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REGULAR ARTICLE

# Effect of boron and zinc application on *HXK1* and *MAKR6* gene expression in strawberry

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## ABSTRACT

Boron (B) and zinc (Zn) are important microelements for normal plant activity; their effect have long been studied in numerous agronomical and molecular researches, whereas the interaction of B-Zn remains poorly understood at genomic and transcriptomic levels. Strawberry (*Fragaria* x *ananassa*) plants were analyzed after single B (80 g/Hl of boron ethanolamine) or Zn (40 g/Hl of Zn-EDTA), and combined B + Zn (80 g/Hl of boron ethanolamine + 40 g/Hl of Zn-EDTA) foliar applications. Leaves, white and red fruits of two genotypes (Candonga and PZ600F13P2 breeding line) were studied. The microelement effects were observed in two conditions: short-term (1 day after treatment) and long-term (20 days after treatment). The differential transcriptomic profiles were analyzed applying RAPD-PCR method. Two genes, *HXK1* and *MAKR6* have been recognized in differently expressed sequenced amplicons and their expression level was measured using quantitative Real-Time PCR. Fertilizing had an influence on studied genes, their expression differed in relation to the genotype, time course and kind of treatment. Reaction of *HXK1* and *MAKR6* varied regarding the singular microelement treatments in front of Control (without any treatment) as well as relatively to the combined fertilizing, even if often the zinc effect was "masked" by boron influence in B + Zn treatment. The present study confirmed an initial hypothesis of a significant effect of B and Z in strawberry fruit development. The obtained results, even if they increase the knowledge about the B and Zn effects, could be considered as a first step to detail this physiological mechanism in strawberry fruit development.

**Keywords:** B x Zn interaction; *Fragaria x ananassa; Hexokinase 1; Kinase regulator*

## INTRODUCTION

The strawberry (*Fragaria* x *ananassa* Duch.) is the most popular type of berry fruits and is widely appreciated for its flavour and fragrance, likewise for the health benefits attained by its consuming. The strawberry growth is one of the important commercial activities in South of Italy. Observation of local strawberry producers during the last decades underlined a positive effect of boron (B) and zinc (Zn) application at beginning and middle of productive season. Combined boron plus zinc treatment provoked a 20% increase of fruit size, a positive effect on yield and fruit texture (data not shown).

The significance of boron, zinc and phosphorus and the influence of their interaction on strawberry yield components, as fruit count per inflorescence and individual fruit weight, were also noted by May and Pritts (1993). Albregts and Howard (1978) have concluded that boron and zinc are two of nine important microelements for strawberry growing. They are presented more in fruit respect to leaves, but also in other kinds of tissues.

The best known site of boron actions in plants is connected to the structural role in cell walls and membranes (Cakmak and Volker, 1997; Blevins and Lukaszewski, 1998; Brown et al., 2002). Several researchers have recognized the role of boron in pollen germination and fruit set (Nestby et al., 2005), as well as vitamin C accumulation in a final stages of strawberry fruit ripening (Cheng, 1994). Shkol'nik (1974) has noted an important role of boron in prevention of phenolic accumulation which inhibits auxin activity. He also has attributed to boron an important role in carbohydrate and RNA metabolism. On the other hand, Kessler (1961) has demonstrated an important role of zinc in regulation of protein synthesis in plants. It has been specified that Zn could be involved as a metal component of enzymes or as a functional, structural or regulatory cofactor (Abdollahi

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et al., 2011). Abd and Mona (2013) have reported an evidence of direct participation of zinc in biosynthesis of growth substances such as auxin.

Previously, for other crops the interaction of boron and zinc has been described ambiguously. Sinha et al. (2000) noted how the high level (toxicity) of both microelements in mustard (*Brassica campestris* L.) induces a negative effect on biomass production, economic yield, and decreases the carbonic anhydrase activity. On the other hand, Hosseini et al. (2007) in presence of not excessive microelement contribution reported the synergic effects of B and Zn on growth of corn (*Zea mays* L.) and antagonistic interaction on nutrient concentration. Rajaie et al. (2009) described a reducing of B toxicity effect applying Zn-based micronutrient in lemon (*Citrus Aurantifolia* L.) seedlings. The same observation was made by Nasim et al. (2015) on two barley (*Hordium vulgare* L.) cultivars. For potato plants, Puzina (2004) in her study has presented the effects of microelement treatments on phytohormones. She reported that boric acid mainly stimulated auxin synthesis while zinc sulphate for the most part increased cytokinin and tryptophan content. In potato leaves, the combined treatment (zinc sulphate and boric acid together) increased both, the auxin and cytokinins content (Puzina, 2004). However, the effect of B and Zn application on strawberry fruit development remains poorly investigated.

To better understand the impact of microelements in plant development a large number of agronomic studies were carried out in different conditions: excess microelement level, or toxicity (Sinha et al., 2000; Rajaie et al., 2009; Nasim et al., 2015), fertilizing applications (Hu and Brown, 1994; Matoh, 2001; Abdollahi et al., 2011; Esringü et al. 2011), or deficiency (Dell and Huang, 1997). Unfortunately, often derived results are closely related to growth conditions, soil quality, agronomic practices or climate characteristics, and, consequently, are not reproducible for another production system. Conversely, structural and functional genetic investigations offer the possibility to characterize the microelement effects on physiological pathways reducing the interaction with environmental influences. It should be noted that because of complexity of plant genomes, fundamental molecular researches are not more rapid and much more expensive compared to agronomic studies and often genome-dependent.

Genetic investigation on strawberry (*Fragaria x ananassa*) could be defined as not easy considering its octoploid genomic structure. In fact, it is derived by natural breeding between two wild octoploid strawberry species (*Fragaria virginiana* and *Fragaria chiloensis*). Taking into account that several studies have demonstrated the high synteny level between *Fragaria vesca* and *Fragaria x ananassa*, genomic studies, in *Fragaria* spp, normally are planned at lowest ploidy level. Furthermore the genome of *Fragaria vesca* has been sequenced and it could be considered as model not only in *Fragaria* spp. but for all the species included in the *Rosaceae* family (Shulaev et al., 2011). At the other hand, considering the numerous copies of the same gene that could be present in a polyploid genome as this of *Fragaria x ananassa*, the difficulty could manifest the choice of genes of reference (or constitutive) which expression remains stable in different stages and tissues. These genes are used as calibrators for the expression of genes of interest. Thus, for strawberry the *FaACTIN* (*actin-97-like*), *FaCHP1* (*protein BPS1, chloroplastic-like*) and *FaGAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) genes have been reported as constitutive in previous studies (Amil-Ruiz et al., 2013; Clancy et al., 2013; Galli et al., 2015).

From botanical point of view the strawberry berry is not a true fruit because it derived by development of the floral receptacle. The true strawberry fruits are the achenes which are located on the external surface of the berry (Coombe, 1976).

In Metaponto area (Basilicata, South of Italy) in spring season the time required for the berry development, from bloom to ripeness, occurs in approximately 25-32 days. The main phases of this process are: cell division, cell expansion, maturation and ripening. Knee et al. (1977) reported the duration of cell division for about 7 days after petal fall, when the cell expansion starts in petal fall phase and follows a logarithmic increase. During the first stage of the berry development, the fruit is characterized by a rapid growth, then numerous metabolites (such as sucrose) start their translocation to the receptacle and fruit changes the colour gradually from green to white (18 - 20 days after pollination). In the last 7-10 ripening days the berry changes the colour gradually from white to red.

Regarding the primary metabolism, the major soluble sugars in strawberry are glucose, fructose, and sucrose (Hancock, 2000). Forney and Breen (1986) have reported that the hexose sugars (glucose and fructose) level remains practically constant during all the period of fruit development process (investigation in whole fruit). Interestingly, Fait and collaborators (2008) measuring different metabolites accumulation in various developmental stages of strawberry fruit (from small green to red ripe), separately for achene and receptacle tissues, have noted that the content of majority of measured metabolites is tissue- and stage- dependent. Sucrose, glucose and fructose increased in receptacle, and in the same time, decreased by 50% their initial levels in the achenes.

In plant only two sugar-phosphorylating enzyme families, hexokinases (HXKs) and fruktokinases (FRKs), are able to transform hexose sugars and permit their utilization (Granot et al., 2013). Interestingly as only FRKs are specific to fructose whereas HXKs catalyze the transformation of different sugars and are subdivided in two groups: group A and group B. The group A is characterized by having of chloroplast transit peptide, whereas the group B has been reported to be associated with membranes activities (Olsson et al, 2003). The roles of HXKs could be summarized in participation to the juvenile-to-adult transition, to the uptake of minerals, mainly phosphates, to the short-term (reversible) sugar-sensing regulation (Kelly et al., 2013) and to the long-term growth-inhibiting effect in case of constitutive expression of *AtHXK1* under the *35S* promoter in *Arabidopsis* and tomato plants (Granot et al., 2013). However, recent studies with citrus, tomato and *Arabidopsis* have shown that HXK is involved in guard cells control and provokes stomatal closure independent of its osmotic effect (Kelly et al., 2013; Lugassi et al., 2015). It is not yet known if in strawberry the *MAKRs* (*membrane associated kinase regulators*) could be directly involved in the same metabolic pathway. In *Arabidopsis thaliana* the members of the *MAKR* family are supposedly involved in abscisic acid-activated signalling pathway, response to brassinosteroid and brassinosteroid mediated signalling pathway, as well as lipid metabolic process (Schmid et al., 2005; Jaillais et al., 2011; Jiang et al., 2015).

This study is one of first efforts to understand, at molecular level, the influences that boron and zinc and their combination could produce on metabolic pathways involved in ripening process of strawberry fruits (*Fragaria x ananassa*).

## MATERIALS AND METHODS

### Plant material and treatment design

Strawberry plants were grown in Southern Italy (Metaponto area, Basilicata). Daily average temperatures during the strawberry cultivation were included between 12 and 24 °C with approximately a 11.5-12-h photoperiod (spring time). The applied plasticulture method provided the strawberry cultivation within the narrow beds (25 cm of height), covered with a black plastic mulch and included two rows of plants with one drip line running between them. Plastic tunnels, or unheated greenhouses, with removable transparent plastic covering (5 m height, 35 m length, included 6 narrow beds) were governed with aim to avoid critical temperatures.

Two genotypes, Candonga and PZ600F13P2 (breeding line) of strawberry (*Fragaria* x *ananassa* Duch.) were analyzed in this study (see Fig. 1). The Candonga cultivar was chosen because of relevant economic interest and its diffusion in Metaponto area, and the breeding line PZ600F13P2 for its agronomic characteristics. Those two genotypes were well adapted to the Metaponto area, but they are a lot divergent for several physiological traits. The blooming and the ripening processes are more fast for Candonga variety, whereas the PZ600F13P2 completes blooming about 5 days later and fruit ripening about 7-8 days later respect to Candonga. A fruit number, conversely, higher in breeding line, due to its stronger flower ramification (branching up to 3d-4$^{th}$ order, when Candonga is characterized by 1$^{st}$-2d order) with consecutive bigger (a mean Candonga fruit is about 1.5 g more heavy) and sweeter (a mean breeding line fruit have less 0.5 Brix degrees) fruits for Candonga cultivar.

No microelement deficiency was noted in soil at moment of planting. The fertilization regime for all the plants included NPK complex applied through fert-irrigation system in proportion of 90/80/140 units/ha/year with seasonal adding (in period from the end of February until April) of 2 kg/week of Ca-EDTA. Furthermore, an organic foliar fertilization (500 g/Hl of 8%N, for total concentration of 3 kg/ha) was applied twice with distance of one week before blooming (last week of January – first week of February). The irrigation system included a drop-by-drop module (capacity 4 l/hour) with annual water consumption of about 2300 m$^3$/ha. The irrigation regime was strongly dependent on climatic conditions.

The B and Zn application was carried out in March 2014 (first half of productive season) in addition to normal soil fertilization and irrigation, common for all the plants. The boron and zinc in the form of boron ethanolamine and zinc-EDTA were applied once through foliar spray according to following treatments: C = Control (distilled water spray), B = Boron (mix of 80g of boron ethanolamine in 100 l of water for a final concentration of 7.6263 mM), Zn = Zinc (mix of 40g of Zn-EDTA in 100 l of water for a final concentration 1.1313 mM), B+Zn = combined application (mix 80g of boron ethanolamine (7.63 mM) and 40g of Zn-EDTA (1.13 mM) in 100 l of water). A total of about 120 plants in randomized block design were involved. The mix of young and mature leaves, white and red fruits (see "W" and "R" in Fig. 1a respectively) were collected 1 day after the treatment (T1) and 20 days after the treatment (T2).

### Characterization of transcriptional activity and individuation of target genes

Total RNA was extracted from the pull of 6 fruits or leaves for each treatment and time of sampling in two replicates using the CTAB-based method as described (Gambino et al., 2008). The DNase treatment and synthesis of the complementary DNA (cDNA) from 1 μg RNA were

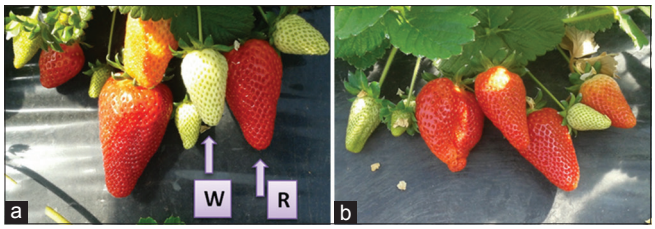


**Fig 1.** Candonga (a) and PZ600F13P2 breeding line (b) strawberry genotypes. Maturation stage and visible aspect for white (W) and red (R) fruits at moment of sampling.

**Table 1: RAPD primers used to detect variations in transcriptional profiles**

| Primer | Sequence | N amplified fragments |
|---|---|---|
| MG8 | GGGGGCCTCA | 4 |
| MG10 | CCGCCCCACT | 5 |
| MG11 | AGGAGCTGCC | 8 |
| MG92 | CCGCTGGGAG | 3 |
| MG109 | GACGGAGGTC | 3 |
| MG111 | GGGCGAGTGC | 2 |
| MG115 | CGGACCGCGT | 6 |
| MG116 | GAGAACTGGC | 3 |
| MG124 | AGACGTACTC | 7 |
| Total | | 41 |

carried out using Fisher BioReagents™ Optizyme™ Recombinant DNase I (rDNase I) and Bio-Rad iScript ™ cDNA Synthesis Kit respectively and according to their protocols.

Transcriptional activity variations supposedly induced by B-Zn treatment were detected in cDNA of leaves using Applied Biosystems AmpliTaq Gold ® DNA Polymerase in RAPD-PCR reaction (Random Amplification of Polymorphic DNA). Primers used for this experiment are presented in Table 1. All amplified fragments were loaded in 1.2% agarose gel with Ethidium bromide; the image analysis (Kodak MI v. 4.0.4) was also carried out. Polymorphic and differential density fragments were isolated from the gel and re-amplified to verify their length. The fragments were also sequenced by external service (Gatc Biotech, Germany) in order to identify putative target genes related to the differential expression. Sequence analysis was performed using NCBI services (nucleotide BLAST by selecting *Fragaria* taxid). Only highly similar sequences (more than 200 alignment score NCBI) were considered to reveal the genes of interest. Specific primers (Table 2) for two genes of interest were designed using free online software: Primer 3 (v. 0.4.0) from Whitehead Institute for Biomedical Research, Primer-Blast from NCBI and NetPrimer from Premierbiosoft services. The specificity of primers was checked by evaluation of melting curve and presence of single amplicon as PCR reaction products. Quantitative real time PCR (qRT-PCR) was performed for all the tissues in two technical replicates using Bio-Rad iTaq ™ Universal SYBR ® Green Supermix kit.

To define the better housekeeping genes, three genes (*FaACTIN*, *FaCHP1* and *FaGAPDH*) previously described respectively by Clancy et al. (2013), Amil-Ruiz et al. (2013) and Galli et al. (2015) were tested. The ratio between all of them was calculated. Only the two reference genes with better correlation were chosen and subsequently used (*FaACTIN* and *FaCHP1*). All the data was elaborated using LinRegPCR, version 2015.3, free software based on Ruijter et al. (2009, 2014) and Tuomi et al. (2010). In qRT-PCR the replications of RNA extraction were treated as technical, for final analysis included 4 technical replications. The statistical and descriptive analysis was performed by PRISME ® version 5, 2007. Non-treated tissue for each time of sampling (control) was chosen as calibrator, non-cDNA template was used as negative control.

## RESULTS AND DISCUSSION

### Transcriptional activity and differently expressed genes

The boron and zinc application visibly didn't affect strawberry growth or functionality. All plants (treated and control) were in good shape and no difference was noted during a simple phenotypic analysis. On the other hand, RAPD-PCR method applied at molecular level in leave tissue highlighted clear changes in expression related to treatment, genotype, as well as the sampling time. A total of 41 different amplified fragments were observed using 9 non specific primers.

RAPD-PCR products for one of the most characterizing primers MG11 is showed in Fig. 2. As follows from the figure, two kinds of variability could be observed: the same fragment (considering its length) repeated for all samples, but with some changes in concentration (brightness of fragment), or singular amplicon which presence/absence changed with time course or depending on treatment. The first isolated fragment is indicated by an arrow. The length of corresponding sequence was of 524 nucleotides, 100% of cover and 99% of identity matched to predicted *Fragaria vesca Hexokinase-1* gene (*HXK1*) placed in NCBI under LOC101297661 in RefSeq *Fragaria vesca* Annotation Release 101 database (Shulaev et al., 2011). According to the KEGG (Kyoto Encyclopedia of Genes and Genomes), this gene has been annotated to be involved in a large number of physiological processes and pathways, such as amino sugar and nucleotide sugar metabolism, biosynthesis of antibiotic and secondary metabolites, galactose, fructose and mannose metabolism, glycolysis and other functions

**Table 2: Specific primers for quantitative real time PCR**

| Primer | Sequence forward | Sequence reverse | Amplified fragment length |
|---|---|---|---|
| *FaHXK1* | TGCGGCTTATGTAGAACGTG | TGCTCTCCAGGGTTCAAACT | 168 |
| *FaMAKR6* | AGCAGCCTCTCTCCATTGAA | TGAAATCCTGCTGGGAAATC | 186 |

(Kawai et al., 2005). Xie et al., (2007) has reported that in strawberry the *HXK1* actively participates in sugar accumulation process during the fruit ripening.

The second isolated fragment was observed in RAPD-PCR amplification with leaves 20 days after the treatment (PZ600F13P2 breeding line) using MG109 (Fig. 3). In this case the chosen amplicon corresponded to the combined B and Zn application (shown by an arrow) and was not expressed in the control and boron treated plants. In case of zinc fertilized leaves the same length of amplified fragment was found with much lower concentration (according to the brightness). The high score (confident) sequence was available for a 607 nucleotides chain showing a similarity in 54% of cover and 86% of identity with predicted *membrane associated kinase regulator 6* (*MAKR6*), annotated in *Fragaria vesca* genome under LOC101301825 (Shulaev et al., 2011). The homology with *MAKR* genes of other species of *Rosaceae* family, such as *Prunus mume*, *Prunus persica*, *Malus x domestica* and *Pyrus x bretschneideri,* was observed (NCBI database). In *Arabidopsis thaliana* the *MAKR6* gene has been annotated (Tabata et al., 2000). According to Schmid et al., (2005) *MAKR* family in *Arabidopsis* is composed by 6 different genes and their biological function is supposed to be related to the putative kinase interacting motifs and membrane localization signals (Heyndrickx and Vandepoele, 2012). Similarly to the *HXK1* gene, the *MAKR6* has been found to be expressed in guard cells of *Arabidopsis,* but in this case related to the biotic stress (Obulareddy et al., 2013). In strawberry the information about *MAKR6* expression has not been found.

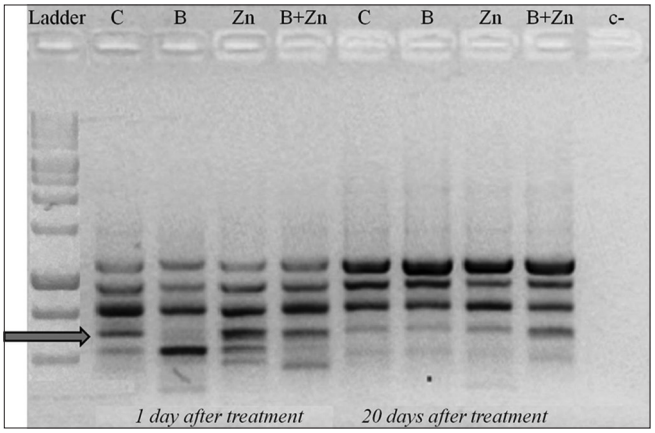


**Fig 2.** RAPD-PCR products with MG11 primer at PZ600F13P2 strawberry genotype (leaves) treated with boron (B), zinc (Zn), combined boron and zinc (B+Zn) and without treatment (C). Isolated fragment N1 is indicated by an arrow.

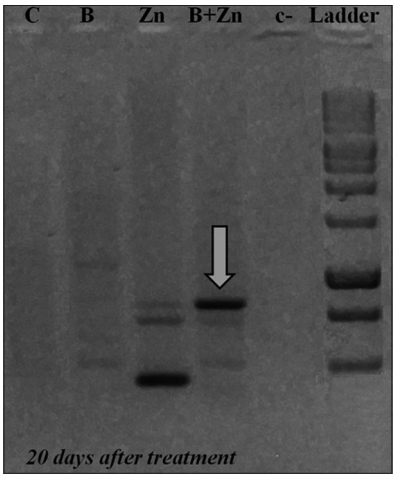


**Fig 3.** RAPD-PCR products with MG109 primer at PZ600F13P2 strawberry genotype (leaves) treated with boron (B), zinc (Zn), combined boron and zinc (B+Zn) and without treatment (C) observed 20 days after treatment. Isolated fragment N2 is indicated by an arrow.

### *Hexokinase 1 (HXK1)* and *Membrane associated kinase regulator 6 (MAKR6)* relative expression

The relative expression for predicted *hexokinase 1* (*HXK1*) and *membrane associated kinase regulator 6* (*MAKR6*) is reported in Fig.s 4 and 5. The shown data includes 2 times (1 and 20 days after treatment) and 3 kinds of tissue: red fruits, white fruits and leaves. The difference could be observed depending on treatment, genotype and time course for both tested genes. All the values have been normalized to the corresponding control, which was calibrated to 1.

A curious observation could be made for "one day after treatment" in Candonga variety (Fig. 4a): the *HXK1* expression in leaves is down regulated by 3 treatments (boron, zinc and combined application). At the same time red and white fruits shown a completely opposite reaction considering the treatment applied: in white fruits the gene of interest is highly overexpressed under boron and combined fertilizing, and much less overexpressed under only zinc application, conversely, in red fruits the strong positive influence seemed to be induced only by zinc application, and weaker over expression depending on boron and boron plus zinc treatments. At T2 (20 days after treatment) the boron influence seemed to become weaker for white fruits and leafs, and oppositely, provoked a high overexpression level in the red fruits. At the other hand, no overexpression was induced by zinc, which effect in B+Zn treatment (red fruits) seemed to be masked by boron. Interestingly, in PZ600F13P2 genotype (Fig. 4b) the

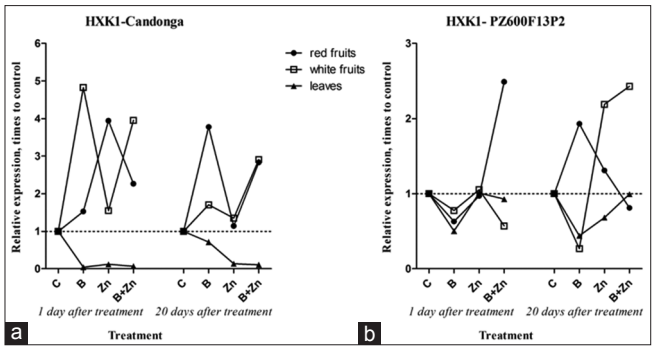


**Fig 4.** Relative expression of *HXK1* under B and Zn treatment in Candonga and PZ600F13P2 genotypes. Effect of boron (B), zinc (Zn) and boron + zinc (B+Zn) applications on *hexokinase 1* (*HXK1*) expression tested in distance of 1 and 20 days after treatment on leaves, white and red fruits of Candonga (a) and PZ600F13P2 (b) strawberry genotypes. All the data are normalized to each control (C) which expression is calibrated to 1.

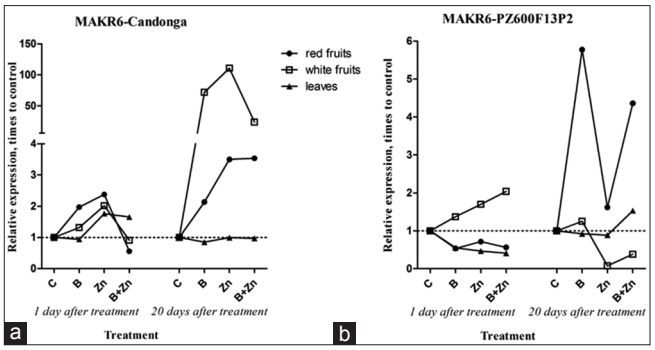


**Fig 5.** Relative expression of *MAKR6* under B and Zn treatment in Candonga and PZ600F13P2 genotypes. Effect of boron (B), zinc (Zn) and boron + zinc (B+Zn) applications on predicted *membrane associated kinase regulator 6* (*MAKR6*) expression tested in distance of 1 and 20 days after treatment on leaves, white and red fruits of Candonga (a) and PZ600F13P2 (b) strawberry genotypes. All the data are normalized to each control (C) which expression is calibrated to 1.

opposition between the *HXK1* reaction of red and white fruits is fixed 20 days after the treatment, whereas 1 day after microelement application *hexokinase 1* expression was practically identical for all the tissues treated with single elements: down-regulation for B, without any effect for Zn. Regarding the combined microelements application – all the tissues have demonstrated the different comportments: red fruits highly overexpressed, leaves without changing and white fruits down-regulated. At T2 the singular B and Zn influence seemed to became stronger for white fruits and leaves, whereas the red fruits changed completely the reaction. B+Zn application provoked the overexpression just in white fruits.

This finding could be explained by considering the time of plant reaction on microelement application (Knee et al., 1977; May and Pritts, 1990). Evidently, in case of *HXK1* expression there was also the strong effect of genotype. Thus, in Candonga cultivar the microelement effect seemed to be more rapid and probably directed by different mechanisms respect to the PZ600F13P2 breeding line. Thus, the boron effect one day after the treatment have induced a strong and completely different reaction in various tissues of Candonga, whereas for the breeding line red, white fruits and leaves seemed to be coherent. Similarly, the zinc application provoked highly different reaction in Candonga and had no effect in PZ600F13P2. Apparently, the differences observed by comparative analysis suggest the presence of different kind of physiological regulation mechanisms depending on genotype (Padula et al., 2012). At the same time, it is evident that in white and red stages the development of fruit is governed by distinct physiological processes (Perkins-Veazie, 1995).

Regarding the *MAKR6* expression (Fig. 5), an important difference between T1 and T2 could be noted in both genotypes. In Candonga variety (Fig. 5a) one day after the treatment the reactions of white and red fruits seemed to be similar (increase of activity with singular boron and zinc and weak decrease in case of combined treatments), instead for 20 days the over expression for all the fertilizers is much higher (except leave tissue).

Interestingly, in this example it can be seen that even if fertilizers were applied as foliar spray, one day after the treatment boron has the effect only in fruit tissues, but not in leaves, whereas 20 days after the treatment all the effects of the microelements applied became not detectable in leave tissue. This effect proved the complexity of plant regulation system (Coombe, 1976; Zaccagnino et al., 2010).

Similarly to the *HXK1* dynamics, *MAKR6* gene reaction in two genotypes was completely different, but for both of them the long-term effect of treatments was stronger. It could be partially explained by the time course of fruit development: the analyzed at T2 red fruits were enlarged green fruits with the cell division yet stopped at moment of treatment, whereas the analyzed at T2 white fruits were small green fruits with active cell division process (Knee et al., 1977). Thus, the importance of developmental stages must be considering in fertilization process: during the initial stages the base of fruit growth is initiating, when the white stage processes are responsible for the main characteristics (sweetness, flavour etc.) of final product – red fruit. So, logically, applying B and Zn in green stage their impact on particular processes of white fruit could be observed. Similarly, previous agronomic studies have demonstrated that the final shape of fruit is affected by the duration of cell division and enlargement, and those processes are correlated with boron availability (Cohen, 1977) which in its turn influence the cell wall plasticity (Hu and Brown, 1994).

Regarding the interaction between the microelements, for both, Candonga and PZ600F13P2 genotypes, the

expression of studied genes was different related to singular microelement treatments in front of combined fertilizing, even if often the zinc effect was "masked" by boron influence in B+Zn treatment. In some cases, the additive effect of combined microelement application was observed (see the *HKX1* expression in white fruits of Candonga at T2 (Fig. 4a) and red fruits of PZ600F13P2 at T1 (Fig. 4b), as well as *MAKR6* in PZ600F13P2 genotype for white fruits at T1 and leaves at T2 (Fig. 5b)). Several authors (Epstein, 1972; Shkol'nik, 1974; Barr and Crane, 1991; Abd and Mona, 2013) suggest that the action of both microelements investigated, B and Zn, could be ascribed as an interaction with the endogenous auxin production.

As mentioned earlier, the *hexokinase 1* and *membrane associated kinase regulator 6*, being observed to be commonly expressed in all the tissues and vegetative stages, could be influenced, directly or not, by a general plant shape due also to fertilizing. In the same time it is not evident which mechanism could link those genes with boron and zinc in plant. The further studies will help to understand the dynamics of gene expression and their relationships with microelement availability.

## CONCLUSIONS

Food quality and sustainable use of natural resources is of great importance for plant growers over the world. Clever use of fertilizers is a part of this process. This study confirmed the initial hypothesis of B and Zn importance and especially the interest on interaction between them. RAPD-PCR method was useful to observe existing differences at transcriptomic level and have permitted to characterize two differently expressed genes: *HKX1* and *MAKR6*. The quantitative analysis of gene expression has demonstrated the influence of applied microelements on tested genes in singular and combined application for short (1 day) and long-term (20 days) action. The observed results put in evidence the effects derived by the genome structure (cultivar related), as well as the tissues specific response. With aim to increase the knowledge, future research action will be direct, by RNA seq analysis, to detail the different metabolic pathways involved in boron and zinc metabolism. It could be interesting to test the boron and zinc influence on auxin pathway and to complete this experimental scheme with a larger time scale to define a possible intermediate action of microelements.

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### Authors' contributions

All authors contributed extensively to the work presented in this paper. G.M. designed the study and interpreted data. Y.K. designed and performed experiments, analyzed and interpreted data and wrote the manuscript. M.C.P. and R.R helped in analytical tools development and critically revised the paper.

## REFERENCES

EL-Kader, A. and G. Mona. 2013. Effect of sulfur application and foliar spraying with zinc and boron on yield, yield components, and seed quality of peanut (*Arachis hypogaea* l.). Res. J. Agric. Biol. Sci. 9(4): 127-135.

Abdollahi, M., S. Eshghi, E. Tafazzoli and N. Moosavi. 2011. Effects of paclobutrazol, boric acid and zinc sulfate on vegetative and reproductive growth of strawberry cv. Selva. J. Agric. Sci. Technol. 14(2): 357-363.

Albregts, E. E. and C. M. Howard. 1978. Elemental composition of fresh strawberry fruit. J. Am. Soc. Hortic. Sci. 103(3): 293-296.

Amil-Ruiz, F., J. Garrido-Gala, R. Blanco-Portales, K. M. Folta, J. Muñoz-Blanco and J. L. Caballero. 2013. Identification and validation of reference genes for transcript normalization in strawberry (*Fragaria* × *ananassa*) defense responses. PLoS One. 8(8): e70603.

Barr, R. and F. L. Crane. 1991. Boron stimulates NADH oxidase activity of cultured carrot cells. In: Current Topics in Plant Biochemistry and Physiology 'Plant Biochemistry and Physiology Symposium'. Vol. 10. Publisher the University of Missouri, Columbia.

Blevins, D. G. and K. M. Lukaszewski. 1998. Boron in plant structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49(1): 481-500.

Brown, P. H., N. Bellaloui, M. A. Wimmer, E. S. Bassil, J. Ruiz, H. Hu and V. Römheld. 2002. Boron in plant biology. Plant Biol. 4(2): 205-223.

Cakmak, I. and R. Volker. 1997. Boron deficiency-induced impairments of cellular functions in plants. Plant Soil. 193(1-2): 71-83.

Cheng, B. T. 1994. Ameliorating Fragaria ssp. and *Rubus idaeus* L. Productivity through boron and molybden addition. Agrochemica. 38(3): 177-185.

Clancy, M. A., H. G. Rosli, S. Chamala, W. B. Barbuzuk, P. M. Civello and K. M. Folta. 2013. Validation of reference transcripts in strawberry (*Fragaria* spp.). Mol. Genet. Genomics. 288: 671-681.

Cohen, M. S. 1977. Effect of boron in cell elongation and division in squash roots. Am. Soc. Plant Biol. 5: 884-887.

Coombe, B. G. 1976. The development of fleshy fruits. Ann. Rev. Plant Physiol. 27: 507-528.

Dell, B. and L. Huang. 1997. Physiological response of plants to low boron. Plant Soil. 193: 103-120.

Epstein, E. 1972. Mineral Nutrition of Plants: Principles and Perspectives. John Wiley and Sons, Inc., New York, London, Sydney, Toronto.

Esringü, A., M. Turan, A. Gunes, A. Eşitken and P. Samboc. 2011.

Boron application improves on yield and chemical composition of strawberry. Acta Agric. Scan. B. 61(3): 245-252.

Fait, A., K. Hanhineva, R. Beleggia, N. Dai, I. Rogachev, V. J. Nikiforova, A. R. Fernie and A. Aharoni. 2008. Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. Plant Physiol. 148: 730-750.

Forney, C. F. and P. J. Breen. 1986. Sugar content and uptake in strawberry fruit. J. Am. Soc. Hortic. Sci. 111: 241-247.

Galli, V., J. M. Borowski, E. C. Perin, R. da Silva Messias, J. Labonde, I. dos Santos Pereira, S. D. dos Anjos Silva and C. V. Rombaldi. 2015. Validation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. Gene. 554(2): 205-214.

Gambino, G., I. Perrone and I. Gribaudo. 2008. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. Phytochem. Anal. 19: 520-525.

Granot, D., R. David-Schwartz and G. Kelly. 2013. *Hexose kinases* and their role in sugar - Sensing and plant development. Front. Plant Sci. 4: 44.

Hancock, J. F. 2000. Strawberries. In: Erez, A., (ed.), Temperate Fruit Crops in Warm Climates. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Heyndrickx, K. S. and K. Vandepoele. 2012. Systematic identification of functional plant modules through the integration of complementary data sources. Plant Physiol. 159(3): 884-901.

Hosseini, S. M., M. Maftoun, N. Karimian, A. Ronaghi and Y. Emam. 2007. Effect of zinc × boron interaction on plant growth and tissue nutrient concentration of corn. J. Plant Nutr. 5: 773-781.

Hu, H. and P. H. Brown. 1994. Localization of boron in cell walls of squash and tobacco and its association with pectin. Evidence for a structural role of boron in the cell wall. Plant. Physiol. 105: 681-689.

Jiang, J., T. Wang, Z. Wu, J. Wang, C. Zhang, H. Wang, Z. X. Wang and X. Wang. 2015. The intrinsically disordered protein BKI1 is essential for inhibiting BRI1 signaling in plants. Mol. Plant 8(11): 1675-1678.

Jaillais, Y., M. Hothorn, Y. Belkhadir, T. Dabi, Z. L. Nimchuk, E. M. Meyerowitz and J. Chory. 2011. Tyrosine phosphorylation controls brassinosteroid receptor activation by triggering membrane release of its kinase inhibitor. Gene. Dev. 25(3): 232-237.

Kawai, S., T. Mukai, S. Mori, B. Mikami and K. Murata. 2005. Hypothesis: Structures, evolution, and ancestor of glucose kinases in the hexokinase family. J. Biosci. Bioeng. 99(4): 320-330.

Kelly, G., M. Moshelion, R. David-Schwartz, O. Halperin, R. Wallach, Z. Attia, E. Belausov and D. Granot. 2013. Hexokinase mediates stomatal closure. Plant J. 75: 977-988.

Kessler, B. 1961. Ribonuclease as a guide for the determination of zinc deficiency in orchard trees. Plant Analysis and Fertilizer Problems. Vol. 3. American Institute of Biological Science, Washington, DC, Pp. 314-322.

Knee, M., J. A. Sargent and D. J. Osborne. 1977. Cell wall metabolism in developing strawberry fruits. J. Exp. Bot. 28(2): 377-396.

Lugassi, N., G. Kelly, L. Fidel, Y. Yaniv, Z. Attia, A. Levi, V. Alchanatis, M. Moshelion, E. Raveh, N. Carmi and D. Granot. 2015. Expression of arabidopsis hexokinase in citrus guard cells controls stomatal aperture and reduces transpiration. Front. Plant Sci. 6: 1114.

Matoh, T. 2001. Boron in plant nutrition and cell wall development. In: Plant Nutrient Acquisition. Springer, Japan, Pp. 227-250.

May, G. and M. Pritts. 1990. Strawberry nutrition. Adv. Strawb. Prod. 9: 10-23.

May, G. and M. Pritts. 1993. Phosphorus, zinc, and boron influence yield components in 'Earliglow' strawberry. J. Am. Soc. Hortic. Sci. 118(1): 43-49.

Nasim, M., Z. Rengel, T. Aziz, B. D. Regmi and M. Saqib. 2015. Boron toxicity alleviation by zinc application in two barley cultivars differing in tolerance to boron toxicity. Pak. J. Agric. Sci. 52(1): 151-158.

Nestby, R., F. Lieten, D. Pivot, C. Raynal Lacroix and M. Tagliavini. 2005. Influence of mineral nutrients on strawberry fruit quality and their accumulation in plant organs. Int. J. Fruit. Sci. 5(1): 139-156.

Obulareddy, N., S. Panchal and M. Melotto. 2013. Guard cell purification and RNA isolation suitable for high-throughput transcriptional analysis of cell-type responses to biotic stresses. Mol. Plant Microbe. Int. 26(8): 844-849.

Olsson, T., Thelander, M. and Ronne, H. 2003. A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. J. Biol. Chem. 278: 44439-44447.

Padula, M. C., L. Lepore, L. Milella, J. Ovesna, N. Malafronte, G. Martelli and N. De Tommasi. 2012. Cultivar based selection and genetic analysis of strawberry fruits with high levels of health promoting compounds. Food Chem. 140: 639-646.

Perkins-Veazie, P. 1995. Growth and ripening of strawberry fruit. Hortic. Rev. 17(8): 267-297.

Puzina, T. I. 2004. Effect of zinc sulfate and boric acid on the hormonal status of potato plants in relation to tuberization. Russian J. Plant Physiol. 51(2): 209-215.

Rajaie, M., A. K. Ejraie, H. R. Owliaie and A. R. Tavakoli. 2009. Effect of zinc and boron interaction on growth and mineral composition of lemon seedlings in calcareous soil. Int. J. Plant Prod. 3(1): 1735-6814.

Ruijter, J. M., C. Ramakers, W. M. Hoogaars, Y. Karlen, O. Bakker, M. J. van den Hoff and A. F. Moorman. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic. Acids Res. 37(6): e45.

Ruijter, J. M., P. Lorenz, J. M. Tuomi, M. Hecker and M. J. van den Hoff. 2014. Fluorescent-increase kinetics of different fluorescent reporters used for qPCR depend on monitoring chemistry, targeted sequence, type of DNA input and PCR efficiency. Mikrochim. Acta. 181(13-14): 1689-1696.

Schmid, M., T. S. Davison, S. R. Henz, U. J. Pape, M. Demar, M. Vingron, B. Schölkop, D. Weigel and J. U. Lohmann. 2005. A gene expression map of Arabidopsis thaliana development. Nat. Genet. 37(5): 501-506.

Shkol'nik, M. 1974. General conception of physiological role of boron in plants. Fiziol. Rast. Mosk. 21(1): 174-186.

Shulaev V., D. J. Sargent, R. N. Crowhurst, T. C. Mockler, O. Folkerts, A. L. Delcher, P. Jaiswal, K. Mockaitis, A. Liston, S. P. Mane, P. Burns, T. M. Davis, J. P. Slovin, N. Bassil, R. P. Hellens, C. Evans, T. Harkins, C. Kodira, B. Desany, O. R. Crasta, R. V. Jensen, A. C. Allan, T. P. Michael, J. Carlos Setubal, J. M. Celton, D. J. G. Rees, K. P. Williams, S. H. Holt, J. J. Ruiz Rojas, M. Chatterjee, B. Liu, H. Silva, L. Meisel, A. Adato, S. A. Filichkin, M. Troggio, R. Viola, T. L. Ashman, H. Wang, P. Dharmawardhana, J. Elser, R. Raja, H. D. Priest, D. W. Bryant, S. E. Fox, S. A. Givan, L. J. Wilhelm, S. Naithani, A. Christoffels, D. Y. Salama, J. Carter, E. Lopez Girona, A. Zdepski, W. Wang, R. A. Kerstetter, W. Schwab, S. S. Korban, J. Davik, A. Monfort, B. Denoyes-Rothan, P. Arus, R. Mittler, B. Flinn, A. Aharoni, J. L. Bennetzen, S. L. Salzberg, A. W. Dickerman, R. Velasco, M.

Borodovsky, R. E. Veilleux and K. M. Folta. 2011. The genome of woodland strawberry (*Fragaria vesca*). Nat. Genet. 43: 109-116.

Sinha, P., R. Jain and C. Chatterjee. 2000. Interactive effect of boron and zinc on growth and metabolism of mustard. Commun. Soil Sci. Plant Anal. 1-2: 41-49.

Tabata, S., T. Kaneko, Y. Nakamura, H. Kotani, T. Kato, E. Asamizu, N. Miyajima, S. Sasamoto, T. Kimura, T. Hosouchi, K. Kawashima, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, S. Nakayama, N. Nakazaki, K. Naruo, S. Okumura, S. Shinpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, S. Sato, M. de la Bastide, E. Huang, L. Spiegel, L. Gnoj, A. O'Shaughnessy, R. Preston, K. Habermann, J. Murray, D. Johnson, T. Rohlfing, J. Nelson, T. Stoneking, K. Pepin, J. Spieth, M. Sekhon, J. Armstrong, M. Becker, E. Belter, H. Cordum, M. Cordes, L. Courtney, W. Courtney, M. Dante, H. Du, J. Edwards, J. Fryman, B. Haakensen, E. Lamar, P. Latreille, S. Leonard, R. Meyer, E. Mulvaney, P. Ozersky, A. Riley, C. Strowmatt, C. Wagner-McPherson, A. Wollam, M. Yoakum, M. Bell, N. Dedhia, L. Parnell, R. Shah, M. Rodriguez, L. H. See, D. Vil, J. Baker, K. Kirchoff, K. Toth, L. King, A. Bahret, B. Miller, M. Marra, R. Martienssen, W. R. McCombie, R. K. Wilson, G. Murphy, I. Bancroft, G. Volckaert, R. Wambutt, A. Düsterhöft, W. Stiekema, T. Pohl, K. D. Entian, N. Terryn, N. Hartley, E. Bent, S. Johnson, S. A. Langham, B. McCullagh, J. Robben, B. Grymonprez, W. Zimmermann, U. Ramsperger, H. Wedler, K. Balke, E. Wedler, S. Peters, M. van Staveren, W. Dirkse, P. Mooijman, R. K. Lankhorst, T. Weitzenegger, G. Bothe, M. Rose, J. Hauf, S. Berneiser, S. Hempel, M. Feldpausch, S. Lamberth, R. Villarroel, J. Gielen, W. Ardiles, O. Bents, K. Lemcke, G. Kolesov, K. Mayer, S. Rudd, H. Schoof, C. Schueller, P. Zaccaria, H. W. Mewes, M. Bevan and P. Fransz. 2000. Sequence and analysis of chromosome 5 of the plant Arabidopsis thaliana. Nature. 408(6814): 823-826.

Tuomi, J. M., F. Voorbraak, D. L. Jones and J. M. Ruijter. 2010. Bias in the Cq value observed with hydrolysis probe based quantitative PCR can be corrected with the estimated PCR efficiency value. Methods. 50(4): 313-322.

Xie, M., J.W. Chen, Q. P. Qin, G. H. Jiang, C. B. Sun, H. Q. Zhang and H. X. Xu. 2007. The control of sugar accumulation within strawberry aggregate fruit by invertase and hexokinase. J. Plant. Physiol. Mol. Biol. 33(3):213-8.

Zaccagnino, N., L. Milella, M. C. Padula, I. Greco and G. Martelli. 2010. Study and characterization of the gene expression regulation in *Fragaria vesca* mediated by DNA methylated under abiotic stress condition. J. Biotechnol. 150(1): 499.