Adv. Hort. Sci., 2017 31(4): 319-327 DOI: 10.13128/ahs-20545

AHS
Advances in Horticultural Science

Phenolic metabolism and antioxidant activity during endodormancy of Kiwifruit buds

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Key words: *Actinidia deliciosa,* biochemistry, chilling, enzyme.

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Citation:
ABEDI GHESHLAGHI E., RABIEI V., GHASEMI M., FATTAHI J., RAZAVI F., 2017 - *Phenolic metabolism and antioxidant activity during endodormancy of Kiwifruit buds.* - Adv. Hort. Sci., 31(4): 319-327

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 16 April 2017
Accepted for publication 19 October 2017

Abstract: Bud dormancy is an adaptability process in woody plants that enables them to survive in unfavorable conditions. In the present study, the phenols, antioxidant capacity, and activity of three enzymes were evaluated during endodormancy phases in two Hayward and Tomuri cultivars and two female and male Golden genotypes of kiwifruit buds. The buds were collected from ten-year-old own-rooted vines from the end of October 2015 until the end of January 2016 in the north of Iran. The results revealed that phenols, antioxidant capacity (RSA), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) activities of buds significantly increased at the beginning of endodormancy and subsequently decreased at the end of the endodormancy. The POD activity increased in Hayward and Tomuri from the onset of endodormancy and continued for two weeks after the endodormancy release. The total phenol had a positive and significant correlation with RSA and PAL enzyme activity. Furthermore, higher antioxidant capacity and phenols in both male and female Golden genotypes were attributed to the higher PAL enzyme activity in both genotypes. This study proposes that the RSA%, PAL activity, and phenol concentration could be employed as a biomarker to indicate bud dormancy phases in kiwifruit.

1. Introduction

The first axillary buds are initiated on the developing shoots of kiwifruit shortly after bud break in the first growing season (Walton *et al.,* 1997). Similar to temperate fruits, the axillary buds of kiwifruit can be induced into endodormancy by short days and low temperatures at the end of the summer or the beginning of autumn (McPherson *et al.,* 1995).

Bud endodormancy is an adaptability process in woody plants that

enables trees to survive in unfavorable conditions such as drought and extremely hot and cold weather (Arora *et al.,* 2003); in addition, more importantly, it encourages the reproductive processes such as the formation of flowers and fruit set to be accomplished in favorable condition and guarantees the reproductive growth and survival of the plants (Campoy *et al.*, 2011). Endodormancy (winter dormancy) is a true dormancy in which the bud growth is prevented by an inhibitory system within the bud (Horvath *et al.,* 2003). Overcoming endodormancy and maximum bud break and flowering in favorable environmental conditions are achieved by accumulating the minimum amount of chilling hours (McPherson *et al.,* 1997). After that endodormancy is released, the growth of buds is prevented directly by external environmental factors. This type of dormancy mainly occurs in late winter and is named ecodormancy (Horvath *et al.,* 2003).

Generally, horticultural specialists determine the time of bud break by chilling and heat units. However, this method is based on ambient temperature, varies according to the environmental conditions, and cannot express the internal situation of buds (Dennis, 2003). During bud endodormancy, there is no visible growth, but physiological changes in respiration, growth regulators, carbohydrate metabolism, the amount of water, and other compounds occur, influencing bud endodormancy control (Ben Mohamed *et al.,* 2010). A series of the changes occurring in the biochemistry of the buds appear to indicate the shift from the endodormancy stage to the ecodormancy stage (Pakish *et al.,* 2009; Szecskó *et al.,* 2002). Richardson *et al.* (2010) stated that high and stable sucrose concentrations are likely to be a good indicator of the true dormancy of the buds in kiwifruit (*Actinidia deliciosa*).

Investigating the relationship between biochemical compounds and the beginning of the endodormancy in nine varieties of apricot revealed seasonal changes in phenolic compounds and peroxidase (Laslo and Vicas, 2012). These changes are caused by the accumulation of chilling units during the endodormancy, that is necessary for the development of some phenological stages. Pakish *et al.* (2009) studied peroxidase and oxidase activities in the varieties of pistachio buds during the winter and observed seasonal variation in the November-March interval.

There is a significant difference in terms of the phenols of buds in different dormancy stages within cultivars of the same species. The total phenol of the flowering buds in peach (Szalay *et al.,* 2005) and apricot (Laslo and Vicas, 2012) increased at the beginning of endodormancy, showed a gradual increase in dormancy period, and disappeared during flowering. Large variations in the content of polyphenols can be observed in certain varieties of pistachio (*Pistacia vera* L.) in the November-March interval. The phenol of all cultivars was reduced in the swollen buds (Pakish *et al.,* 2009).

Factors controlling the onset, maintenance, and termination of endodormancy are varied and have not been studied much (Luedeling *et al.*, 2009). Moreover, the starting point of endodormancy, the end of endodormancy, and the start of ecodormancy are not clearly defined in plants; therefore, the study of changes in biochemical compounds in this field could be useful (Pakish *et al.,* 2009; Ben Mohamed *et al.,* 2010). A number of studies have been done on the changes in carbohydrates (Richardson *et al.,* 2007, 2010), nitrogen, and amino acid (Walton *et al.*, 1991) of kiwifruit in the endodormancy period; however, the activities of enzymatic and non-enzymatic antioxidants such as phenol and its metabolism have not been studied yet. Thus, the aim of this study was to inspect the changes in antioxidant capacity, total phenol, and activity of three enzymes during endodormancy and the early ecodormancy in four cultivars and genotypes of kiwifruit.

2. Materials and Methods

Plant material and sampling

Bud samples of Hayward and Tomuri kiwifruit cultivars (*Actinidia deliciosa*) and two male and female Golden genotypes (*A. chinensis*; mass selection of Golden kiwifruit cultivar seedlings) were collected from canes of ten-year-old own-rooted vines from the end of October 2015 until the end of January 2016 with a 7-day interval at 11 steps. From the first to the last sampling, the vines received 0, 222, 309, 443, 570, 740, 864, 1003, 1125, 1272, and 1450 chilling units, respectively (Richardson *et al.,* 1974). Kiwifruit vines were located at the National Citrus and Subtropical Research Institute of Iran (latitude 75.36° North and longitude 33.51° East) and were trained on a T-bar training system with a planting distance of 4×6 m. The samples were immediately frozen in liquid nitrogen and kept for subsequent analyses at -80°C. At every sampling date, 30 buds were collected from 1-year-old canes at nodes 6 to 20 starting from the basal end of canes. Ten buds

were selected randomly in three replications for further analyses of biochemical compounds (Richardson *et al.*, 2010). The activities of phenylalanine ammonia-lyase (PAL), peroxides (POD), polyphenol oxidase (PPO), antioxidant capacity (RSA), and total phenol content were determined in buds during endodormancy.

Estimation of endodormancy period

Chilling requirements and bud endodormancy period of kiwifruit cultivars and genotypes were estimated via single-node cuttings test. Simultaneously, fifteen cuttings in three replications of each cultivar and genotype were collected and the buds were taken for biochemical measurements. Cuttings from each treatment were transferred into a forcing chamber at 25°C, with 16 h of light (Wall *et al.,* 2008). The lowest mean time budburst (MTB) for half of the buds were considered as an endodormancy release (Tisne-Agostini *et al.,* 1992).

Extraction and determination of total phenol and antioxidant capacity

The total phenol of each extract was determined according to the Folin-Ciocalteu procedure reported by Meyers *et al.* (2003). Moreover, the spectrophotometric method introduced by Wettasinghe and Shahidi (2000) was employed for the chemical determination of antioxidant.

Extraction and assay of PAL

One hundred mg of kiwifruit buds powdered by liquid nitrogen were mixed with 2 ml of 1.0 mM borate buffer containing 1.0% of polyvinyl pyrrolidone. After homogenization by a homogenizer (model IKA-T8, Germany), the samples were centrifuged for 15 min at 4°C and 13,000 rpm. The supernatants were slowly transferred into the tubes by pipette. Extracts for the ensuing measurements were maintained at -80°C. PAL (EC 4.3.1.24) activity was determined according to the study carried out by Yu *et al.* (2012).

Extraction and assay of PPO and POD activities

The two hundred-mg samples of fresh buds, which were collected, were ground in liquid nitrogen and homogenated with 2 ml of potassium phosphate buffer (50 mM, pH= 7.0) containing 1% polyvinyl pyrrolidone (PVP) (W/V) and 0.05% EDTA at 4°C. The homogenate was centrifuged at 14000 rpm for 15 min at 4°C. The supernatant was used as a crude enzyme solution for assay and was maintained at -80°C for the following measurements.

The activity of PPO enzyme (EC 1.14.18.1) was quantified by the method described by In *et al.* (2007). The activity of POD enzyme (E.C 1.11.1.7) was measured by the method employed by Srivastava *et al.* (1983). The activities of these enzymes were calculated, using the Beer-Lambert law on the basis of a single enzyme unit (µmol) per mg of fresh weight according to the following formula:

$$\text{U/g FWmin} = \text{absorption changes per minute} \times \text{reaction mixtur} / \text{supernatant volume} \times \text{extinction coefficient}$$

Statistical analyses

This study was conducted as a two-factor factorial in a completely randomized design. The first factor is 11 sampling dates, and the second factor is four cultivars having three replications in the period of 2015-2016. The ANOVAs and standard errors of the mean (SE) were generated, using SAS 9. All significant means were separated, using the Duncan ($P \leq 0.01$). The correlation between the total phenol and antioxidant capacity, PAL, PPO, and POD activities was calculated via the software SPSS 22.

3. Results and Discussion

The beginning date for the chilling accumulation was considered to be when a stable chilling accumulation occurred and the temperatures causing a negative effect were infrequent (Richardson *et al.,* 1974; Guerriero *et al.,* 1990). This date corresponded with 27th October 2015. The first samples were conducted on this date. Mean time budburst was more evident on this date than on the date of the endodormancy release; thus, the kiwifruit axillary buds may inter endodormancy sooner than the end of the summer or the beginning of autumn (McPherson *et al.,* 1995). The results revealed that the maximum depth of endodormancy in this study was in late November. The duration of bud endodormancy was different in the cultivars and genotypes. The end of endodormancy was 21st December 2015 for female Golden genotype (740-unit chilling), 28th December 2015 for male Golden genotype (864-unit chilling), and 4th January 2016 for Hayward and Tomuri cultivars (1003-unit chilling) as shown in figure 1.

The antioxidant capacity indicated a significant difference between the buds of genotypes and cultivars sampled on different dates ($P \leq 0.01$). The highest value in the antioxidant capacity was observed in female Golden genotype on 28th December 2015 (Fig. 2); however, there were no significant differences in the antioxidant capacity of all buds samples

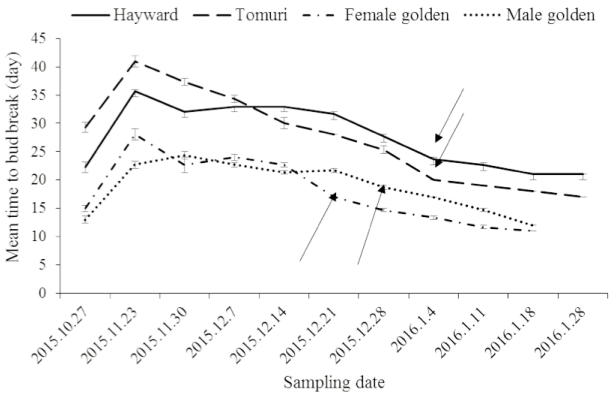

Fig. 1 - Effect of sampling date of cuttings on average of 50% bud break and endodormancy end period in four kiwifruit cultivars and genotypes during dormant season in 2015-2016. The arrows show the endodormancy end date for each cultivar and genotype.

collected in December (i.e. 07th, 14th, 21st and 28th). The lowest value in the antioxidant capacity was observed in Tomuri cultivar on 11th January after endodormancy release (Fig. 2). The antioxidant capacity of buds changed considerably at the beginning and during the maintenance and release of endodormancy (P≤0.01) (Fig. 2). The increase in antioxidant capacity from the end of October to the early November was simultaneous in four cultivars and genotypes; however, the peak period of the antioxidant capacity of buds was different between these cultivars and genotypes (Fig. 2). The antioxidant capacity showed a significant reduction (P≤0.01) in the male and female genotypes at the end of December, being simultaneous with the end of bud endodormancy of both genotypes. The reduction in the antioxidant capacity in Hayward and Tomuri cultivars occurred about two weeks later, coinciding with

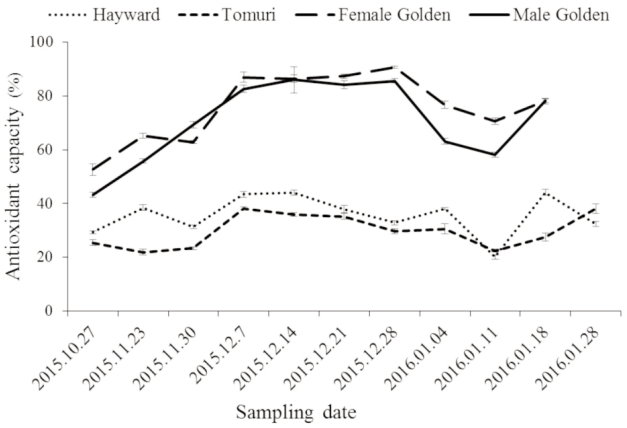

Fig. 2 - Effects of kiwifruit cultivars and different sampling dates on antioxidant capacity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

the completion of their chilling requirements and the end of endodormancy in the buds (Fig. 2).

The activity of PAL enzyme has been shown in figure 3 and demonstrated a significant difference in buds of all kiwifruit cultivars and genotypes (P≤ 0.01). The activity of this enzyme in the male and female Golden genotypes was more than that of the Hayward and Tomuri cultivars (Fig. 3). Female Golden genotype had the highest PAL activity in mid-December. The lowest activity of this enzyme was in Tomuri cultivar on 30th November 2015. PAL enzyme activity in all four kiwifruit cultivars and genotypes began an upward trend at the end of November (P≤0.01) (Fig. 3). It remained relatively stable during endodormancy in cultivars and genotypes, but was associated with a fluctuation in male and female Golden genotypes during ecodormancy. However, the enzyme activity of all cultivar showed a significant decrease (P≤0.01) at the end of endodormancy compared to the stable periods of endodormancy.

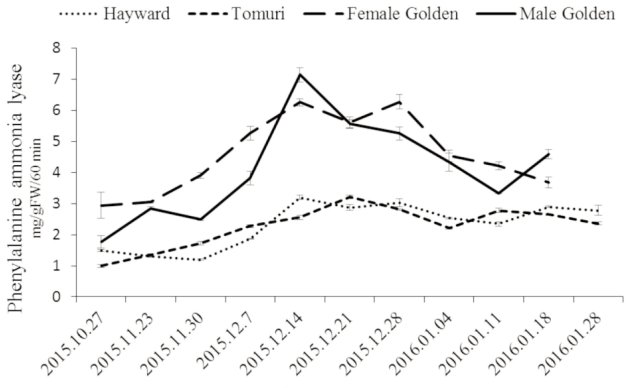

Fig. 3 - Effects of kiwifruit cultivars and different sampling dates on phenylalanine ammonia-lyase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds Moreover, ± the standard error of mean is shown on the vertical bar.

Plants release hydrogen peroxide (H_2O_2) in response to the environmental stress. Low temperature stress has also been shown to induce H_2O_2 accumulation in cells (Okane *et al.,* 1996). Hydrogen peroxide, as the second messenger of the increase in the activity of PAL enzyme, can activate PAL enzyme activity, as a key enzyme in the phenylpropanoids pathway, ultimately leading to higher total phenol and accumulation of flavonoids (Wang *et al.,* 2015). Oxidative stress caused by chilling during the endodormancy of kiwifruit buds increased the activity of this enzyme and the production of antioxidant compounds such as phenols, resulting in a higher antioxidant capacity in the buds during the endodormancy. It has been reported that cold acclimation of

plants leads to a remarkable increase in PAL activity, depending upon the range of low temperature to which the plants are subjected (Stefanowska *et al.,* 2002).

The amount of total phenol have been shown in figure 4 and varied between cultivars and genotypes of kiwifruit, and there were significant differences in their buds (P≤0.01). Male and female Golden genotypes had higher total phenol than Hayward and Tomuri cultivars (Fig. 4). Total phenol content showed substantial changes at the beginning, during the maintenance, and at the end of the endodormancy of kiwifruit buds (P≤0.01) (Fig. 4). The amount of total phenol showed an increasing trend from the late October. In the late November-early December period, coinciding with the onset of true endodormancy, the amount of total phenol reached its maximum (Fig. 4) and its value remained at a high level in the buds of cultivars and genotypes during this period. By reaching the end of endodormancy, the amount of phenol had decreased significantly at the end of December and at the beginning of January in the buds of male and female Golden genotypes and Hayward and Tomuri cultivars, respectively (P≤0.01).

Phenolic compounds are a valuable piece of evidence used in determining the differences between diverse varieties of *Myrtus communis* and *Pistacia lentiscus* and have a key role in detecting the genetic differences in biochemical methods (Tattini *et al.,* 2006). Thus, it appears that the significant differences in terms of the total phenol content between Hayward and Tomuri cultivars and male and female Golden genotypes (P≤0.01) (Fig. 4) are the result of their genetic differences.

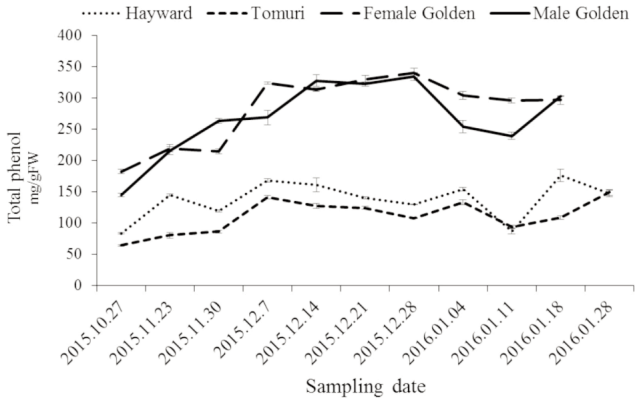

Fig. 4 - Effects of kiwifruit cultivars and different sampling dates on phenol content of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

Endodormancy is developed gradually after the cessation of growth; additionally, the severity of the endodormancy deepens in autumn and then gradually disappears by removing the physiological barriers of growth through the chilling process (Dennis, 2003). Total phenol concentration and antioxidant capacity enhance along with the development of endodormancy and reach their maximum value in the deepest stage of endodormancy (Fig. 2, 4). The changes in these two variables are similar in the establishment, maintenance, and release of endodormancy. A high total antioxidant activity in male and female Golden genotypes may be attributed to the high amount of phenol. Phenolic compounds are synthesized in plant cells in favorable environmental conditions, but environmental stresses change their levels in cells (Kliebenstein, 2004). Mid-autumn cold and the start of endodormancy period causes an increase in oxidative stress in plants. This stress results from reactive oxygen species that affect the growth of plants (Scalabrelli *et al.,* 1991; Mittler *et al*., 2004). Plants possess a protective system composed by the enzymatic antioxidant system such as peroxidase and catalase (Anderson *et al.,* 1995) and the non-enzymatic systems (Agarwal and Pandey, 2004). Phenols are non-enzymatic antioxidants and their antioxidant activities are mainly due to their redox properties which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quencher (Huda-Faujan *et al.,* 2009).

Phenols play an important role not only during cold resistance, but also during breaking the endodormancy of peach (Siller-Cepeda *et al.,* 1992) and apricot (Viti and Bartolini, 1998) buds as an antioxidant. Total phenol changes in kiwifruit buds are in agreement with the results of the studies on peach flower buds (Szalay *et al.,* 2005), apricot vegetative buds (Laslo and Vicas, 2012), and pistachio flower buds (Pakish *et al.,* 2009).

Correlation analyses showed a positive significant correlation between total phenol, antioxidant capacity and PAL activity in kiwifruit cultivars and genotypes (Table 1). Reduction in antioxidant capacity (Fig. 2) and total phenol (Fig. 4) after receiving chilling and a stable period in winter could be considered as a biomarker of endodormancy release in the cultivars which were studied.

In cultivars and genotypes of kiwifruit buds, PPO activity changed substantially (P≤0.01) as shown in figure 5. This enzyme had the highest activity in the Tomuri variety on 7th January 2016 and the lowest activity in male and female Golden genotypes at the

Table 1 - Correlation coefficient of kiwifruit cultivars and genotypes between phenol, antioxidant capacity (RSA %), phenylalanine ammonia-lyase (PAL), peroxidase (POD), and Polyphenoleoxidase (PPO)

Cultivars and genotypes		Phenol	PAL	RSA	POD	PPO
Hayward	Phenol	1	0.25 NS	0.95 **	-0.11 NS	-0.34 NS
	PAL	0.25 NS	1	0.17 NS	0.24 NS	-0.18 NS
	RSA	0.95 **	0.17 NS	1	-0.22 NS	-0.27 NS
	POD	-0.11 NS	0.24 *	-0.22 NS	1	0.44 NS
	PPO	-0.34 NS	-0.18 NS	-0.27 NS	0.44 NS	1
Tomuri	Phenol	1	0.38 NS	0.94 **	0.38 NS	0.28 NS
	PAL	0.38 NS	1	0.42 NS	0.20 NS	-0.22 NS
	RSA	0.94 **	0.42 NS	1	0.27 NS	0.28 NS
	POD	0.38 NS	0.20 NS	0.27 NS	1	0.28 NS
	PPO	0.28 NS	-0.22 NS	0.28 NS	0.28 NS	1
Female Golden	Phenol	1	0.76 **	0.93 **	0.20 NS	0.16 NS
	PAL	0.76 **	1	0.67 *	-0.19 NS	-0.16 NS
	RSA	0.93 **	0.67*	1	0.19 NS	0.14 NS
	POD	0.20 NS	-0.19 NS	0.19 NS	1	0.88 **
	PPO	0.16 NS	-0.16 NS	0.14 NS	0.88 **	1
Male Golden	Phenol	1	0.75 *	0.83 **	0.36 NS	0.26 NS
	PAL	0.75 *	1	0.74 *	0.40 NS	0.37 NS
	RSA	0.83 **	0.74 *	1	0.61 NS	0.49 NS
	POD	0.36 NS	0.40 NS	0.61 NS	1	0.95 **
	PPO	0.26 NS	0.37 NS	0.49 NS	0.95 **	1

** Correlation is significant at the 0.01 level.

first sampling date (Fig. 5). At the end of October 2015 and contemporaneous with the development of bud endodormancy, the activity of PPO enzyme, in all cultivars except Hayward, increased and reached its peak in mid-December and then decreased (P≤0.01; Fig. 5). PPO activity in the Hayward variety peaked a week earlier than the other cultivars. In Tomuri cultivar, PPO activity was stable for three weeks in the dormancy period and reduced at ecodormancy.

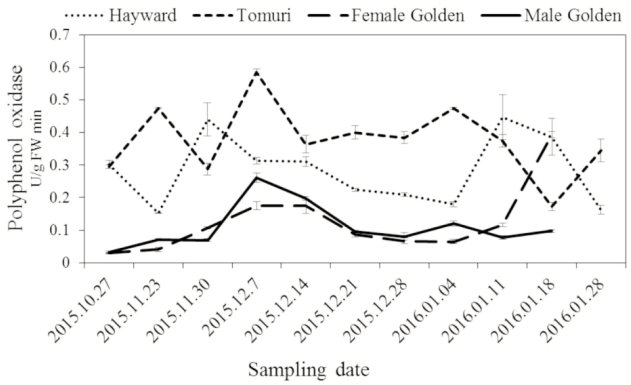

Fig. 5 - Effects of kiwifruit cultivars and different sampling dates on polyphenol oxidase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten buds. Moreover, ± the standard error of mean is shown on the vertical bar.

PPO is a copper-containing enzyme which catalyzes the oxidation of phenolic compounds to quinone or quinine-like compounds in the presence of molecular oxygen. A high PPO enzyme activity after endodormancy is probably due to the removal of some growth-inhibiting phenols (Wang *et al.,* 1991), and the phenolic substances such as inhibitors or stimulants change enzyme activity (Thirugnanasambantham *et al.,* 2013).

The increased activity of the PPO enzyme at the early stage of endodormancy period and its declined activity at the end of the endodormancy of grape buds (Scalabrelli *et al.,* 1991), plums (Szecskó *et al.,* 2002), and pistachio flower buds (Pakish *et al.,* 2009) were reported, corresponding with the results of this experiment. It appears that the effect of antioxidant compounds is to inhibit free radicals and reactive oxygen species in cultivars and genotypes during stress.

POD activity was notably different in buds (P≤0.01). This enzyme had the highest activity in the Tomuri cultivar after the endodormancy release (Fig. 6). The lowest activity was observed at all cultivars and genotypes at the first sampling date (the 27th

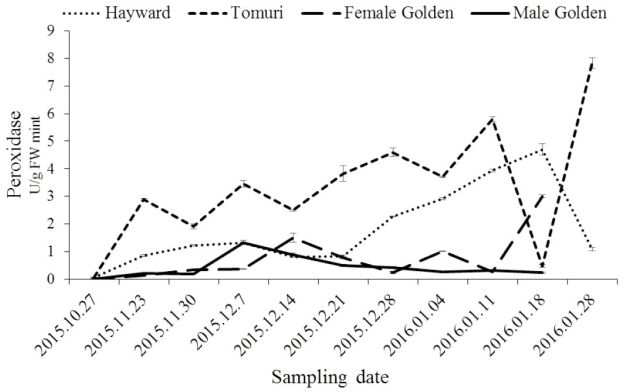

Fig. 6 - Effects of kiwifruit cultivars and different sampling dates on peroxidase activity of axillary buds during dormant season in 2015-2016. Each data point represents the mean of three replicates, each containing ten bud. Moreover, ± the standard error of mean is shown on the vertical bar.

October). The POD activity pattern was not constant during the endodormancy of buds in the cultivars and genotypes which were studied (Fig. 6). POD activity increased in Golden genotypes later than that in the Hayward and Tomuri cultivars. The activity of this enzyme increased significantly in early December and mid-December 2015 in male and female Golden genotype buds, respectively (P≤0.01). However, POD activity decreased significantly in both genotypes at the bud endodormancy release (Fig. 6). The increased activity of POD at the end of October, in

early endodormancy of buds, for Hayward and Tomuri cultivars was in agreement with results of studies on grape buds (Scalabrelli *et al.,* 1991), onion bulbs (Benkeblia and Shiomi, 2004), and floral buds of apricots (Laslo and Vicas, 2012). The increasing activity of this enzyme continued after an endodormancy breakdown in Hayward and Tomuri cultivars; moreover, these results concurred with the antioxidant enzyme changes in pear flower buds, where POD activity increased both during and after the end of endodormancy (Hao and Feng-Wang, 2004).

There are reports on the POD activity associated with susceptibility to cold in pistachio (Pakish *et al.,* 2009) and peach (Szalay *et al.,* 2005), suggesting that the varieties resistant to cold have higher POD activity than the susceptible cultivars. Tomuri had the highest POD activity and its activity significantly increased at the ecodromancy period (P≤0.01) (Fig. 6). Therefore, Tomuri cultivar could be the most tolerant to cold, that needs to be investigated further.

A significantly positive correlation was observed between phenols, PAL, and antioxidant capacity in Golden genotypes, and between phenols and antioxidant capacity in Hayward and Tomuri cultivars (Table 1). The lowest PPO and POD activities (P≤0.01) (Figs. 5, 6) and the insignificant changes in these activities during early endodormancy could result from the higher non-enzymatic antioxidant capacity (for example, total phenol and PAL) in Golden genotypes (Fig. 2). The buds of Hayward and Tomuri cultivars showed lower PAL activity and total phenol and higher POD and PPO activities than those in male and female Golden genotypes from the early stage of endodormancy to the ecodormancy stage (Figs. 4-6). However, there was a significantly positive correlation between total phenol content and antioxidant capacity in two cultivars (Table 1). It was concluded that the antioxidant capacity in Hayward and Tomuri cultivars may be due to total phenol rather than POD and PPO activities. Gur *et al.* (1988) reported the difference in PPO enzyme activity and phenol content in apple cultivars.

4. Conclusions

Peroxidase activity increased with the onset of the buds endodormancy, but continued for two weeks after the end of the endodormancy in Hayward and Tomuri cultivars. This is probably due to the resistance of these cultivars to cold compared to the male and female genotypes and, therefore, requires further investigation. This study indicated a significant and stable increase in the PAL and RSA activities. Furthermore, we found that phenol concentration could be associated with the transition to, and maintenance of, bud in true endodormancy. Due to the positive and significant correlation between total phenol, antioxidant capacity, and PAL activity, we concluded that antioxidant capacity, in both male and female Golden genotypes, is attributed to the high phenol which was a result of high PAL enzyme activity.

Acknowledgements

The paper is resulted of Ebrahim Abedi Gheshlaghi's PhD thesis. This research was funded by the University of Zanjan, Iran, which is gratefully acknowledged.

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