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RESEARCH PAPER

Response of pre harvest foliar application of micronutrients on yield and quality attributes of peach cv. Early Grand

Allah Bakhsh[1]*, Fiaz Hussain[1], Muhammad Kashif Raza[1], Attiq Akhtar[1] and Hafiz Wasif Javaad[1]

[1]Horticultural Research Station, (Soon Valley), Nowshera, District Khushab, Pakistan

***Corresponding author: Allah Bakhsh (allahbakhsh64@gmail.com)

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Key message: Micronutrients play a pivotal role in the improvement of fruit yield and quality. In the present investigation, foliar application of 0.5% of each micronutrient i.e. boron, zinc, iron and copper application depicted remarkable development in physical and quality parameters of peach.

Abstract: Peaches are produced in different climatic regions of Pakistan. Peach growth and yield is affected by many factors but nutrition is the most influential element in this regard as nutrients tend to improve photosynthetic efficiency. This two years (2018, 2019) study was performed to evaluate the foliar application of micronutrients on quality and yield features of nine years old peach cv. Early Grand by using five combinations of micronutrients (0, 0.5, 1.0, 1.5 and 2% each of $ZnSO_4$, $FeSO_4$, H_3BO_3 and $CuSO_4$). Physio-chemical analysis of soil was done before the application of micronutrients. Foliar application of micronutrients significantly improved quality and yield as compared with control in both years. Results depicted that fruit obtained from the plants having application of 0.5% each of $ZnSO_4$, $FeSO_4$, H_3BO_3 and $CuSO_4$ showed increased fruit diameter (5.04 cm, 5.03 cm), fruit firmness (11.66 lb inches^{-2}, 12.16 lb inches^{-2}) yield per tree (75.60 kg, 76.10 kg), juice TSS (13.58 $^{\circ}$Brix, 13.35 $^{\circ}$Brix), Vitamin C (6.08 mg/100g, 5.96 mg/100g) and reduced fruit acidity percentage (0.28%, 0.26%) and juice pH (3.83, 3.91). Overall, application of 0.5% micronutrients concentration (each of zinc, iron, boron and copper) was considered as the optimum doze to boost the quality of fruit and yield in peach cv. Early Grand.

Keywords: Micronutrients, Foliar application, Peach, Qualitative traits, Fruit growth.

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Introduction

A descendent of Rosaceae family, peach (*Prunus persica* L.) is a commercial fruit crop in China, Italy, Spain, USA and Pakistan. Peach in addition to other species of Rosaceae family are cumulatively recognized as stone fruits (Ali, 2014a). It has an attractive appearance, delicious taste and superior quality. Peach fruit is a rich source of Vitamin A and C, and also contains a considerable proportion of potassium and fiber (Habib, 2015). This fruit is native to China where it was domesticated and cultivated since ancient times. China ranks first among all peach growing countries in the world with total production of 15.21 Million Metric Tons. This is followed by Italy, Spain and USA (Food and Agriculture Organization [FAO], 2018). After citrus, mango, guava, apple, date palm, banana and apricot, peach is the most extensively cultivated fruit crop in Pakistan having 87,864 tonnes total produce from an area of 15,032 hectares (Government of Pakistan [GoP], 2019). In Pakistan, peaches are mainly cultivated in Khyber Pakhtunkhwa and Balochistan, while screened low chill and early maturing cultivars are in vogue in Pothwar areas of Punjab. More than fifty peach varieties were subjected to production trials in Khyber Pakhtunkhwa but only a few of them were recommended for commercial cultivation. Early Grand, Florida King, Florida Gold, Plain 4, A-6 and A-669 are some low chill cultivars of peaches. These cultivars require low chilling hours (<7 ºC for 275 hour) for bud burst and growth (Zeb & Khan, 2008; Habib, 2015).

Climatic and edaphic factors of Punjab are well suited for quality production of low chilling peach cultivars. Crop nutrition along with other management practices, is among those leading factors which enhances growth, yield and subsequently overall quality (fruit size, weight, juice%, TSS etc.) of fruit crops. Fruit trees have more nutritional requirements as compared with annual crops. Peaches, being an important fruit crop, require a specific amount of nutrient application. Different visual symptoms of plant nutrient deficiencies cannot be assessed until soil and plant analysis are conducted (Shah & Shahzad, 2008). Micronutrients play pivotal functions in growth physiology and hence improve the quality of fruits (Zaman & Schumann, 2006). Micronutrients are usually required in minute quantities, nevertheless are vital to the growth of plants. They improve general conditions of the plant and are known to act as catalyst in augmenting organic reactions taking place in plants (Patil et al., 2010).

Imbalanced nutrient application (excess or deficient) adversely affects the yield. Owing to depletion of micronutrients in the soil by high yielding fruit crops, foliar application of micronutrients is very important to get commercial crop as well as this method also helps to avoid disease occurrence and enhance insect pest resistance and drought tolerance (Tariq et al., 2007). Growth and yield response of micronutrients is enhanced by 10 to 20 times by foliar application method in contrast to soil application (Zaman & Schumann, 2006). Direct application of Zn in alkaline soils results in adsorption or precipitation on the soil surface and does not move readily to the root zone. Foliar application of micronutrients is very effective to recover these deficiencies in soils of Pakistan. Dhotra et al. (2018) observed that unbalanced fertilization, micronutrients deficiencies, poor tree management and inadequate cultural practices are mainly responsible for peach orchard related quality issues. Quick response, low application rate and uniform distribution of micronutrients are the leading advantages of foliar application. Many biological processes occurring in plants depend upon micronutrients such as zinc, boron, copper and iron, which are connected with overall quality and yield in fruit crops (Shoeib & El-Sayed, 2003). High rate of photosynthesis leads towards improved sugars accumulation and decreased acidity (Abedy, 2001).

Boron is helpful in promoting physiological functions and metabolic activities in plants which results in improved growth and development (Shireen et al., 2020). Adequate amount of boron in plants increases the process of pollination as it helps in pollen grain germination and elongation of pollen tubes, resulting in heavy bearing (El-Sheikh et al., 2007). Foliar spray of micronutrients was found more effective than soil application in curing B deficiency (Shireen et al., 2018a). Similarly, copper stimulates enzyme activity which results into protein metabolism, lignin and vitamin A synthesis (Henry, 2009). Iron deficiency limits quantitative factors i.e. yield, fruits per tree, quality and size of several fruit crops. Hence its application has a conducive impact on the said attributes (Àlvarez-Fernàndez et al., 2006). El-Boray et al. (2015) noticed that fulvic acid, boron, calcium and zinc application through spray on apple trees significantly improved fruit weight, yield, fruits per tree, TSS and sugars. Similarly owing to zinc, boron, iron and NPK application contributed towards improved peach quality and yield (Yadav et al., 2013).

In Pakistan, about 6% of the peach orchards soils are deficient in boron, while 2% soils in zinc (Samiullah et al., 2013). Zn is a component of almost 60 enzymes. It has a role in producing the growth hormone IAA. Zn plays a key role in nitrogen metabolism of plants and Zn deficient plants have reduced protein contents (Mengel et al., 2001). Additionally, Zn application improves the production of hormones like auxins, gibberellins and melatonin as well as helps to increase the activities of antioxidant systems which ultimately supports to enhance photosynthetic processes (Hassan et al., 2020). High soil pH also hinders availability of boron, zinc, copper and iron to plants (Rashid et al., 2008), similarly seasonal changes in temperature and soil moisture also alter availability of nutrients to the plants (Dhotra et al., 2018). Keeping in view the unfavorable physico-chemical conditions of local soils, it becomes necessary to supply micronutrients in balanced proportion through foliar spray to increase peach production. The given account of scientific facts prompted us to assess the impact of trace minerals application (Zn, B, Fe and Cu) on growth and quality of peach as no such type of study has been previously reported on locally grown peach cv. Early Grand.

Materials and Methods

A two years field experiment was conducted during 2018 and 2019 at Horticultural Research Station, Nowshera (Soon Valley), District Khushab (32° 33' 52.2" N, 72° 08' 28" E), on Peach plants of cv. Early Grand, budded on local almond rootstock. The meteorological data regarding chilling hours, annual rainfall, average minimum and average maximum temperature shown in Fig. 1, 2 and 3, respectively during the experimental period 2018-19. Nine years old plants of uniform health, size and vigor growing at 3×3 m distance were selected for the study. Foliar application of micronutrients was made twice, first during last week of February at petal fall stage and then repeated after 15 days of the first application in 2018 and 2019. The micronutrients boron, zinc, iron and copper in the form of H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$ were formulated in the laboratory and applied in following combinations:

T_1= Control
T_2 = 0.5% H_3BO_3 + 0.5% $ZnSO_4$ + 0.5% $FeSO_4$ + 0.5% $CuSO_4$
T_3 = 1% H_3BO_3 + 1% $ZnSO_4$+ 1% $FeSO_4$ + 1% $CuSO_4$
T_4 = 1.5% H_3BO_3 + 1.5% $ZnSO_4$ + 1.5% $FeSO_4$ + 1.5% $CuSO_4$
T_5 = 2% H_3BO_3 +2% $ZnSO_4$ + 2% $FeSO_4$ + 2% $CuSO_4$

Aforesaid treatments were applied in four replications according to Randomized Complete Block Design and a sole plant was considered as an experimental unit. Soil analysis of the orchard was carried out prior to the application of treatments, following a procedure adopted by Ashraf et al. (2010) with minor modifications. Three soil samples (2 feet away from the main stem of each experimental tree) were collected. Each individual sample consisted of 200 g soil taken from three depth levels (0-15 cm, 16-30 cm and 31-45 cm). Twenty samples of the same depths were combined into one composite sample. These samples were dried, pulverized by mortar and sieved through 2 mm mesh size before analysis. Soil analysis was conducted at soil and water testing laboratory Khushab, Pakistan. The average values of physico-chemical parameters regarding three different depths of composite samples (mixture of 20 samples from same depth) are presented in Table 1.

Fruits were harvested in morning hours on clear sunny days at a firm mature stage during June for both years and fruit weight per tree was recorded. Five representative fruits samples of uniform size and shape were randomly selected in the field from each treatment. Fruit harvested was washed to remove field heat/debris. After drying, the fruit was packed in hardboard cartons, shifted to the laboratory and stored at room temperature for data collection of different physical and quality parameters. For biochemical parameters, juice was extracted in an experimental laboratory from five fruits from each treatment.

Table 1 Physio-chemical parameters of soil samples collected from experimental orchard

Soil characteristics	Depth		
	0-15 (cm)	16-30 (cm)	31-45 (cm)
Texture	Loam	Loam	Loam
pH	8.0	8.1	8.5
EC	1.67	1.44	1.62
Organic matter (%)	1.2	0.59	0.43
Available phosphorus (mg/Kg)	7.9	5.05	4.42
Available potassium (mg/Kg)	181.41	158	1.52
Saturation (%)	41	41	41
Zinc (mg/Kg)	1.57	1.32	1.1
Iron (mg/Kg)	3.8	2.9	3.2
Copper (mg/Kg)	3.8	2.23	1.98
Boron (mg/Kg)	0.43	0.31	0.2

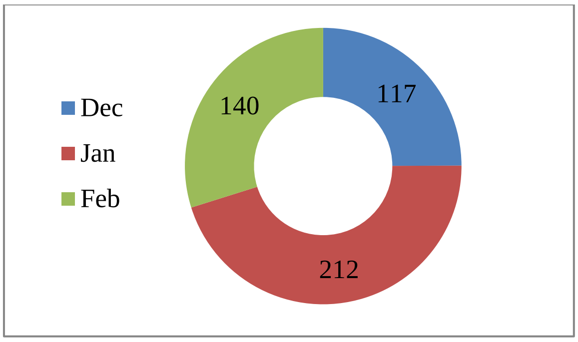

Fig. 1 Chilling hours (0-10 ^{o}C) of Soon Valley, district Khushab from December 2018 to February, 2019

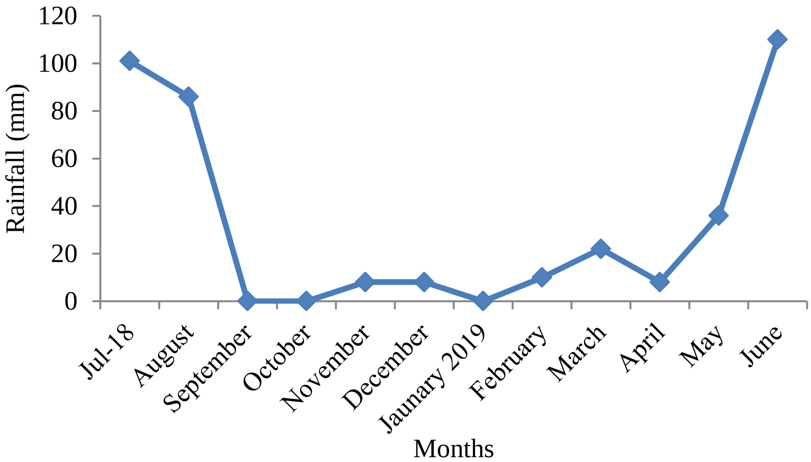

Fig. 2 Rainfall data for 2018-19 of Soon Valley, District Khushab, Pakistan

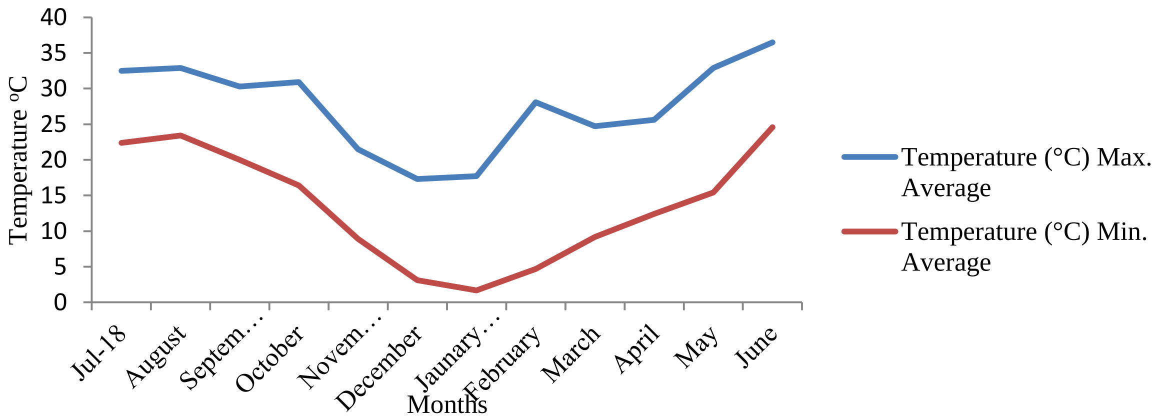

Fig. 3 Average maximum and average minimum temperature for 2018-19 of Soon Valley, District Khushab Pakistan

Physical parameters

Fruit weight (g)

Plant yield calculation is based on a single fruit weight assessment. Average of five randomly selected fruits was measured by weighing balance (Sartorius Laboratory, Model Cubis ii). Single fruit weight was recorded and was expressed as average fruit weight in gram (g).

Fruit diameter (cm)

Fruit diameter is the foundation to access the quantitative standard of a single fruit. Randomly selected samples of five fruits were taken to determine the fruit diameter's values with digital vernier caliper (Model: HT0403-A1, Cingda Industry Co., Ltd. China) and mean values were evaluated.

Fruit firmness (lb inches^{-2})

Fruit firmness: average of five randomly selected fruits was measured by using Wagner Penetrometer Model FT 327 and expressed as lb-inches^{-2} described by Sharif et al. (2019).

Yield/tree (kg)

A sample of five ripened fruits was selected to find out fruit weight average with a digital balance. It assisted to find out the yield (kg) per plant basis. Average fruit weight was multiplied by with the total number of fruits counted on the tree at the harvesting time.

Acidity (%)

For assessment of acidity %, a sample of 10 ml of extracted juice was titrated against 0.1 N NaOH. With the addition of 2-3 drops of phenolphthalein were used as indicator with achieving pink color as end point. Following formula was exercised to determine the avidity percentage.

$$\text{Acidity (\%)} = \frac{0.1\text{N NaOH} \times 0.0064}{\text{ml of juice used}} \times 100$$

TSS ($^{\circ}$Brix)

Digital refractometer (Model MASTER-α, Atago, Japan) was used to assess TSS value. A sample of five fruits was taken for juice extraction. One drop of fruit juice was put on the prism of refractometer thus TSS was measured and its value was expressed in $^{\circ}$Brix.

Vitamin C (mg/100 g)

To find out the vitamin C strength, a method described by Ruck (1961) was used by taking 10 ml juice in 100 ml volumetric flask in which 0.4% oxalic solution was added. A 5ml aliquot sample was measured and titrated against 2, 6-dichlorophenol indophenol dye till the achievement of light pink color being the terminal point which lasted for 15 seconds. Estimation of Vitamin C was measured with the below given formula:

$$\text{Vitamin C (mg/100 ml)} = \frac{1 \times R1 \times V \times 100}{R \times W \times V1}$$

Where
R_1 = ml dye used in titration of aliquot
R = ml dye used in titration of 1 ml of standard ascorbic acid solution prepared by adding 1 ml of 0.1% ascorbic acid + 1.5 ml of 0.4% oxalic acid
V_1 = ml of juice used in titration
V = Volume of aliquot made by addition of 0.4% oxalic acid
W = ml of aliquot used for titration

pH

pH meter (Tacussel, Villeubanne, France) was used to determine juice pH. 200 µl of juice was used to determine juice pH by the method illustrated by Moing et al. (1998).

Statistical analysis

The trail was laid according to RCBD. Response of foliar applied micronutrients on fruit attributes of peach was determined by statistical analysis of data with software Statistix 8.1. The collected data was analyzed by using two way analysis of variance techniques (ANOVA), while treatment means were compared using LSD test ($P \leq 0.05$) (Steel et al., 1997).

Results

Soil analysis report

Peach orchard soil analysis showed that the loam textured soil was alkaline in reaction, where organic matter, available P and micronutrients (Zinc and Boron) were found low as per criteria compiled by Estefan et al. (2013).

Physical parameters

Fruit weight (g)

Foliar application of micronutrients significantly improved the fruit size of peach during both years of study. Data pertaining to fruit weight (Table 2; Fig. 4) indicated a significant difference among treatments. Maximum mean fruit weight (98.34 g and 94.21 g in 2018 and 2019, respectively) was obtained in 1% of each $H_3BO_3 + ZnSO_4 + FeSO_4 + CuSO_4$ followed by 0.5% concentration of each nutrient ($H_3BO_3 + ZnSO_4 + FeSO_4 + CuSO_4$) compared to lowest fruit weight in control in both years (60.87 g and 72.58 g, respectively).

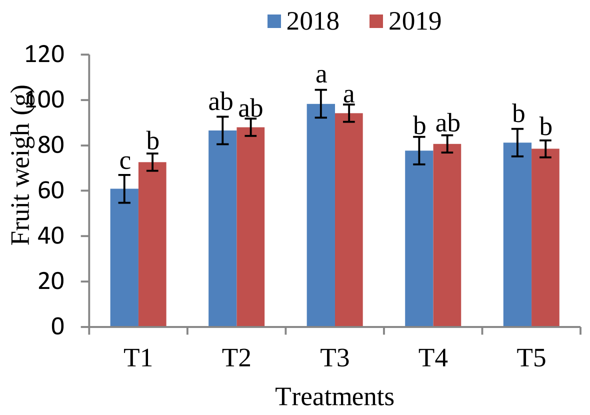

Fig. 4 Response of different combinations of zinc, iron, copper and boron through foliar spray on fruit weight of Peach cv. Early Grand

Fruit diameter (cm)

Data in Table 2 and Fig. 5 indicated a prominent significant behavior after application of micronutrients on fruit diameter during both years of study. Maximum mean fruit diameter (5.04 cm and 5.03 cm in 2018 and 2019 respectively) was attained by T_2 holding mixture of 0.5% of boric acid, zinc sulfate, ferrous sulfate and copper sulfate of each), while lowest fruit diameter (3.69 and 3.90 cm) was noted in T_1 (Control) in both years.

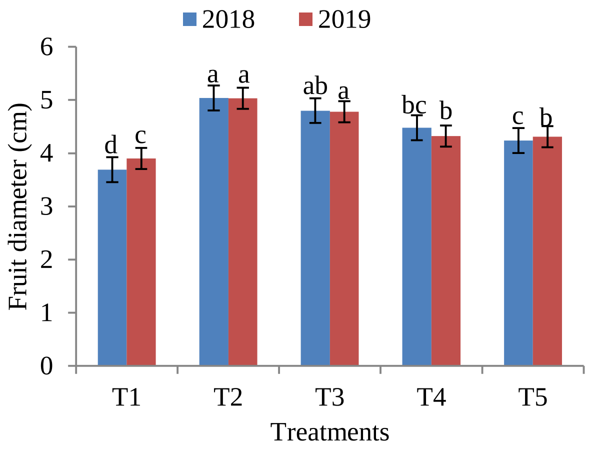

Fig. 5 Response of foliar fertilization of zinc, iron, boron and copper on fruit diameter of peach cv. Early Grand

Fruit firmness (lb inches^{-2})

Fruit firmness is an indicator of good shelf life and micronutrients spray had positive impact thus increasing its market opportunity and reducing losses. Fruit firmness significantly improved through foliar use of micronutrients during both years of study when compared with control (Table 2 and Fig. 6). Maximum mean fruit firmness (11.66 and 12.16 lb inches^{-2} during 2018 and 2019, respectively) was obtained by the application of 0.5% concentration of H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$ in combination, followed by 2% each of H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$.

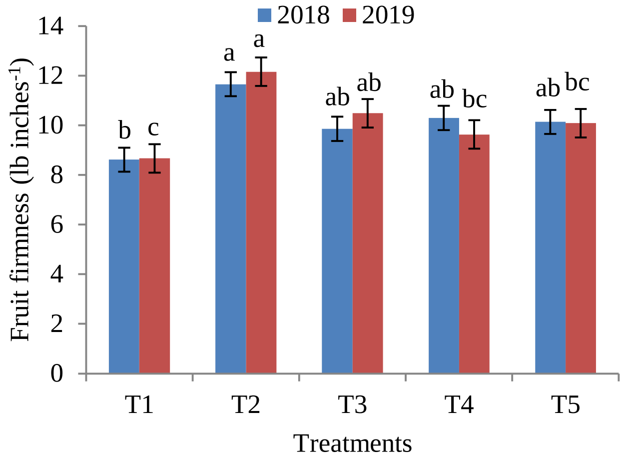

Fig. 6 Response of foliar fertilization of zinc, iron, boron and copper on fruit firmness of peach cv. Early Grand

Yield/tree (kg)

The data regarding fruit yield (Table 2 and Fig. 7) showed a little significant influence of micronutrients. The highest yield per tree (75.60 kg and 76.10 kg during 2018 and 2019, respectively) was obtained in T_2 (0.5% boric acid, zinc sulfate, ferrous sulfate and copper sulfate) followed by T_3 (1% boric acid, zinc sulfate, ferrous sulfate and copper sulfate), while the minimum yield (49.63kg and 42.13 kg) was obtained from T_1(Control) in both years. Foliar application of micronutrients significantly increased yield.

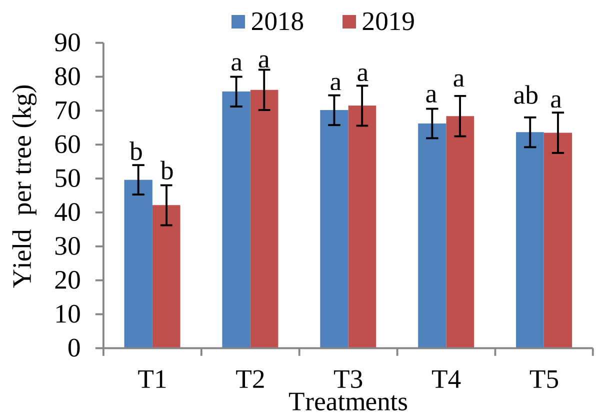

Fig. 7 Response of foliar fertilization of zinc, iron, boron and copper on yield/tree of peach cv. Early Grand

Biochemical parameters

Acidity (%)

The acidity percentage was slightly significant among the treatments during both years of study as shown in Table 3and Fig 8. Lowest mean acidity (0.28% and 0.26% during 2018 and 2019, respectively) was observed in those fruits which were sprayed at the rate of 0.5% of each H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$, while maximum acidity was recorded in untreated fruits (Control) in both years.

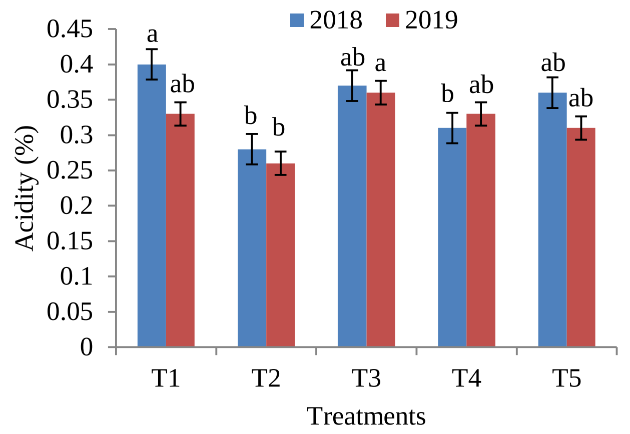

Fig. 8 Response of foliar fertilization of zinc, iron, boron and copper on acidity of peach fruit (*Prunus persica* L.) cv. Early Grand

Total soluble solids (˚Brix)

Results of TSS during both years of study (Table 3 and Fig. 9) depicted that micronutrients use as a foliar method significantly enhanced TSS in peach fruit. Highest TSS (13.58 and 13.35 ˚Brix during 2018 and 2019, respectively) was observed in those fruits, which were treated with 0.5% each of H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$, while lowest value of TSS (11.76 and 12.2 ˚Brix) were observed in control during both years.

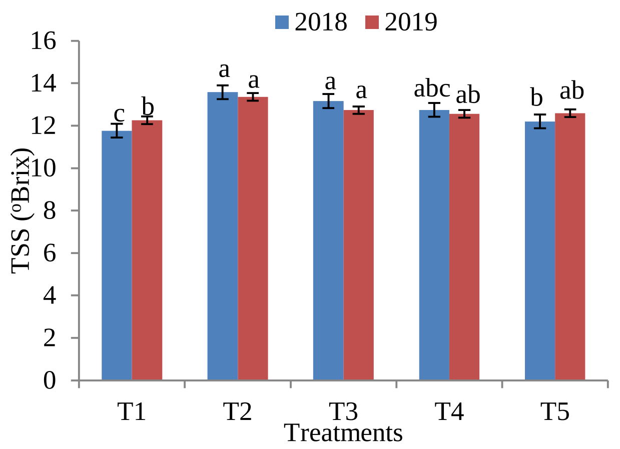

Fig. 9 Response of foliar fertilization of zinc, iron, boron and copper on TSS of peach cv. Early Grand

Vitamin C (mg/100g)

A profound effect of micronutrients was observed in increased vitamin C contents in both years 2018-2019 as compared to control as shown in Table 3 and Fig. 10. The highest vitamin C values (6.08 and 5.96 mg/100g) were noted in T_2 (0.5% each of boric acid, zinc sulfate, ferrous sulfate and copper sulfate), followed by T_4 (1.5% boric acid, zinc sulfate, ferrous sulfate and copper sulfate), while lowest values of Vitamin C (4.65 and 4.90 mg/100 g) were noted in control in both years.

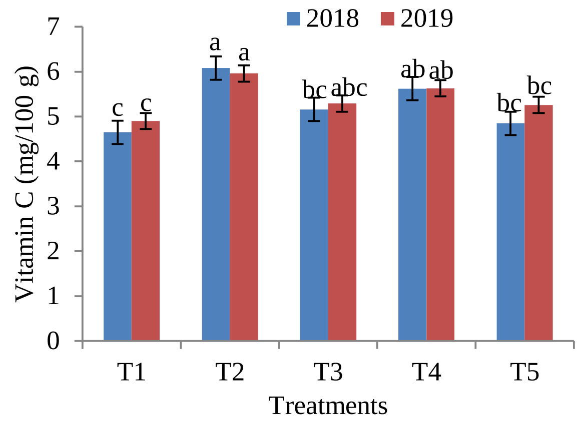

Fig. 10 Response of foliar fertilization of zinc, iron, boron and copper on vitamin C of peach cv. Early Grand

pH

Data regarding pH of juice (Table 3; Fig. 11) showed slight significant results in both years of study. Foliar application of micronutrients decreased juice pH. The lowest pH (3.83 and 3.91 during 2018 and 2019, respectively) was measured in those fruits which were treated by 0.5% H_3BO_3, $ZnSO_4$, $FeSO_4$ and $CuSO_4$ each, while highest pH was reported in control in both years.

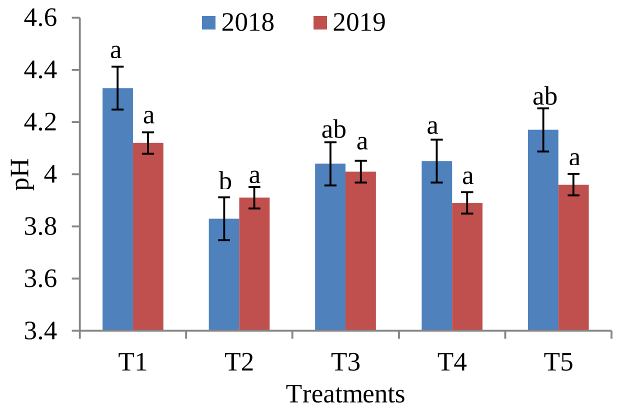

Fig. 11 Response of foliar fertilization of zinc, iron, boron and copper on pH of peach cv. Early Grand

Discussion

Micronutrients are well recognized regarding their pivotal role in the augmentation of physical and quality attributes of peach. Fruit weight is key quality characteristics because it is directly associated with grower profit and consumer preference (Nawaz et al., 2008; Khan et al., 2010). Increase in weight of fruit could be owing to enhanced plant metabolism and fruit development as a result of micronutrient application (Tripathi et al., 2015). Similar results on peaches have also been reported by Dhotra et al. (2018) through the application of B, Zn and Fe.

Sourour (2000) revealed that micronutrient's application by foliar method in Valencia orange resulted in enhanced fruit weight. Fruit diameter is also a major parameter in terms of quality of produce. It is a marketable index since consumers prefer good size peaches and ultimately correlated with fetching high profit from the market. Boron helps in cell wall synthesis and elongation so fruit diameter may have been enhanced due to its application (Tripathi et al., 2015). The results in this study are in conformity with Ali et al. (2014a&b) who ascertained that foliar fertilization of zinc, manganese, iron, boron and copper enhanced diameter of fruit both in persimmon and peach. Similar results were also obtained by Dhotra et al. (2018) as they found that application of zinc, boron and iron application increased fruit length of peach cv. Shan-e-Punjab.

Fruit firmness is an indicator of good shelf life and micronutrients spray had positive impact thus increasing its market opportunity and reducing losses (Nakano et al., 2020). Increase in fruit firmness by foliar application of micronutrients may be due to the combined effect of boron, zinc and copper. Boron which helps in membrane integrity and lignification (Matas et al., 2009), zinc regulates biological membranes (Sinclair & Kramer, 2012) and copper helps in cell wall metabolism (Yruela, 2005). Cronje et al. (2009) found the same trend in fruit firmness when litchi was sprayed by zinc, copper, boron and potassium nitrate.

Fruit yield is the ultimate goal of farmers, as higher yield and good quality of fruits provide an opportunity to gain higher profit (Ahmed et al., 2006). This effect may be due to increased process of pollination, cell elongation and cell division, along with sugar metabolism and accumulation of carbohydrates by the application of boron (Sourour, 2000; El-Sheikh et al., 2007). Similarly, eminent role of zinc in regulation of enzymes activity (Broadley et al., 2007) and pivot role of iron in biosynthesis of chlorophyll leading to increased photosynthesis (Chatterjee et al., 2006) might have contributed to increasing yield. Results regarding fruit yield were in agreement with Mishra et al. (2016), who found increased fruit yield in strawberry by foliar method of micronutrient's application. In the same way, Singh et al. (2017) depicted that yield/plant in mango can be increased by applying micronutrients through foliar method. Our results are also supported by Liu et al. (2020) who reported increased grain yield of maize through zinc fertilization.

Total soluble solids (TSS) are indirect calculation of sugar contents in fruits which is one of the basic tools to measure quality of fruit. In addition to this sugars are the basic source of energy providers (Shireen et al., 2018b). Citrate and malate are the major organic acids present in peach, they found 62% and 22.6%, respectively and largely contribute towards the acidity percentage of peaches (Baccichet et al., 2020).The improvement in total soluble solids contents and decrease in acidity percentage may be due to the reason that zinc provides assistance in hydrolysis of complex polysaccharides into simple sugars which resulted in improved TSS (Rawat et al., 2010). Similar findings have also been reported by Ferdosi and Farooq (2017); Ambaliya et al. (2018) that application of $ZnSO_4$ and H_3BO_3 and multi-micronutrient increased TSS of mango and Aonla (*Emblica officinalis* Gaertn.) cv. Gujarat Aonla-1, respectively. Similarly results were obtained when application of micro elements (manganese, boron, iron and zinc) suppressed the acidity percentage in mango cv. Dusehri and Valencia oranges (Abd-El-Motty et al., 2006; Anees et al., 2011). Vitamin C (ascorbic acid) is necessary for many important functions of human life. It is known as water soluble, a potent anti-oxidant which takes part in the immune system and helps to fight against skin diseases (Padayatty et al., 2003). Similar findings have also been reported by Tariq et al. (2007), where they noticed foliar use of zinc as well as boron appreciably increased vitamin C contents in sweet oranges. It was also reported by Hassan et al. (2010) that micronutrients sprayed on plum trees improved vitamin C proportion in fruit. Previous findings (Ali et al., 2014a; Dhotra et al., 2018) are also comparable with these results that foliar application of trace elements decreased pH of peach juice.

Conclusion

The results obtained from this study revealed that foliar applied micronutrients improved physical and biochemical traits in peaches. It is concluded from the results that application of 0.5% of each boron, zinc, iron and copper showed increased fruit weight, fruit diameter, fruit firmness, yield per tree, juice, TSS, Vitamin C and reduced acidity percentage and juice pH. On the basis of current investigation, use of 0.5% foliar fertilization of copper, boron, iron and zinc can be suggested to peach growers of Soon Valley to improve the yield and fruit attributes of peach fruit cv. Early Grand.

Authors Contributions: F.H. planned and performed the experiment. A.B. wrote the manuscript in addition to supervision of research experiment. M.K.R and A.A. helped in literature citation and edited the manuscript. H.W.J. helped in statistical analysis of data.

Conflict of Interest: The authors declare that they have no conflict of interest.

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