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# Physiological and Agronomic Responses of Peanut (*Arachis hypogaea* L.) to Melatonin Seed Pretreatment and Gamma-Aminobutyric Acid Foliar Application under Accelerated Aging

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## ABSTRACT

This research aims to investigate the effects of melatonin and gamma-aminobutyric acid (GABA) on improving plant quality from aged peanut seeds, a factorial experiment was conducted in 2021–2022 using a randomized complete block design (RCBD) with three replications. Treatments included two seed quality levels (normal and aged), three melatonin pretreatment concentrations (0, 50, or 100 µM), and two GABA foliar application levels (0, or 1 mM). Seed aging was induced at 40°C and 96–100% humidity for 96 hours, followed by 8-hour melatonin soaking. Aging reduced pegs and pods per plant, 100-seed weight, and relative water content while increasing MDA, anthocyanin, and antioxidant enzyme activities (SOD, CAT). Pretreatment with 100 µM melatonin improved yield traits and enzyme activity in aged seeds. Both 50 and 100 µM melatonin increased anthocyanin and reduced MDA. GABA foliar application (1 mM) enhanced pegs per plant, 100-seed weight, RWC, shell yield, and antioxidant enzyme activity, while reducing MDA. Seed aging reduced yield by 45.81%, but melatonin pretreatments (50 and 100 µM) improved it by 9.91% and 11.33%, respectively. In normal seeds, these treatments increased yield by 7.53% and 14.66%. GABA application improved yield by 6.53%. Path analysis showed that pod number and 100-seed weight had the strongest positive effects on yield, while SOD and CAT had indirect adverse effects. Overall, pretreatment with 100 µM melatonin and 1 mM GABA foliar application is recommended to mitigate seed aging effects and improve peanut yield.

# 1. INTRODUCTION

Seed deterioration is one of the most important factors that reduce the physiological quality of seeds, and its damage increases under adverse environmental conditions (Zhang et al., 2021). Seed deterioration begins with a chain of biochemical events, including damage to the membrane and disruption of biochemical reactions, as a result of which many vital properties of the seed, the rate of germination and seedling establishment reduce, and abnormal seedlings increase, leading to the death of the seed (Arab et al., 2022). Membrane damage is considered as a basic and primary factor in seed deterioration. Loss of integrity of the cell membrane and increase in membrane permeability causes an increase in the leakage of seed compounds and loss of seed vitality. As a result, seed cells cannot maintain their natural physical conditions and function properly (Ebone et al., 2019). The composition inside a seed affects its aging rate. The more oxidizing substances such as oil are in the seeds, the more they are subject to deterioration and require special storage methods and treatment. Therefore, oilseeds are more sensitive (Wang et al., 2021). For example, peanut is an annual plant belonging to the legume family, which has an essential place among industrial products and is one of the most important oil plants in the world. Peanut seed contains 44-56% oil and 22-30% protein and is a valuable source of vitamins E, K, and B (Li et al., 2022a). Peanut seed is an oil seed that contains saturated fatty acids such as palmitic acid and stearic acid and unsaturated fatty acids such as oleic acid and linoleic acid. The rapid oxidation of unsaturated fatty acids of peanut causes rapid seed deterioration and reduction in seed vigor during the period of drying and storage, and one of the problems of its cultivation is the reduction in the percentage of germination and the emergence of seedlings of peanut in the field (Lin et al., 2022). Therefore, it is necessary to find solutions to reduce or improve the damage caused by peanut seed deterioration.

Today, there are many solutions to reduce seed deterioration. Seed pretreatment is one of the important methods of seed empowerment (Kaya et al., 2022) So that the seeds are first watered using different methods and then dried again for ease of transportation and planting. This practice increases the rate and uniformity of germination and greening, increases germination in a wider range of environmental factors, and improves the growth and establishment of seedlings (Elsanatawy et al., 2021). Melatonin can be one of these substances. Melatonin (N-acetyl 5-methoxytryptamine) is an indole compound derived from tryptophan that is synthesized in the chloroplast and mitochondria of plant cells (Aydın et al., 2021) One of the important functions of melatonin in plants is its significant effect on auxin, gibberellin, cytokinin, and abscisic acid hormones (Jiang et al., 2017). Melatonin also activates the antioxidant response of cells and antioxidant enzymes including SOD, CAT and ascorbate peroxidase (APX) and scavenges reactive oxygen species (ROS) and thereby reducing the effects of stress on plants (Li et al., 2022b). Pretreatment using 200 µM of melatonin improves the germination percentage, seed vigor index, germination index, seedling length, seedling vigor index, and seedling weight in the deteriorated oat seeds (Yan et al., 2020).

Sugars and amino acids are the most frequent organic metabolites that play an important role in signal transmission, osmotic regulation, energy supply, and free radical inhibition in plants (Sharma et al., 2019). Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is produced in plants to activate defense responses and regulate biochemical functions, especially under stress (Malekzadeh et al., 2014). Treatment with bioactive compounds such as GABA can be considered as a practical method to increase the seedling establishment potential in old seeds. The GABA is a specific plant growth regulator that acts as an internal signal in response to environmental stresses (Hu et al., 2015; Rousta et al., 2023). Under salinity stress, increasing the accumulation of GABA in pumpkin (*Cucurbita pepo*) leaves significantly reduced lipid peroxidation and improved the activity of antioxidant enzymes (Nejad-Alimoradi et al., 2019). In a study, researchers investigated the use of GABA on the deteriorated seeds of pumpkin. The results showed that by increasing the deterioration the percentage of saturated fatty acids increases and the amount of unsaturated fatty acids reduces. The use of GABA reduces the harmful effects of the deterioration on seedling growth and fatty acid compounds (Tavakkol Afshari and Seyyedi, 2020).

Studies have shown that the occurrence of any undesired conditions such as environmental stress (Delijani et al., 2022; Matinizadeh et al., 2024; Moshki et al., 2024) in the way of seed production or the use of inappropriate methods of production and after that seed storage can cause early deterioration and reduce seed vigor, which will cause irreparable damage (Demir et al., 2023). Therefore, it is necessary to find an efficient solution to reduce the negative impacts caused by seed deterioration for any reason. In this study, the use of melatonin and GABA as compounds that have shown positive effects on inhibiting the aging process of plants have been considered. The effect of these substances was investigated in the form of seed pretreatment and foliar application in peanut seeds and plants.

## 2. MATERIALS AND METHODS

### *Experimental overview*

This study was conducted in peanut field in Astaneh Ashrafieh and the genetics and breeding laboratory of Tea Research Institute on peanut during two years (2021 and 2022). The cultivar used for planting was North Carolina 2 ($NC_2$). A factorial experiment was performed based on a RCBD with three replications. Planting operation was done in the first year on May 14, 2021 and in the second year on May 15, 2022 at a depth of 4 cm. Each plot consisted of 4 lines with a length of 6 meters. The distance between the rows was 40 cm and the distance on the row was 40 cm

The experiment was conducted using a factorial arrangement that included: (1) two seed quality levels (normal and aged seeds); (2) three melatonin seed pretreatment concentrations (0, 50, and 100 µM); and (3) two GABA foliar application levels (0 and 1 mM). These treatments were applied in combination to evaluate their individual and interactive effects. To apply the seed deterioration treatment, peanut seeds were placed in an incubator at 40 °C and 96–100% relative humidity for 96 hours. This method was used to simulate aging conditions, following the guidelines of ISTA (1999). Prior to the deterioration treatment, the seeds were pretreated by soaking in different concentrations of melatonin. For this purpose, seeds were placed in containers and soaked in melatonin solutions at the desired concentrations. After soaking, they were aerated for 8 hours to allow for oxygen exchange and then dried in the shade under ambient conditions.

Foliar application of treatments was performed early in the morning at the beginning of the flowering stage, which occurred 38 days after planting, under optimal environmental conditions. Care was taken to ensure that the leaves were completely wetted during application. Trait measurements were conducted two weeks after foliar application. Tables 1 and 2 present the physicochemical properties of the field soil and the meteorological data for the two study years, respectively.

**Table 1.** Physico-chemical properties of the experimental soil

| Characteristics | Units | Values |
|---|---|---|
| Organic carbon | % | 1.1 |
| Phosphorus (P) | mg $kg^{-1}$ | 10.5 |
| Potassium (K) | mg $kg^{-1}$ | 229 |
| Electronic content (EC) | $dSm^{-1}$ | 0.506 |
| pH | | 7.3 |
| Sand | % | 55 |
| Clay | % | 12 |
| Silt | % | 33 |
| Soil texture | | Silty-loam |

**Table 2.** Meteorological parameters for the field sites during the experiment (Astaneh Ashrafieh Meteorological Office)

| | Mean temperature (°C) | | Relative humidity (%) | | Precipitation (mm) | |
|---|---|---|---|---|---|---|
| **Month** | **2021** | **2022** | **2021** | **2022** | **2021** | **2022** |
| March | 14.95 | 13.7 | 75.82 | 75.20 | 0.67 | 1.1 |
| April | 18.51 | 17.5 | 83.5 | 82.30 | 2.65 | 1.4 |
| May | 23.82 | 22.4 | 80.62 | 76.10 | 0.52 | 0.1 |
| June | 26.79 | 25.6 | 78.72 | 74.00 | 1.35 | 0.2 |
| July | 27.35 | 26.7 | 75.33 | 77.8 | 2.82 | 1.3 |
| August | 24.98 | 24.9 | 79.04 | 76.4 | 3.22 | 2.7 |
| Long-term average value | Mean temperature (°C) | | Relative humidity (%) | | Precipitation (mm) | |
| | 20 | | 72 | | 1500 | |

### *Measured traits*

#### *Leaf RWC*

Leaf RWC was measured two weeks after foliar application and before irrigation. The leaves were weighted by a scale with accuracy of 0.001 (fresh weight), then cut into equal pieces and stored in disposable plastic cups for 24 hours in 150 mL of double distilled water and in a refrigerator at 4˚C. The lids of the glasses were covered entirely using aluminum foil (Kramer, 1983). Next, the leaves were removed from the distilled water and after the water was dried with filter paper, they were weighed again (saturated weight). Then, they were placed in an oven at 70˚C for 72 hours and weighed (dry weight). Leaf RWC was calculated using Equation (1).

$$\mathrm{RWC} = \left(\frac{\mathrm{Fw} - \mathrm{Dw}}{\mathrm{Sw} - \mathrm{Dw}}\right) \times 100 \qquad (1)$$

Where Fw: leaf fresh weight, Dw: leaf dry weight, Sw: leaf turgid weight.

### *Lipid peroxidation*

Lipid peroxidation was assessed by measuring the concentration of MDA, following the method of Du and Bramley (1992). 0.25 g of plant sample was extracted with 5 ml of 0.1% trichloroacetic acid (w/v). The homogenate was centrifuged at 20 °C and 10,000 g for 20 minutes. 2 mL of 0.25% thiobarbituric acid was added to 250 µl of the extract. The resulting mixture was placed in a 100 °C water bain-marie for 30 minutes, and then the tubes were removed and cooled in ice for 15 minutes. It was immediately centrifuged at 20 °C at 10,000 g for 10 minutes. The absorbance was read at wavelengths of 440, 532 and 600 nm. To calculate the concentration of MDA, the extinction coefficient equal to 157 M cm $^{-1}$ was used.

### *Leaf anthocyanin*

The leaf anthocyanin was measured using the method of Mita et al. (1997). 0.2 g of fresh plant tissue was homogenized in liquid nitrogen, and then 4 mL of 1% hydrochloric acid solution in methanol was added and placed in the refrigerator for 24 hours. The resulting mixture was centrifuged at 13,000 g for 10 minutes. The absorbance in the supernatant was read using spectrophotometry at wavelengths of 657, and 530 nm.

### *Antioxidant enzymes*

### *SOD activity*

The reaction mixture for measuring SOD activity included 1.5 mL of potassium phosphate buffer (pH = 7.8), 0.1 mL of 2.25 mM *nitro* blue *tetrazolium* chloride (NBT), 0.1 mL of 3 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mL of 200 mM methionine, 0.1 mL of 1.5 mM sodium carbonate, 1 mL of distilled water, and 0.05 mL of enzyme extract. A blank was used to zero the device and a control was used for the standard curve. The reaction in the extracts prepared at 25 °C was started by turning on the fluorescent lamp (15 W) and adding 0.1 mL of 60 µM riboflavin, and after 15 minutes, the reaction was stopped by turning off the lamp. The blank tube contained the above substances, with the difference that the enzyme extract was not added and it was not placed in the light. In the control tube, there was also the above mixture and the enzyme extract was not added, but it was placed in the light. The absorbance was recorded at the wavelength of 560 nm. The difference in absorbance of the samples and the control at the wavelength of 560 nm indicates the inhibition of NBT photo-reduction in the presence of SOD. By calculating the absorbance difference, the enzyme unit of the samples was calculated and the enzyme activity was expressed in enzyme unit/total protein (mg) in 50 microliters of the extract (Sairam et al., 2002).

### *CAT activity*

CAT activity was measured at 25°C by the method of Cakmak and Horst (1991). A spectrophotometer at the wavelength of 240 nm was used to measure CAT enzyme activity. The substances used included 3000 microliters of 50 mM potassium phosphate buffer (pH=7), 5 microliters of 3.41 M hydrogen peroxide, and 100 microliters of enzyme extract. CAT converts $H_2O_2$ into $O_2$ and H2O without the need for a reducing agent. The maximum absorbance of hydrogen peroxide occurs at the wavelength of 240 nm. Therefore, with the start of the reaction by CAT, the amount of hydrogen peroxide in the reaction mixture gradually reduces, and as a result, the amount of absorption at the wavelength of 240 nm also reduces. The specific activity of CAT was calculated based on micromoles of hydrogen peroxide per minute per milligram of soluble protein.

### *APX activity*

The reaction medium for measuring APX activity contained 600 microliters of 0.1 mM EDTA, 1500 microliters of 50 mM phosphate buffer (pH=7), 400 microliters of 0.5 mM ascorbic acid, 400 microliters of 30% $H_2O_2$, and 100 microliters of enzyme extract. Enzyme activity measurement was recorded for 1 minute at the wavelength of 290 nm with an extinction coefficient of 2.8 mM cm$^{-1}$ (Nakano and Asada, 1981).

### *Grain yield*

The number of pegs per plant, the number of ripe pods per plant, the shell yield and 100 seed weight were also measured by taking 10 plants in 1 m² and finally reported in g m$^{-2}$.

### *Statistical analysis*

For the data of two years, Bartlett's test was performed first. Bartlett's test was used to ensure the homogeneity of variances, which is a key assumption for conducting ANOVA. When an experiment is conducted across two locations and/or years, it is essential to test the homogeneity of variances prior to performing combined analysis of variance (ANOVA). For this purpose, Bartlett's test was used to assess the equality of error variances across environments (Steel et al., 1997). This test is based on the error mean squares obtained from each environment separately. If Bartlett's test is non-significant ($P > 0.05$), the variances are considered homogeneous, and a combined ANOVA can be validly performed. However, if the test is significant ($P \leq 0.05$), indicating heterogeneity of variances, a combined analysis is not statistically valid, and the data are analyzed separately for each environment. For the traits studied in this article, this test was not significant and the homogeneity of the experimental error was confirmed, as a result, composite analysis was performed. Data was analyzed using SAS 9.1 and mean comparison of data was done by LSD method at the probability level of 5%.

## 3. RESULTS AND DISCUSSION

The effect of year and foliar application at the probability level of 5% and the effect of deterioration, the interaction of pretreatment ×foliar application and year × deterioration at the probability level of 1% had an effect on leaf RWC. The effect of year, deterioration, pretreatment, and foliar application, and the interaction of deterioration × pretreatment, deterioration× foliar application, and year× deterioration was significant at the probability level of 1% on MDA. At the same time, year × pretreatment was significant at the probability level of 5% on MDA. Seed deterioration and melatonin pretreatment at the probability level of 1% and GABA foliar application at the probability level of 5% were effective on the amount of leaf anthocyanin. Seed deterioration, melatonin pretreatment and the interaction of these two factors affected CAT activity at the probability level of 1%. The number of pegs per plant was affected by the year, seed deterioration, melatonin pretreatment, GABA foliar application and interactions of deterioration×pretreatment, year×pretreatment, year×deterioration×pretreatment and year×deterioration. The year at the probability level of 5% and the treatments of seed deterioration, pretreatment and the interaction of year × deterioration at the probability level of 1% significantly affected the number of pods in the peanut plant. According to the analysis of variance, all main effects and interaction of deterioration × melatonin, deterioration × GABA, year × deterioration, year × melatonin, and deterioration × melatonin × GABA on 100 seed weight were significant. The results showed that the seed deterioration ($P<0.01$), melatonin and GABA ($P<0.05$), and the interaction of melatonin × GABA ($P<0.01$) had an effect on shell yield. Seed deterioration, seed pretreatment with melatonin and GABA foliar application influenced grain yield at the probability level of 1%. The interaction of seed deterioration × melatonin also influenced grain yield at the probability level of 5% on grain yield (Table 3).

### *Leaf RWC*

All treatment compounds resulting from the interaction of seed pretreatment by melatonin × foliar application by GABA caused an increase in leaf RWC compared to the control (Figure 1). In the first and second years, seed deterioration reduced leaf RWC by 6.86 and 11.60% (Figure 2). Seed aging caused a reduction in leaf RWC in the plants grown from these seeds. Melatonin pretreatment and GABA foliar application caused a significant increase in leaf RWC. Melatonin and GABA can improve leaf RWC by reducing lipid peroxidation, various ROS and maintaining membrane stability (Farouk and Al-Amri, 2019).

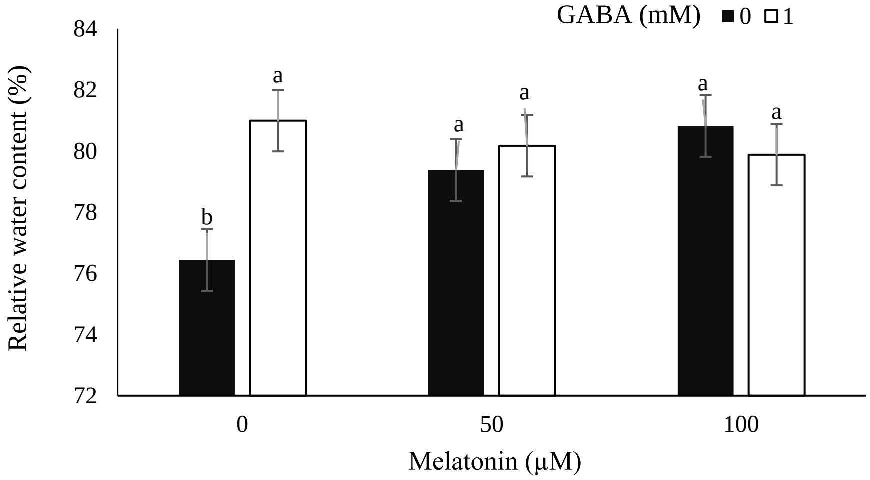

**Figure 1.** Effect of pretreatment with melatonin and foliar application with gamma-aminobutyric acid (GABA) on relative water content in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

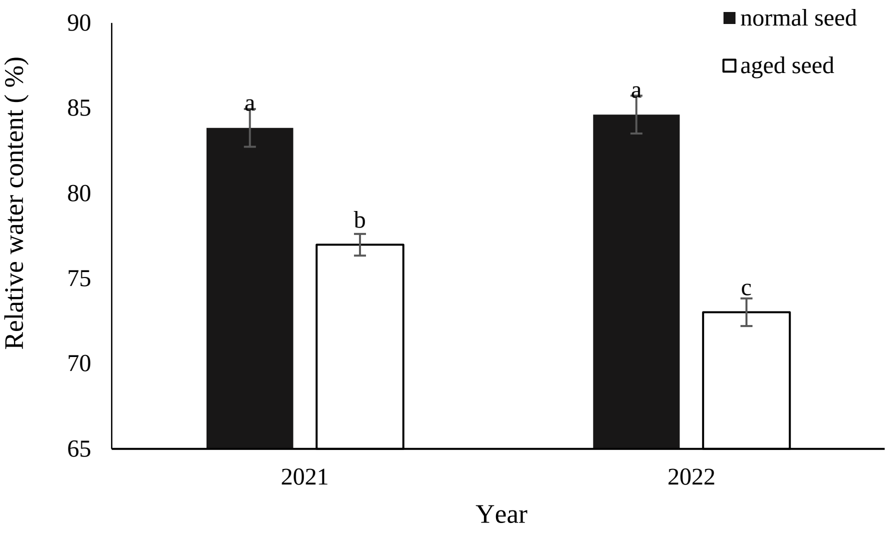

**Figure 2.** Effect of year and seed deterioration on relative water content in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at p<0.05

### *MDA*

The amount of MDA in the leaves of plants grown from aged seeds was 92.57% higher than that of normal seeds. The use of 50 and 100 µM of melatonin as seed pretreatment under deterioration caused a significant reduction by 10.66 and 23.78% respectively, in the amount of MDA in the leaves of plants obtained from these seeds, but melatonin had no effect in normal seeds (Figure 3).

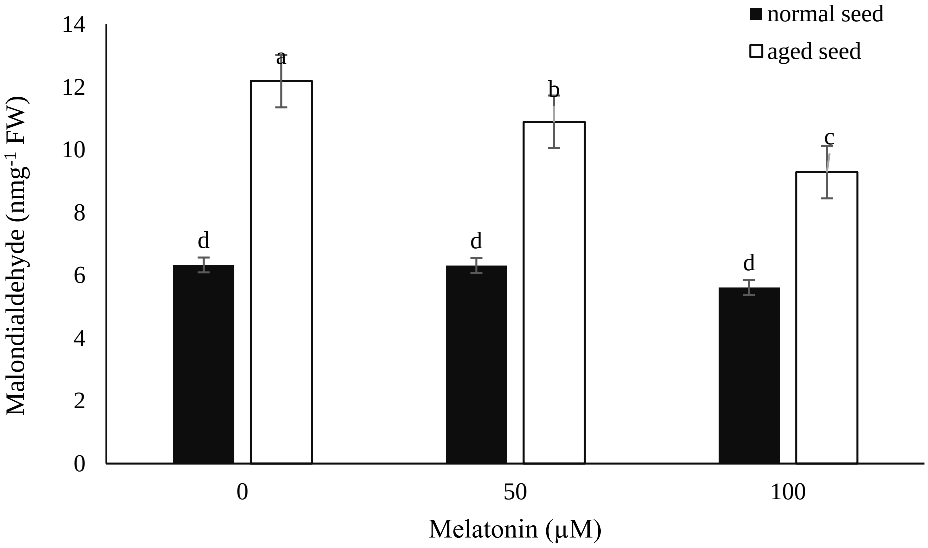

**Figure 3.** Effect of seed deterioration and pretreatment with melatonin on malondialdehyde in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at p<0.05

Mean comparison of MDA under the influence of deterioration and GABA foliar application showed that foliar application of 1 mM of GABA reduced the amount of MDA by 12.01%. in the leaves of plants grown from aged seeds compared to the conditions of not using this substance (Figure 4). Seed aging increased the amount of MDA in the leaves of tested plants, indicating that deterioration caused membrane damage and increased lipid peroxidation. The increase in the amount of MDA under deterioration can be considered as the result of the increase in ROS production, which may be caused by the weakness of the antioxidant system in plants (Liu et al., 2023).

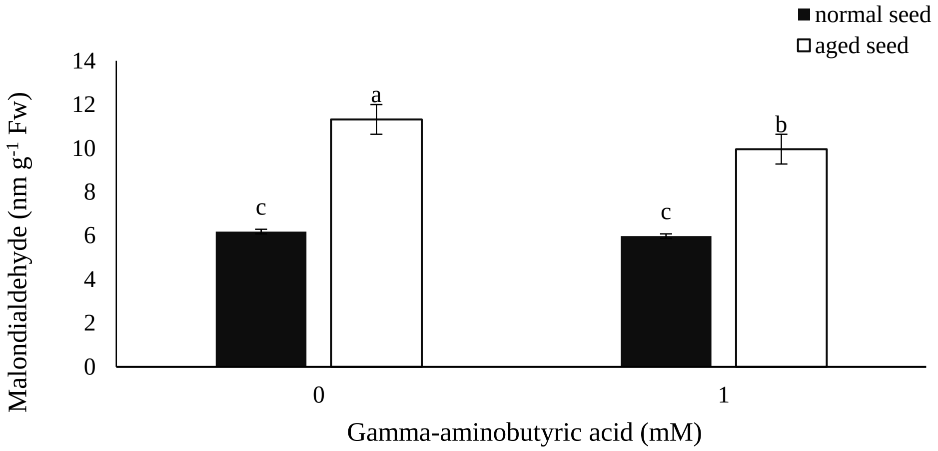

**Figure 4.** Effect of seed deterioration and foliar application with gamma-aminobutyric acid (GABA) on malondialdehyde in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at p<0.05

Melatonin and GABA have probably prevented the peroxidation of cell membrane lipids and the increase of MDA by cleaning ROS. The reduction in cell membrane damage in response to receiving these two substances can indicate the induction of the antioxidant defense system by these substances. By removing free radicals, these two substances reduce the damage caused, and as a result, membrane lipid peroxidation reduces. The physiological role of GABA in stress tolerance is related to osmotic regulation, membrane stability, and removal of ROS (Shelp et al., 2012).

### *Anthocyanin*

The results showed that deterioration increased the amount of anthocyanin in the leaves by 84.61%. Seed pretreatment by 50 and 100 μM of melatonin increased anthocyanin by 6.89 and 14.94%, respectively, which was statistically significant. The GABA spraying also increased the amount of anthocyanin in the leaves by 5.26% (Table 4).

The effect of seed deterioration, melatonin pre-treatment, GABA foliar application, interaction of deterioration × melatonin and deterioration × GABA was significant at the probability level of 1% on SOD activity (Table 3). Seed aging increased SOD activity by 11.04, 27.15, and 32.91 percentage in the 0, 50, and 100 μM levels of melatonin, respectively. Soaking normal seeds in 100 μM of melatonin increased anthocyanin by 9.66%, and in aged seeds, the use of 50 and 100 μM of melatonin increased the activity of this enzyme by 20.60 and 31.26% compared to the non-use of this substance in plants grown from these seeds (Figure 5). Anthocyanins are a group of water-soluble flavonoids, and their increase indicates an increase in the main pathway of flavonoid production. In the present study, seed deterioration increased the amount of anthocyanin and the use of melatonin and GABA increased anthocyanin. The increase in the amount of anthocyanin under seed deterioration in this study can also be due to the activation of the internal mechanisms of the plant to reduce the destructive effects of deterioration stress. Researchers found that GABA stimulates the activity of phenylalanine ammonia lyase (PAL) and starts the phenylpropanoid pathway, as a result of which the synthesis of phenolic compounds and the antioxidant activity increases (Soleimani Aghdam et al., 2015). The reason for the increase in anthocyanin by using melatonin is related to the increased expression of genes involved in the synthesis of flavonoids by using this substance (Liang et al., 2018).

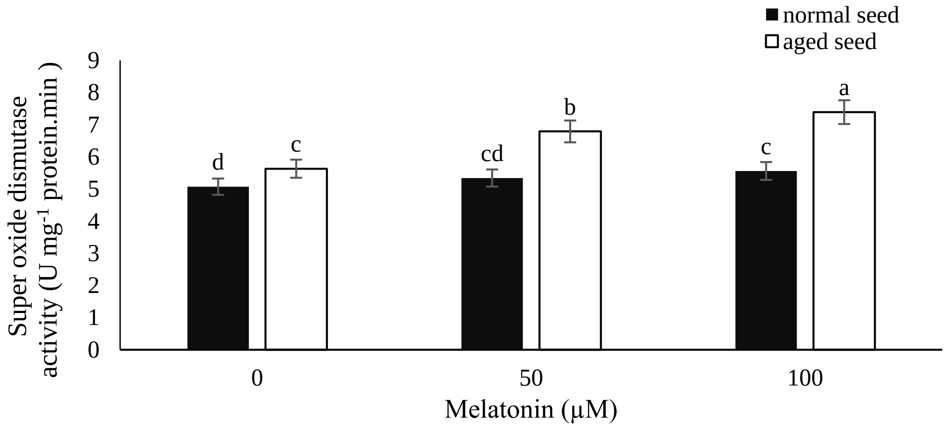

**Figure 5.** Effect of seed deterioration and pretreatment with melatonin on super oxide dismutase in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at p<0.05

**Table 3.** Variance analysis of measured traits in peanut affected by seed deterioration, seed pretreatment with melatonin and gamma-aminobutyric acid (GABA) foliar application

| S.O.V | d.f. | RWC | MDA | Anthocyanin | SOD | CAT | APX | Number of pegs per plant | Number of pods per plant | 100 seed weight | Shell yield | Grain yield |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | 1 | 45.51* | 12.56** | 0.0001 | 0.205 | 0.001 | 0.060 | 125.34** | 113.37* | 110.95** | 2.02 | 5635.68 |
| Error | 4 | 67.17 | 6.52 | 0.0001 | 0.428 | 0.017 | 0.003 | 5.11 | 27.76 | 11.75 | 1849.95 | 7.91 |
| Seed deterioration (A) | 1 | 1533.79** | 399.23** | 0.055** | 29.49** | 2.81** | 0.060 | 292.82** | 2116.29** | 10594.34** | 12695.75** | 1644.68** |
| Melatonin (B) | 2 | 16.47 | 20.17** | 0.001** | 7.77** | 0.16** | 0.056 | 221.99** | 583.64** | 412.03** | 1549.69* | 652538.89** |
| GABA (C) | 1 | 36.68* | 15.67** | 0.0005* | 1.35** | 0.01* | 0.047 | 20.69* | 66.99 | 141.68** | 2402.59* | 460000.35** |
| A*B | 2 | 2.02 | 7.19** | 0.00007 | 2.57** | 0.06** | 0.013 | 42.60** | 30.25 | 100.33** | 259.90 | 116180.17* |
| A*C | 1 | 1.27 | 9.40** | 0.00002 | 1.38** | 0.00008 | 0.018 | 5.12 | 34.37 | 47.04* | 5.60 | 8646.12 |
| B*C | 2 | 47.08** | 0.74 | 0.00010 | 0.07 | 0.001 | 0.020 | 0.54 | 17.76 | 11.97 | 2081.71** | 19500.72 |
| A*B*C | 2 | 8.38 | 0.37 | 0.00011 | 0.05 | 0.003 | 0.005 | 1.20 | 4.07 | 0.38 | 530.50 | 30472.17 |
| Y*A | 1 | 101.44** | 23.15** | 0.00015 | 0.10 | 0.003 | 0.0008 | 13.17* | 214.76** | 200.60** | 0.0001 | 4884.01 |
| Y*B | 2 | 8.10 | 4.89* | 0.00006 | 0.09 | 0.002 | 0.009 | 16.34** | 13.10 | 62.29** | 273.06 | 2206.89 |
| Y*C | 1 | 13.91 | 1.06 | 0.000008 | 0.21 | 0.008 | 0.022 | 0.09 | 15.17 | 0.02 | 139.11 | 52542.01 |
| Y*A*B | 2 | 5.29 | 1.00 | 0.00005 | 0.012 | 0.009 | 0.001 | 20.50** | 5.60 | 84.42** | 105.99 | 49268.72 |
| Y*A*C | 1 | 22.39 | 0.15 | 0.00003 | 0.08 | 0.003 | 0.001 | 0.72 | 0.19 | 0.22 | 17.91 | 95120.68 |
| Y*B*C | 2 | 21.90 | 1.90 | 0.000015 | 0.005 | 0.0004 | 0.003 | 0.97 | 13.09 | 1.65 | 254.12 | 10518.72 |
| Y*A*B*C | 2 | 12.76 | 2.68 | 0.000006 | 0.04 | 0.0005 | 0.013 | 0.50 | 7.43 | 2.04 | 304.36 | 9108.72 |
| Error | 44 | 7.36 | 1.09 | 0.00008 | 0.13 | 0.003 | 0.02 | 3.09 | 19.19 | 8.39 | 352.39 | 26302.29 |
| C.V (%) | - | 3.40 | 12.39 | 10.15 | 6.22 | 9.77 | 10.40 | 3.72 | 16.44 | 4.21 | 5.51 | 6.24 |

**Table 4.** Mean comparison of measured traits in peanut affected by year, seed deterioration, seed pretreatment with melatonin and gamma-aminobutyric acid (GABA) foliar application

| Treatment | RWC | MDA | Anthocyanin | SOD | CAT | APX | Number of pegs per plant | Number of pods per plant | 100 seed weight | Shell yield | Grain yield |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (%) | (nm g$^{-1}$ leaf Fw) | (µm g$^{-1}$ leaf Fw ) | (U mg$^{-1}$ protein.min) | | | | | (g) | (kg ha$^{-1}$) | (kg ha$^{-1}$) |
| Year | | | | | | | | | | | |
| 2021 | 80.41 a | 8.02 b | 0.094 a | 6.02 a | 0.55 a | 1.41 a | 46.00 b | 25.38 b | 67.46 b | 340.78 a | 2605.44 a |
| 2022 | 78.82 b | 8.85 a | 0.091 a | 5.91 a | 0.56 a | 1.37 a | 48.63 a | 27.89 a | 69.94 a | 340.44 a | 2587.25 a |
| LSD 5% | 1.289 | 0.497 | N.S. | N.S. | N.S. | N.S. | 0.836 | 2.08 | 1.376 | N.S. | N.S. |
| Deterioration | | | | | | | | | | | |
| normal seeds) | 84.23 a | 6.087 b | 0.065 b | 5.32 b | 0.36 b | 1.40 a | 49.33 a | 32.06 a | 80.83 a | 353.89 a | 3371.72 a |
| aged seeds | 75.00 b | 10.797 a | 0.120 a | 6.60 a | 0.76 a | 1.37 a | 45.30 b | 21.21 b | 56.57 b | 327.33 b | 1821.47 b |
| LSD 5% | 1.289 | 0.497 | 0.004 | 0.176 | 0.026 | N.S. | 0.836 | 2.081 | 1.376 | 8.917 | 77.04 |
| Melatonin (µM) | | | | | | | | | | | |
| 0 | 78.71 b | 9.26 a | 0.087 c | 5.35 c | 0.47 c | 1.38 a | 44.13 c | 21.23 c | 64.81 c | 331.71 b | 2420.21 c |
| 50 | 79.78ab | 8.60 b | 0.093 b | 6.06 b | 0.56 b | 1.36 a | 47.63 b | 27.78 b | 68.22 b | 342.79 a | 2622.71 b |
| 100 | 80.35 a | 7.45 c | 0.100 a | 6.47 a | 0.64 a | 1.37 a | 50.19 a | 30.90 a | 73.06 a | 347.33 a | 2746.88 a |
| LSD 5% | 1.579 | 0.609 | 0.005 | 0.216 | 0.032 | 0.085 | 1.024 | 2.549 | 1.686 | 10.921 | 94.35 |
| GABA (mM) | | | | | | | | | | | |
| 0 | 78.88 b | 8.90 a | 0.090 b | 5.83 b | 0.55 b | 1.38 a | 46.78 b | 25.67 a | 67.29 b | 334.83 b | 2516.67 b |
| 1 | 80.35 a | 7.97 b | 0.095 a | 6.10 a | 0.57 a | 1.43 a | 47.85 a | 27.60 a | 70.10 a | 346.38 a | 2676.53 a |
| LSD 5% | 1.289 | 0.497 | 0.004 | 0.176 | 0.026 | N.S. | 0.836 | N.S. | 1.376 | 8.917 | 77.04 |

### *Superoxide Dismutase (SOD) Activity*

Mean comparison of SOD activity affected by seed aging and GABA foliar application showed that only under seed deterioration conditions, the use of GABA caused a significant increase in SOD activity. In contrast, there was no significant difference under normal conditions compared to the control (Figure 6). GABA spraying also had a considerable effect on SOD activity at the 5% probability level (Table 3). Overall, the activity of SOD was higher in the leaves of plants grown from aged seeds. Under seed aging, an imbalance occurs between reactive oxygen species (ROS) production and antioxidant scavenging capacity, leading to oxidative stress. In such conditions, enhancing SOD activity is crucial to dismutase superoxide radicals into hydrogen peroxide and mitigate oxidative damage (Govindaraj et al., 2017).

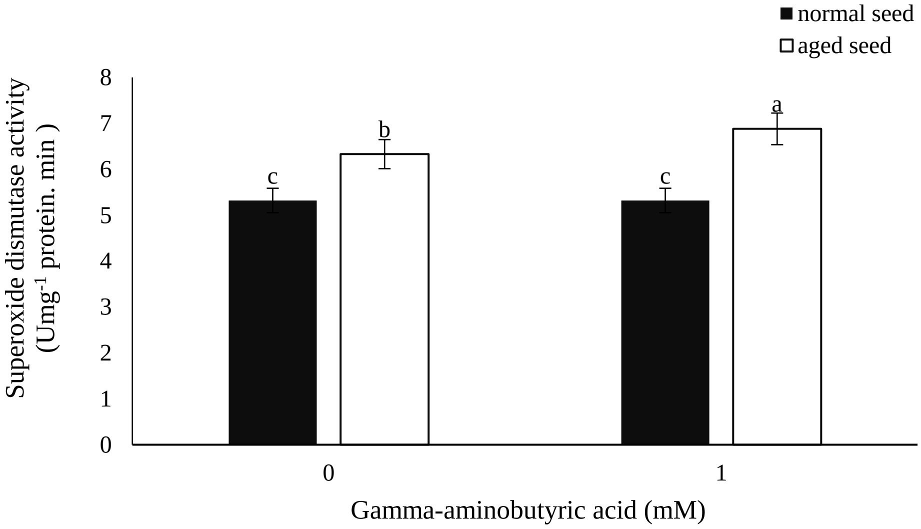

**Figure 6.** Effect of seed deterioration and foliar application with gamma-aminobutyric acid (GABA) on super oxide dismutase in peanut

Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Catalase (CAT) Activity*

Seed aging caused a 79.41% increase in CAT activity at zero melatonin level, and this effect was also evident at two other melatonin concentrations (Figure 7). Pretreatment of normal seeds with a low concentration of melatonin had no significant effect on CAT activity; however, the application of 100 µM melatonin significantly increased CAT activity by 44.11%. The highest catalase activities were observed in leaves of plants grown from aged seeds pretreated with melatonin, where concentrations of 50 and 100 µM increased CAT activity by 27.86% and 32.78%, respectively (Figure 7). Foliar application of GABA led to a 3.63% increase in CAT activity compared to the control (Table 3). Catalase is essential for decomposing hydrogen peroxide into water and oxygen, thereby preventing oxidative damage caused by ROS accumulation (Govindaraj et al., 2017). One possible reason for the increase in CAT under seed aging may be the elevated levels of hydrogen peroxide under such stress.

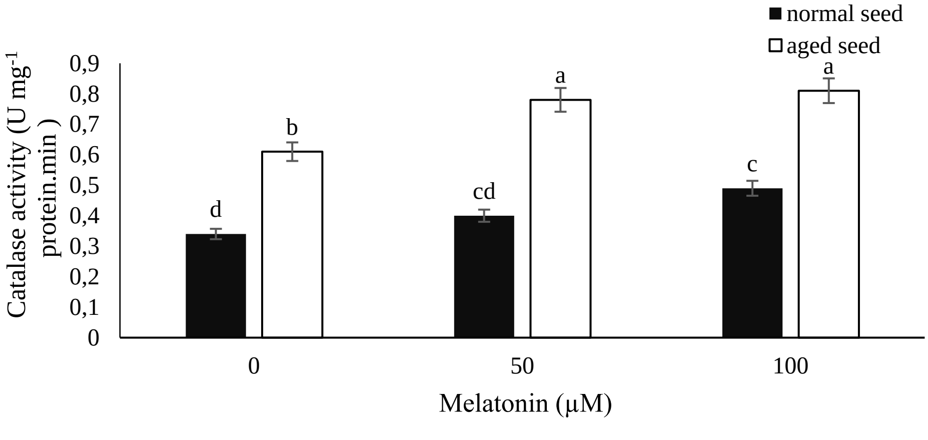

**Figure 7.** Effect of seed deterioration and pretreatment with melatonin on catalase activity in peanut.

Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Ascorbate Peroxidase (APX) Activity*

In contrast to SOD and CAT, APX activity was not significantly affected by any of the experimental treatments (Tables 2 and 3), indicating that this enzyme may play a less prominent role under the specific stress and treatment conditions tested.

It is known that melatonin can enhance antioxidant capacity via several mechanisms, including direct scavenging of free radicals, upregulation of antioxidant enzymes such as SOD and CAT, protection of enzymes from oxidative damage, and boosting mitochondrial efficiency to reduce ROS production (Gong et al., 2017). Additionally, GABA has been shown to increase antioxidant enzyme activity under stress conditions. This effect is likely due to GABA's role in enhancing polyamine biosynthesis and preventing their degradation, which in turn supports CAT activity by reducing lipid peroxidation and enhancing ROS scavenging (Wang et al., 2014). GABA-treated corn plants have exhibited elevated SOD activity (Shi et al., 2010), and this activation of the antioxidant defense system is considered vital for improving plant tolerance to oxidative stress (Ramalho et al., 2018; Shi et al., 2010).

### *Number of pegs per plant*

During both years, the number of pegs in plants grown from aged seeds was significantly lower than plants grown from normal seeds. The highest number of pegs was recorded in plants whose seeds were pretreated by 100 µM of melatonin. Interestingly, in the second year, the number of pegs in the plants obtained from normal and aged seeds that were pretreated by 100 µM of melatonin was at the first statistical level. Investigation of the interaction of year×deterioration×pretreatment showed that in aged seeds, both levels of melatonin increased the number of pegs in the plant to a significant level in both years, while in normal seeds, only the application of 100 µM of melatonin increased Number of pegs per plant (Figure 8). Melatonin likely enhanced peg formation by mitigating oxidative damage during early seedling development and supporting reproductive tissue differentiation. Its known role in modulating auxin signaling and improving root/shoot architecture may also indirectly support peg initiation in peanuts (Arnao & Hernández-Ruiz, 2015; Zhang et al., 2022).

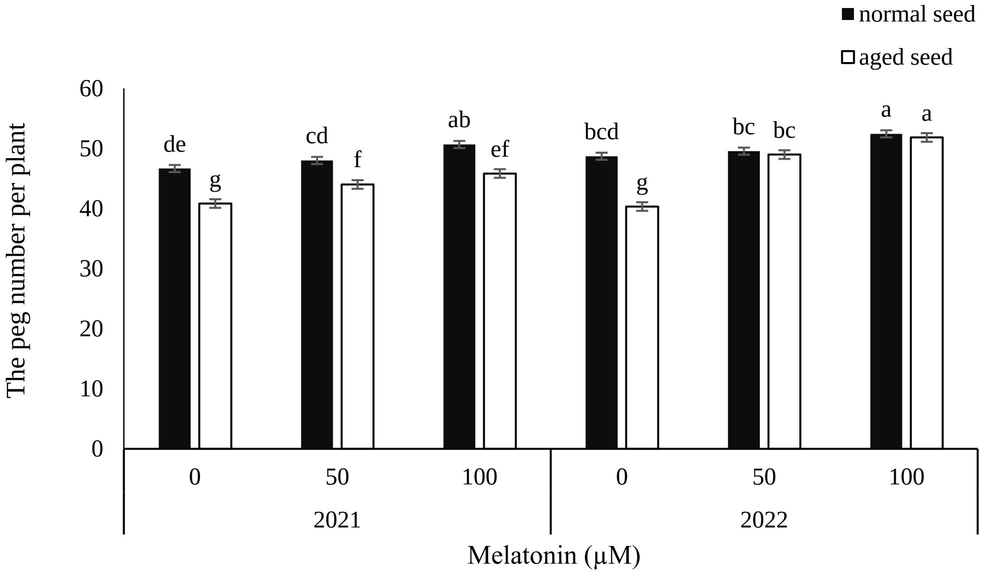

**Figure 8.** Effect of seed deterioration and pretreatment with melatonin on the peg number per plant in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Number of pods per plant*

The results of mean comparison of the number of pods per plant under the influence of the interaction of year × deterioration showed that seed aging in the first and second year caused a significant reduction by 43.95 and 23.36% in Number of pods per plant, respectively (Figure 9). The decline in pod number may result from impaired floral and peg development due to reduced seed vigor. Stress-induced disruption in hormonal balance and increased lipid peroxidation in aged seeds likely impair resource allocation to reproductive growth (Bewley et al., 2013). This highlights the critical role of seed quality in determining yield components.

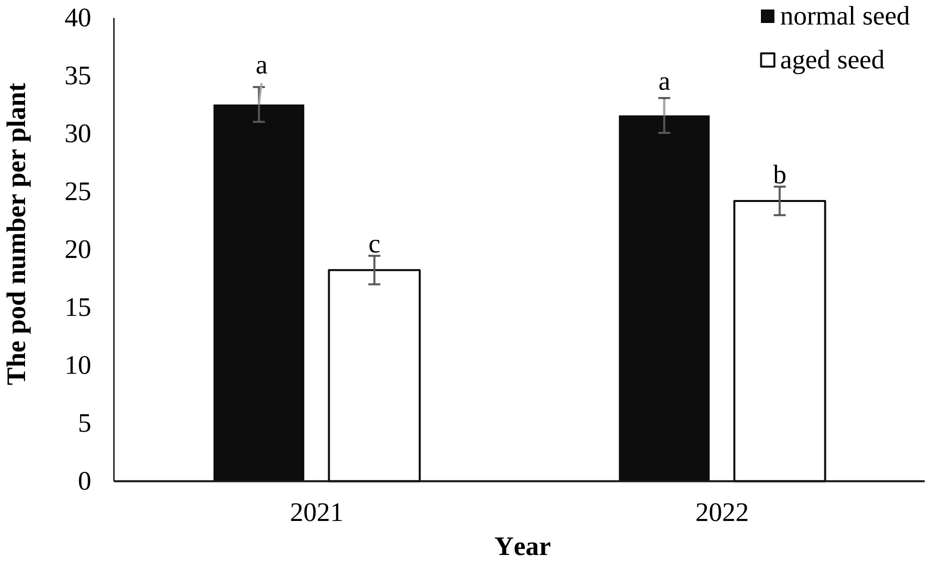

**Figure 9.** Effect of seed deterioration and year on the pod number per plant in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$.

*100 seed weight (HSW)*

The highest 100 seed weight in the plants was obtained from normal seeds with pretreatment by 100 µM of melatonin in the second year (94.22 g) and compared to the control (normal seeds without pretreatment in the second year) showed an increase by 24.53%. In both years, the application of deterioration caused a significant reduction in 100 seed weight. Soaking normal seeds in 50 and 100 µM of melatonin in both years increased 100 seed weight, but in aged seeds, only the use of 100 µM of melatonin in the first year could improve 100 seed weight and in other cases showed no difference with the control (Figure 10). Melatonin's antioxidant properties may have protected cellular structures and enhanced seed filling by preserving photosynthetic capacity and improving assimilate transport, especially in high-concentration treatments (Li et al., 2016). In aged seeds, only higher melatonin doses may overcome the threshold of damage.

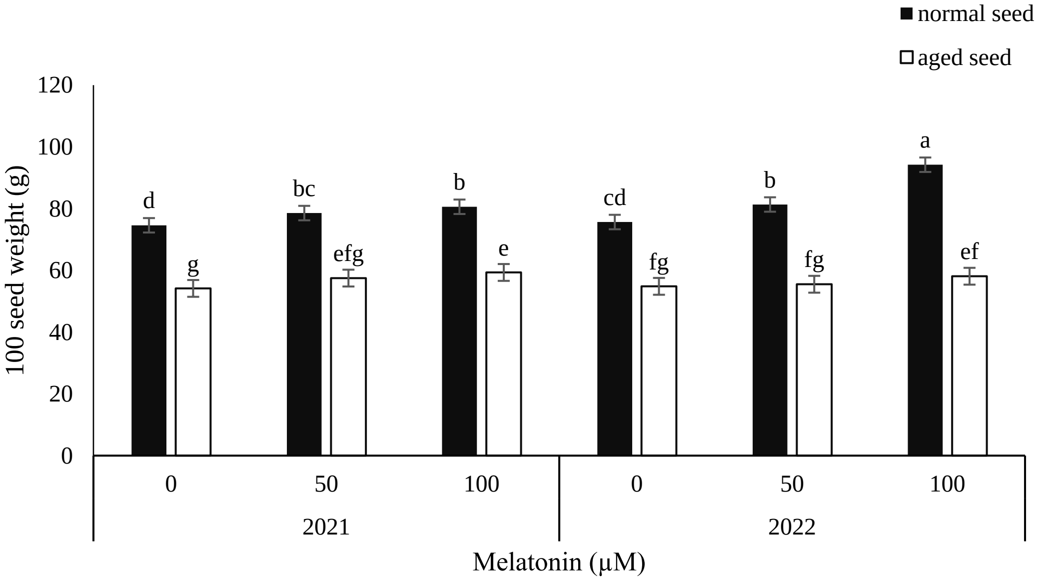

**Figure 10.** Effect of seed deterioration, pretreatment with melatonin and foliar application with gamma-aminobutyric acid (GABA) on 100 seed weight in peanut
Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Shell yield*

Seed aging caused a 50.7% reduction in shell yield (Table 4). The interaction of melatonin×GABA showed that all the treatment combinations increased shell yield compared to the control. Shell yield

increase varied between 8.25 and 10.44 percent but was statistically similar (Figure 11). Shell yield reduction likely mirrors overall plant biomass reduction under stress. The observed improvement due to combined treatment suggests potential synergism between melatonin and GABA in enhancing stress tolerance and biomass allocation (Bouché & Fromm, 2004; Hasan et al., 2021).

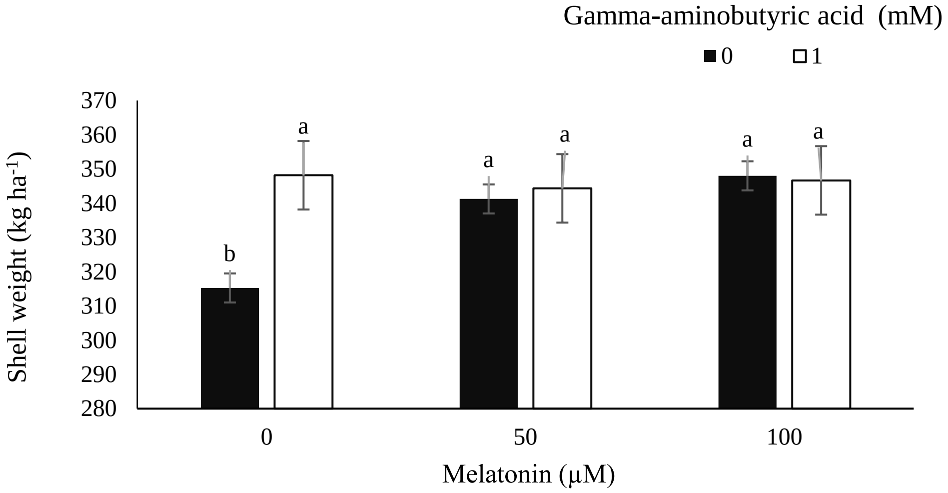

**Figure 11.** Effect of seed pretreatment with melatonin and foliar application of gamma-aminobutyric acid (GABA) on shell weight in peanut

Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Grain yield*

Mean comparison of grain yield showed that seed aging caused a 45.81% reduction in grain yield in control without melatonin. Pretreatment of normal seeds with 50 and 100 µM of melatonin increased 7.53 and 14.66% grain yield. Whereas, pretreatment of aged seeds by 50 and 100 µM of melatonin could improve grain yield by 9.91% and 11.33% under such conditions, respectively (Figure 12). GABA foliar application increased grain yield by 6.53% compared to the control (Table 4). Melatonin and GABA may contribute to yield improvement by reducing oxidative stress, preserving chlorophyll content, and enhancing enzymatic antioxidant defense. These effects collectively sustain photosynthesis and seed filling, especially under the oxidative stress associated with seed aging (Zhang et al., 2019; Hasan et al., 2021).

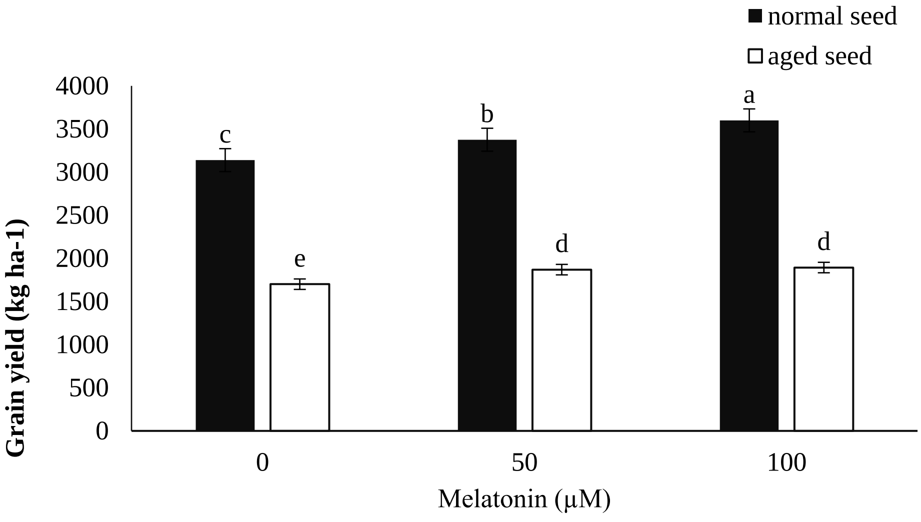

**Figure 12.** Effect of seed deterioration and pretreatment with melatonin on grain yield in peanut

Error bars represent the standard error (n=3). The mean values in a group with the same letter are not significantly different at $p<0.05$

### *Path analysis*

Path analysis was used to determine the contribution of direct and indirect effects of variables. As shown in Table 5, when the trait of seed yield was considered as a dependent variable in the plants obtained from normal seeds, the number of pods per plant (0.71), 100 seed weight (0. 67) and SOD (-0.52) were entered the model as the main variables. According to the coefficient of determination ($R^2$), these three traits explained 61.57% of grain yield changes. The results of causal analysis of seed yield under normal conditions (Table 5) showed that the number of pods per plant and 100 seed weight had the most direct and positive effect. SOD indirectly caused a reduction in the yield through the reduction in the number of pods per plant and 100 seed weight.

**Table 5.** Path analysis of yield- related traits in plants derived from normal seeds

| Plant traits | Number of pods per plant | 100 seed weight | Super oxide dismutase | Total effect |
|---|---|---|---|---|
| Number of pods per plant | **0.4829** | 0.2138 | 0.0151 | 0.7120 |
| 100 seed weight | 0.2677 | **0.3858** | 0.0150 | 0.6686 |
| Super oxide dismutase | -0.2756 | -0.2184 | **-0.0265** | -0.5206 |
| R-Square | 0.6157 | | | |

Causal analysis of plants obtained from aged seeds showed that traits of 100 seed weight (0.45), SOD (0.34) and CAT (-0.32) were entered the model as the main variables. $R^2$ showed that 23.06% of the variable changes in grain yield in plants obtained from aged seeds were explained by these three traits. 100 seed weight had the most direct and positive effect on grain yield. SOD had the most negative impact on Path. CAT also caused a reduction in grain yield through an indirect effect on 100 seed weight (Table 6).

**Table 6.** Path analysis of yield- related traits in plants derived from aged seeds

| Plant traits | 100 seed weight | Super oxide dismutase | Catalase | Total effect |
|---|---|---|---|---|
| 100 seed weight | **0.4316** | 0.1403 | -0.1122 | 0.4597 |
| Super oxide dismutase | -0.2413 | **-0.2510** | 0.1425 | -0.3498 |
| Catalase | -0.2867 | -0.2117 | **0.1689** | -0.3295 |
| R-Square | 0.2306 | | | |

The reduction in HSW in this study due to the deterioration of the seeds may be due to the reduction in the growth of the photosynthetic organs due to the low quality of the seeds. Therefore, the decrease in the leaf area under the deterioration through the reduction in the net photosynthesis rate has finally reduced HSW significantly. Researchers stated that HSW depends on the amount of photosynthetic matter available and the capacity of the seeds for storage, and the reduction in each reduces HSW (Li et al., 2022). Researchers reported that HSW and the number of pods in *Vigna radiata* L. grown from seeds pretreated with 50 μM of melatonin were higher than the control (MariaJanas and Maria Posmyk, 2013). Other studies have also shown the positive effect of melatonin pretreatment on increasing the number of pods per plant in soybean plants (Wei et al., 2015).

Peanut grain yield in this study showed a reduction under deterioration, which can be due to the decrease in the number of pods per plant, the number of pegs per plant, leaf RWC and HSW, and the increase in the activity of antioxidant enzymes, anthocyanin and MDA. Melatonin and GABA also increased grain yield in this plant by increasing leaf RWC, the number of pods per plant and HSW, and reducing MDA. The researchers announced that seed priming by melatonin improves seed quality, seedling development, plant growth, reduces the accumulation of ROS, protecting photosynthetic pigments, improves the performance of photosynthetic components, higher leaf RWC, and finally increases the yield of the product (Szafranska et al., 2016). Researchers found that indole compounds such as melatonin affect the growth and performance of plants by affecting different physiological mechanisms such as photosynthesis, respiration, ion absorption, membrane permeability, enzyme activity and hormones (Zhang et al., 2015). GABA increases growth and yield in various crops. Through increasing the level of endogenous plant hormones, this substance plays a positive role in improving factors related to vegetative and reproductive growth, as well as physiological traits and plant performance (Ramos-Ruiz et al., 2018).

## 4. CONCLUSIONS

According to the study results, accelerated aging of seeds caused changes in physiological processes of peanut, such as increasing the activity of antioxidant enzymes, anthocyanins, and reducing 100 seed weight, the number of pods per plant, and seed yield. The use of melatonin as seed pre-treatment and GABA as foliar application had a positive effect on normal and aged seeds and reduced the effects of seed deterioration. Finally, within the scope of the research, pretreatment of aged seeds with 100 µmol of melatonin and foliar application with 1 mM of GABA is suggested to improve the yield of peanut seeds under normal and seed aging conditions.

## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest or personal relationships.

## STATEMENTS AND DECLARATIONS

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