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ORIGINAL ARTICLE

INFLUENCE OF SPRAYING OF NANO-FERTILIZERS WITH BORON AND CALCIUM ON VEGETATIVE GROWTH CHARACTERISTIC AND *PGAUX* GENE EXPRESSION OF POMEGRANATE TRANSPLANT

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Abstract: A lath experiment was carried out during season 2018-2019 to assess the influence of foliar spraying with nano-boron applied with three concentrations (0.5, 1 and 1.5 g.L^{-1}) and nano-calcium, with three concentrations (1, 2 and 3 g.L^{-1}) and *pgAUX* gene expression on pomegranate transplants. The experiment was layout as Randomized Complete Block Design (RCBD) and then Duncan's multiple range test was performed for mean comparison at 5% probability level by GenStat program. Results showed that the nano-calcium concentrations were superior compared with boron concentration sprayed and control especially length increment, stem diameter, leaf increment and leaf area. Moreover, the chemical mineral analyses of leaves were highly significant in transplant leaves when treated with nano-calcium. The relative gene expression measurements were not significant among control and each nano-boron concentration but the nano-calcium treatments caused a raise *pgAUX* gene expression.

***Keyword*:** Nano-fertilizers, Boron, Calcium, Gene expression, Pomegranate.

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1. Introduction

Pomegranate (*Punica granatum* L.) is a significant and popular fruit as commercial fruits that are planted in many countries like Iran, Egypt, Spain, Afghanistan, Morocco, China, USA, Burma, Russia, Bulgaria, Japan and Italy. Northern India represents a native area from Iran to the Himalayas for pomegranate trees that have been cultured and adapted since ancient times in the Mediterranean and the region of Caucasus in Asia [Gumienna *et al.* (2016)]. Pomegranate is consumed frequently as fresh fruit and is also used in jam, jelly, vinegar and juice [Sheikh and Manjula (2012)]. In tropical and subtropical areas, it is one of the most valuable fruit crops. The pomegranate is synonymous with the Middle East's most ancient civilization and was mentioned in religious books [Mohamed *et al.* (2020)]. Ali *et al.* (2020) reported that boron (B) deficiency is a popular micronutrient problem in agriculture, resulting in reduced yields and declining crop quality. The role of boron in plants includes effects on fruit set and yield, pollen tube elongation, pollen grain germination. Besides there are some indirect effects of boron responsible for sugar translocation, dehydrogenase enzyme activation plant hormones and nucleic acids [Maitham *et al.* (2020)]. Boron is an important nutrient and while leaves can withstand this element's toxic levels, boron deficiency can cause serious problems such as poor fruit growth, lower yield and poor fruit quality [Sheikh and Manjula (2012)]. Calcium is a vital element for physiological resistance in fruits [Marschner (2012)], also alleviates the cell membrane and boosts cell turgor pressure [Ramezanian *et al.* (2009)]. In many fruit species, calcium disorders inhibit physiological maturity prior to harvesting, such as delays and declines in fruit quality [Hernandez-Munoz *et al.* (2006)]. Applications of foliar calcium can substantially prolong the aging

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process, nevertheless, little is studied about the impact of calcium as a foliar application on quality and yield traits [Ramezanian *et al.* (2009)]. Foliar fertilizers also help prevent signs of toxicity that can occur after the application of the same microelements to the soil [Obreza *et al.* (2010)]. Alternatively, utilizing nanotechnologies [Scott and Chen (2003)], which are nowadays used for the development, processing and implication of nanoscale complexes, several problems have been solved in multidisciplinary sciences and industries. Materials that are smaller than 100nm are typically known as nano-materials, at least in a single dimension. Applications of this new technology have been established in agriculture and nano-technologies are already being used to produce, process, store, pack and transport agricultural products [Wiesner *et al.* (2006)]. The nano-fertilizer market, which can gradually feed plants in a regulated manner, is the most significant application of nanotechnology in crop production, in contrast to what is happening in the case of common fertilizers. These nano-fertilizers may be more effective, reducing soil contamination and other threats to the environment that could occur when chemical fertilizers are used [Naderi *et al.* (2011)]. One of the benefits of using nano-fertilizers is that it is possible to add smaller quantities than when common fertilizers are used [Subramanian *et al.* (2015)]. Therefore, the goal of this work is to illustrate the effect of nano-boron and nano-calcium on the growth and *pgAUX* gene expression of pomegranate transplants.

2. Materials and Methods

Selected pomegranate transplants similar in size and aged (2 years) from Najaf station for fruit production of the Iraqi agriculture ministry. The plants were cultivated in plastic pots containing soil 3Kg, sandy clay, pH 7.88, EC 0.78 dS/m. Transplants were grown up in a lath for the period from 1 February 2019 to 1 November 2019. The layout of the experiment setup as Randomized Complete Block Design (RCBD) with two variables, the first factor is nano-boron (nB) with three concentrations (0.5, 1 and 1.5 $g.L^{-1}$) and the second factor was nano-calcium (nCa) with three concentrations (1, 2 and 3 $g.L^{-1}$) were sprayed as foliar application as well as the control (without spray) and all doses repeated two times at 15/3/2019 and 15/4/2019. Each experimental unit comprised five transplants 15 treatments for each foliar application plus the control (7 concentrations) and then triplicated to provide 105 transplants as a total number. The data analyzed by GenStat program for ANOVA and mean separation using Duncan's Multiple Range Test at a probability level of 0.05.

2.1 Vegetative growth parameters

Measurement recorded on 2 November 2019. The measurement included the mean of transplant length increment (cm) by using the measuring tape, mean of transplant stem diameter increment (mm) using Vernier, mean of leaf number increase per transplant, average leaf area of the 5-9th leaves using graphic lines with scanner according to Arnon 1949. The macro and micronutrients (N, P, K, Ca, B) were determined using Kjeldahl method to determine N, spectrophotometry method to determine P, flame emissions to determine K. B concentrations were measured using atomic absorption spectrophotometry (AAS).

2.2 Gene expression measurement

Total RNA extraction and cDNA synthesis: Total RNA for samples were isolated from different treatment leaves by uses (SV Total RNA Isolation kit/ Promega, USA). The quality of RNA was verified by the demonstration of intact ribosomal bands following agarose gel electrophoresis. DNA was removed from RNA samples using the DNase I Mix/ Promega, USA (DNase I, $MnCl_2$, yellow core buffer). First-strand cDNA was synthesized from (16µl) of total RNA using the (power cDNA Syntheses kit/IntronBio. Inc. USA) with Oligo $(dT)_{15}$ primer, following the manufacturer's instructions and quantified using gel electrophoresis.

Quantitative Real-Time PCR (qRTPCR): Relative gene expression was made by qRT-PCR using the kit from IntronBio, USA company (Master Mix SYBR Green QPCR). The primers are available in Gen Bank (www.ncbi.nih.nlm.gov/Gen Bank/EMBL/ DDBJ. the primer sequences pgAUXgene is responsible for *Punica granatum* auxin response factor 3-like. The primers were two parts (Forward 5-CCACGAAGGCATT TG CTCAC -3) (Reverse 5-GCTGTG CCTATGGCTTGAGA -3), GC %55, T.m (60.11-60.11), product length 785. Real-time Reaction mix (22.5µl per well) contained 12.5µl Master Mix SYBR Green, 2.5µl forward and reverse primers, 7.5µl DEPC-D.W and 2.5µl of c DNA. Real-time PCR program according: initial denaturation step of 95C for 10min, followed by 40 cycles of 95C for 30s, 60C for 1min and 72C for 30s. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR products were also checked for purity via agarose gel electrophoresis using actin gene of pomegranate (pgActin) as reference gene that sequence primers to normalize (Forward 5-AGTCCTCTTCCAGCCATCTC-3) (Reverse 5-CACTGAGCA CAATGTTTCCA-3), T.m (60.04-59.82), GC% 55. The data of relative gene expressions were normalized and analyzed by the GeneX program [Al-Janabi and Al-Rawi (2018)].

3. Results

3.1 Vegetative growth parameters

Table 1 showed that the add nano-calcium with 2g.L^{-1} concentration caused high signification in length increment of pomegranate transplants reached 17.96 cm and nonsignificant in length increment among others concentration of nano-calcium (1, 2 g.L^{-1}) and nano-boron treatments (1, 1.5 g.L^{-1}) reached (17.36, 17.23) cm and (16.93 and 17.30) cm, respectively. While control treatment was low value and nonsignificant in length increment in leave of pomegranate transplants reached 12.36 cm. The increment of the stem diameter of pomegranate transplants was highly significant of stem diameter increment showed in transplants which treated with nano-calcium 3g.L^{-1} reached 3.5233mm. While the low increment value of stem diameter of pomegranate transplant using 1 g.L^{-1} of nano-boron treatment reached 2.33mm (Table 1). The increment of leaves was statistically significant when transplant treated with 1 g.L^{-1} nano-calcium reached 38.733 leaves.transplant^{-1} and control treatment was the least value reached 27.000 leaves.transplant^{-1}. Table 2 displayed that 3 g.L^{-1} of nano-calcium treatment gave high significant leaf area reached 692.30 cm, while the transplants treated with control (water only) appeared low significant value reached 405.57cm.

The chemical mineral analysis in leaf contents showed in Table 2 refers that the high nitrogen percent reached (1.8933, 1.900 and 1.9166%) when pomegranate transplants treated with nano-calcium (1, 2 and 3 g.L^{-1}), respectively, but all other treatments had low significant mean. The same table showed no significant effects in phosphor and potassium percent among all treatments by the application of foliar nano fertilizers from boron and calcium. Table 2 exhibited high significance of calcium percent when transplants treated with nano-calcium (3 and 2 g.L^{-1}) reached (2.97667 and 2.78000 %) consecutively, while the low significant value of calcium percent was by the control

Table 2: Influence of foliar nano-boron and nano-calcium fertilizers on nutrient composition percent of pomegranate (*Punica granatum*) transplants.

Treatment	N%	P%	K%	Ca%	B%
Control	1.75667b	0.3800a	0.88000a	2.18667c	21.567d
B0.5	1.70333b	0.1267a	0.76333ab	2.67000b	24.100cd
B1	1.74667b	0.1300a	0.88000a	2.70000b	26.867ab
B1.5	1.74400b	0.1300a	0.77667ab	2.63667b	28.967a
Ca 1	1.89333a	0.1733a	0.76667ab	2.67667b	24.067cd
Ca 2	1.90000a	0.1733a	0.87333a	2.78000ab	25.100bc
Ca 3	1.91667a	0.1533a	0.83333a	2.97667a	23.900cd

The same letters in columns mean no significant differences based on Duncan's Multiple Range Test at a probability level of 0.05.

Table 1: Influence of foliar nano-boron and nano-calcium fertilizers on the vegetative growth parameters of pomegranate (*Punica granatum*) transplants.

Treatment	The Stem Length Increment (cm)	The Stem Diameter Increment (mm)	The Leaf No. Increment/Transplant	The Leaf Area (cm)
Control	12.36b	2.5567bc	27.000c	405.57c
B0.5	13.56b	2.6867abc	34.900ab	507.20bc
B1	16.93a	2.3800c	34.100abc	618.63ab
B1.5	17.30a	2.9400abc	31.100bc	643.30ab
Ca 1	17.36a	3.4467ab	38.733a	595.10ab
Ca 2	17.96a	3.2733abc	34.900ab	581.37ab
Ca 3	17.23a	3.5033a	34.100abc	692.30a

The same letters in columns mean no significant differences based on Duncan's Multiple Range Test at a probability level of 0.05.

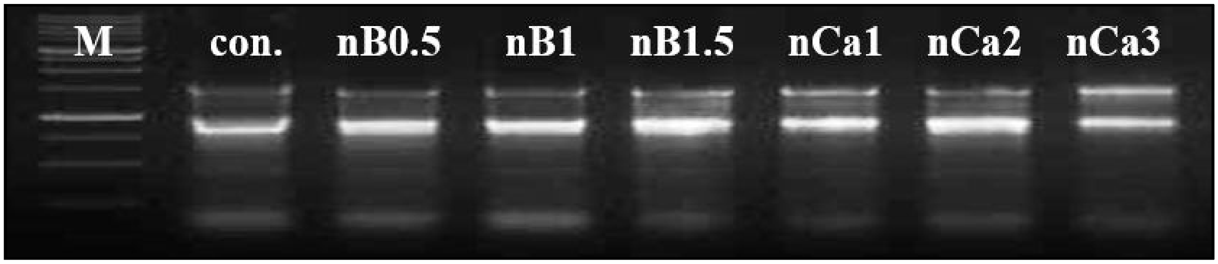

Fig. 1: Total RNA extraction of the pomegranate leaves for the treatments using agarose gel 1.5% and 100 voltage for 20 minutes, M:marker, con.: control, nB:nanoboron (0.5, 1, 1.5 g.L^{-1}), nCa: nanocalcium (1, 2, 3 g.L^{-1})

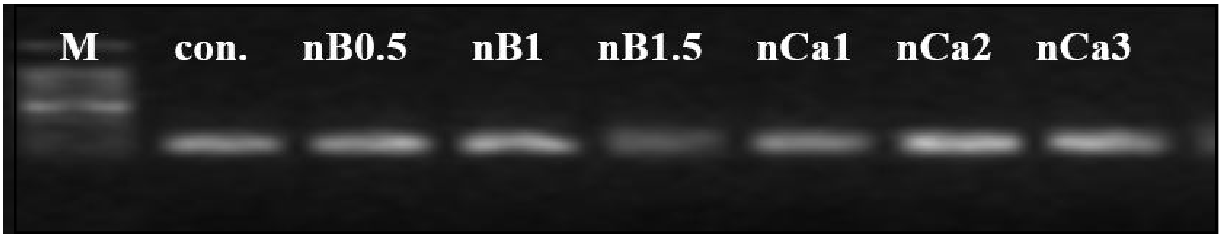

Fig. 2: cDNA synthesis from RNA of the pomegranate leaves for the treatments using agarose gel 1.5% and 100 voltage for 20 minutes, M: marker, con.: control, nB: nano-boron (0.5,1, 1.5 g.L^{-1}), nCa: nano-calcium (1,2,3 g.L^{-1})

treatment, also the others treatments were in the middle among low and high values.

The results in Table 2 showed no significant variations in the average spraying of foliar and nano fertilizers of boron and calcium on the chemical properties. The lowest average for all chemical parameters in leaf contents was by the control treatment. The treatment of spraying nano-boron with (0.5 g.L^{-1}) showed the least ratio of calcium reached 2.18667% and 1.70333%. Besides, it appears from the same table that the increase in the percentage of K% when spraying with foliar nano-boron (1 g.L^{-1}) and nano-calcium (2 g.L^{-1}) provided the highest ratios 0.88000 and 0.87333, respectively. In contrast, spraying with nano-boron (0.5 g.L^{-1}) revealed the lowest percentage reached 0.76333%. The same table showed that an increase in the averages of B% when spraying with foliar nano fertilizers of boron using treatment of B 1.5 which gave the highest ratio and reached 28.967% in comparison with the control that had the lowest average of B% reached 21.567%.

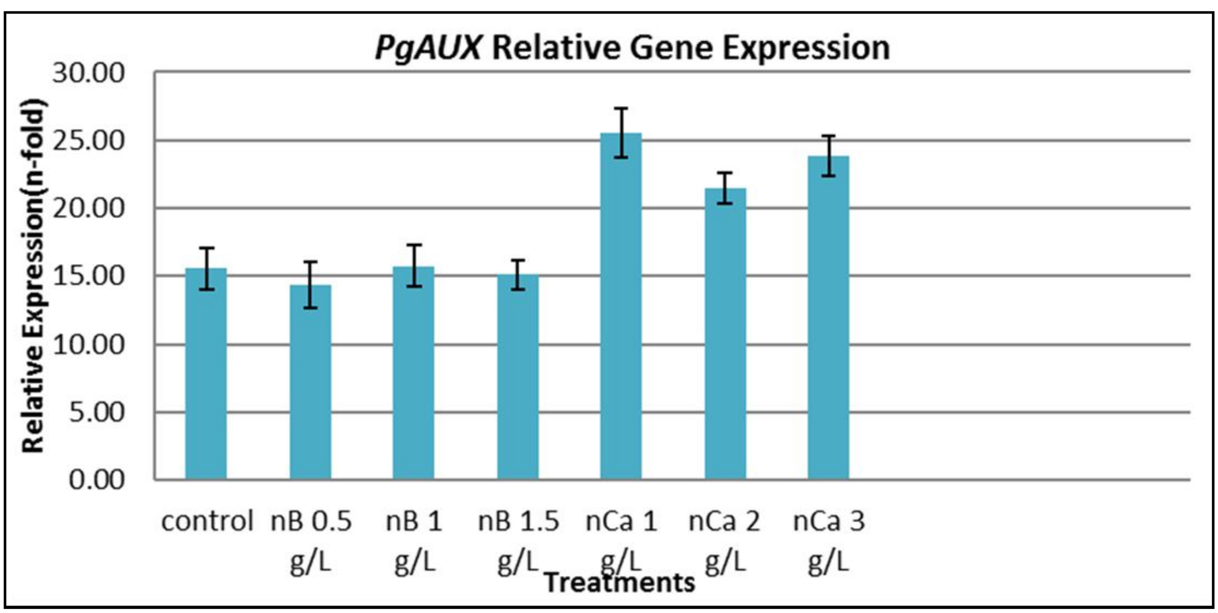

Fig. 3: Relative gene expression for *pgAUX* gene under different treatments control: without spray nB: nano-boron, nCa: nano-calcium

3.2 *pgAUX* Gene expression

Proved the gel electrophoresis appeared in Figs. 1 and 2 that successful extraction of total RNA from all leaves was for treated transplants and convert total RNA to cDNA for all samples were high of quality and successful and this reflected on result clarity.

The analysis *pgAUX* gene expression responsible for auxin hormone unit synthesis appeared that no significant effects appeared among all concentration of nano boron and control treatments, while the gene expressions were highest when transplants treated with nano-calcium (1, 2 and 3 $g.L^{-1}$) reached 25.58, 21.50 and 23.86 fold, respectively.

4. Discussion

This study is in agreement with Sheikh and Manjula (2012), Obreza *et al*. (2010), Ali *et al*. (2020) and Maitham, *et al*. (2020). When compared to control plants, the study found that foliar Ca application resulted in an increase in Ca leaf concentration. Calcium's important roles in the cell wall, affecting the mechanical properties of plant tissues, may have an influence on fruit cracking. Boron and calcium and are two elements that, if present in insufficient quantities, can cause fruit to fracture. Foliar fertilizers also help prevent signs of toxicity that can occur after the application of the same microelements to the soil [Obreza *et al.*(2010)]. Boron deficiency can cause serious problems such as poor fruit growth, lower yield and poor fruit quality [Sheikh and Manjula (2012)].

5. Conclusion

The spraying with Nano-fertilizer boron and calcium showed that the nano-calcium concentrations were superior compared with boron concentration sprayed and control especially length increment, stem diameter, leaf increment and leaf area. Moreover, the chemical mineral analyses of leaves were highly significant in transplant leaves when treated with nano-calcium. The relative gene expression measurements were not significant among control and each nano-boron concentration but the nano-calcium treatments caused a raise *pgAUX* gene expression.

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