

## Full Length Research Paper

# Free endogenous growth regulators in Pistachio (*Pistacia vera* L.)

Yesim Okay\*, Nurdan Tuna Güne and A. İlhami Köksal

Department of Horticulture, Faculty of Agriculture, Ankara University, 06110 Ankara, Turkey.

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This research was carried out in order to observe seasonal changes of internal plant growth regulators in pistachio. This research was conducted during three years at a commercial pistachio orchard located in Fırat valley in Nizip, Gaziantep. 'Kırmızı' pistachio cultivar trees grafted on *Pistacia vera* L. were used as plant material. Changes in auxin (IAA), cytokinin (Zeatin), gibberellin (GA<sub>3</sub>) and abscisic acid (ABA) were observed in leaves in May, July and September of the first (heavy cropping year - 'on'-year) and second (subsequent light cropping year 'off'-year) years. Besides, yield and flower bud abscission rate were observed during three years to determine the relationships among the level of plant growth regulator, yield and flower bud abscission. It was observed that the IAA, zeatin and ABA levels in the leaf were high in the first year, and was low in the second year. The annual GA<sub>3</sub> levels were lower in the 'on' year and higher in the alternate bearing, 'off' year. The correlation analysis between the hormones and fruit bud abscission rates showed that increase in the ABA levels caused also increase in fruit bud abscission at all months in the first year.

**Key words:** Auxin, zeatin, gibberellic acid, abscisic acid, pistachio.

## INTRODUCTION

Pistachio nut is a common fruit species grown especially in the South East Anatolia of Turkey which is the third important pistachio producer after Iran and USA in the world. In Turkey, low and irregular yield are major problems due to alternate bearing of trees and insufficient cultural managements (Ak and Parlakçı, 2006).

In pistachio growing, alternate bearing is an undesirable physiological character (Johnson and Weinbaum, 1987), severely affecting yield and as a result management factors such as cash flow, labor needs etc. A general characteristic in species which tend to alternate bearing is that flower buds do not occur the next year, hence yield is insufficient, because of heavy fruit load in previous year, 'on' year (Monselise and Goldschmidt, 1982).

It has been reported that alternate bearing mechanism in pistachio is different from other fruit species such that although the tree had many flower buds for the next year they have dropped off during summer period suggesting that the main reason for alternate bearing in this species

is the drop off flower buds (Crane and Nelson, 1971; Crane et al., 1973; Porlingis, 1974; Crane and Iwakiri, 1985; Barone et al., 1995).

However the actual mechanism in alternate bearing is still not sufficiently clear in pistachio. Genetic factors (Esmailpour and Khezri, 2006), some environmental and physiological factors, cultural managements (Ferguson et al., 1995), carbohydrate and nitrogen balance of the tree (Crane and Al-shalan, 1974, 1977; Crane et al., 1976, 1982; Nzima et al., 1977; Weinbaum et al., 1994; Boler, 1998; Vemmos, 1999a; Ünlü et al., 2005; Baninasab and Rahemi, 2006), plant nutrients (Brown et al., 1995; Picchioni et al., 1997; Rosecrane et al., 1998; Zeng et al., 1998; Vemmos, 1999b; Zeng and Brown, 1999), rootstock (Crane and Iwakiri, 1986) and plant growth regulators (Gaspar et al., 1985; Ferguson and Maranto, 1989; Lavee, 1989; Lovatt and Ferguson, 1998, 2001) are all associated with alternate bearing. However, the level of the effects of these factors is still unclear.

Previous studies report that some physiological events such as flower bud and embryo formation, development, bud abscission, fruit set and growth is regulated via plant growth regulators (Takeda and Crane, 1980; Marine and Greene, 1981; Chen, 1987; Vemmos et al., 1994).

\*Corresponding author. E-mail: [okay@agri.ankara.edu.tr](mailto:okay@agri.ankara.edu.tr). Tel: +90 312 5961319. Fax: +90 312 3179119.

Moreover it has been mentioned that the availability of plant growth regulators in plant tissues affect the occurrence and also severity of alternate bearing (Yılmaz, 1990; Westwood, 1993).

Five groups of natural growth regulators including three auxins, several cytokinins, many gibberellins, abscisic acid and ethylene are believed to play an important role in plant physiology. Among these, auxins, gibberellins and cytokinins are known as stimulators, and others are known as growth retardants (Westwood, 1993; Davies, 2004). The primary natural auxin, indole-3-acetic acid, is produced mostly in subapical regions of actively growing shoots, young leaves, and developing embryos. It controls cell enlargement by affecting the extensibility of the cell wall and may induce or retard abscission of mature fruit. Gibberellins are produced mainly in very young leaf and embryos, and in roots. They function in cell elongation, aid in breaking rest of seeds and dormant buds, prevent flower initiation and seem to interact with auxin to prevent abscission of young fruits. Cytokinins are responsible for the regulation of nucleic acids, apical dominance, stimulating bud initiation, enhancing seed germination, influencing transport of nutrients and metabolites, inhibiting abscission and flower, fruit and leaf senescence, and root initiation (Westwood, 1993). Occurrence, changes in their levels or interactions among these growth regulators in fruit trees change during vegetation period. Moreover, they are synthesized at different times.

In many studies, plant growth regulators were externally applied in pistachio in order to obtain regular yield, increase flower bud formation and prevent flower bud abscission (Takeda and Crane, 1980; Lin et al., 1984a, b; Ferguson and Maranto, 1989; Pontikis, 1990; Lovatt, 1996; Lovatt and Ferguson, 1998, 2001; Acar et al., 2006; Lovatt et al., 2006). But effective results could not be obtained in most of them (Ülger, 1997) because of insufficient data on the change of endogenous plant growth regulators in this species. Therefore in this research, it was aimed to observe seasonal changes in endogenous plant growth regulators in pistachio. Additionally, this research may help further studies to be performed on how cultural applications such as irrigation, fertilization, pruning affect changes in plant regulators and help to express the effect of endogenous growth regulators on yield and fruit quality.

## MATERIALS AND METHODS

This research was conducted during three years in a commercial pistachio orchard located in Firat valley in Nizip, Gaziantep in which pistachio is the mostly produced species. During the research period, annual precipitation in the region was 48.38 mm and precipitation values were higher than 60 mm from November to April. The average relative humidity and annual average temperature was 55.42% and 16.44°C, respectively (Anonymous, 2009). Pistachios were grown by using traditional methods, common in this region. The plant material was 'Kırmızı' pistachio

cultivar trees of 35 years old and grafted on *Pistacia vera* L. At the beginning of this research, leaf and soil samples were analyzed for nitrogen and phosphorus content (Jackson, 1960; Bremner, 1965), texture characteristics (Bouyoucos, 1951), pH and CaCO<sub>3</sub> content (Jackson, 1960), plant available phosphorus (Olsen et al., 1954) and total nitrogen (Bremner, 1965).

During experimental period, flooding irrigation was applied from the second half of July with 20 days intervals by using ca. 450 mm of water as in Kanber et al. (1993) for whole irrigation period. All trees were pruned according to traditional pruning technique. Fertilization was not performed.

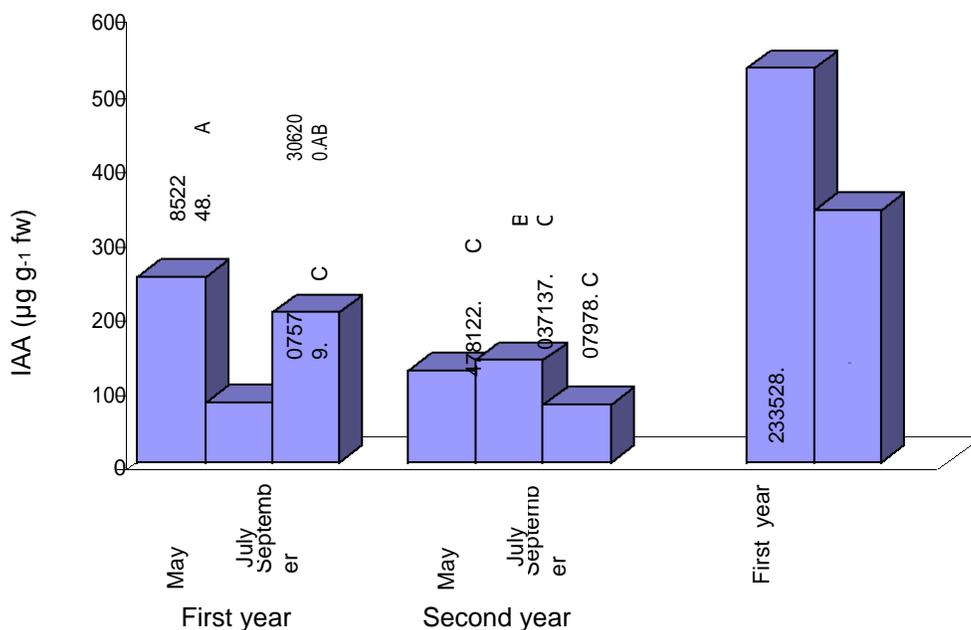
Changes in free endogenous growth regulators such as indole-3-acetic acid (IAA), zeatin, gibberellic acid (GA<sub>3</sub>) and abscisic acid (ABA) were analyzed in leaf samples taken from May, July and September of the first (heavy cropping, 'on' year) and second subsequent light cropping ('off' year). Besides, yield and flower bud abscission rate were observed during three years in order to determine the relationships among plant growth regulator amounts, yield and flower bud abscission. For flower bud abscission rates, flower buds were marked at the beginning of the vegetation period on four directions of each tree and were counted at the beginning of June and also in November. In this research, yield per tree (kg<sup>1</sup> per tree) was determined by weighing of fresh and red-unshelled nuts (Ak and Ka ka, 1992).

## Analyses of free endogenous growth regulators

In May, July and September of the yield (1<sup>st</sup> year) and periodicity (2<sup>nd</sup> year) years leaf samples were taken and, frozen in liquid nitrogen and kept at -80 C up to the extraction stages. In these samples, auxin, zeatin, gibberellic acid and abscisic acid content were determined with three steps such as extraction from plant tissues, separation by thin layer chromatography and quantification by high performance liquid chromatography (Gunes et al., 2010).

1 g fresh weight of leaf tissue was weighed and put in amber bottles with cover. 20 ml extraction solvent (methanol:chloroform:2 N ammonium hydroxide, 12:5:3, V:V:V, % 0.001 BHT) was added and bottles wrapped in aluminum foil while cover tightly closed to protect the hormones from the light and were kept at -18°C for the first week. Extraction solvent was transferred to other bottles, 40 ml extraction solvent was added and both of the bottles were kept at the same conditions for the second week. Extraction solvent was filtered and 60 ml extraction solvent (combined extract) were kept at the same conditions for the third week. Combined extract were transferred to the separation funnel, 25 ml distilled water was added, shaken vigorously, the aqueous methanol phase extract on top of the balloon was taken. All solvent substances in extraction bubble was evaporated at 45°C under vacuum until the water phase is remained. The remaining water phase was adjusted to pH 2.5 using 0.1 N HCl. Extraction process was performed three times by adding 15 ml ethyl acetate each time. These phases included the free form of IAA, GA<sub>3</sub> and ABA. The remaining water phase was adjusted to pH 7 using 1 N NaOH. Extraction process was done three times by adding 15 ml of ethyl acetate each time and ethyl acetate phases were collected. These phases included the free form of zeatin. Ethyl acetate phase which includes all ethyl acetate phases, free forms of IAA, zeatin, GA<sub>3</sub> and ABA was evaporated at 45°C under vacuum. Residue was dissolved in 1 ml methanol in the dark and stored at -18°C until run through thin layer chromatography.

For the separation of each endogenous growth regulator, 20 µl was taken from the extract dissolved in methanol, run through thin layers for 8 h with isopropanol:2 N ammonium hydroxide: distilled water (84:8:8) used as the carrier phase and each hormone was determined according to R<sub>f</sub> value under UV light. Consequently, hormone bands were excised and dissolved in 1ml methanol, maintained at -18°C in the dark until HPLC analysis.



**Figure 1.** The indole acetic acid amounts determined in the 1<sup>st</sup> ('on' year) and 2<sup>nd</sup> ('off' year) of the research (µg g<sup>-1</sup> fw).

Free endogenous growth regulator contents in pistachio leaves were determined with HPLC equipped with UV detector (Bio Rad UV-1806) on C<sub>18</sub> column (Phenomenex Luna, 5 µ). Each hormone was identified at different wavelengths (Gunes et al., 2010). The samples were filtered through 0.45 µm filters (Millipore, SLGV013NL) prior to analysis and a volume of 20 µl sample was injected for the analysis. The results were quantified as µg g<sup>-1</sup> fresh weight according to the external standards for each growth regulator. All the analysis were carried out at room temperature. Identification of the peaks was done according to the retention times of external standards for each hormone such as indole-3-acetic acid (IAA), gibberellic acid (GA<sub>3</sub>), zeatin and abscisic acid (ABA) and was quantified in comparison with peak areas of the external standards. External standards used in this study were IAA (Sigma, I-2886), GA<sub>3</sub>, (Sigma, G-7645), zeatin (Sigma, Z-0164) and ABA (Sigma, A-1049).

#### Statistical analyses

This research was planned as a randomized block design. Multifactorial variance analysis (ANOVA) was performed on the data by Minitab Software (MINITAB Inc.) and data in each year were separately evaluated. Nitrogen and phosphorus rates were taken into consideration as variables for all parameters, apart from hormones. Besides these variables, sampling months were evaluated for hormone concentrations. Means were compared by Duncan's Multiple Range Test (P 0.05) in MSTAT-C programme. Arcsin transformations were used for all percentage data.

## RESULTS

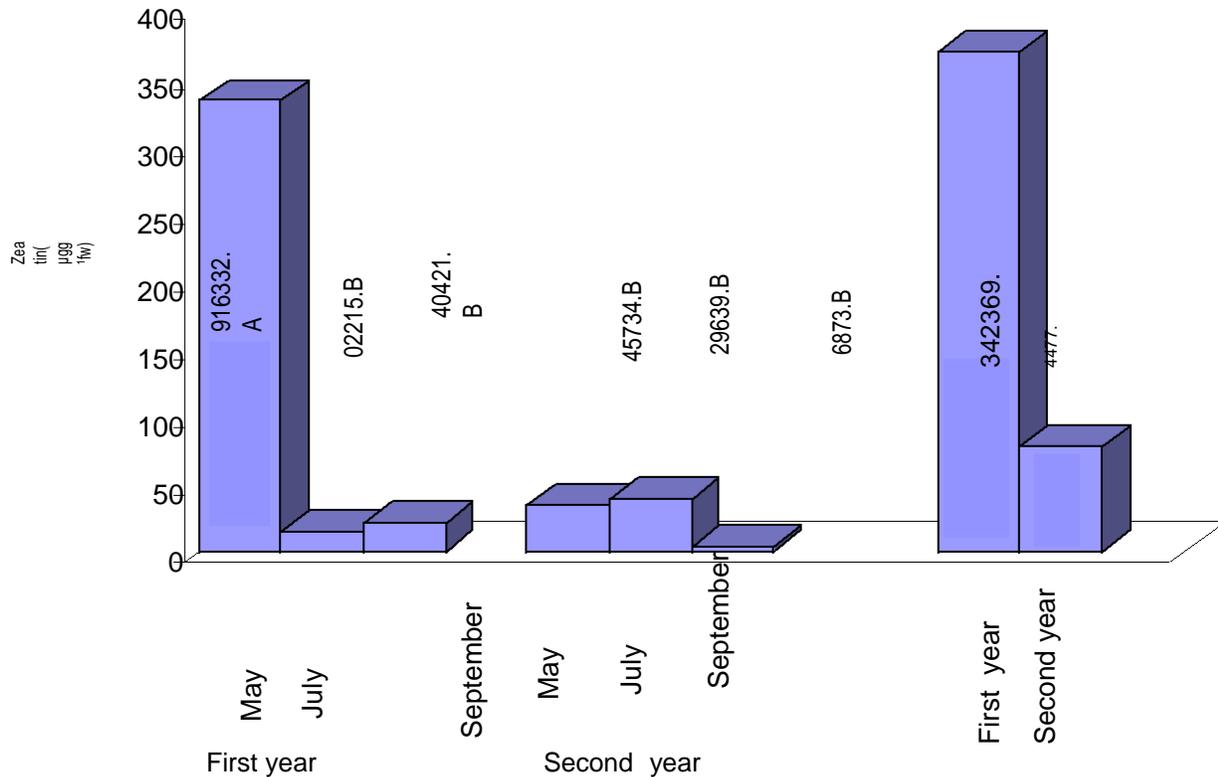
The soil characteristic of experimental area is suitable for cultivation of pistachios, salt, lime does not pose problems in terms of such factors have been identified.

#### The amounts of indole acetic acid, zeatin, gibberellin and abscisic acid (µg g<sup>-1</sup>)

The differences between IAA, zeatin, GA<sub>3</sub> and ABA amounts in the leaf samples taken in May, July and September of the first ('on' year) and second ('off' year) year and the dates on which the samples taken were significant. The differences between hormone concentrations at the same date and different date were evaluated separately (LSD = 70.63).

It was observed that the IAA level (248.852 mg g<sup>-1</sup>) was highest in May at the 1<sup>st</sup> year, decreased in July (79.075 mg g<sup>-1</sup>) and again increased in September (200.306 mg g<sup>-1</sup>). In the second year of experiments, IAA level increased in May (122.478 mg g<sup>-1</sup>) and in July (137.037 mg g<sup>-1</sup>), and decreased in September (78.079 mg g<sup>-1</sup>). The differences between the IAA levels at these two years were not significant (P<0.05). When the total annual IAA level was evaluated, it was observed that the IAA value in the leaf was higher in the first year than in the second year (Figure1).

The zeatin levels of the leaf also showed the same tendency as in IAA levels with lower values. The variation at the efficiency and alternate bearing years were different. The highest zeatin level (332.916 mg g<sup>-1</sup>) was recorded in May and decreased to 15.02 mg g<sup>-1</sup> in July. It was also low in September (21.404 mg g<sup>-1</sup>). The zeatin level increased in May (34.457 mg g<sup>-1</sup>) and July (39.296 mg g<sup>-1</sup>) in the following alternate bearing year but it was low in September (3.687 mg g<sup>-1</sup>). The zeatin levels recorded for three months were grouped together



**Figure 2.** The zeatin amounts determined in the 1<sup>st</sup> ('on' year) and 2<sup>nd</sup> ('off' year) of the research ( $\mu\text{g g}^{-1} \text{fw}$ ).

statistically. The total annual zeatin amounts were higher in the 'on' year than in the 'off' year (Figure 2).

The GA<sub>3</sub> levels showed the same trend in the following two years. The GA<sub>3</sub> level was determined as 462,916 mg g<sup>-1</sup> in the 1<sup>st</sup> ('on') year in May; decreased in July (297,496 mg g<sup>-1</sup>) and increased in September (815,562 mg g<sup>-1</sup>). Similar changes were observed in the 2<sup>nd</sup> ('off') year. In both years, changes in the GA<sub>3</sub> levels were significant for all months. In contrast to changes in IAA and zeatin levels, the annual GA<sub>3</sub> levels were lower in the 'on' year than in the alternate bearing, 'off' year (Figure 3).

The ABA showed different changes in the 'on' and 'off' years. The ABA was at the highest level in the 'on' year in May (121.683 mg g<sup>-1</sup>), showed a regular decrease (in turn 55.038 and 27.715 mg g<sup>-1</sup>). The ABA which was determined at the lowest level (6.496 mg g<sup>-1</sup>) in May in the following alternate bearing year, increased to the highest level in July (126.087 mg g<sup>-1</sup>), and decreased to the lowest level in September (6.565 mg g<sup>-1</sup>). The ABA amount was high in the efficiency year, and low in the alternate bearing year (Figure 4).

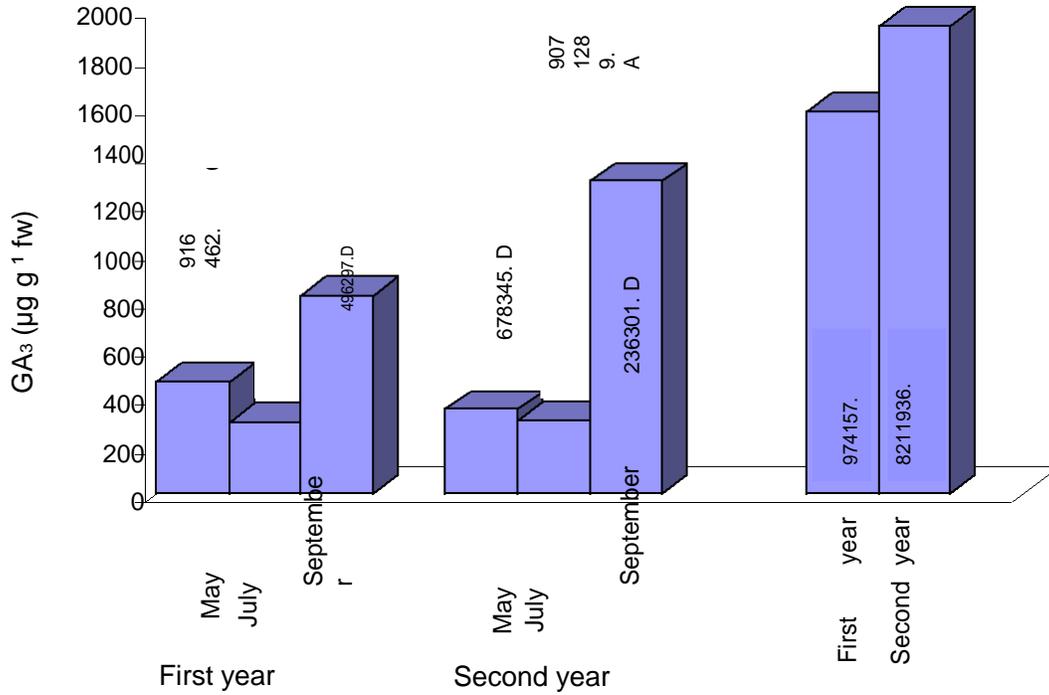
When the differences between the hormone levels were determined in the same sample taking date, it was determined that the hormones showed the same ranking in the 'on' and 'off' years. According to this, the GA<sub>3</sub> and IAA amounts of the leaves were high. This redundancy was found to be statistically important. At both years, the

ABA amount was higher than that of zeatin. This redundancy is not important in the efficiency year for July, but is important in the alternate bearing year. In September the difference between ABA and zeatin amounts were not significant. When the values of May, July and September were analysed, it was seen that the auxin (IAA) and cytokinin (zeatin) levels decreased in July and September. The gibberellin (GA<sub>3</sub>) amount increased in July. In the second year of the research in which the alternate bearings was seen, especially the IAA and zeatin amounts were at lower levels (Figure 5).

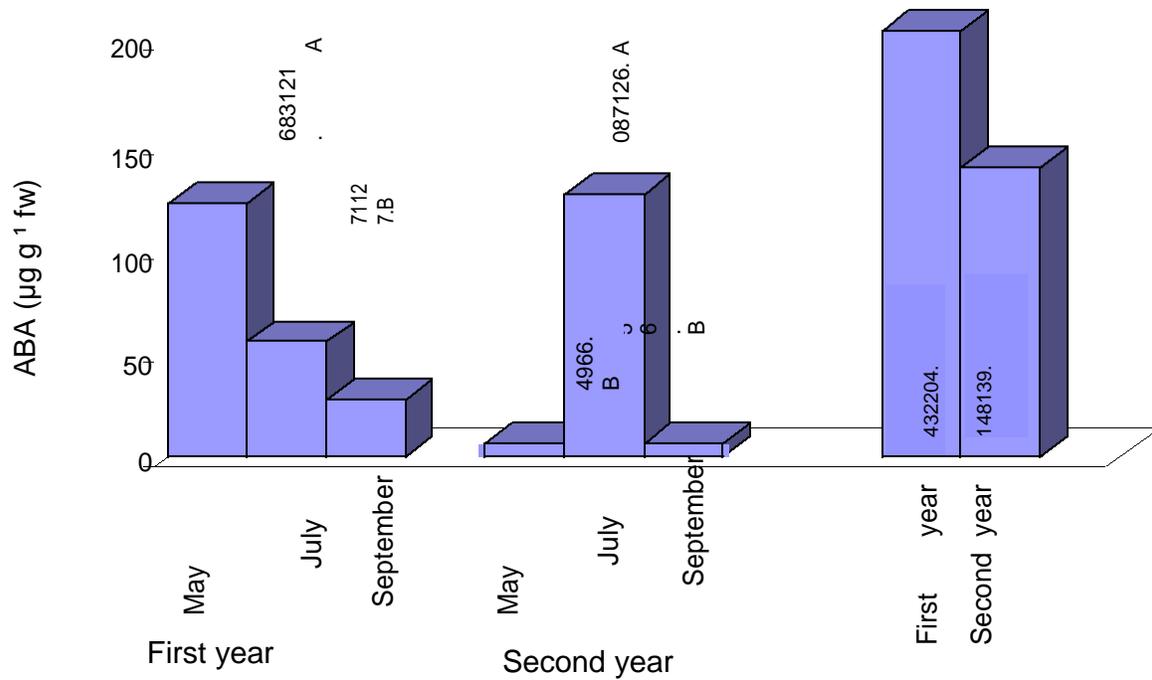
### **The correlations with yield (kg tree<sup>-1</sup>), fruit bud abscission rates (%) and growth regulator amounts (mg g<sup>-1</sup>)**

The differences between the yield and fruit bud abscission rates in the years when the research was conducted were found to be statistically unimportant. The efficiency determined was low and the fruit bud abscission rate was found high in the alternate bearing year of the research (Table 1).

The correlation between the IAA, zeatin, GA<sub>3</sub> and ABA amounts and the efficiency values of these years determined in the leaves were found unimportant. When the correlations between the hormones and fruit bud abscission rates were analyzed only some of the



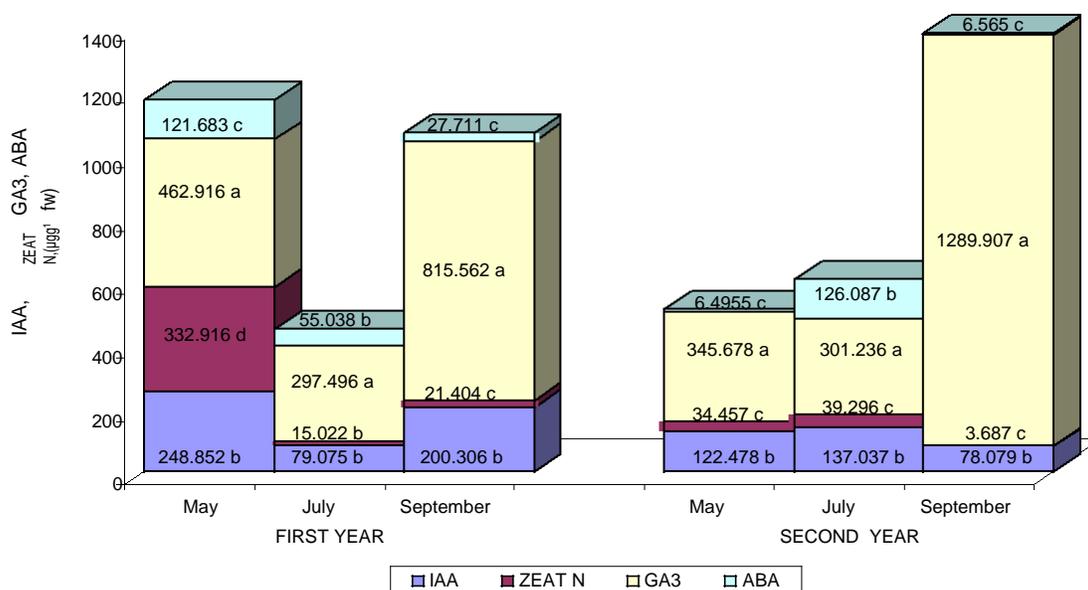
**Figure 3.** The gibberellin amounts determined in the 1<sup>st</sup> ('on' year) and 2<sup>nd</sup> ('off' year) of the research (µg g<sup>-1</sup> fw).



**Figure 4.** The abscisic acid amounts determined in the 1<sup>st</sup> ('on' year) and 2<sup>nd</sup> ('off' year) of the research (µg g<sup>-1</sup> fw).

correlations in the first year of the research were significant ( $r = 0.998$ ). The direct relationship between fruit bud abscission and hormone amounts of leaves in

the second year of research was not significant. Accordingly, the IAA amount of the leaves in March and July in the first year of the research, when the zeatin



**Figure 5.** The indole acetic acid, zeatin, gibberellin and abscisic acid amounts determined in the 1<sup>st</sup> ('on' year) and 2<sup>nd</sup> ('off' year) of the research ( $\mu\text{g g}^{-1}\text{ fw}$ ).

**Table 1.** The correlations with efficiency ( $\text{kg tree}^{-1}$ ), fruit bud abscission rates (%) and hormone amounts.

Yield and flower bud abscission rates			
Experimental years	Yield ( $\text{kg}^{-1}$ per tree)	Flower bud abscission (%)	
1 <sup>st</sup> ('on' year)	2,1	10	
2 <sup>nd</sup> ('off' year)	1,5	12,5	
3 <sup>th</sup> ('on' year)	3,91	11,11	

Flower bud abscission rates and hormone amounts			
	First year		
	May	July	September
IAA	+	+	-
Zeatin	-	+	-
GA <sub>3</sub>	-	-	+
ABA	+	+	+

amount in July, and the GA<sub>3</sub> amount in September increases the fruit bud abscission rates increased. When the zeatin and GA<sub>3</sub> amounts in May, GA<sub>3</sub> amount in July, IAA and zeatin amounts in September increase, the fruit bud abscission rates decreased. When the ABA amounts increase in all the months of the first year the fruit bud abscission rates increased (Table 1).

## DISCUSSION

The flower buds on the pistachios have been developed in May of the previous year. Flowering changes according to the weather conditions occurs in the first

period of April and May, there is a 20 to 28 h period between the inflorescence and fertilization. The period in which a rapid development occurs in the pericarp and after 5 to 7 days of the inflorescence rests is called the 1<sup>st</sup> development period. At the end of this period, the 2<sup>nd</sup> development period, the pericarp development slows down and the embryo develops rapidly from the middle of June to the beginning of August. It has been stated that the embryo develops rapidly after 6 to 8 weeks of the pollination and the grain reaches its total size at the 16<sup>th</sup> week and it matures after 4 to 6 weeks (Whitehouse et al., 1964; Ayfer, 1967; Lin et al., 1984c; Ayfer et al., 1990).

It has been determined that a (S) type growth curve

has been seen in the fruit and embryos, and that the embryo development of the pistachio starts at the end of June and at the beginning of July (Köro lu and Köksal, 1999). Four months after pollination, the fruits fill the shell and they are harvested (Kuru et al., 1988; Tekin et al., 2001). According to this, it is understandable that the ABA amount decreases in May, July and September in which the development and maturation in the plants continues. In the I and II growth periods of pistachio especially in the hypertrophy and filling periods, the substances stimulating the growth are too much. The GA<sub>3</sub> amount increased towards the fruit maturation (Figure 5). It has been stated in the other researches that, the GA<sub>3</sub> and similar components have been increased until the development phase of the fruit (Lilov and Christov, 1978; Gaspar et al., 1985).

Eri (1985) has reported that the auxin amount is in a positive relationship with the meristematic activities, that the amount in the plant changes according to seasons and the amount is at the maximum level at the beginning of the vegetation. In our research it has also been determined that the IAA and GA<sub>3</sub> amounts were high in May in which the active development continues, and the ABA and zeatin amounts were found low. Almost at all months of the two years in our research, the GA<sub>3</sub> levels were found high and IAA amounts followed this. During the trials, the ABA and zeatin amounts were determined at lower levels. As a result, it has been thought that the months samples were taken to have played a role in the period in which the active growth and developments continues. Our findings, are in agreement with hormone functions which have been synthesized in the plant and with the growth periods of the pistachio fruits (Salisbury and Ross, 1991; Westwood, 1993; Davies, 2004).

In a different research in which the hormone amounts of the fruit, leaves and flower buds of the pistachio were analyzed (Çetinkaya, 2004), it has been reported that the IAA level in June in the leaves has increased in July, and has decreased in August and the IAA amount in the fruit bud has reached the highest level in June and the lowest level in July. In contrast to our findings the IAA amount in the leaves is high in the year in which efficiency is low, and is low in the year in which efficiency is high. Despite this, in the year in which efficiency is high more IAA has accumulated in the fruit bud, and less IAA has been accumulated in the year in which efficiency is low. It has been determined that the IAA is in a relation with efficiency and has an effect on alternate bearing, and the IAA amount is higher in the leaves. It has been thought that the IAA synthesized in the leaves head towards the fruit buds, and so that the pouring increases. The GA<sub>3</sub> amount in the leaves has increased in July to August similar our findings (Figure 3). In the fruit filling phase, the GA<sub>3</sub> amount in the fruits increases in July, and the highest rates in the fruit bud has been reached in June, and the lowest rates has been reached in August. The ABA level in the leaves has increased in July, and decreased in

August. In our research a same curve has been seen in the

alternate bearing year (Figure 4). In the efficiency year a regular decrease in the ABA amounts of the leaves was observed from May to September. It can be concluded that the ABA had a significant relationship with alternate bearing, it played a role in the pouring of the fruit bud, it is much more synthesized in the efficiency year and head towards fruits and on the contrary lower ABA has been found in the flower bud. Lovatt and Ferguson (1998, 2001) have been determined that the pouring has been realized as a result of the ABA concentration increase in the flower buds.

The pouring of the fruit buds starts in June and increases in July when the filling of the fruit starts. The flower bud competes with the fruits in terms of carbohydrate nutrition and this has an effect on the alternate bearing. In the competence between fruit, fruit bud and leaf for using nutrition, fruits overrides. The buds and leaves could not be nourished enough and so that they form the source of the alternate bearing (Crane and Nelson, 1971; Crane et al., 1976; Çetinkaya, 2004). It was determined that the ABA amount was at the highest level in July in the alternate bearing year.

It is very clear that the hormones which prevent pouring produced by the leaves and the hormones produced by the fruit increasing pouring have an effect on the alternate bearing mechanism (Crane and Nelson, 1972; Crane et al., 1973; Navarro et al., 1990; Okuda, 2000; Çetinkaya, 2004). Thus a positive correlation was determined between the ABA amounts of the leaves and fruit bud abscission rates, the flower bud pouring rate was increased when the ABA amount was increased (Table 1). In addition to the separate amounts of the hormones carried by being synthesized in the different parts of the plant, their rates in compliance with each other and the fact that the plant is synthesized in which development period actively are also important (Ülger, 1977). Therefore, it is very important to know the seasonal changes of the plant growth regulators for the explanation of the physiologic mechanisms like formation of flower buds, flower and fruit pouring and alternate bearing. Especially, the successful external growth regulator applications made in the periods in which the related hormone synthesis is slow and low is required in terms of the determination of the effective application periods.

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