Effects of Melatonin Applications on Anatolian Black Pine (*Pinus nigra* J. F. Arnold. subsp. *pallasiana* (Lamb.) Holmboe) Afforestation Performance in Semi-Arid Areas

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Melatonin, a substantial hormone, is a natural antioxidant agent that functions as a protector against the harmful effects of free radicals. Studies have found that "exogenous melatonin" applications have a positive effect on the growth and development of plants. This study investigated the adaptation of the seedlings that were transported from the nursery to the afforestation site for the process of planting. In 2019 the 2+0 aged bare-rooted Kastamonu/Taşköprü Anatolian Black pine seedlings, which are suitable for planting in semi-arid areas, were selected as research materials. Four different doses of "exogenous melatonin" (250, 500, 1000, and 1500 µM) were administered through two different methods (root-dipping and needle-spraying). Morphological seedling characteristics and bioactive chemical variables were measured for the control group and the seedlings treated with different doses of melatonin. Antioxidant enzyme activities were identified. When both the needle-spraying and root-dipping methods for melatonin application were evaluated in terms of morphological and biochemical variables, the best results were determined in low doses (250 to 500 µM). The results suggest that melatonin provides support to the metabolic process for the resistance of seedlings to low temperatures and semi-arid climatic conditions.

DOI: 10.15376/biores.18.2.2551-2572

Keywords: Exogenous melatonin; Pinus nigra subsp. pallasiana; Reactive oxygen species; Needle-spraying; Root-dipping

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INTRODUCTION

A number of adaptation challenges are seen with the lifting and transfer of bare-rooted seedlings raised in nursery conditions and planting them at afforestation sites. This challenge is more common in afforestation in arid, semi-arid, and anthropogenic steppe areas (Ayan *et al*. 2021). The adaptation of the seedling to the new environment of its planting is defined as acclimatization (Dirik 1990; Kijowska-Oberc *et al*. 2020; Yasmeen *et al*. 2022). The factors affecting planting success can be environmental and depend on the timely and intensive implementation of the applied technical processes. The rate of seedling viability is the direct result of the interaction of morphological, physiological, and genetic qualities that the seedling has. In particular, root damage during the lifting of bare-

rooted seedlings on the nursery bed and planting them at the afforestation site negatively affect the root-to-shoot ratio (Harris 1992; Ledo *et al.* 2018).

Special operations have been implemented to enhance planting success, particularly on arid and semi-arid lands. Preservatives applied to the root region of the seedling prior to planting are one of these applications. "Exogenous melatonin" applications have been reported to have a positive effect on plant growth and development and the acquisition of tolerance to abiotic stress conditions (Li *et al*. 2012; Turk *et al*. 2014; Bajwa *et al*. 2014; Wei *et al*. 2015; Lee and Back 2017; Nawaz *et al*. 2018; Shen *et al*. 2021). *Melatonin (N-acetyl-5-methoxytryptamine)*, a natural antioxidant, is an important hormone that performs the function of protecting biological tissues from the harmful effects of free radicals. Melatonin is known to be a preserved molecule in the evolutionary process in all species of living things and was first discovered in plants in 1995 (Van Tassel *et al*. 1995, 2001). Initial research in plants focused on *Chenopodium rubrum* (lamb's quarters), *Eichhornia crassipes* (water hyacinth), *Vitis vinifera* (grape vine), *Prunus avium* (cherry), and *Ulva* sp. (green algae) (Wolf *et al*. 2001; Tan *et al*. 2007; Boccalandro *et al*. 2011; Tal *et al.* 2011; Zhao *et al*. 2013).

Melatonin functionally delays leaf abscission in plants. It is tasked with the role of growth regulator, such that it enables the development of tissues such as root and shoot. It was also determined to be a natural stimulus against various stressors (Shen *et al*. 2021; Zhao *et al*. 2021). It has been shown by researchers that it is an important signal molecule against abiotic stresses such as drought, salt, cold, frost, temperature, *etc*. It is a critically important antioxidant molecule that is tasked with rapidly scavenging free radicals formed at the cellular level by stress (Galano *et al*. 2011; Arnao 2014; Bajwa *et al*. 2014; Zhang *et al*. 2014; Arnao and Hernandez-Ruiz 2015; Jiang *et al.* 2016; Chen *et al*. 2018; Kanwar *et al*. 2018; Shen *et al*. 2021). Furthermore, the application of "exogenous melatonin" against oxidative stress regulates the plant defense mechanism (Tan *et al*. 2012; Wang *et al*. 2015; Zhang *et al*. 2017). Melatonin neutralizes hydroxyl ($OH^{-}\cdot$), superoxide ($O_2^{-}\cdot$), and $NO^{-}\cdot$ radicals, all of which show high impact. It increases the activity of various antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and guaiacol peroxidase (GPx) enzymes (Baydaş *et al*. 2001). Melatonin functions as an antioxidant by scavenging free radicals, stimulating the activity of antioxidant enzymes, reducing electrical conductivity, and increasing mitochondrial oxidative phosphorylation (OXPHOS) (Reiter *et al.* 2003). Externally applied melatonin inhibits drought stress by increasing the activity of antioxidant enzymes (Wang *et al*. 2013; Li *et al*. 2015). Li *et al*. (2016) determined that the external application of melatonin of 0.05 mmol/L to canola (*Brassica* ssp.) seeds increased the content of soluble sugar and protein and prevented growth decline caused by drought stress. Cao *et al*. (2019) investigated the effectiveness of the melatonin hormone against frost harm in harvested peach berry. Frost harm was decreased in melatonin-treated fruits. Melatonin stimulates the contents of the hydrogen peroxide (H_2O_2) at the first stage but inhibits it in the subsequent process; it increases the expression of genes responsible for antioxidants. In brief, melatonin increases synthesis genes, reduces oxidative stress, restores redox balance, and improves the activity of antioxidant enzymes (Xin *et al*. 2017; Wei *et al*. 2018; Gholami and Zahedi 2019; Gholami *et al*. 2022).

Seedlings grown in open-field nursery conditions experience certain levels of stress during the transplant and planting stages to afforestation sites, failing to demonstrate the desired growth performance. With the aim of increasing the success of seedlings at plantation sites and preventing the stress experienced by seedlings during the planting

process, this study investigated whether external melatonin applied to the seedling will be successful with hormone use. The effect of melatonin applications on afforestation performance of bare-rooted 2+0 aged Anatolian Black pine seedlings (*Pinus nigra* J. F. Arnold. subsp. *pallasiana* (Lamb.) Holmboe) was determined.

EXPERIMENTAL

This study was conducted using 2+0 aged bare-rooted provenances of Kastamonu/Taşköprü Anatolian black pine in a 76.8 ha plantation site in Karasapaca Village (Latitude: 41048626; Longitude: 3407123) within the borders of Kastamonu-Tosya Forest Enterprise Directorate between 2018 and 2020 in Turkey. The area has an average altitude of 910 m a.s.l. and the annual rainfall amount is 467 mm. The study area is south-facing, with a slope ranging from 31 to 60%. The soil structure is sandy-clay and its bedrock has a sediment rock structure.

Anatolian black pine seedlings were grouped as to four different doses (250 μM - 500 μM - 1000 μM - 1500 μM) and Control group (Wang *et al*. 2013) and planted in the site on 07 March 2019. Two different applications forms (root-dipping / needle-spraying) were used (Yer Celik 2021). Before planting, the plants' roots were soaked in melatonin for 30 minutes (root-dipping). In the second application method, melatonin was applied to the needles of the seedlings by spraying every month during the vegetation period. Biochemical measurements were made using the seedlings three times during the vegetation period (beginning (April), mid (July), and end (November) of the vegetation period). As of the end of vegetation, morphological measurements were conducted on 30 seedlings in total (10 seedlings x 3 replications).

As morphological measurements, root collar diameter (RCD) was measured with a 0.1 mm precision digital caliper; seedling height (SH) was measured with a steel tape; The fresh weight of the shoot (FWS), the fresh weight of the root (FWR), and the total fresh weight of the seedlings (FWSD) were measured with a precision balance of 0.001 g. After drying, the values for the dry weight of the shoot (DWSH), the dry weight of the root (DWR) and the total dry weight of the seedlings (DWS) and number of buds (NB), number of branches per seedling (NBS), and root length (RL) parameters were measured. Besides, the following values were calculated from these obtained values,

$$(\%Root) = \frac{DWR}{DWS} * 100 \quad (1)$$

$$SI = \frac{SH\ (mm)}{RCD\ (mm)} \quad (2)$$

$$LI = \frac{DWS\ (g)}{DWR\ (g)} \quad (3)$$

$$DQI = \frac{DWS}{(LI + SI)} \quad (4)$$

where %Root, SI, LI and DQI represent the root percentage, sturdiness index (Aphalo and Rikala 2003), layering index and Dickson quality index (Ayan 2002).

As biochemical variables, H_2O_2, MDA, chlorophyll, proline, protein, sugars, as well as, antioxidant enzyme activities such as SOD and POD were investigated. The detection of proline amount was made according to the method of Bates *et al*. (1973), protein content according to Bradford (1976) method, malondialdehyde (MDA) content

according to Lutts *et al.* (1996), and hydrogen peroxide (H_2O_2) according to Velikova *et al.* (2000). The method of Witham *et al*. (1971) was used to determine photosynthetic pigments. SOD activity was measured according to nitro blue tetrazolium chloride (NBT) reduced by O_2^- under light (Agarwal and Pandey 2004). POD activity was determined according to Yee *et al*. (2002). All analyses were performed with three replications. Data of morphological and biochemical variables obtained as a result of the measurements were subjected to variance analysis (ANOVA). The Duncan test was used for intergroup binary comparisons when statistically significant differences ($p<0.05$) were found in terms of measured values. Statistical analyses were done using IBM SPSS Statistics 22 package software.

RESULTS

Effect of Melatonin Applications at Different Doses on Morphological Characters

The effects of exogenous melatonin, applied at different doses to different parts of the plants, on root collar diameter, number of branches per seedling, number of buds, root length, fresh weight of the shoot, dry fresh weight, the total fresh weight of the seedlings, the dry weight of the shoot, and sturdiness index values of the seedlings were found to be significant according to the variance analysis results ($p<0.05$). Variance analysis of morphological characters and results of the Duncan's Test are presented in Table 1. The highest values in terms of RCD and RL compared to the control were determined in the 500 μM melatonin treatment applied to seedling roots before planting. The highest NBS compared to control was detected in seedlings at the treatment doses of 250 and 500 μM sprayed on seedling shoots. According to the statistical values of the number of buds in the seedlings at the end of vegetation, the highest NB was recorded in the seedlings treated with 250 μM melatonin to root zone. In terms of FWS, FWR, and FWSD values; the highest values compared to the control group were detected in the dose of 500 μM melatonin spraying, respectively. The highest DWSH was achieved in the 250 μM dose of melatonin applied by needle-spraying. In terms of its effect on morphological seedling parameters, low doses of melatonin (250 μM, 500 μM) yielded more positive results in both the needle-spraying and root-dipping.

Effect of Different Doses of Melatonin Application on Bioactive Chemical Components

Its effect on nitrogen compounds (proline, total soluble protein)

The variation of proline quantity because of applying melatonin to plant parts at different doses through different ways of application is given in Table 2. The dose, time, and way of application were determined to cause statistically significant variations ($p<0.05$) on the studied parameters. Analyses conducted at the beginning of the vegetation period (April), mid-vegetation period (July), and at the end of the vegetation period (November) found that proline contents increased in all treatments compared to the control group in April and July.

Table 1. Statistical Values of Sapling Morphological Characters

Dose* Appl.	Control	250 µM Needle-spraying	250 µM Root-dipping	500 µM Needle-spraying	500 µM Root-dipping	1000 µM Needle-spraying	1000 µM Root-dipping	1500 µM Needle-spraying	1500 µM Root-dipping	*F-value / P-level*
SH (cm)	11.13±0.485a	11.62±0.425a	10.85±0.570a	11.53±0.339a	11.22±0.361a	11.29±0.377a	11.31±0.291a	11.38±0.351a	11.50±0.276a	*0.345 ns*
RCD (mm)	4.44±0.165b	4.50±0.119b	4.77±0.136ab	4.56±0.223b	5.04±0.174a	4.52±0.146b	4.82±0.127ab	4.69±0.105ab	4.79±0.113ab	*1.629**
NBS	2.46±0.290b	4.33±0.231a	2.50±0.317b	4.16±0.407a	2.50±0.177b	3.03±0.297b	2.83±0.249b	3.16±0.472b	2.16±0.328b	*5.724****
NB	2.50±0.223cd	3.50±0.141b	5.50±0.733a	3.16±0.249bc	3.00±0.185bc	2.96±0.188bc	3.16±0.127bc	2.33±0.087cd	1.86±0.171d	*11.951****
RL (cm)	28.33±0.74b	27.63±0.622b	29.76±0.816ab	27.70±0.618b	31.26±0.434a	28.40±0.743b	29.13±0.869ab	28.20±0.770b	29.53±0.774ab	*2.620 **
FWS (gr)	4.73±0.310bc	5.32±0.249ab	3.26±0.222d	6.33±0.951a	3.93±0.296cd	4.73±0.206bc	3.55±0.200cd	5.62±0.180ab	3.27±0.307d	*7.710****
FWR (gr)	2.03±0.176c	2.77±0.216ab	1.63±0.167c	3.41±0.564a	1.95±0.089c	2.19±0.173bc	1.73±0.091c	1.83±0.052c	1.41±0.089d	*7.221****
FWSD (gr)	6.76±0.309bc	8.10±0.347b	4.89±0.326e	9.74±1.488a	5.88±0.337cde	6.97±0.357bcd	5.29±0.257de	7.45±0.147bc	4.69±0.378e	*8.127****
DWSH (gr)	2.98±0.096ab	3.09±0.098a	3.06±0.091ab	3.07±0.104ab	2.80±0.177ab	3.05±0.089ab	2.76±0.140ab	3.05±0.098ab	2.70±0.121b	*1.741***
DWR (gr)	1.68±0.124a	1.39±0.090a	1.38±0.112a	1.54±0.165a	1.39±0.139a	1.41±0.134a	1.39±0.121a	1.40±0.121a	1.43±0.121a	*0.587 ns*
DWS (gr)	4.66±0.149a	4.48±0.127a	4.45±0.128a	4.61±0.216a	4.20±0.241a	4.46±0.170a	4.15±0.205a	4.46±0.136a	4.13±0.193a	*1.195 ns*
%Root	35.08±1.951a	30.64±1.632a	30.51±1.931a	31.38±2.169a	33.29±2.245a	30.22±2.029a	32.46±2.039a	30.68±1.947a	33.44±1.856a	*0.721 ns*
LI	2.54±1.035ab	2.60±1.017ab	2.36±1.548b	2.72±1.660a	2.35±0.728b	2.53±1.060ab	2.38±0.772ab	2.44±0.771ab	2.33±0.758b	*1.512**
SI	20.18±0.226a	20.62±0.250a	20.69±0.247a	20.67±0.265a	20.46±0.256a	20.84±0.310a	20.41±0.197a	20.64±0.226a	20.29±0.200a	*0.772 ns*
DQI	0.17±0.010a	0.16±0.008a	0.19±0.014a	0.17±0.012a	0.16±0.009a	0.16±0.010a	0.16±0.009a	0.16±0.006a	0.16±0.010a	*0.856 ns*

Seedling height (SH); Root collar diameter (RCD); number of branches per seedling (NBS); number of buds (NB); root length (RL); The fresh weight of the shoot (FWS); the fresh weight of the root (FWR). FTA: the total fresh weight of the seedlings (FWSD). dry weight of the shoot (DWSH). dry weight of the root (DWR). total dry weight of the seedlings (DWS). Root percentage (%Root). Layering index (LI); Sturdiness index (SI); Dickson quality index (DQI) Significant at *** 0.001 level. ** 0.01 level . * 0.05 level. ns Non-significant. a.b.ab.….bcd There is a significant difference at 0.05 level between groups shown with different letters

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According to all melatonin applications in November, higher proline was determined in control seedlings. In April, while vegetation was in its beginning, the melatonin dose of 1,500 µM applied to seedling roots constituted a higher proline value compared to control and all other treatments. In the mid-vegetative month of July, the application of melatonin in the form of spraying increased the proline value, also the highest proline value was ensured with the 500, 1000, and 1500 µM doses applied with the needle-spraying to shoots.

In terms of protein values (Table 2), the highest values were determined in the 250 µM treatment applied to the root part of the seedling in April and the 500 µM melatonin treatment applied to the seedling shoots in July. The control seedlings had a higher protein value in all treatment doses and administration ways in November after the vegetation period was completed.

Table 2. The Effect of Melatonin Applications at Different Doses on Nitrogenous Compounds

Dose * Application	Proline (µmol/g)			Protein (mg/g)		
Mounts	April	July	November	April	July	November
Control	7.45±0.109 ı	12.98±0.017 ef	15.87±0.008 8a	11.08±0.058 c	8.91±0.099 d	13.32±0.105 1a
250 µM/ Needle-spraying	8.27±0.028 h	14.85±0.050 d	13.25±0.018 2e	8.16±0.222 f	10.02±0.12 8c	6.09±0.0365 ı
250 µM/ Root-dipping	9.13±0.065 e	13.47±0.541 ef	14.15±0.074 6c	14.33±0.066 a	10.01±0.09 4c	12.19±0.142 1b
500 µM/ Needle-spraying	8.57±0.049 g	18.51±0.057 a	11.97±0.013 5f	9.60±0.028 e	12.4±0.047 a	6.78±0.0366 h
500 µM/ Root-dipping	9.55±0.025 d	17.18±0.053 b	8.62±0.0150 h	10.46±0.050 cd	8.50±0.046 e	8.98±0.0484 d
1000 µM/ Needle-spraying	8.92±0.010 f	18.32±0.051 a	14±0.0362 d	13.45±0.448 b	10.36±0.17 3b	9.65±0.0433 c
1000 µM/ Root-dipping	10.59±0.13 7c	13.64±0.053 e	15.23±0.043 7b	9.56±0.101 e	7.88±0.151 f	7.95±0.0465 f
1500 µM/ Needle-spraying	10.93±0.04 6b	18.43±0.076 a	11.92±0.036 2f	10.22±0.062 de	7.64±0.151 f	7.38±0.0340 g
1500 µM/ Root-dipping	12.61±0.08 0a	16.40±0.120 c	11.77±0.026 4g	8.55±0.322 f	7.08±0.090 g	8.41±0.0455 e
Average ± Std. error	9.56±0.159	15.98±0.235	12.98±0.219 6	10.60±0.220	9.21±0.172	8.97±0.2425
F value / P value	*465.727***	*142.353***	*3727.973***	*84.721***	*206.053***	*1205.913***

*** Significant at the 0.001 level. a.b....de There is a significant difference at 0.001 level between groups shown with different letters.

Its effect on enzyme activities (superoxide dismutases/SOD and peroxidase/POD)

When studying the effects of melatonin on antioxidant enzyme activities, no significant differences were detected in April SOD activity (Table 3). In July, SOD activity showed high value in all melatonin applications compared to control. The application of 250 µM melatonin, specifically by needle-spraying, showed the highest enzyme activity (16.61 EU). A significant decrease in SOD was detected in November compared to July (Table 3). As for POD activity, the highest POD values were determined in the Control treatment in November, 1000 µM needle-spraying in April, and 500 µM in July (Table 3).

Table 3. Effect of Melatonin Applications at Different Doses on Enzyme Activities

Dose * Application	SOD (EU /mg protein)			POD (EU /mg protein)		
Mounts	April	July	November	April	July	November
Control	2.67±0.001	4.83±0.125f	3.85±0.023 d	0.37±0.003c	0.26±0.004 d	0.51±0.006 a
250µM/ Needle-spraying	2.68±0.001	16.61±0.094 a	3.93±0.022c	0.11±0.002f	0.32±0.006c	0.14±0.001ı
250µM/ Root-dipping	2.67±0.002	10.78±0.140 c	1.15±0.041 h	0.58±0.004 b	0.31±0.004c	0.44±0.008 b
500µM/ Needle-spraying	2.67±0.001	14.33±0.292 b	1.14±0.022 h	0.11±0.003f	0.46±0.002 a	0.17±0.001 h
500µM/ Root-dipping	2.68±0.000 9	14.15±0.335 b	2.54±0.020 e	0.32±0.016 d	0.24±0.002 e	0.26±0.002 d
1000µM/ Needle-spraying	2.67±0.001	10.93±0.016 c	2.33±0.015f	1.29±0.006 a	0.33±0.009 b	0.3±0.002c
1000µM/ Root-dipping	2.67±0.000 9	6.79±0.068e	5.01±0.027 b	0.29±0.005 e	0.21±0.006f	0.22±0.002f
1500µM/ Needle-spraying	2.67±0.000 0	8.41±0.050d	1.55±0.016 g	0.33±0.003 d	0.21±0.006f	0.19±0.001 g
1500µM/ Root-dipping	2.68±0.001	10.59±0.063 c	5.34±0.023 a	0.12±0.007f	0.18±0.003 g	0.24±0.002 e
Average ± Std. error	2.67±0.000 6	10.83±0.382	2.98±0.160	0.39±0.036	0.28±0.008	0.27±0.012
F value / P value	*0.343^{ns}*	*507.594****	*5910.510****	*2687.038****	*227.811****	*938.935****

*** Significant at the 0.001 level. $^{a.b....h}$ There is a significant difference at 0.001 level between groups shown with different letters.

Its effect on carbon compounds (glucose, sucrose)

The highest values of sucrose were determined in all of the months of April, July, and November, following the application at the dose of 250 µM to the root. On the other hand, it was determined that different treatment combinations in different periods created the highest glucose content value.

The highest glucose content was determined by the spraying of 1000 µM melatonin to the needle leaves in April, while 250 µM melatonin application to the roots and needle leaves in July produced the highest glucose value, and in November, the highest dose was achieved by 1500 µM melatonin treatment applied to the root within the scope of the research (Table 4).

Its effect on oxidative stress (H_2O_2, MDA)

When the effects of different doses of melatonin applications on the amount of H_2O_2 were examined, the H_2O_2 concentration was found to be higher in all melatonin treatment groups compared to the control in both April and July. On the other hand, a higher H_2O_2 value was detected in the control treatment in the dormant phase of the seedlings in November compared to all melatonin treatment combinations (Table 5). In April, the H_2O_2 content was the highest in seedlings that were treated with both needle-spraying and root-dipping at the melatonin dose of 500 µM.

In July, H_2O_2 was at the highest level at 500 µM melatonin applications through needle-spraying and 250 µM through root-dipping (Table 5). The MDA concentration was the highest in the control in April, while it was detected that the spray application of melatonin at the dose of 250 µM to the needle leaves in July and November caused the highest concentration (Table 5).

Table 4. Effect of Melatonin Application at Different Doses on Carbonated Compounds

Dose * Application	Sucrose (mg·g^{-1})			Glucose (mg·g^{-1})		
Mounts	April	July	November	April	July	November
Control	66.32±1.587 d	70.51±0.055 bc	72.73±0.279 f	54.85±0.085 cd	62.13±0.036 b	52.98±0.0393 e
250µM/ Needle-spraying	76.38±1.336 c	74.15±0.085 b	79.51±0.022 c	57.33±0.0716 bc	65.08±0.059 a	60.87±0.016 d
250µM/ Root-dipping	86.33±1.699 a	78.98±0.443 a	98.55±0.261 a	60.38±0.0551 ab	64.92±0.017 a	59.06±0.024 e
500µM/ Needle-spraying	64.69±1.533 de	68.83±0.071 c	79.50±0.025 c	54.47±0.1112 cd	61.05±0.048 bc	57.41±0.015 f
500µM/ Root-dipping	79.96±0.434 bc	65.47±0.111 c	81.89±0.016 b	60.30±0.0867 ab	57.07±0.058 d	63.43±0.017 b
1000µM/ Needle-spraying	83.73±1.246 ab	68.05±0.028 c	74.55±0.152 e	63.02±0.1059 a	60.20±0.019 c	60.86±0.011 d
1000µM/ Root-dipping	64.40±1.576 de	66.00±0.087 c	79.48±0.034 c	52.60±0.0964 d	57.95±0.058 d	62.70±0.020 c
1500µM/ Needle-spraying	61.47±1.541 e	65.60±0.082 c	79.43±0.038 c	53.14±0.0774 d	58.48±0.060 d	63.50±0.030 b
1500µM/ Root-dipping	65.09±1.504 de	65.35±0.045 c	77.29±0.033 d	54.15±0.2257 cd	58.16±0.034 d	66.87±0.037 a
Average ± Std. error	72.04±1.055	69.21±0.070	80.33±0.740	56.69±0.0514	60.56±0.033	60.85±0.403
F value / level	*44.444****	*8.171****	*2816.839****	*11.106****	*41.216****	*24973.304****

*** Significant at the 0.001 level. [a.b....de] There is a significant difference at 0.001 level between groups shown with different letters

Table 5. Effect of Melatonin Applications at Different Doses on Oxidative Stress

Dose * Application	H_2O_2 (µmol/g)			MDA (µmol/g)		
Mounts	April	July	November	April	July	November
Control	6.77±0.054 h	25.58±0.081 h	69.06±0.108 4a	4.37±0.110 a	12.65±0.076 d	19.48±0.094 b
250µM/ Needle-spraying	8.17±0.052de	30.61±0.175 e	50.38±0.204 3f	3.98±0.243bcd	16.58±0.071 a	20.94±0.012 a
250µM/ Root-dipping	6.79±0.042 g	36.90±0.170 a	43.76±0.205 8g	3.75±0.065 de	9.75±0.088 g	18.85±0.028 d
500µM/ Needle-spraying	8.83±0.058 b	35.03±0.085 b	53.85±0.085 7e	3.55±0.089 e	11.5±0.035 f	18.18±0.085 f
500µM/ Root-dipping	9.39±0.084 a	27.02±0.181 g	59.33±0.109 8c	4.34±0.076 ab	12.34±0.115 e	19.29±0.033 c
1000µM/ Needle-spraying	7.55±0.073 e	33.58±0.099 c	57.99±0.082 4d	3.94±0.105 cd	12.44±0.093de	18.86±0.042 d
1000µM/ Root-dipping	7.13±0.028 f	32.18±0.160 d	66.95±0.085 0b	3.72±0.066 de	15.35±0.089 b	16.32±0.094 h
1500µM/ Needle-spraying	8.00±0.256 d	27.76±0.286 f	41.66±0.045 7h	4.30±0.157 abc	14.09±0.198 c	17.47±0.033 g
1500µM/ Root-dipping	8.42±0.100 c	33.72±0.136 c	53.79±0.183 3e	4.22±0.048 abc	15.32±0.053 b	18.93±0.006 d
Average ± Std. error	7.89±0.097	31.38±0.392	55.20±0.932	4.02±0.049	13.34±0.219	18.70±0.131
F value / P level	*75.223****	*561.341****	*4746.064****	*6.262****	*459.503****	*505.536****

*** Significant at the 0.001 level. [a.b....h] There is a significant difference at 0.001 level between groups shown with different letters.

Its effect on photosynthetic pigment content (chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a/b, total carotenoid)

In April, the highest amount of chlorophyll *a* was obtained from 1500 µM root-dripping application and chlorophyll *b* content from 500 µM needle-spraying application (Table 6). In July, both chlorophyll *a* and chlorophyll *b* contents were lower in both needle spraying and root dipping applications of 1500 µM than the control. When the November values of chlorophyll *a* and *b* amounts were examined, it was seen that higher values were obtained for both pigment contents at 250 µM melatonin dose in both needle-spraying and root-dipping applications compared to control and other melatonin treatment combinations.

When the effects of the applications on the total chlorophyll values (Table 7) were examined, 500 µM melatonin dose in April and July and 250 µM melatonin dose in November gave the highest total chlorophyll amount in needle-spraying (Tables 6, 7). When the chlorophyll *a* /chlorophyll *b* ratio was examined in terms of the values in November when the dormancy phase started in seedlings, it was determined that 250 µM melatonin dose in the needle-spraying application gave higher results than the control group. Changes in the total amount of carotenoids depending on the months and doses are given in Table 7. In November, a dose of 1500 µM melatonin applied as a spray to the needles resulted in higher total chlorophyll content than in the control and all other melatonin treatment combinations (Table 7). According to Table 8 data, the highest carotenoid value was determined in the 500 µM melatonin dose application given by spraying the needles in April and July.

Table 6. The Effect of Melatonin Applications at Different Doses on Photosynthetic Pigments: Chlorophyll *a*, Chlorophyll *b*

Dose * Application	Chlorophyll *a* (mg·g^{-1})			Chlorophyll *b* (mg·g^{-1})		
Mounts	April	July	November	April	July	November
Control	0.008±0.00003 h	0.047±0.001 c	0.081±0.0001 b	0.013±0.00006 h	0.029±0.0008 c	0.145±0.0005 c
250µM/ Needle-spraying	0.051±0.0001 b	0.055±0.0003 b	0.109±0.0002 a	0.076±0.00128 de	0.038±0.0013 ab	0.178±0.0002 a
250µM/ Root-dipping	0.042±0.00005 e	0.047±0.0014 c	0.081±0.0000 b	0.077±0.00042 d	0.042±0.0052 a	0.146±0.0000 b
500µM/ Needle-spraying	0.050±0.00004 c	0.064±0.0002 a	0.08±0.0006 c	0.090±0.00018 a	0.039±0.0005 a	0.143±0.0002 d
500µM/ Root-dipping	0.046±0.00004 d	0.055±0.0001 b	0.081±0.0000 b	0.075±0.00034 e	0.033±0.0003 bc	0.145±0.0001 c
1000µM/ Needle-spraying	0.032±0.00015 g	0.048±0.0001 c	0.081±0.0000 b	0.048±0.00021 g	0.033±0.0002 bc	0.143±0.0000 d
1000µM/ Root-dipping	0.051±0.00003 b	0.034±0.002 d	0.08±0.0001 c	0.078±0.00008 c	0.019±0.0019 d	0.143±0.0000 d
1500µM/ Needle-spraying	0.041±0.00008 f	0.033±0.0001 d	0.077±0.0000 d	0.063±0.00018 f	0.02±0.00018 d	0.136±0.0000 e
1500µM/ Root-dipping	0.053±0.00005 a	0.031±0.003 d	0.077±0.0000 d	0.082±0.00031 b	0.017±0.0010 d	0.137±0.0000 e
General Average	0.042±0.0014	0.046±0.0012	0.083±0.0009	0.067±0.00236	0.0304±0.0011	0.146±0.0012
F value / P level	*36546.579***	*50.177***	*1710.641***	*2316.034***	*22.450***	*3012.704***

*** Significant at 0.001 level. [a.b....e] There is a significant difference at 0.001 level between groups with different letters.

Table 7. The Effect of Melatonin Applications at Different Doses on Photosynthetic Pigments: Total Chlorophyll, Chlorophyll *a*/b, Total Carotenoid

Dose * Application	Total Chlorophyll (mg·g^{-1})			Chlorophyll *a*/*b* (mg·g^{-1})			Total Carotenoid (mg·g-1)		
	April	July	November	April	July	November	April	July	November
Control	0.021±0.00006ı	0.076±0.0018d	0.226±0.0005c	0.646±0.00469c	1.598±0.0197bc	0.561±0.0022cd	1.469±0.00327h	4.519±0.130cd	1.523±0.0360d
250 µM/Needle-spraying	0.127±0.00118d	0.094±0.0015b	0.287±0.0004a	0.682±0.01420a	1.452±0.0480cd	0.61±0.0006a	5.469±0.00525b	4.55±0.064cd	1.484±0.0014e
250 µM/Root-dipping	0.119±0.00038f	0.09±0.0067bc	0.228±0.0001b	0.550±0.00366e	1.323±0.1853d	0.555±0.0004f	5.109±0.00280d	4.25±0.068de	1.533±0.0052d
500 µM/Needle-spraying	0.140±0.00016a	0.104±0.0005a	0.223±0.0004d	0.559±0.00143e	1.649±0.0230bc	0.558±0.0052def	5.840±0.00244a	5.786±0.008a	1.485±0.0024e
500 µM/Root-dipping	0.121±0.00030e	0.088±0.0004bc	0.226±0.0002c	0.620±0.00338d	1.645±0.0141bc	0.559±0.0007def	4.82±0.00215e	5.274±0.009b	1.419±0.0006f
1000 µM/Needle-spraying	0.080±0.00009h	0.081±0.0003cd	0.225±0.0001d	0.665±0.00583b	1.45±0.0093cd	0.566±0.0006bc	3.912±0.01480g	4.828±0.006c	2.201±0.0006b
1000 µM/Root-dipping	0.129±0.0008c	0.053±0.0046e	0.223±0.0001d	0.651±0.00079bc	1.881±0.0489a	0.556±0.0011ef	5.34±0.00706c	3.843±0.138f	2.211±0.0025b
1500 µM/Needle-spraying	0.104±0.00013g	0.053±0.0002e	0.214±0.0001e	0.646±0.00299c	1.644±0.0123bc	0.568±0.0004b	4.394±0.00410f	3.724±0.006f	2.305±0.0043a
1500 µM/Root-dipping	0.136±0.00026b	0.049±0.0045e	0.214±0.0000e	0.654±0.00307bc	1.717±0.0941ab	0.563±0.0004bcd	5.845±0.00265a	3.983±0.249ef	2.166±0.0035c
General Average	0.109±0.00376	0.076±0.0022	0.230±0.0022	0.630±0.00495	1.596±0.0287	0.566±0.0018	4.690±0.136	4.529±0.076	1.814±0.0390
F-value. P-level	*7209.636****	*38.950****	*5838.993****	*62.616****	*5.015****	*72.245****	4813.238***	38.862***	*991.313****

*** Significant at the 0.001 level. $^{a.b....def}$ There is a significant difference at 0.001 level between groups shown with different letters.

Discussion

In semi-arid areas, root collar diameter is a more important criterion than seedling height in determining seedling quality (Ayan *et al*. 2021). Because thick-root collar diameter seedlings are richer in nutrient content, the amount of leaves is higher. The plant has the capacity to absorb and hold more water, provided that it has a thicker cross-section surface, hence the sufficient root system. In addition, since they have a thick cuticula and wood layer, they are more resistant to mechanical stresses (Yahyaoğlu and Genç 2007). From the results obtained within the scope of this research, it was determined that melatonin had a positive effect on RCD characteristics compared to the Control seedlings (Table 1).

Aphalo and Rikala (2003) define the seedlings with SI < 50 as "quality seedlings". It was observed that the mean SI value was <50 in all of the seedlings. In addition, LI value below 3 is a parameter that indicates good root growth of the seedlings (Ayan *et al*. 2021). In this study, LI values were also found below 3 (Table 1). Also, according to the root/shoot ratio of the *Pinus nigra* Arnold subsp. *pallasiana* (Lamb.) [sinonim *P. nigra* var. *caramanica*] Holmboe two-years-old seedlings were generally considered to be of better-quality (Akgül 2010).

If bare-rooted seedlings are used in afforestation of semi-arid lands, especially for coniferous species, the stem to root ratio should be ideally between 2 and 2.5 (ÇEMGM 2013). The S:R ratio is effective on the water stress, in other words, on the physiological condition of the seedling in environments in which the seedling is grown. Therefore, seedlings with a S:R ratio of a maximum 3 have a higher success rate in drought areas. This is because the seedlings are strong enough to meet the water they lose by transpiration with their roots. For these reasons, it is recommended to use seedlings with a G:D ratio of max 3, especially in afforestation to be made in arid regions (Eyüboğlu *et al.* 1992). In afforestation, seedlings with a low SH:RCD ratio should be used as in the S:R ratio. Deligöz *et al*. (2009) stated that *P. nigra* seedlings in quality class 4 (minimum collar diameter is 4 mm and minimum seedling height is 10 cm) were the most suitable to outplanting as they had the best survival and growth. Moreever, morphological features better forecasted field performance in the first three years at the shallow soil site, with the number of the first order lateral roots being the best single morphological variable. Height and root dry weight were the best attributes to forecast growth during the first three years after planting on both sites (Ivetić *et al*. 2016).

The fact that the seedlings in the Tosya plantation site with a semi-arid climate have a positive effect on the characteristics of seedlings roots, especially LI, RCD, RL, and FWR, at the end of the first year shows that the seedlings can maintain their success performance in the subsequent years. Researchers commonly use root cutting in the production of bare-rooted seedlings for semi-arid areas. For example, the highest percentages of the seedlings roots of Anatolian black pine are found as 37.04% in twice lateral pruning with undercutting (Çetinkaya and Deligöz 2012) and 18.25% in once root pruning (Avanoğlu *et al*. 2005). The production of seedlings with root systems that meet better morphological and physiological standards is essential to enable them to rapidly establish and thrive upon field performance (Davis and Jacobs 2005). In addition Çömez and Gezgin (2019) suggested that higher root-collar diameter and number of lateral roots in seedlings of the subspecies can be obtained with the application of potassium nutrition in the nursery phase for the success in afforestation of semi-arid areas. Mycorrhiza inoculation at the nursery stage was found to be effective only on root length. Especially,

Rhizopogon Fr. (1817) inoculation was suggested to obtain healthy and well-growing seedlings of the subspecies (OGM 2013). Lazarević *et al*. (2012) reported that the use of vegetative and spore inoculums of autochthonous *Suillus granulatus* (L.) Roussel (1796) proved to be an effective method of obtaining containerized ectomycorrhizal *P. nigra* seedlings under open field conditions after 11 months. As can be understood from the result of this research, in summary; melatonin application has a positive effect on the root development of seedlings such as root cutting, mycorrhiza inoculation, and potassium fertilization. Zhao *et al*. (2021) stated that melatonin has an important role as a growth regulator and enables the development of tissues such as root and shoot.

The Effect of Different Doses of Melatonin Treatments on Bioactive Chemical Components

Deligöz *et al*. (2018), studying seasonal and needle age-related variations in the biochemical characteristics of *P. n.* subsp. *Pallasiana*, owing to the closely linked associations between plant growth and photosynthesis, reported that needle age and seasons affected carbohydrate and pigment contents. As can be seen in Table 2, proline values were higher than the control in April and July. However, the amount of proline in the treatment groups was highest in July compared to the control, and lower in November. This situation during the vegetation period is an indication that the activity of metabolic reactions is rapid, especially in the months of active growth (Howe 1990; Oliveira *et al*. 1994). As a matter of fact, the low amount of proline in November confirms this result. Researchers have reported that amino acid, protein, and carbohydrate metabolism slows down at low temperatures due to the decrease in the kinetic energy and the slowdown of metabolic activity (Drossopoulos and Niavis 1988; Reich *et al.* 1998). The proline content of plant samples is higher in the needle-spraying application than in the root-dipping method in July when the metabolism is accelerated (Table 2). High dose (1500 μM) proline content increased more in both needle- spraying and root-dipping applications in April. This situation has been associated with the fact that the application of high doses of melatonin stimulated the metabolic activities in the plant, induced the synthesis events in both roots and leaves, and started the growth and development process in the seedlings (Maier *et al*. 1998; Kumar *et al*. 2003). In parallel with the increase in soil and atmospheric temperature at the beginning of vegetation, it is stated that melatonin may stimulate adaptation in root and leaf cells during the temperature change process in seedlings (Aasamaa *et al*. 2004; Sun *et al*. 2021). Indeed, many researchers have reported that the amount of proline in plant tissues increases under stress conditions caused by environmental changes, which play an active role in the preservation of osmotic potential in developing root and leaf cells, stimulation of cell cycle, strengthening of cells by accumulating proline-rich proteins in the walls, and detoxification of toxic compounds such as MDA and ROS that may occur during metabolic reactions (Bohnert and Sheveleva 1998; Jovanovic-Galovic *et al*. 2004; Dichio *et al*. 2006). Taking into account the protein values, the values in November were found to be the highest in the control group. This condition has been associated with the termination of vegetation in seedlings and the termination of phenological activities (Oliveira *et al*. 1994; Poorter 1999). It was concluded that decreased air and soil temperature during these months caused metabolism to stop in both leaves and root cells, and melatonin applications in November did not stimulate metabolic activity in plant tissues (Berry and Björkman 1980; Barbaroux and Breda 2002). It is thought that the increase in air temperature, the change in the water ratio in the air and

in the soil, and the acceleration of physiological processes in seedlings at 500 and 1000 µM spraying doses in July may be effective in the formation of the highest protein content compared to the control (Staswick 1994). Proline and soluble protein are important osmolytes (Morgan 1984; Shao *et al*. 2006). As a matter of fact, the results obtained in protein in July and November coincide with the results of proline accumulation (Table 2). The SOD and POD enzyme activity values obtained from different doses and ways of melatonin administration were parallel to the total soluble protein and proline values. The protein values are also high in the needle-spraying applications and in the melatonin doses of 500 and 1000 µM, where POD measurements in July showed the highest increase (Table 3). In November, when metabolic activities slowed down, melatonin of 1500 and 1000 µM in root-dipping applications had a positive effect on SOD values (Gindaba *et al*. 2004; Millard *et al*. 2007). This can be explained by the protective role of antioxidant enzymes in maintaining homeostasis in cells under environmental change conditions (Vitasse *et al*. 2011). In this study, it was observed that melatonin has a positive effect on soluble sugars (Sucrose and Glucose) at different doses and in different application processes. It can be said that these compounds are effective in regulating osmotic regulation in cells, maintaining the structure and components of cellular membranes, especially during periods when metabolic reactions slow down and the temperature drops (Schrader and Sauter 2002; Barbaroux *et al*. 2003). In April and July, the highest sucrose and glucose values were observed in the application of 250 µM *via* the root-dipping (Table 4).

H_2O_2 is an important compound that acts as a signaling molecule in cellular tissues and cells and can accumulate in the cell in normal metabolic reactions (Barba-Espin *et al*. 2010). However, the amounts of this compound above the threshold value are toxic (Aasamaat *et al*. 2004). The amount of this compound in plant tissues may vary depending on plant age, development of organs, and environmental factors (Halliwell 2006; Bazot *et al*. 2013). In the study, while the H_2O_2 content increased in July when the metabolism was active, it decreased in November when the metabolism slowed down, confirming this result. With the warming of the weather at the beginning of the vegetation in April, the needle-spraying dose of 500 µM melatonin was high in both root tissue and leaves, which can be explained by stimulating photosynthesis rate and root respiration (Ogren 2000; Atkin *et al*. 2000). In the process of growth and development in plant tissues, mitotic divisions in stem cells and leaf tissue increase, the osmotic regulation and, in addition, cell wall activity also accelerate. In these processes, the accumulation of MDA in the cell is a normal condition. However, high doses of MDA have a toxic effect on cells and tissues. In the study, it was observed that melatonin applications generally reduced the MDA content in seedlings (Table 5). Especially in April, the MDA content was low compared to the control. However, in this month, the H_2O_2 content in the seedlings was high. In other studies, it has been emphasized that the higher the H_2O_2, the higher the MDA content in the cells (Halliwell 2006; Quan *et al*. 2008). However, the low content of MDA at a high concentration of H_2O_2 suggested in this study that melatonin applications protect the structure of cell membrane lipids, inhibit peroxidation reactions, and suppress the accumulation of MDA in tissues.

Photosynthetic pigments are the most functional elements of photosystems localized in the layers of granules (inner membrane tissue) and phospholipids of chloroplasts. Photosystems are complex structures formed by chlorophyll *a*, chlorophyll *b*, and carotenoids with proteins. In photosynthesis, auxiliary pigments absorb the energy under visible light to provide energy, and ATP and NADPH are synthesized due to energy

flowing in the ENB system (Halliwell 2006; Quan *et al*. 2008). However, although each pigment absorbs sunlight in photosystems, the energy of sunlight is transported only by electrons that break off from chlorophyll-*a* molecules and are converted into usable energy (Alyammahi and Gururani 2020). In addition to absorbing light, carotenoids and chlorophyll *b* molecules also play an active role in protecting the chlorophyll *a* molecule from photooxidation, protecting membrane lipids in which pigment systems settle, and eliminating ROS damage (Berger *et al*. 2001; Jahns *et al*. 2009). When the effect of exogenous melatonin applications on photosynthetic pigments at different doses and ways of application was examined, pigment amounts showed significant changes according to time, dose, and application processes. The amount of chlorophyll *a* and chlorophyll *b* was higher in all application groups in April compared to the control. In July, the content of both chlorophyll *a* and chlorophyll *b* was the lowest in both needle-spraying and root-dipping at the melatonin dose of 1500 µM, compared to the control (Table 6). In November, the application of 250 µM in both needle-spraying and root-dipping applications increased both pigment content compared to the control. In light of the data, it can be said that chlorophyll *a* and chlorophyll *b* data are negatively affected by 1500 µM high-dose melatonin applications when the temperature increases, but low-dose affected pigment content positively when the temperatures decrease (Ohtsuka *et al*. 1997; Ougham *et al*. 2008). Many researchers have noted that chlorophyll *a* and *b* molecules at high temperature or high light intensity are broken down by oxidation or their synthesis is blocked (Kauppi, 1991; Johnson and Ruban 2009). However, both pigments were generally high in April and July, which was associated with melatonin application preventing pigment destruction by preserving the phospholipid layer in the grana membranes (Arnao and Hernandez-Ruiz, 2015; Ougham *et al*. 2008). The total chlorophyll content in plants (Table 6) increased with an increasing dose in root-dipping in April and showed an increase in low doses (especially 500 and 250 µM) in needle-spraying. In July, the total amount of chlorophyll (1500 µM) decreased at high doses. In November, the lowest (250 µM) dose reduced the total amount of chlorophyll compared to the control (Table 7). An increase in the total amount of chlorophyll in high-dose root applications in April has been associated with the fact that melatonin stimulates pigment synthesis and inhibits the destruction of chlorophyll molecules (Arnao and Hernández-Ruiz, 2015; Liang *et al*. 2015). Melatonin may play an active role in protecting pigment systems by increasing the stability of chloroplast membrane lipids due to its strong reaction with lipids (Arnao and Hernandez-Ruiz 2015; Wang *et al*. 2013). Chlorophyll a:b is the most important parameter showing the ratio of chlorophyll *a* to chlorophyll *b* in leaves. The amount of chlorophyll *b* may be higher in shade-loving plants, and the transformation between the two pigments in light conditions plays an important role in the adaptation of light intensity changes (Demming-Adams and Adams 1996; Alyammahi and Gururani 2020). Chlorophyll *a:b* ratio was high in 250 µM and 1000 µM spraying doses in April and November and 1500 µM spraying in each of three months (Table 7). In April and July, it was high at 1000 µM dose (Table 7). These results show that melatonin application positively affects the pigment content in general during periods when vegetation is active. However, high doses have been more effective in hot periods. This indicates that melatonin applications protect chloroplast membranes from photooxidation in high light intensity and drought conditions (Berger *et al*. 2001; Wang *et al*. 2013; Liang *et al*. 2015). Also, the positive effect of melatonin applications was noticeable in November. This result has been associated with the fact that melatonin prevents peroxidation events occurring in phospholipids in chloroplast membranes not only

in summer but also in seasons dominated by low temperature (Hardeland *et al*. 2000; Kolar and Machackova 2005). Melatonin applications affected the carotenoid content in leaf samples differently based on the dose and method of administration (Table 7). In needle-spraying applications, 250 and 500 μM doses in April and July and 1000 and 1500 μM doses in November increased the carotenoid content. In root application, an increase was observed in April for all doses and in July only for 500 μM, and in November for 1000 μM, 1500 μM, and 250 μM (Table 7). In line with the carotenoid data, both spraying and root-dipping melatonin applications stabilized the lipid activities in the chloroplast membranes against temperature changes in April, during which an increase in carotenoids was observed (Kolar and Machackova 2005; Arnao and Hernández-Ruiz 2013; Bajwa *et al*. 2014). Indeed, high-dose melatonin (1000 and 1500 μM) administration in November increased the carotenoid content, confirming this result. These doses may have prevented oxidation of fatty acids in chloroplast membrane lipids in the period when growth and metabolic activity slowed down and may have been effective in increasing resistance to low temperature (Hardeland *et al*. 2000; Kolar and Machackova 2005; Bajwa *et al*. 2014). The employed melatonin substance provides support for the metabolic process for the resistance of seedlings to low temperatures and semi-arid climatic conditions as well as melatonin applications in the form of spraying in the planting area during the summer drought period will increase the success of the plantation.

CONCLUSIONS

1. When both the needle-spraying and root-dipping methods for melatonin application were evaluated in terms of morphological and biochemical variables, the best results were determined in low doses.
2. Compared to control group seedlings, the statistically significant results ($p < 0.05$) were achieved in 250 μM > 500 μM > 1000 μM > 1500 μM application dose groups, respectively.

ACKNOWLEDGMENTS

Funding support from the Kastamonu University (Science Research Project Foundation KÜ-BAP01/2018-50 Kastamonu, Turkey) is gratefully acknowledged.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Declarations

The authors declare no conflict of interest.

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Article submitted: November 29, 2022; Peer review completed: January 14, 2023; Revised version received and accepted: January 30, 2023; Published: February 6, 2023.
DOI: 10.15376/biores.18.2.2551-2572