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Effect of preharvest applied brassinosteroid on "Anna" apple fruit retention, coloration and quality

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Fruit set, quality and coloration of “Anna” apple (*Malus domestica* L.) fruits were studied after preharvest treated with different concentrations of brassinolide. Four concentrations of brassinolide 1, 2, 3 and 4 mg/l were applied at full bloom, after fruit set and at color change through the two seasons of study. The results proved that all BRs treatments significantly increased fruit set and fruit retention at harvest, vitamin c, peel anthocyanin contents, PAL enzyme activity and PAL, CHI genes expression (Chitinase) as compared with control treatment. In addition, BRs-treated apples at 3 mg/l increased fruit diameter, weight, firmness, total sugars and color. On the other hand, Anna apple fruits treated with brassinosteroid at 1 and 2 mg/l showed no significant effect on fruit diameter, weight, TSS, sugars and firmness as compared with control treatment. In conclusion, this study recommended using brassinolide at 3 or 4 mg/l at different stages of growth to improve the production of “Anna” apples.

Keywords: Brassinolide, Fruit set, coloration, PAL enzyme.

INTRODUCTION

Apple cultivations under Egypt conditions faced different production problems such as poor fruit set, low yield, preharvest fruit drop, unsatisfied fruit color and a number of physiological disorders such as bitter pit, soft scald and sun scald (Espley et al. 2007: Aly et al. 2010 and Farag and Attia, 2018). Thus, several factors and treatments are involved in improving fruit production and quality of apple fruits included environmental factors, plant nutrition and using plant growth regulators (Honda and Moriya, 2018: Arseneault and Cline, 2016). Brassinosteroids are a novel group of hormones that regulate plant growth, development and plant responses to various environmental stresses when applied at appropriate stage of growth, gene expression, increased cell division and expansion, ethylene production, vascular differentiation, alter photosynthetic pigment loss and promote fruit ripening (Clouse and Sasse, 1998: Clouse, 2002: Vardhini and Rao, 2002: Domagalska et al. 2007: Wang et al. 2013 and Bartwal et al. 2013: Symons et al. 2006). Studies have provided evidence that exogenous applications of brassinosteroids have specific physiological influences on different aspects of fruit crop such as root and shoot development, retard fruit abscission, enhanced flowering, and increased fruit set, enhanced fruit growth and development, fruit yield, fruit quality and fruit ripening (Baghel et al. 2019). Preharvest brassinosteroid-treated sweet cherry maintained fruit firmness, increased fruit anthocyanin contents and phenolic compounds (Roghabadi and Pakkish, 2014). Furthermore, Chumpookam et al. (2017) and Champa et al. (2015) showed that preharvest application of brassinosteroid increased fruit weight, diameter, length, firmness and total soluble solids of pineapple and table grape fruits, respectively. Anthocyanins,

condensed tannins, and flavonols are synthesized via the flavonoid pathway, a branch of the phenylpropanoid pathway (Takos et al. 2006, Deluc et al. 2006 and Winkel-Shirley. 2001). The flavonoid pathway consists of a number of enzymatic steps, each catalyzed by a sequential reaction for flavonoid synthesis (Takos et al. 2006). In apple it has been determinated that the accumulation of anthocyanins is majorly correlated to the expression and activity of the enzymes including phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), dihydroflavonol4-reductase (DFR) and UDP-Glucose: flavonoid-3-O-galactosyl transferase (UFGT) (Wang et al. 2000, Lister and Lancaster, 1996 and Ban et al. 2009). Chalcone isomerase (CHI is one of the key enzymes in the flavonoid biosynthesis pathway, and catalyzes the stereospecific isomerization of chalcones into their corresponding (2S)-flavanones. According to the above discussion, the objectives of the present study was to evaluate the effects of the exogenous applications of different brassinoloide concentrations on fruit set, retention at harvest and final fruit quality of "Anna" apples.

MATERIALS AND METHODS

2.1. Plant materials and treatments:

The present study was conducted during 2019 and 2020, seasons at EL-Nubaria region, Beheira governorate, Egypt on five-years-old "Anna" apple trees budded on Balady rootstock grown in sandy soil under irrigation system. The trees were spaced at 4m× 4m and in end of December of both years trees were treated with Dormex at the rate of 1%. The experiment was designed as randomized completely block design (RCBD) and the following five foliar spray treatments were obtained with three replicates for each treatment: water only (control), 1 mg/l brassinolide (Brassinolide 96.8%, Argent Techno Library, Philippines), 2 mg/l brassinolide, 3 mg/l brassinolide, and 4 mg/l brassinolide. The treatments were sprayed at full bloom (19, 25 February during 2019 and 2020, respectively), after fruit set with ten days (7, 10 March during 2019 and 2020, respectively) and at the beginning of fruit color change (8, 5 May during 2019 and 2020, respectively), the surfactant Tween 80 at the rate of 0.5cm/ L was added to all treatments and all treatments were sprayed to the point of run off by using handheld sprayer in the morning.

2.2. Fruit set percentage:

Fruit set % = Number of fruits at pea stage /Number of flowers ∗ 100.

2.3. Number of fruits/ spur at harvest:

Fifteen spur from each tree were labeled and number of retention fruit at harvest was counted.

2.4. Fruit quality characteristics at harvest:

2.4.1. Physical quality characteristics:

At harvest day (29, 22 may during 2019 and 2020, respectively) ten fruits were randomly taken from each replicate in both seasons and the following characteristics were determined: Fruit length and fruit diameter (cm) were measured using a mastercraft (electronic caliper with digital display).Average fruit weight (g), fruit volume (cm^3). Fruit firmness was determined as (lb/in^2) using Effigi pressure tester (mod. FT 011).

2.4.2. Chemical quality characteristics:

In "Anna" apple fruit juice, the percentage of total soluble solids (TSS) was measured using a hand refractometer, acidity as malic acid was determined according to (A.O.A.C., 1985). Total sugars were determined by using the phenol sulfuric acid method (Smith, 1956). Vitamin C (mg/ 100ml) was determined according to (A.O.A.C., 2007). Anthocyanin pigment (mg/ 100g) was determined according to Fuleki and Francis (1968).

2.4.3. Estimation of Phenylalanine Ammonia-Lyase (PAL) Activity:

One gram of apple fruit tissue was homogenized in 3 ml of ice-cold sodium borate buffer (0.1 M) pH 7.0 containing 2-mercaptoethanol (1.4 mM) and insoluble polyvinylpyrrolidone (PVP) (0.1 g). The extract was filtered through cheese cloth and the filtrate was centrifuged at 15,000 g for 15 minutes at 4 oC (Universal 32R, Hettich Zentrifugen model D-78532, Germany). The supernatant was used as enzyme source. Phenylalanine ammonia-lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Jenway UV/VIS spectrophotometer, Model 6305, Bibby Scientific Limited, Staffordshire, UK). About 0.4 ml of enzyme was incubated with 0.5 ml of borate buffer (0.1 M) pH 8.8 and 0.5 ml of L-phenylalanine (12 mM) for 30 min at 30 oC. The amount of trans-cinnamic acid generated was calculated using its extinction coefficient of 9630 M-1cm-1. Enzyme activity was expressed as µmol

trans-cinnamic acid min-1g-1 fresh weight (Dickerson et al. 1984).

2.4.4. Quantification of the PAL and CHI Genes Expression (Chitinase) Using Real-time PCR:

2.4.4.1. RNA isolation protocol:

Total RNA was extracted from apple fruits tissue using GStract™ RNA Isolation kit II (Guanidium Thiocynate) according to the manfacture procedures.

2.4.4.2. Reverse transcription-polymerase chain reaction (RT-PCR) of mRNA:

Reverse transcription (RT) or first strand reaction was performed for converting the mRNA to complementary DNA (cDNA) in the presence of dNTPS and reverse transcriptase. The components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 42°C. The exponential amplification via reverse transcription polymerase chain reaction provides a highly sensitive technique, where a very low copy number of RNA molecules can be detected.

Reverse transcription reaction was performed using oligo (dT) primer (5'-TTTTTTTTTTTTTTT-3'). Each 25 µl reaction mixture contained 2.5 µl (5x) buffer with $MgCl_2$, 2.5 µl (2.5 mM) dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2mg/ml) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler programmed at 42 °C for 1 hr, 72 °C for 10 min (enzyme killing) and the product was stored at 4 °C until use.

2.4.4.3. Estimation of Quantitative of PAL and CHI genes expression using RT-qPCR:

Two specific primers were used in this study (Table1), obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech., UK Limited, HP 79NA, England).

Samples were analyzed using the Fermentase kit: Each reaction contained 12.5 µl of 2x Quantitech SYBR® Green RT Mix, 1µl of 25 pm/µl forward primer, 1 µl of 25 pm/µl reverse primer,1 µl of the cDNA (50ng), 9.25 µl of RNase free water for a total of 25 µl. Samples were spun before loading in the Rotor's wells.

The real time PCR program was as follows : initial denaturation at 95 °C for 10 min.; 40 cycles of at °C for 15 sec.; annealing at 60°C for 30 sec and extension at 72 °C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene-6000-system (Qiagen, USA).

Table 1: Sequence of primers used in the real-time PCR

Primers	Primer sequence 5→3	Annealing (°C)
PAL **(F)**	TTCAAGGCTACTCTGGC	60
PAL **(R)**	CAAGCCATTGTGGAGAT	
CHI **(F)**	GTA GTG ATC AAR GAR ATIAAIGG	60
CHI **(R)**	TCN ACN ACR TTN GCI TTI TC	
18S rRNA(F)	TTCCATGCTAATGTATTCAGAG	60
18S rRNA(R)	ATGGTGGTGACGGGTGAC	

2.4.4.4. Real-time PCR Data analysis:

The reaction was performed using a Rotor-Gene 6000 (Qiagen, ABI System, USA). Relative quantification of gene expression was performed by ΔCq=Cq–reference gene, ΔΔCq=Cq – control, and ΔΔ C q expression = $2^{(-\Delta\Delta Cq)}$ (Livak and Schmittgen 2001). The expression levels of the target genes were normalized relative to 18S rRNA gene and relative expression of untreated control plants at each time were set as 1.

2.5. Statistical analysis:

Treatments and enzyme activity were arranged in the field as a complete randomized design (CRD) with three replicates. The data were analyzed for variance by using Statistical Analysis System (SAS, 2000). The Least Significant Differences (LSD) at 0.05 levels according to Sendecor and Cochran (1980) were used to separate treatments means.

RESULTS AND DISCUSSION

3.1. Fruit set percentage:

The data in Table 2 indicated that fruit set percentage increased with preharvest foliar application of brassinolide as compared with control. The trees treated with 0, 1.2,3,4 mg/l brassinolide showed fruit set of 42.33, 42.96, 51.32, 56.79 and 55.49 %, respectively in the first season and 41.62, 43.72, 43.44, 47.74 and 49.78 in the second season.

The positive increase in fruit set percentage of "Anna" apples (Table 2) by brassinolide could be attributed to its influence on promoting stem elongation, stronger attachment with the pedicel (Clouse et al. 1996: Haubrick and Assmann, 2006). Furthermore, the application of brassinolide at 0.01 ppm when applied at anthesis increased fruit set of Japanese persimmon by

76.2% and 70.60%, respectively (Suzuki et al. 1988: Watanabe et al. 1997)

3.2. Number of fruit retention/ spur at harvest:

The data in Table 2 showed that fruit retention percentage was significantly increased by using preharvest treatment with brassinolide.The concentration of 3, 4 mg/l were greater at harvest as compared with control and other brassinolide concentrations.

The increase in fruit retention at harvest may be due to improve photosynthetic process, increased fruit set, reduced fruit abscission and delayed senescence (Braun and Wild, 1984: Khripach et al. 1999: Gomes et al. 2003). In addition, brassinolides have been shown to increase fruit number per plant of passion fruit (Gomes et al. 2006).

3.3. Physical quality characteristics:

At harvest, the data in Table 3 illustrated that, there were no significant change in fruit length as a result of applying brassinoloide as compared with the control treatment except the application of 1 mg/l brassinoloide in both seasons of study. The changes of fruit diameter at harvest, in response to various brassinoloide treatments were reported in Table 3. The data indicated that there were no significant changes in fruit diameter as compared with the control treatment except the application of 3mg/l brassinoloide. The trend of data for fruit weight, fruit size and fruit firmness were similar to that obtained with fruit diameter in both seasons (Table 3). The results were consistent in both seasons of study. The role of brassinoloide in improving physical quality of “Anna” apples such as fruit diameter, fruit weight and fruit firmness could be attributed to a direct effect of brassinoloide on cell division, elongation, increased carbohydrate supply, prevention loss of pigments and alleviation stress conditions which result in better crop growth (Iwahori et al. 1990: Sasse, 1997; Musing, 2005; Hayat et al. 2010, 2012: Chai et al. 2013). Furthermore, Bhat et al. (2011) reported an increase in grape berry size, berry length, diameter, berry number and cluster weight by exogenous application of 0.4 mg/1 brassinoloide. Meanwhile, sweet cherry fruit-treated with brassinosteroid at 0.75 ppm increased fruit firmness (Roghabadi and Pakkish, 2014: Mandava and Wang 2016).

3.4. Chemical quality characteristics:

The data in Table 4 illustrated that there were no significant differences were observed in TSS of “Anna” apples at harvest especially in the second season. Moreover, brassinoloide treatments possessed greater acidity percentage as compared with control treatments. Also, the data indicated that there were no significant changes in total sugars percentage as compared with the control treatment except the application of 3mg/l brassinoloide possessed high percentage. Vitamin C percentages of “Anna” apples were significantly higher than those in the control, the application of brassinoloide at 3 mg/l possessed greater Vitamin C as compared with other concentration and control treatments. The data in Table 3 also indicated that brassinoloide application led to higher anthocyanin contents in the peel of “Anna” apples. brassinoloide at 3mg/l was greater than other brassinoloide concentrations and control treatments. The positive effect of brassinoloide on enhancing chemical quality of fruits was previously reported (Mandava and Wang, 2016: Vergara et al. 2018). Furthermore, preharvest brassinoloide application enhanced grape color, reducing the rate of soluble solids and titratable acidity degradation (Champa et al. 2015), increased ascorbic acid contents (Roghabadi and Pakkish 2014). Color improvement with brassinoloide treated fruits has been attributed to promote ethylene synthesis, enhanced chlorophyll enzyme degradation and carotenoid accumulation without impairing fruit quality (Schlagnhaufer *et al*. 1984: Zhu et al. 2010: Zaharah and Singh, 2010). Preharvest application of 24-epibrassinolide increased anthocyanin contents in grape berries, phenolics and antioxidant activity (Xi et al. 2013: Xu et al. 2015: Luan et al. 2016).

PAL and CHI gene expression:

The ratio of PAL and CHI genes expression was quantified in in apple fruits treated with BR concentrations in two season using quantitative real-time PCR.Expression profile of these genes (Fig. 1 and 2).Expression of PAL gene were induced by all applied treatments with different ratio expression levels among treatments compared with untreated control.Fig 1 showed that the level of PAL gene expression in apple fruits treated with BR concentrations in two season. the greatest expression level was recorded on the treatment with concentrate 4 mg(T5) compared to expression levels of control, followed by concentrate 3 mg(T4), while the low PAL expression level was recorded on the treatment with concentrate 1 mg(T2)compared to control in both season.The level of PAL expression was slightly increase in season two in

all treatments compared with season one.

Table 2: Effect of various preharvest applied concentrations of Brassinolide on fruit set percentage and number of fruit retention/ spur of "Anna" apples during 2019 and 2020, seasons

Treatments	Fruit set %		Number of fruit retention/ spur at harvest	
	2019	2020	2019	2020
Control	42.33b	41.62b	1.73c	1.73c
1 mg/ L Brassinolide	42.96b	43.72ab	1.73c	1.87c
2 mg/ L Brassinolide	51.33a	43.44ab	1.93bc	1.87c
3 mg/ L Brassinolide	56.79a	47.74ab	2.07b	2.27b
4 mg/ L Brassinolide	55.49a	49.78a	2.93a	2.87a

*Values, within each column, of similar letter (s) were not significantly different according to the least significant difference (LSD) at 0.05 levels.

Table 3: Effect of various preharvest applied concentrations of Brassinolide on physical quality of "Anna" apples during 2019 and 2020, seasons

Treatments	Fruit length (mm)		Fruit diameter (mm)		Fruit weight (g)		Fruit volume (cm^3)		Firmness (lb/in^2)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Control	68.84b	69.54b	65.50b	66.48b	143.54b	143.40b	172.67b	180.33c	10.03abc	9.60b
1 mg/ L Brassinolide	73.21a	73.08a	66.37b	66.65b	150.66b	152.30ab	188.33a	190.33b	9.93c	9.77b
2 mg/ L Brassinolide	68.79b	68.22b	65.68b	66.02b	144.19b	149.68ab	185.33a	185.33bc	10.00bc	9.70b
3 mg/ L Brassinolide	68.53b	68.65b	72.64a	72.47a	163.43a	155.66a	190.00a	198.00a	10.17a	10.50a
4 mg/ L Brassinolide	69.99ab	70.18b	67.43b	67.96b	147.05b	147.30ab	184.67a	185.67bc	10.10ab	9.76b

*Values, within each column, of similar letter (s) were not significantly different according to the least significant difference (LSD) at 0.05 levels.

Table 4: Effect of various preharvest applied concentrations of Brassinolide on chemical quality of "Anna" apples during 2019 and 2020, seasons

Treatments	TSS %		Acidity %		Total sugars %		Vitamin C (mg/100ml)		Anthocyanin (mg/100g)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Control	12.53b	12.30a	0.88d	0.84b	9.07bc	9.13c	8.51d	8.98c	9.62c	10.98d
1 mg/ L Brassinolide	12.57ab	12.10a	0.903c	0.88b	8.97c	9.27bc	8.84cd	10.12b	11.05b	11.69bc
2 mg/ L Brassinolide	12.50b	12.20a	0.903c	0.93a	9.03bc	9.20bc	9.18c	10.38b	11.47b	11.98b
3 mg/ L Brassinolide	12.80a	12.20a	0.923a	0.95a	9.30a	9.57a	10.72a	11.26a	12.05a	12.97a
4 mg/ L Brassinolide	12.70ab	12.30a	0.913b	0.95a	9.20ab	9.37b	9.98b	10.99a	11.05b	11.40cd

*Values, within each column, of similar letter (s) were not significantly different according to the least significant difference (LSD) at 0.05 levels.

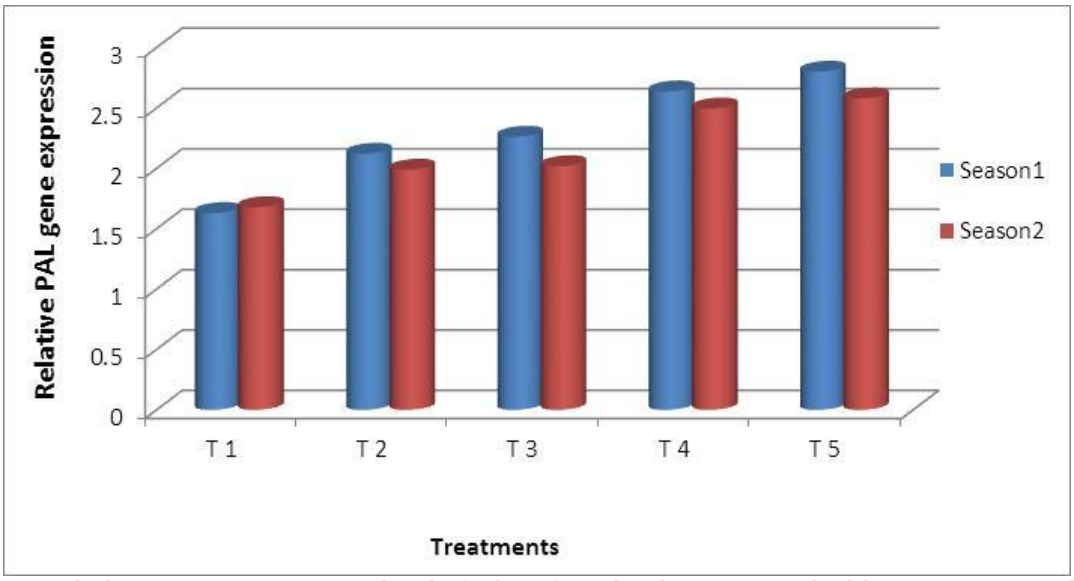

Figure 1: Relative PAL gene expression in fruits of apple plants treated with BR concentrations in two season. Untreated plants (T1), treated with concentrate 1mg (T2), treated with concentrate 2mg (T3), treated with concentrate 3mg (T4) and treated with concentrate 4mg (T5). The expression level of the target genes were normalized relative to 18s gene and relative expression of untreated control plants at each treatment. Each value represents mean ± S.E (n = 3). LSD $_{0.05}$ (Treat.) = 0.07

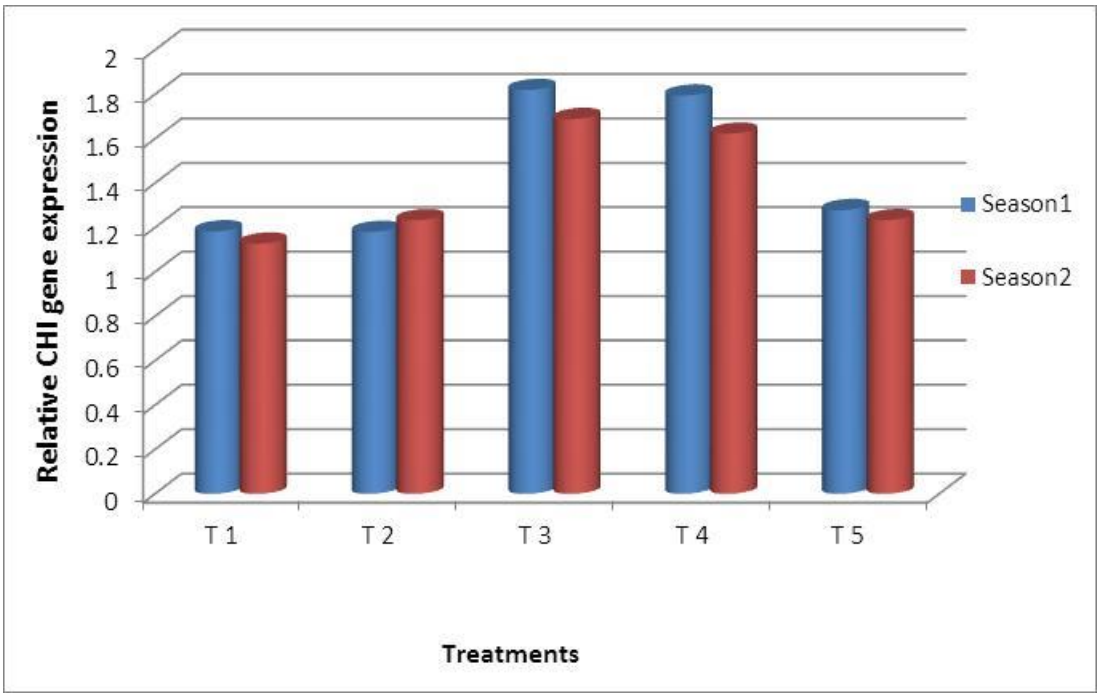

Figure 2: Relative CHI gene expression in fruits of apple plants treated with BR concentrations in two season. *Untreated plants (T1), treated with concentrate 1mg (T2), treated with concentrate 2mg (T3), treated with concentrate 3mg (T4) and treated with concentrate 4mg (T5).* The expression level of the target genes were normalized relative to 18s gene and ratio expression of untreated control plants at each treatment. Each value represents mean ± S.E (n = 3). LSD$_{0.05}$ (Treat.) = 0.10

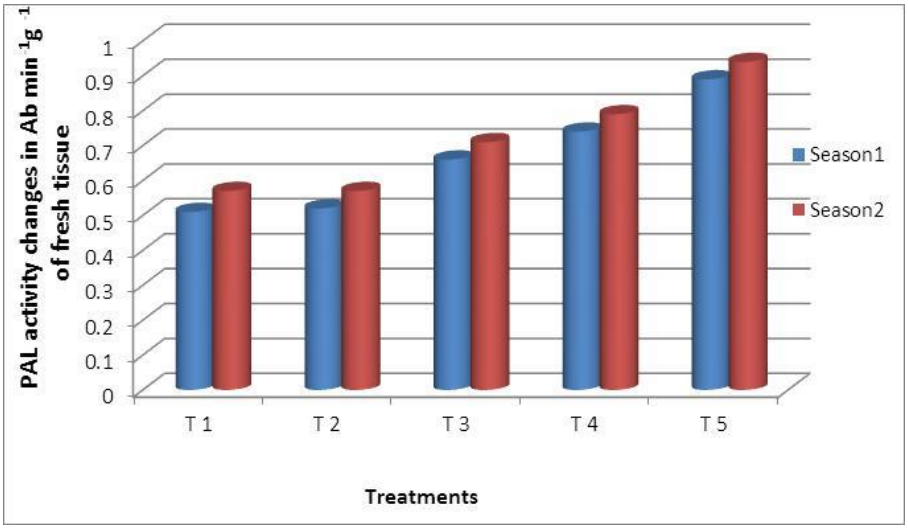

Figure 3. Activities of PAL (Phenylalanine ammonia-lyase) in apple fruits plants treated with BR concentrations in two season. *Untreated plants (T1),* treated with concentrate *1mM* (T2), treated with concentrate *1mM* (T3), treated with concentrate *1mM* (T4) and treated with concentrate *1mM* (T5). Values are the mean of three replicates ± SE. * Values are means of three replications. $LSD_{0.05}$ (Treat.) = 0.04

Fig 2 showed that the level of CHI gene expression in apple fruits treated with BR concentrations in two seasons. The high level of CHI gene expression was recorded on the treatment with concentrate 2 mg (T3) compared to expression levels of control, while the level of CHI gene expression was decreased with increasing of BR concentrations. The level of gene expression was decreased gradually at concentrate 3 mg(T4) then the concentrate 4 mg(T5) in both season. The level of CHI gene expression was slightly increased in season two in all treatments. RT-qPCR using gene-specific primer showed that all CHI gene family were expressed a in 'Royal Gala' leaves. CHI expression was also observed in three apple cultivars tested: 'Red Delicious', 'Holly' and'Camoesa de Llobregat'. In contrast, expression of the two Type I PcCHI genes in pear cultivars 'Comice', 'Bartlett', 'Cangxili' and 'Syhue' was significantly higher than in apple (Dare et al. 2020).

The results illustrated in Figure (Fig. 3) showed that PAL enzyme activity in apple fruits treated with BR concentrations in two seasons. The treated with concentrate 4 mg(T5) was high increase of activity, followed by the concentrate 3 mg(T4), while the low PAL activity was observed at the low concentrate 1 mg(T2) compared with the control (T1) in both season. The concentrate 2 mg (T3) was moderately enzyme activity. The result of PAL enzyme activity in season two was slightly increased in all treatments compared with season one.

Anthocyanin synthesis is a process that involves many steps (Lancaster et al. 1992). PAL, CHI, CHS, F3H, DFR, ANS and UFGT are closely related with anthocyanin biosynthesis in apple (Wang et al. 2000, Lister and Lancaster, 1996 and Ban et al. 2009). PAL activity has been reported to positively correlate with anthocyanin synthesis in grapes, strawberries and apples, but its role in regulating anthocyanin formation in apples remains controversial (Ju, 1998, Ritenour, et al. 1997, Kim et al. 2003, Cheng and Breen, 1991, 33, 34). In this study the results illustrated in Figure (Fig. 3) showed that PAL enzyme activity in apple fruits treated with BR concentrations in two seasons. The treated with concentrate 4 mg(T5) was high increase of activity, followed by the concentrate 3 mg(T4), while the low PAL activity was observed at the low concentrate 1mg(T2) compared with the control (T1) in both season. The concentrate 2mg (T3) was moderately enzyme activity. The result of PAL enzyme activity in season two was slightly increased in all treatments compared with season one.

CONCLUSION

Our study concluded that preharvest foliar application of brassinoloide at 3 or 4 ppm enhanced physical and chemical characteristics of "Anna" apples such as fruit set, fruit retention,

diameter, weight and color.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Said Attia did the experimental treatments, determination of growth parameters, wrote the Manuscript and reviewed the article. Ibrahim Adss did the determination of enzyme activity and genes expression, wrote the Manuscript, and reviewed the article.

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