). Polyamines protective proteins can stabilize cellular structures, such as thylakoid membranes, by binding to proteins (Tiburcio *et al*., 1994). Polyamines also participate as radical scavengers (Zhao *et al*., 2007) and ethylene antagonist (El Meskaoui1 and Trembaly, 2009). In this study, the copper stress increased carbohydrate in the pistachio plants. SA and SPM decreased the carbohydrate in the pistachio under copper stress. This finding is similar to the findings of Alaoui-Sosse (2004) and Guzel (2013) regarding SA treatment in maize under salinity (Khodary, 2004) and spermidine treatment in Nympoides peltatum undercopper stress (Wang, 2007).

The increase of carbohydrate can be a result of the aggregation of sugar by reducing their load of phloem or reducing transport and their utilization (Alaoui-Sosse, 2004). Sinniah *et al*. (1998) demonstrated that carbohydrate contributes to the regulation of internal osmolarity and the biomolecules. Khodary (2004) reported that SA helps by using soluble sugar to generate new cells and regulate growth in plants and may speed up the conversion of soluble to insoluble sugar. Polyamines increase proteins biosynthesis or decrease protein decomposition by the elimination of reactive oxygen species in plants (Wang, 2007). In this experiment, copper stress reduced growth parameters and protein amount and increased carbohydrate in pistachio plants. The effects of copper stress were more significant in the Qazvini cultivar compared to the Badami cultivar. Sa and Sp decreased the effects of copper stress in pistachio cultivares (Badami and Qazvini).

Conclusions

This experiment showed that copper stress reduced growth parameters and protein amount and increased the amount of carbohydrates. The interaction of SP and SA reduced the effects of copper stress in pistachio plants. SP and SA had synergistic effects in alleviating stress induced by excess copper. Based on our data, it is suggested that the Badami cultivar was more tolerant to copper stress compared to the Qazvini cultivar.

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