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Polyphenol Oxidase Enzyme Activity of Mulberries Grown in Iğdir Ecological Conditions

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Abstract

The presence of polyphenol oxidase enzyme (PFO) in black and white mulberry varieties grown in Iğdır province were determined. In the study, no polyphenol oxidase enzyme was detected in the white mulberry varietie, therefore the study was conducted only with black mulberry varieties. The mulberry samples were collected from Iğdır Center, Melekli and Karakoyunlu districts during June and July, both in 2015 and 2016, when the fruits were fully ripe. Mulberries were stored at -20 °C, and the crude enzyme extract was stabilized with pH 5.0 sodium-acetate buffer containing 1% Polyethylene glycol (PEG). To weigh with a precision scale, 20 grams of frozen mulberries were taken into mortars. After the mulberries were thoroughly crushed in these mortars, 40 ml of crude enzyme extract preparation solution was added to each variety and mixed homogeneously. These mixtures were filtered through a cheesecloth and rotated at 4 °C, 10,000 rpm for 30 minutes. The obtained supernatants were used for enzyme solubilization, and the enzyme activity of the solution was determined spectrophotometrically. The PFO content depended on the genotype. There was a significant difference among the tested mulberry genotypes.

Keywords: Black mulberry (*Morus nigra*), Polyphenol oxidase enzyme, Iğdır

INTRODUCTION

Mulberry is a type of fruit that can grow in temperate, tropical and subtropical climate zones due to its high adaptability to different climate and soil conditions. Mulberry (*Morus* spp) belongs to the Morus genus of the *Moraceae* family of the *Urticales* order. The number of species included in the *Morus* genus is estimated by Freeman (1978) as 12, Huo (2002) as 14, Koidzumi (1917) as 24 and 1 subspecies (Machii *et al*., 2001), Martin et al. (2002) report it as more than 30, and Datta (2002) reports it as 68. *Mulberry* is especially common in East, West and Southeast Asia, Southern Europe, southern North America, northwest of South America and some parts of Africa (Datta, 2002). *Mulberry* species whose fruits are used and widely cultivated are *Morus alba*, *Morus nigra*, and *Morus rubra*. *Morus alba* is native to China, Japan, Thailand, Malaysia and Burma, *Morus nigra* is from Türkiye, Iran, Arabia, parts of Russia in South Asia and Syria, and Morus rubra is from North America (Bellini *et al.*, 2000). However, the natural distribution areas of mulberry have been greatly changed by human interventions (Zheng *et al*., 1988). As with many fruit species, Anatolia is the homeland of mulberry and one of the oldest cultural areas (Özbek, 1977). In our country, 50,000 tons of product are obtained from mulberry trees that produce 2,130,000 fruits (MEB, 2013).

Mulberry is considered to symbolize wisdom and patience, as cultivated fruit species never begin to develop their buds in cold weather conditions (Grieve, 2002). Mulberry is a type of fruit that can be grown even in our regions where the continental climate prevails. Our country looks like a collection garden with mulberry trees grown in all regions. Mulberry is produced in almost every province in our

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country, but only 7 provinces do not produce enough mulberry to be included in the statistics (Erdoğan and Pırlak, 2005). While Erzurum province ranks first in mulberry production in our country with a production of 5,921 tons and an average yield per tree of 102 kg, this is followed by Erzincan province with a production of 5,134 tons and an average yield per tree of 40 kg, with a production of 3,941 tons and an average yield per tree of 28 kg. and Malatya province (TUIK, 2014). Although our country, whose fruit-growing culture dates back to ancient times, is one of the homelands and natural distribution areas of mulberry, this genetic potential cannot be adequately evaluated. Many genotypes, which have very superior characteristics in terms of fruit quality, have been cut down and destroyed just to benefit from their timber. Although it has a wide distribution in the world, mulberry fruit is not yet recognized in many countries. It will be possible to bring mulberry fruit into the economy with the development of its consumption range and preservation techniques (Erdoğan and Pırlak, 2005).

Although mulberry is grown in Iğdır province, where the study was conducted, it does not constitute an economic value and mulberry does not get the value it deserves. Enzymes are organic catalysts synthesized intracellularly by living cells under genetic control. The term catalysis is used in Greek to mean effective in chemical reactions, accelerating and facilitating the reaction. Substances that act as catalysts in biological events are called enzymes. In general, enzymes catalyze certain reactions between certain substances. Nutrient elements are used in the body with the help of enzymes and are transformed into body structure as a result of reactions. All vital events require enzymes. Enzymes are synthesized from nucleic acids under the control of DNA (deoxyribonucleic acid). A small change in the DNA structure causes some enzymes to not be synthesized or to be synthesized incorrectly. The defectively synthesized enzyme cannot show activity and causes various problems in body functioning. Without enzymes, the body cannot maintain its vitality. Enzymes play a role in the formation of many physical and chemical events such as digestion, respiration, growth, muscle contraction and photosynthesis.

MATERIAL AND METHOD

Material

Since the study examined the polyphenol oxidase enzyme activity in black mulberries, polyphenol oxidase was referred to when the enzyme was mentioned in the article. To determine the enzyme activities of mulberry varieties, mulberries were marked in the gardens of Iğdır Center (M1, M2, M3) and the villages of Melekli (ME1, ME2, ME3), Karakoyunlu (K1, K2, K3) and samples were taken from each of them in June and July collected. Starting from 2015-2016, samples were brought to the laboratory of Iğdır University Faculty of Agriculture and left in the refrigerator to examine the enzyme activities of mulberry. After the frozen mulberries were taken out of the refrigerator, they were weighed to 20 g using a precision scale and left in the refrigerator (Figure 1).

Figure 1. *Examples used in the research*

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After freezing, the mulberries were thoroughly pounded, and acetate buffer (500 ml) was prepared to determine the enzyme activities of the pounded mulberries. After these processes were completed, the crude extract was prepared, and the mixing process was carried out after pouring the prepared crude extract into the acetate buffer (Figure 2).

Figure 2. *Samples prepared for analysis*

After this stage, the mixture we had was filtered into a closed plastic bottle with the help of cheesecloth. The filtered mixture was weighed on a precision scale and placed in the centrifuge with the same weights. The mixture was removed after being kept in the centrifuge for 30 minutes at + 4℃. To check the enzyme activity, the cuvettes to be used in the research were passed through pure water and separated into blank samples and main samples, and the color change on the spectrophotometer was examined one by one, and the enzyme activity was checked. The centrifuge used in the research can be seen in Figure 3.

Figure 3. *Centrifuge used in the research*

Method

Acetate Buffer Preparation pH=5: In the study, 3,402 g of sodium acetate was used for 500 ml of acetate buffer. 3.402 g of sodium acetate was left in the beaker, 450 ml of pure water was added, and the sodium acetate was dissolved in pure water with the help of a magnetic stirrer. Then, the pH was adjusted to 5 by slowly pouring the acetic acid into the acetate buffer.

Crude Extract Preparation: After the mulberries were weighed as 20 g on a precision scale, 40 ml of acetate buffer, twice the weight of the mulberries, was prepared in a ratio of 1:2. After 100 ml of 0.4 g of PEG (polyethylene glycol) was deposited onto the prepared acetate buffer, the PEG was dissolved with the help of a magnetic stirrer.

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Substrate Freedom: To prepare the substrate used in the curry and sample to measure the enzyme activity, the substrate was weighed as 0.11 g on a precision scale, dissolved in some pure water, and then 10 ml of pure water was added.

Statistical Analysis: Descriptive statistics of the data obtained in terms of polyphenol oxidase enzyme activity are expressed as mean and standard error. In terms of the enzyme activity in question, the main effects of variety (K1, K2, K3, M1, M2, M3, ME1, ME2, ME3), year (2015 and 2016), month (June and July) and variety x year, variety x month, To test the hypotheses regarding the interaction effects of year x month and variety x year x season, analysis of variance technique was used in factorial trial design in random parcels. Tukey's multiple comparison test for variety was used to determine significant differences.

To develop a prediction equation in terms of polyphenol oxidase enzyme activity, a prediction model was developed with the MARS data mining algorithm using variety, year and month variables (Demirel et al., 2023a; Türkoğlu et al., 2023a; Türkoğlu et al., 2023b). Additionally, the CART data mining algorithm was used to obtain a regression tree based on the existing data set (Kovalchuk et al., 2017). Statistical analysis of the data was performed with R software (Demirel et al., 2023b; Eren et al., 2023).

RESULTS and DISCUSSION

In terms of enzyme activity, the main effects of variety (K1, K2, K3, M1, M2, M3, ME1, ME2, ME3), year (2015 and 2016), month (June and July) and variety x year, variety x month, year x Month and variety x year x month interaction effects were found to be statistically significant. Approximately 100% of the total variation in enzyme activity was explained by these effects included in the model. Looking at the introductory statistics regarding the main effects, it was observed that Melekli mulberries (ME1=ME2> ME3) had more enzyme activity. On the other hand, the lowest enzyme activity was obtained from K1 accession. It was determined that enzyme activity was higher in 2015 and June compared to 2016 and July (Table 1).

Table 1. *Introductory statistics for main effects*

Kind	Enzyme activities (Unit)
K1	293.3 ± 61.3h
K2	314.1 ± 60.1g
K3	338.9 ± 65.4d
M1	283.3 ± 56.0ı
M2	323.8 ± 64.7f
M3	337.9 ± 60.1e
ME1	554.9 ± 19.7a
ME2	551.8 ± 35.1b
ME3	465.8 ± 16.4c
Year	
2015	475.8 ± 18.5a
2016	293.9 ± 29.6b
Month	
June	477.4 ± 24.1a
July	292.2 ± 24.9b
General	384.8 ± 19.4

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When we look at the enzyme activity averages of varieties according to years; It was observed that the enzyme activity in 2015 was more than in 2016, Karakoyunlu 3 and Melekli 3 accessions were more than the others in 2015, and Melekli 2 had more enzyme activity in 2016 (Table 2).

When looking at the average enzyme activity of varieties by month; While it was found most in Melekli 2 in June, it was least found in Karakoyunlu 3. In July, the highest enzyme activity was detected in the Melekli 1 accession, while it was found to be the least in Center 1 (Table 3).

Table 2. *Enzyme activity averages of varieties according to years (Unit)*

Kind	2015	2016
K1	438 ± 88.0h	148.3 ± 19.7h
K2	470.9 ± 75.9f	157.2 ± 16.5f
K3	536.6 ± 55.6a	141.1 ± 9.64ı
M1	390.0 ± 69.3ı	176.4 ± 66.5 e
M3	499.0 ± 55.8 c	148.4 ± 54.9g
M3	481.5 ± 51.7e	194.3 ± 70.5d
ME1	497.1 ± 19.4d	612.4 ± 2.10b
ME2	462.9 ± 38.8g	640.6 ± 27.2a
ME3	505.7 ± 23.5 b	426.2 ± 0.64c

Table 3. *Enzyme activity averages of varieties according to months (Unit)*

Kind	June	July
K1	413.8 ± 99.0h	172.9 ± 30.7h
K2	417.4 ± 99.0g	210.8 ± 40.4e
K3	412.0 ± 111.0ı	265.9 ± 65.5d
M1	435.1 ± 49.2f	131.4 ± 46.4ı
M2	447.5 ± 78.9e	200.0 ± 77.9g
M3	474.6 ± 54.8d	201.2 ± 73.6f
ME1	578.8 ± 17.1b	530.7 ± 34.4a
ME2	625.6 ± 34.0a	478.0 ± 45.5b
ME3	492.7 ± 29.4c	439.2 ± 6.22c

When we look at the enzyme activity averages of varieties according to years; It was determined that the month of June 2015 was the highest and the month of July 2016 was the least (Table 4).

Table 4. *Enzyme activity averages for months according to years (Unit)*

Year	June	July
2015	594.6 ± 8.71a	357.0 ± 15.2a
2016	360.3 ± 35.3b	227.4 ± 44.5b

Enzyme activity averages of varieties according to years and months are given in Table 5. According to the results; In 2015, the highest enzyme activity value among the June averages was observed in Karakoyunlu 3, and the highest enzyme activity value among the July averages was observed in Melekli 1. In 2016, the highest enzyme activity was observed in Melekli 2 in June, while it was detected in Melekli 1 in July.

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Table 5. *Samples prepared for analysis*

Kind	2015		2016	
	June	**July**	**June**	**July**
K1	635.2 ± 0.115c	241.5 ± 0.115h	192.3 ± 0.115h	104.2 ± 0.115g
K2	640.7 ± 0.058b	301.1 ± 0.058g	194.0 ± 1.150g	120.4 ± 0.058e
K3	660.9 ± 0.058a	412.3 ± 0.058c	162.6 ± 0.058ı	119.5 ± 0.115f
M1	545.2 ± 0.115h	235.1 ± 0.115ı	325.0 ± 0.580e	27.70 ± 0.115h
M2	623.8 ± 0.058d	374.2 ± 0.115e	271.0 ± 0.580f	25.70 ± 0.058ı
M3	597.2 ±0.115e	365.8 ± 0.0058f	352.0 ± 0.580d	365.0 ± 0.115d
ME1	540.5 ± 0.058ı	453.7 ± 0.115a	617.1 ± 0.058b	607.7 ± 0.058a
ME2	549.6 ± 0.115g	376.2 ± 0.058d	701.5 ± 0.115a	579.7 ± 0.115b
ME3	558.3 ± 0.058f	453.1 ± 0.058b	427.0 ± 1.150c	425.3 ± 0.058c

MARS data mining algorithm was used to determine the factors affecting enzyme activity, and the results are summarized in Table 6. The coefficients of the prediction equation obtained with the algorithm in question are given below. The correlation coefficient (r=0.99) between the predicted values calculated with the prediction equation for enzyme activity and the actual value was found to be statistically significant (t=126, df=106, P<2.2e-16, 95% confidence interval; 0.9951523-0.9977415). Almost all of the total variation in enzyme activity was explained by the elements included in the model.

Enzyme activity for the K1 genotype was estimated at 267 units in July 2015. Enzyme activity was estimated at 93 units for the K1 genotype in July 2016. For the K1 genotype, the enzyme activity was estimated to be at 572 units in June 2015, while the enzyme activity was estimated to be at 180.2 units in June 2016. Enzyme activity for the K3 genotype was estimated at 409.31 units in July 2015. Enzyme activity was estimated at 122.5 units for the K3 genotype in July 2016. For the K3 genotype, the enzyme activity in June 2015 was estimated to be at 664 units, and the activity in June 2016 was estimated to be at 160 units.

Enzyme activity for the M2 genotype was estimated at 350.2 units in July 2015. Enzyme activity was estimated at 26 units for the M2 genotype in July 2016. For the M2 genotype, the enzyme activity in June 2015 was found to be at 655.2 units, and the activity in June 2016 was 264 units. Enzyme activity for the M3 genotype was estimated at 366 units in July 2015. Enzyme activity was estimated at 37 units for the M3 genotype in July 2016. For the M3 genotype, the enzyme activity in June 2015 was estimated to be 597.2 units, and the activity in June 2016 was estimated to be 352 units.

Enzyme activity for the ME1 genotype was estimated as 453.7 units in July 2015. Enzyme activity for the ME1 genotype was estimated as 607.7 units in July 2016. For the ME1 genotype, the enzyme activity in June 2015 was estimated to be 540.5 units, and the activity in June 2016 was estimated to be at 617.1 units. Enzyme activity for the ME2 genotype was estimated at 578 units in July 2015. Enzyme activity for the ME2 genotype was estimated as 579.7 units in July 2016. For the ME2 genotype, the enzyme activity in June 2015 was estimated to be 701.5 units, and the activity in June 2016 was estimated to be at 376.2 units. Enzyme activity for the ME3 genotype was estimated at 453.1 units in July 2015. Enzyme activity for the ME3 genotype was estimated at 425.3 units in July 2016. For the ME3 genotype, the enzyme activity in June 2015 was estimated to be at 571.2 units, and the activity in June 2016 was estimated to be at 427 units. The enzyme activity of the ME3 genotype varies depending on the month and year factor.

Enzyme activity for the K2 genotype was estimated at 303 units in July 2015. Enzyme activity for

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the K2 genotype was estimated at 129 units in July 2016. For the K2 genotype, the enzyme activity in June 2015 was estimated to be 608 units, and the activity in June 2016 was estimated to be 216.43 units.

Enzyme activity for the M1 genotype was estimated at 235.1 units in July 2015. Enzyme activity was estimated at 27.7 units for the M1 genotype in July 2016. For the M1 genotype, the enzyme activity in June 2015 was estimated to be 572 units, and the activity in June 2016 was estimated to be 325 units. When the prediction equation created by MARS data mining is examined, it is noted that the effect of the variety factor on enzyme activity depends on the year and month factors.

Table 6. *Basic functions and coefficients of the MARS model*

Basic function	Coefficients
(Fixed)	572
Kind K2	36
Kind K3	92
Kind M2	83
Kind M3	26
Kind ME1	-31
The Year 2016	**-391**
Month July	**-305**
Kind K3 * Year 2016	-113
Kind K3 * Month July	50
Kind M1 * Year 2016	145
Kind M1 * Month July	-32
Kind M3 * Year 2016	146
Kind M3 * Month July	74
Kind ME1 * Year 2016	468
Kind ME1 * Month July	218
Kind ME2 * Year 2016	521
Kind ME2 * Month July	110
Kind ME3 * Year 2016	247
Kind ME3 * Month July	186
Year 2016 * Month July	**217**
Kind M1 * Year 2016 * Month July	-178
Kind M2 * Year 2016 * Month July	-150
Kind M3 * Year 2016 * Month July	-302
Kind ME1* Year 2016 * Month July	-140
Kind ME2 * Year 2016 * Month July	-144
Kind ME3 * Year 2016 * Month July	-101

GCV 267 GR2 0.99 R^2 0.99 CVR^2 0.98

The CART algorithm was used to determine independent variables affecting enzyme activity. The regression tree diagram created with this algorithm is given in Figure 4. The correlation coefficient

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(0.944) between the enzyme values predicted by the CART algorithm and the measured enzyme values was found to be significant (t=29.323, df= 106, P<2.2e-16, 95% confidence interval; 0.9182926-0.9611332). However, according to the regression analysis results obtained from the CART algorithm, 89% of the total variation in the enzyme in question was explained by the independent variables (variety, year and month) included in the model. The node at the top of the regression tree is called the root node and gave the grand average of enzyme activity (385 units). The child node on the left, located at the first depth of the regression tree, consists of the K1, K2, K3, M1, M2 and M3 genotypes, and the enzyme activity for this node was determined as 315 units. Likewise, the right child node at the first depth of the tree created with the CART algorithm consists of ME1, ME2 and ME3 genotypes, and the average enzyme activity of this node was determined as 524 units. Thus, two genotype groups were obtained in terms of enzyme activity. However, this child node is divided into two new child nodes according to the months (July and June). However, the average enzyme activity of the group consisting of ME1, ME2 and ME3 genotypes in Melekli in June (566 units) was found to be higher than the average enzyme activity in July (483 units). The enzyme activity of the genotypes in Melekli varied by month, but not by year.

On the other hand, the enzyme activity of K1, K2, K3, M1, M2 and M3 genotypes varied according to months and years. The average enzyme activity of the group consisting of K1, K2, K3, M1, M2 and M3 genotypes in 2015 (469 units) was found to be higher than the average enzyme activity in 2016 (161 units). However, the average enzyme activity of the group consisting of K1, K2, K3, M1, M2 and M3 genotypes in July 2016 (72 units) was found to be lower than the average enzyme activity in June of the same year (249 units). The effect of the year factor affecting the enzyme activity of these six genotype groups varied depending on the month factor.

However, the average enzyme activity of the group consisting of K1, K2, K3, M1, M2 and M3 genotypes in July 2015 (322 units) was found to be lower than the average enzyme activity in June of the same year (617 units).

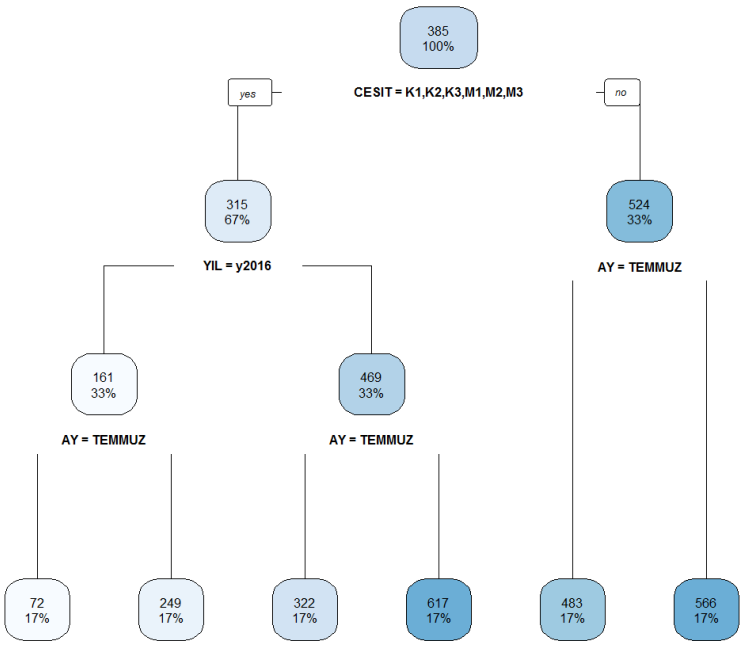

Figure 4. *Regression tree created with CART Algorithm*

In the study, if the enzyme activity for June 2015 is listed from most to least, the K3 variety is 661 units, the K2 variety is at 641 units, the K1 variety is at 635 units, the M2 variety is at 624 units, the M3

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variety is at 597 units, the ME3 variety is at 558 units, the ME2 variety is at 549.7 units, and the M1 variety is at 549.7 units. Type was determined at 545 units, the ME1 was determined as 540.7 units. For July 2015, enzyme activity from most to least: the ME1 variety at 454 units, the ME3 variety at 453 units, the K3 variety at 412 units, the ME2 variety at 376 units, the M2 variety at 374 units, the M3 variety at 366 units, the K2 variety at 301 units, the K1 variety at 241.7 units, the M1 variant is designated at 235 units.

In the study, if the enzyme activity for June 2016 is listed from most to least, the ME2 variety is 701.7 units, the ME1 variety is 617.0 units, the ME3 variety is 427 units, the M3 variety is 352 units, the M1 variety is 325 units, the M2 variety is 271 units, the K2 variety is 194 units, the K1 variety is 194 units. Variety was found to be 192.3 units, and type the K3 was found to be 163 units. Suppose the enzyme activity If the enzyme activity for July 2016 is listed from most to least. In that case the ME1 variety is 608 units, the ME2 variety is 580 units, the ME3 variety is 425 units, the M3 variety is 367 units, the K2 variety is 120.3 units, the K3 variety is 120 units, the K1 variety is 104 units, and the M1 variety is 104 units. 28 units, the M2 type was found to be 26 units.

In this study, the enzyme activity of mulberries grown in Iğdır ecological conditions was determined. Polyphenol oxidase enzyme activity was detected in K1, K2, K3, M1, M2, M3, ME1, ME2 and ME3 mulberry varieties in June and July of 2015 and 2016. Although there are few studies on mulberry enzyme activity in Türkiye and in the world, our study sheds light on future studies.

Although there are few studies on mulberry enzyme activity in Türkiye and in the world, (Ünal, 2007) purified the polyphenol oxidase enzyme from Anamur banana grown in Türkiye in 2007 and studied the characteristic properties of the enzyme. He found the optimum temperature of Banana PFO to be 30 ºC and the optimum pH to be 7.0. In 2003, (Beşel 2003) researched the polyphenoloxidase (PFO) enzyme obtained from Mushroom (*Agaricus bisporus*). It was observed that when 8% TX-114 was used, almost 5-fold purification was achieved, and when polyvinylpolypyrrolidone (PVPP) was used at pH 7.0, it was observed that the purification increased 10-fold and 72% enzyme was recovered. However, when studied at pH 6.0, it was observed that the purification increased to 15.5 times and the recovery approached 100%. In our research, no purification was performed, and there is no similarity in this respect. In another study, the biochemical properties of the PFO enzyme extracted from Victoria grapes grown in South Africa were investigated by (Repeanu et al., 2006). They found the optimum pH for enzyme activity to be 5 and the optimum temperature to be 25 °C with 10 mM catechol substrate in McIlvaine buffer. In many studies, the temperature value for the best activity of the enzyme is estimated to be between 25 °C - 30 °C. In our study, the best enzyme activity was observed in June between 2015 and 2016, and the average temperatures of June were observed to be 24.9 °C and 22.6 °C, respectively. (Mahanta et al., 1993), investigated the formation of brown compounds in fermented (black) tea in the specific activities of two basic enzymes, PFO and peroxidase (POD). PFO and POD activities were determined by spectrophotometric method, and the maximum activities of PFO and POD were observed to be during the rolling process during tea processing. It was observed that the reason for the decrease and subsequent increase in enzyme activity was due to mechanical damage affecting oxidative fermentation (enzymatic browning) in fermented black tea. In our study, PFO enzyme activity of black mulberry was measured by spectrophotometric method.

It is estimated that the increase and decrease in enzyme activity over years and months is due to ecological conditions. In our research and when the studies are analyzed, the temperature and the type of fruit affect the minimum and maximum values of the polyphenol oxidase enzyme. It was estimated that the polyphenol oxidase enzyme was desired in dark-colored fruits, but not in light-colored fruits. In our study, polyphenol oxidase enzyme activity in black mulberry varies according to years and months.

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Determining the ecological conditions and detecting changes in enzyme activity over the years is important for those who grow black mulberry in Iğdır province. We believe that our study will shed light on future studies in terms of comparison and determination of polyphenol enzyme activity in black mulberry.

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