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Effects of zinc on pollen gamete penetration to pistils in some apple crosses assessed by fluorescence microscopy

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Abstract. Zinc is classically the second abundant moveable metal in plants after iron and represented in all enzyme classes. Zinc generally contributes in the biosynthesis of IAA and GA3 phytohormones which play the major role in fertilization and fruit set. Zinc deficiency leads to reduction in leaf and shoot size, photosynthesis and finally decreases fruit set. Foliar Zinc spray was shown to be efficient and fast for improving Zinc deficiency in fruit trees. In this research the effects of Zinc solution by (0, 3000 and 5000 mg. L-1) were studied on pollen penetration to the pistil and ovary in the four apple cultivars crosses which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". Spraying was done on the shoots two weeks before blooming in the spring. Pollen penetration was studied using fluorescent microscopy technique 72 and 120 hours after field pollination. Results revealed that the effects of Zinc, crosses and their interaction were significant on pollen germination on the stigma and tube penetration into the primary, middle and beginning of the ovaries and the highest pollen germination on the stigma (43.5%) was observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zinc 120 hours after pollination and highest pollen tube penetration into the ovary (12/88%) was observed in this cross, respectively. Finally, it was shown that fluorescence microscopy is an accurate technique for nutrition assay in pollination and fruit set. The foliar application of Zinc increased pollen germination and pollen tube growth in all of the crosses.

Keywords. Fruit set, micronutrient, ovary, pollination, pollen tube growth.

INTRODUCTION

Pollen can be used advantageously by breeders, geneticists, physiologists, germplasm supervisors and growers (Dafni and Frimage 2000; Sharafi et al. 2017; Sedgley 1990). In higher plants, pollen grains carry the male gamete on the female part of a flower and play a vital role in breeding program and assist in successful fruit set (Dafni et al. 2012; Hisock and Allen 2008; Sedgley 1990). Generally high crop yield is dependent on viable pollen grains (Dafni et al. 2005). Pollen fertility and viability have dominant prominence in natural and artificial hybridization programs (Sedgley 1990).

Zinc (Zn) is an essential micronutrient in plants and has a vital role in cell division, nucleic acid metabolism, protein synthesis, photosynthesis, carbohydrate metabolism, and phytohormones regulation (Broadley et al. 2007; Chen et al. 2008). Zn directly contributes in the biological synthesis of auxin (IAA) and gibberellin (GA3) which have utmost roles in the plant growth, pollination, fertilization and fruit set in fruit trees (Broadley et al. 2007). Also, Zinc plays a major role as a cofactor in the structure and function of more than 300 enzymes in plants, such as Cu/Zn superoxide (Cu/Zn-SOD), carbonic anhydrase (CA), and sorbitol dehydrogenase (SDH). Zinc toxicity in plants is far less widespread than Zn deficiency (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010). It was reported that about 30% of the agricultural soils in the world show Zn deficiency and Zinc is the most common micronutrient deficient, mostly in high-pH soils (Alloway 2008). Fruit trees which grown in such soils encounter Zn deficiency and show both poor growth and yield quantity and quality. On the other hand, apple (*Malus domestica* L.) commercial cultivars are self- incompatible and therefore, need to be planted along with cross-compatible pollinizer that generates sufficient favorable pollen (Hegedus et al. 2012; Losada and Herrero 2014; Sedgley 1990; Ramirez and Davenpor 2013). On the contrary, apple trees are highly susceptive to Zn deficiency (Alloway 2008). Zn deficiency decreases leaf and shoot size and reduces photosynthetic rates, and thus influences the apple yield and quality (Yan et al. 2010). Zinc deficiency in apple trees is observed as small leaves, late opening of flower and leaf buds, chlorosis between the lateral veins of the leaves, and terminal dieback (Marshner 2011; Macdonal 2000; Sedgley 1990;).

In many fruit trees foliar applications of Zn have been effectively used to promote tree vigor, fruit set, and yield (Wojcik 2007). Some researchers (Golzer and Grant 2006; Qin 1996; Song et al. 2015; Song et al. 2016; Yadav et al. 2013; Zhang et al. 2014) have reported that in the most fruit trees; foliar applications of Zn on mature leaves is unsuccessful and does not provide significant Zn to new leaves produced after spray application or in the following spring. The best time for Zn foliar application is nearly after fruit harvest in the autumn or immediately after pistillate flower senescence followed by two weeks later.

Zhang et al (2013 and 2016) reported that Zinc sulfate spray before bud break increases the activity of carbohydrate metabolic enzymes and regulates endogenous hormone levels in fruits of Fuji apple cultivar. Solar and Stampar (2001) reported that the yield of hazelnut trees was highest in the treatment with 2000 $mg.kg^{-1}$ B + 2000 $mg.L^{-1}$ Zn and lowest in the treatment with 1000 $mg.L^{-1}$ B + 1000 $mg.L^{-1}$ Zn.

Hipps and Davies (2000) reported that foliar application of Zn after blooming could increase the Zn concentration of apple fruit; and spraying Zn on leaves in autumn notably improves the flower Zn content in the coming year. Also, foliar Zn application promotes pollination and cell division.

Moreover, foliar application of Zn was shown to be effective and fast for improving Zn decreasing symptoms in many plants (Sanchez and Righetti 2002). Various studies in palm, citrus and apple showed that foliar application of Zn can significantly increase the Zn concentration, fruit yield and quality (Karimi et al. 2017; Keshavarz et al. 2011; Rodríguez et al. 2005; Neilsen et al. 2004; Neilsen et al. 2005 Khayyat et al. 2007; Zhang et al. 2013). Boron, Iron and Zinc foliar applications have been observed to have a positive effect on chlorophyll contents in B, Fe and Zn deficient plants.

Pollination is one of the most critical stages in the life cycle of a flowering plant, involving a complex series of cell-cell interactions that constitute the pollen-pistil interaction (Dafni et al. 2005; Dafni et al. 2012; Hisock and Allen 2008). In order for fertilization, pollen must first establish molecular compatibility with the stigma and then germinate to produce a pollen tube that penetrates the stigma and grows through the transmitting tissue of the style to locate on the ovule within the ovary (Radonic et al. 2017). Initiation and successful completion of this sequence of events depends upon the stigma and style providing the exact requirements for pollen germination and sustained growth and guidance of the pollen tube through the pistil and ovary. The pollen must therefore be programmed to respond appropriately at every step of this interaction. (Losada and Herrero 2014; Dafni et al. 1998; Nepi and Franchi 2000; Sedgley 1990; Shivanna 2003; Rodriguez-Riano and Dafni 2000). Zn deficiency can have a marked effect on pollination by affecting pollen production, pollen physiology, floral anatomy, and fruit set (Usenik and Stampar 2002).

It has been demonstrated that the first action of stigma is to hook the pollen grains on its surface. For this mechanism receptive stigma must have an adhesive surface. Pollen-stigma interaction is instituted after adhesion of pollen grains on the stigmatic head and multifold incidents occur. The first step is the hydration of the pollen grain and release of wall proteins that bind to receptors on the stigma surface (Radunic et al. 2017; Yellof and Hunt 2005).

Also, florescence microscopy technique accomplished to study pollen tube growth after field and laboratory-controlled pollination and used to identify the

self- and cross-(in) compatibility of cultivars, effective pollination period (EPP), and effects of pollen types on fruit set (Altagic et al. 2012; Kubitscheck 2017). Furthermore, it has not been used for the investigation of the effects of macro and micro nutrients on the pollen germination and tube grow especially in the apple cultivars.

In spite of numerous researches on the response of deficient fruit trees to Zn foliar application, there is no enough information directly appropriate to apple trees. It could be said that because of the five partitions of the apple flower stigma and style; pollen penetration assays in the style have not been used for the screening of nutritional elements on the flower buds (Mularczyk-Oliwa et al. 2017; Sheffield et al. 2005).

The objective of this study was to assess the effects of foliar application of Zn on the apple trees two weeks before bud break in the spring in some crosses by florescence microscopy technique.

MATERIALS AND METHODS

Plant materials, Zn treatments, crosses and pollination

This research was carried out on four apple cultivars which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". All of the trees were 12-year-old on EM126 rootstocks and foliar sprayed by $ZnSO_4$ as the Zn source (0, 3000 and 5000 mg. L^{-1}) two weeks before bud break in the spring. In the beginning volume of each treatment was calculated based on the fruit trees number and then ZnSO4 was dissolved in distilled water and so sprayed with sprayer on the shoots. Spraying was done in the in the morning (7-8 O clock), when the sky was cloudy and the weather moisture was 70%.

Crosses among the cultivars (six crosses) were programmed as ♀Red delicious × Golden delicious♂, ♀Gala× Fuji♂, ♀Red delicious × Fuji♂, ♀Red delicious × Gala♂, ♀Golden delicious × Fuji♂ and ♀Golden delicious × Gala♂. For each cross four repeats in all direction of the trees were considered and, in each repeat, at least 2 branches with 60 – 100 flower buds were labeled in winter. Selected female cultivar's flower buds were bagged at 'Balloon' stage to prevent the entrance of foreign pollens on the closed pistils. Pollens were collected from the male cultivar flower buds and maintained in freezer until usage in the field pollination time. Pollen germination was tested in an *in vitro* medium before field application on the pistils. Selected female cultivar's flowers were pollinated with selected male parent pollen when the pistils were acceptable for pollens and repeated after 24 hours to increase the pollination accuracy.

Florescence microscopy assessment

Based on the apple trees EPP; 72-120 h is enough for the pollen tubes to reach the ovary hence, pollinated flowers were collected at 72 and 120 h after pollination, sliced and fixed in acid FAA solution (5 % v/v Formaldehyde (40%), 90 % v/v Alcohol ethylic 70% and 5 % Acetic acid glacial 96 %). After rinsing with water two to three times, pistils were cleared in 16% NaOH at room temperature for three days. They were then rinsed in water and stained with 0.1% aniline blue in 0.1% K_2HPO_4. Each part of the pistil was placed on a microscope slide with 10% glycerol and squashed under a glass cover slip. The number of pollen tubes and the rate of pollen tube growth in the different parts of the style were measured using fluorescence microscopy (Olympus AX70). In each pistil the number of germinated pollen grains on the stigma, the number of pollen tubes in the upper and middle parts of the style and in the beginning of the ovary were determined by a fluorescent microscope (Fig 1). Pollen germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen on the stigma and expressed as a percentage and normalized by angular transformation. The mean of the pollen tube number was calculated as the average number of pollen tubes in different parts of 10 pistils at least. Due to the five partitions of the apple flower stigma and style; the mean of the 5 parts was evaluated for each of them.

Experimental design and data analysis

The experiment was carried out as a factorial based on completely randomized design with three factors

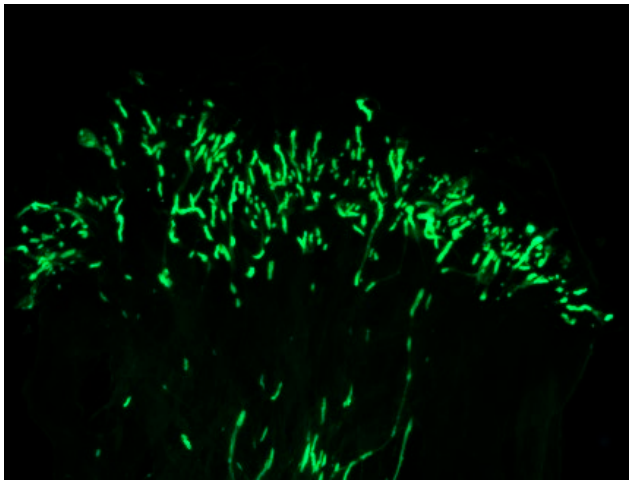

Fig 1. Florescence microscopy photographed from pollens of Red delicious germinated on the Golden delicious stigma and pollen tubes penetrated to the upper part of the style.

including Zn in three levels (0, 3000 and 5000 mg. L^{-1}), time (72 and 120 hrs.' after pollination) and six crosses in five replications (at least ten styles per cross). The data was analyzed using SPSS (24) software. Mean values were analyzed by Duncan's multiple range test.

RESULTS

The analysis of variance showed that the crosses of four cultivars "Golden Delicious", "Red Delicious", "Gala" and "Fuji" had a significant effect on pollen germination percentage on the stigma and pollen tube number which penetrated into the upper and middle parts of the of style and also in the beginning of the ovary at p≤0.01 respectively. Also, Zn concentration and time independently affected the pollen germination percentage on the stigma, the number of pollen tubes which penetrated to the upper and the middle parts of the style and the primary part of the ovary significantly at p≤0.01 (Table 1). The interaction among Zn concentration × crosses was significant on the pollen germination on the stigma, penetration of pollen tubes in the upper and middle parts of the style and so the beginning of the ovaries at p≤0.01 (Table 1), but the interaction of crosses × time was not significant on the studied traits (Table 1).

However, three ways interaction among the time × crosses × Zn concentration was not significant on the pollen germination percentage on the stigma, the number of pollen tubes that penetrated into the middle and the end of the style and the number of pollen tubes in the beginning of the ovary (Table 1).

Based on our findings, the foliar application of Zn on the apple trees enhanced the growth of pollen tubes toward the ovary. In addition, the use of Zn increased the pollen germination on the stigma. About 85 to 90% of pollen germination on the stigma occurred 48 hours after pollination in pollen which was treated by Zn (data not shown). The highest pollen germination percentage on the stigma (43.5%) was observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zn 120 hours after pollination and the highest pollen tube penetration into the ovary (12/88%) was observed in this cross, respectively. These results may be related to the Zn positive effects on the cell division in pollen tube followed by elongation lead to arrival to the ovaries (Fig. 2).

In comparison with 3000 mg. L^{-1} and 5000 mg. L^{-1} concentration of the Zn, pollen germination rate decreased significantly and showed a toxic effect on pollen. In the crosses which both of pollen parents style parents and were treated by 5000 mg. L^{-1} Zn, pollen germination on the stigma was decreased. This could be related to the toxic effect of Zn. The concentration of 3000 mg l^{-1} of Zn has a positive effect on germination percentage and penetration of pollen tubes, and has been effective in maintaining and integrating the membrane of pollen cells. In this research pollen tubes which penetrated into the style and ovary was also affected. It appeared that the use of Zn on both the male and female parents led to increase in the pollen germination on the stigma in the in-*vivo* condition.

However, in trees treated with 5000 mg. L^{-1} Zn, the number of pollen tubes and the swelling of the tip of the tubes were significantly reduced; this was in accordance with some researchers reports regarding the negative effects of Zn on the vital phenomenon in high concentration (Marschner, 2011; Sedgley, 1990; Sharafi et al., 2017; Zhang et al., 2013; Zhang et al., 2016)

Based on the results of Figure. 1, the highest pollen germination percentage was observed in the cross ♀ Red delicious × Gala ♂ with 43.51% and the highest pollen tube penetration in the cross ♀Red delicious × Golden delicious with 19.27% respectively (Fig. 1).

Table 1. Analysis of variance of the effect of time, crosses and Zn on the pollen germination on the stigma and tube penetration to upper and middle part of the style and so beginning of the ovary.

Beginning of ovary	Middle of style	Upper of style	Stigma	Df	Sources of Variation
9.01 ^{ns}	12.10 ^{ns}	**55.07	**149.64	5	Cross
**149.03	**207.02	**239.06	**4120.07	2	Zn concentration
**765.05	**601.04	**974.05	**2245.44	1	Time
7.01 ^{ns}	15.00 ^{ns}	10.01 ^{ns}	55.97^{ns}	5	Cross × time
**32.04	**02.48	96.50**	83.02 **	10	Cross × Zn
*35.07	*52.06	38.07 *	**327.71	2	Zn ×Time
14.01 ^{ns}	38.07 ^{ns}	19.01 ^{ns}	29.01^{ns}	10	Cross × Zn ×Time
9.04	14.01	13.04	27.03 ^{ns}	144	Error
				179	Total

ns= Non Significant, * = Significant at p ≤0.05, ** = Significant at p ≤0.01.

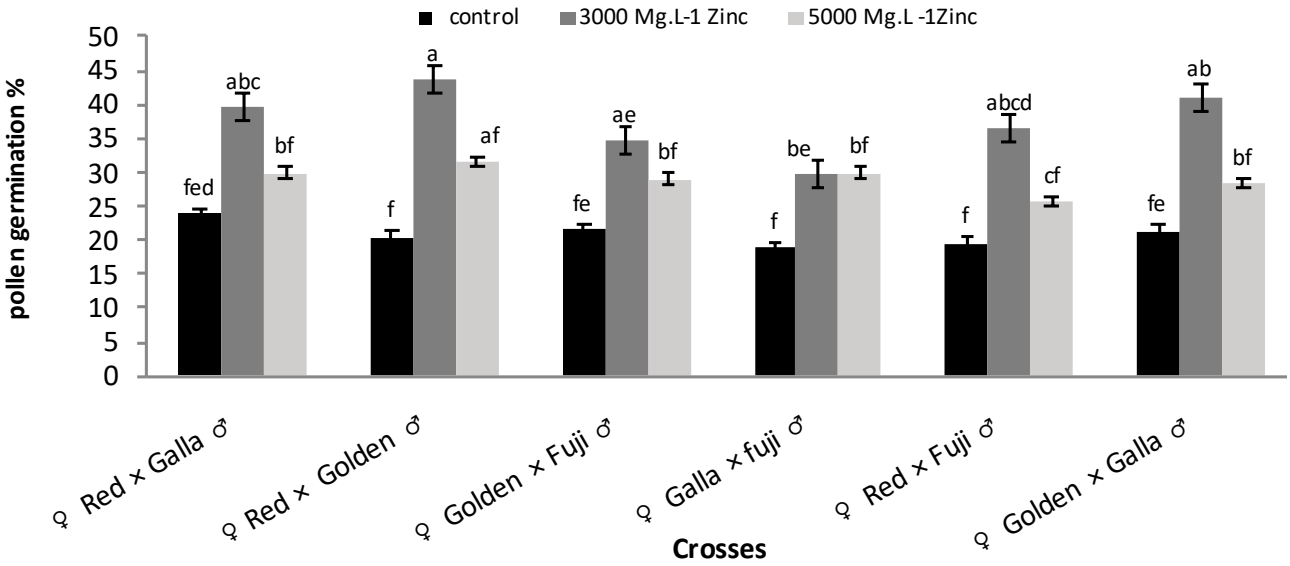

Fig 2. The interaction between Zn concentration and crosses on the pollen germination on the stigma in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

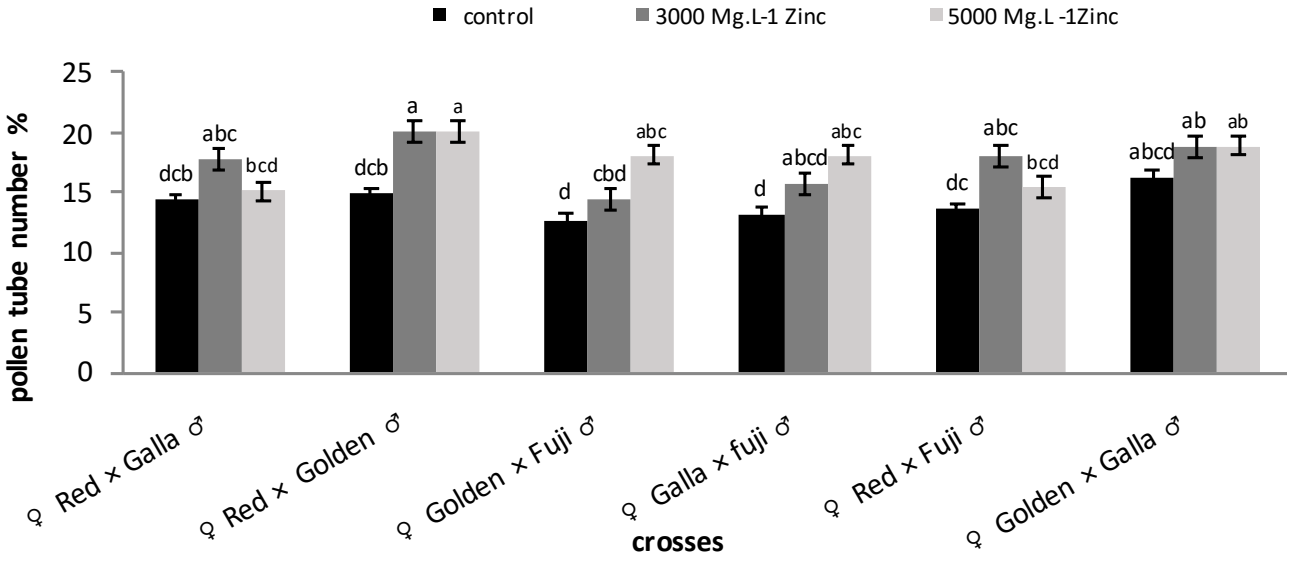

Fig 3. The interactions between Zn concentration and crosses on the pollen tube penetration into the beginning of the style in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

Results were showed that pollens of Golden delicious on the Red delicious lead to increase the ovule fertilization and finally fruit set among all of the studied crosses (data not shown). The results of Figure 3 showed that the interaction of Zn and crosses significantly affected the pollen tube penetration to the beginning of the styles in all of the six crosses. The highest (20.09%) and lowest (15.2%) pollen tube penetration to the beginning of the styles was observed in the cross ♀ Red delicious × Golden delicious ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen tub penetration to the beginning of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses.

It may be connected with the positive effects of Zn on the apple pollen. The positive effects of Zn in high concentration were reported in the most of the plants.

The highest (18.08%) and lowest (13%) pollen tube penetration to the middle part of the styles was observed

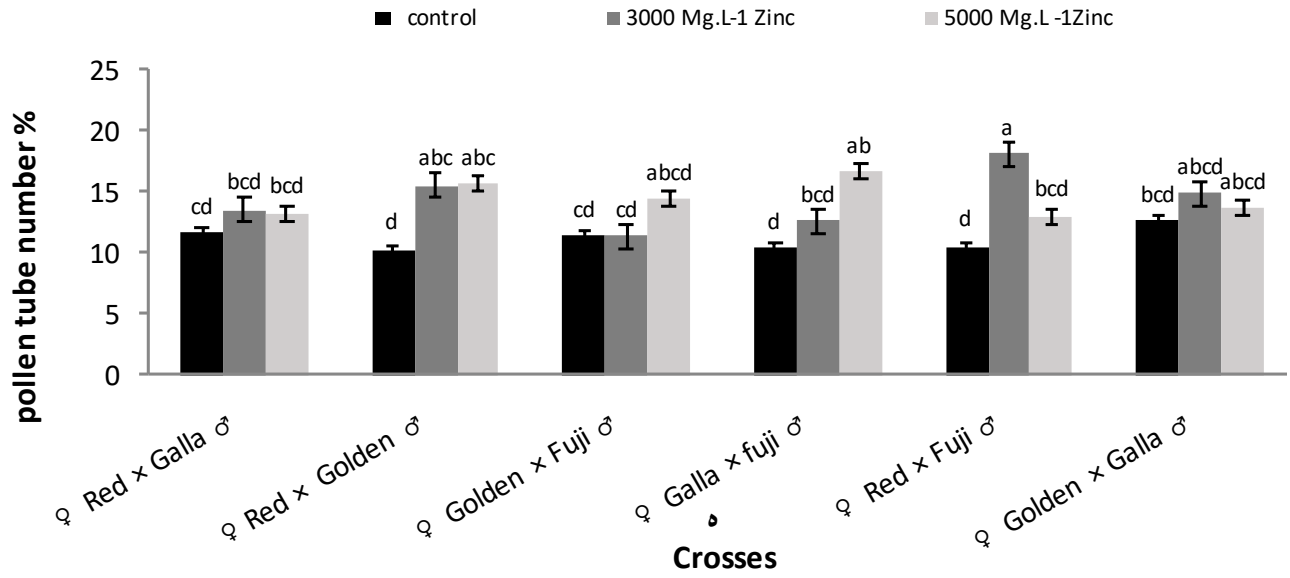

Fig. 4. The interactions between Zn concentration and crosses on the pollen tube penetration into the middle part of the style in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

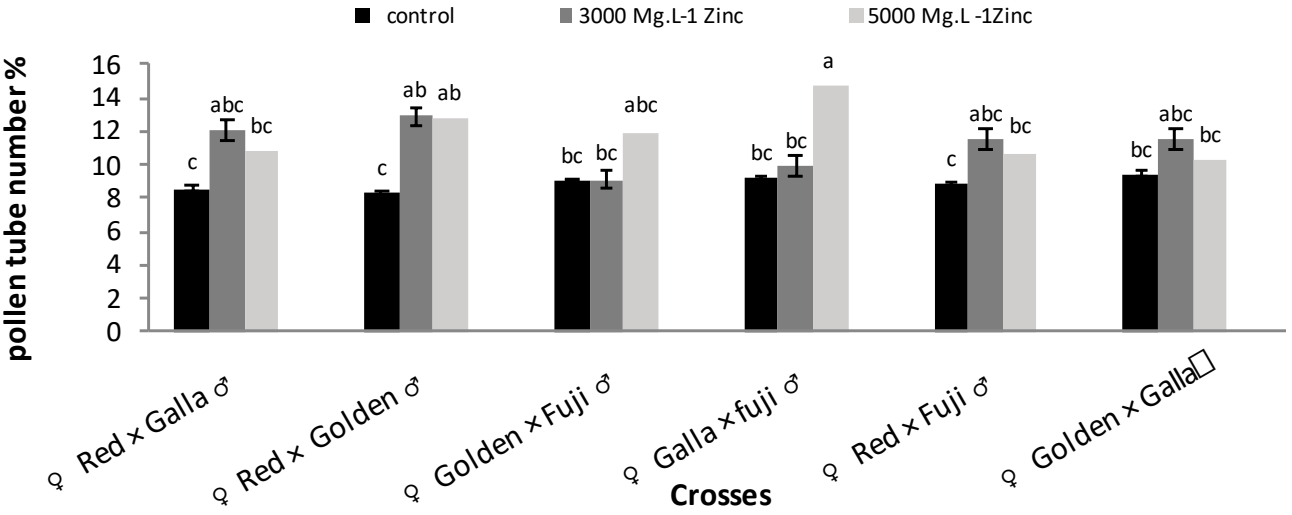

Fig. 5. The interactions between Zn concentration and crosses on the pollen tube penetration into the beginning of the ovary in different crosses. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

in the cross ♀ Red delicious × Fuji ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen tube penetration to the middle part of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 4).

Also, the results of Figure 5 showed that the highest (12.88%) and lowest (10.28%) pollen tube penetration to the middle part of the styles was observed in the cross ♀ Red delicious × Golden delicious ♂ in the 3000 mg. L^{-1} Zn and not treated crosses (control) ♀Golden delicious × Gala ♂ respectively and thus, pollen tub penetration to the middle part of the styles was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses.

Based on the results shown in Figures 6, 7, 8 and 9 the interaction of Zn concentration and the time after pollination significantly affected the pollen germination on the stigma, tube number in the upper and middle parts of the style and also in the beginning of the ovary in all of the crosses. Maximum (34.28%) and minimum

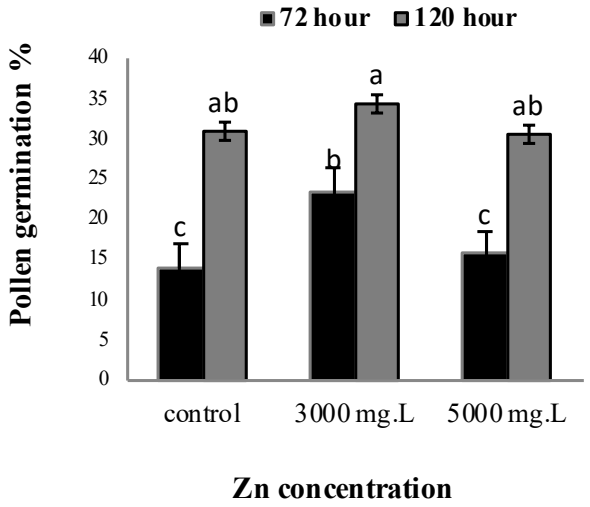

Fig. 6. The interactions between time and Zn concentration on the pollen germination percentage on the stigma. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

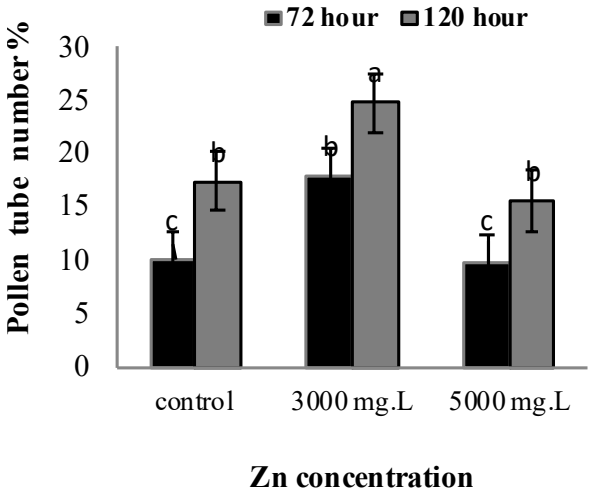

Fig. 7. The interactions between time and Zn concentration on the pollen tube number in the beginning of style. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

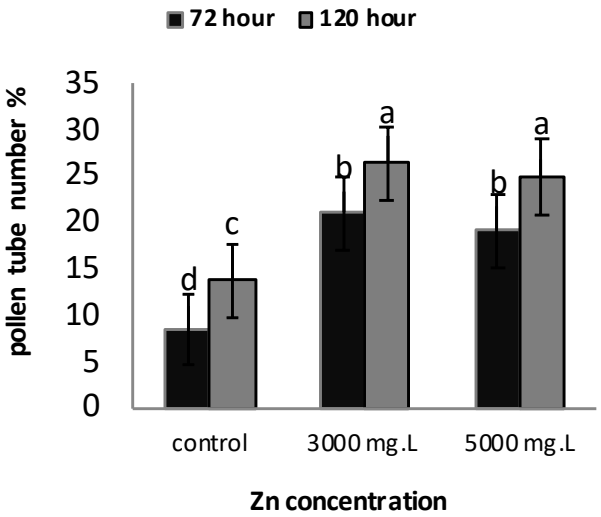

Fig. 8. The interactions between time and Zn concentration on the pollen tube penetration percentage on the middle part of style. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

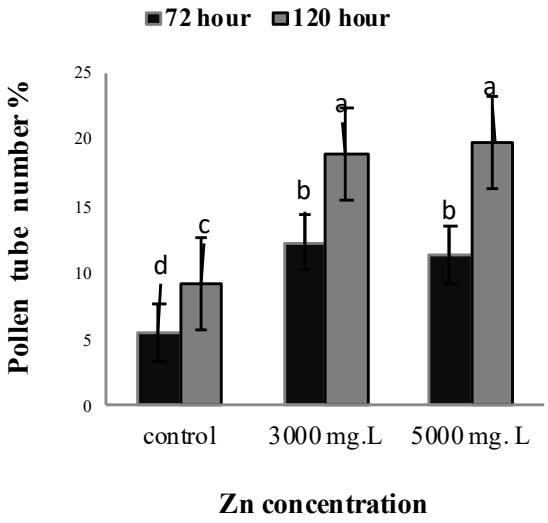

Fig. 9. The interactions between time and Zn concentration on the pollen tube penetration percentage on the beginning of the ovary. Means in each column, followed by similar letter (s) are not significantly different at 1% probability level.

(14.2%) pollen germination on the stigma was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, pollen germination on the stigma was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 6).

Maximum (24.73%) and minimum (9.9%) pollen tube penetration to the beginning of the style was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the beginning of the style was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 7).

Maximum (26.48%) and minimum (8.63%) pollen tube penetration to the middle of the style was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the middle of

Table 2. Pearson correlation coefficients for the effect of the Zn on the pollen germination percentage on the stigma and pollen tube penetration to the upper and middle parts of the style and so the beginning of the ovary.

Correlation	Stigma level	Upper of style	Middle of style	Beginning of ovary
Stigma level	1			
Upper of style	0.59^{ns}	1		
Middle of style	0.45^{ns}	**0.79	1	
Beginning of ovary	**0.57	**0.68	0.85**	1

ns= Non Significant, * = Significant at p ≤0.05, ** = Significant at p ≤0.01

the style was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 8).

However, maximum (19.8%) and minimum (5.47%) pollen tube penetration to the beginning of the ovary was observed in the 3000 mg. L^{-1} Zn and not treated crosses (control) respectively and thus, tube penetration to the beginning of the ovary was decreased in 5000 mg. L^{-1} Zn while it was higher than the controls in all of the crosses (Figure 9).

In all of the crosses the highest pollen tube number in the beginning of the style were observed 120 hr after pollination. This phenomenon demonstrated that pollen germination and tube growth were increased followed by the time which may be related to the nutrition case in the style. However, there was a significant difference between the interaction of time and Zn concentration on the pollen tube number in the middle part of the style and in the beginning of the ovary respectively (Figures 8 and 9). Maximum pollen tube numbers in the middle part of the style and in the beginning of the ovary were observed 120 hr after pollination.

Correllation between Zn and pollen germination and penetration to different parts of the style and ovary is showen in Table 2. There was a significant positive correlation between Zn concentrations and germination percentage of pollen on the stigma and the tube number which penetrated to the upper and middle parts of the style and also to the beginning of the ovary respectively (Table 2).The correlation between pollen tubes penetration to the upper (./68) and middle of the style (./85) and the beginning of the ovaries was positive (Table 2).

DISCUSSION

In this research the pollen tube penetration percentage into the styles and ovaries increased by Zn application two weeks before bud break especially in 3000 mg. L^{-1}.

In this study a large number of pollen grains germinated on the stigma exudate and formed callose, indicating good growth of pollen tubes by Zn treatment in apple crosses. However, few of the pollen tubes were observed to penetrate to the style. The average number of pollen tubes was only slightly higher in some crosses. In addition, 120 h after pollination, the average pollen tube length and growth rate were slightly higher in all of the crosses. Previous studies have suggested that self-pollen tubes can grow slower or have higher rates of abrasion than cross-pollen tubes (Golzer and Grant 2006; Qin 1996; Song et al. 2015; Song et al. 2016; Yadav et al. 2013; Zhang et al. 2014; Sedgley 1990).

In accordance with our results Pandey et al, (2006); observed that Zinc is critically required for pollen function and fertilization in lentil. Also, Neilsen et al (2005) observsd that postbloom humic-and fulvic-based zinc sprays can improve apple zinc nutrition also Neilsen et al (2004) reported positive effects of zinc and boron in fertigated high density apple orchards.

Keshavarz et al (2011); reported that foliar application of Zinc and Boron improves walnut vegetative and reproductive growth. They demonstrated the first report of the benefit of foliar B and Zn on pollen germination in walnut trees. There was clear positive effect of B and Zn applied as individual foliar applications and a synergistic effect when applied in combination on walnut yield and quality parameters.

Fei et al (2016); studied enzyme activities, and expression of Zn/Iron-regulated transporter-like protein (ZIP) family genes in the mild, moderate, and severe Zn deficiency in *(Citrus sinensis* L. Osbeck). They reported that the expression of the ZIP family genes, ZIP1, ZIP3, and ZIP4, was promoted by Zn deficiencies. However, chlorophyll contents and net photosynthetic rate decreased with reduction in Zn contents reduction. Also, comparison of severe Zn-deficient and normal leaves revealed increased significant activities of peroxidase (POD) and catalase (CAT, but significantly reduced Zn-containing enzymes such as Cu/Zn superoxide dismutase (Cu/Zn-SOD).

In plants, about half of the ZIP genes could be induced under Zn deficiency, while ZIP1-4 genes seem to be involved in plant Zn transport. Zinc/iron-regulated transporter-like proteins (ZIPs) play a key role for Zn uptake in plants and currently, over 100 ZIP family members have been recognized in diverse plant species (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Furthermore, Zn is involved in various cellular processes, including photosynthesis, nucleic acid and lipid metabolism, protein synthesis, detoxification of reactive oxygen species (ROS), and membrane stability (Broadley et al. 2007). The main role of Zn is involvement in important procedures of cell division and gene expression associated with nucleic acid and protein metabolism. Zinc play an essential role in processes leading to DNA synthesis, and DNA polymerase contains firmly bound zinc. These roles help to explain the nature of some of the symptoms characteristic of Zn deficiency in fruit trees, for example, 'rosetting' and shoot tip dieback (Broadley et al. 2007; Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Zinc deficiency leads to malfunctioning of some enzymes, for example, alkaline phosphatase carbonic anhydrase. It could lead to decreased starch formation, accumulation of amino acids, and synthesis of Auxin via impaired tryptophan synthesis. It decreases carbonate dehydrogenase activity which has affected the balance of carbon dioxide and carbon acid and thus has indirectly influenced the rate of photosynthesis. Zinc deficiency has also been found to promote formation of abscisic acid causing premature abscission of leaves and flower buds (Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Hence, Zinc is important in DNA and RNA metabolism and protein synthesis and thus, maintains the structural integrity of biomembranes. More than 1,200 protein molecules (Zn metalloprotein) have been identified including a large number of 'zinc-finger'- containing proteins and transcription factors, oxidoreductases and hydrolytic enzymes such as metalloproteases. Furthermore, Zn is a mechanical factor of ribosomes and thus vital for their structural integrity. It plays a major role in carbohydrate metabolism by regulating key enzymes, fructose 1,6-bisphosphatase and aldolase. Synthesis of auxin, and indole acetic acid, is particularly impaired under Zn deficiency (Broadley et al. 2007; Andreini and Bertini 2012; Moghadam et al. 2013; Nosarszewski et al. 2004; Weinthal et al. 2010).

Finally by comparing the effects of the contols with 3000 and 5000 mg. L^{-1} Zn in Figures 1, 2, 3, 4, 5, 6 ,7 and 8 it was demomonstrated that pollen germination and tube penetration to the style and ovary increased until 3000 mg. L^{-1} Zn but, in 5000 mg. L^{-1} mentioned traits were decreased respectively. This could be interpreted that by increasing the Zn concentration it may show toxic effects of pollen tube cell growth. However, in this study, 5000 mg. L^{-1} did not show toxic effect because it showed positive effects on pollen germination and tube penetration to the style and ovary in comparison with the crosses which were not treated with Zn.

CONCLUSION

"It was concluded that fluorescence microscopy technique is a very accurate method for assay of nutrient effects on pollen germination and tube penetration to the pistils in fruit trees in compared with fruit set percentage studies. In this research the mentioned method was used for assay of Zn effects in four apple cultivars crosses which included "Golden Delicious", "Red Delicious", "Gala" and "Fuji". Results showed that the highest pollen germination percentage on the stigma (43.5%) and penetration of pollen tube to the ovary (12/88%) were observed in the cross (♀Golden Delicious × Gala♂), in 3000 mg. L^{-1} of Zn 120 hours after pollination respectively. However, the foliar application of Zn by 1000 mg. L-1 on apple cultivars two weeks before bud break positively affects pollen germination and tube penetration to the ovary in all of the crosses".

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