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Physiological and Biochemical Characterization of the GABA Shunt Pathway in Pea (*Pisum sativum* L.) Seedlings under Drought Stress

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Abstract: The physiological and biochemical role of the γ-aminobutyric acid (GABA) shunt pathway in green pea seedlings (*Pisum sativum* L.) was studied in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Characterization of seeds germination pattern, seedlings growth (plant height, fresh and dry weight, and chlorophyll contents), GABA shunt metabolite (GABA, glutamate, and alanine) levels, total protein and carbohydrate levels, and oxidative damage (MDA level) were examined. Data showed a significant effect of drought stress on seed germination, plant growth, GABA shunt metabolites level, total protein and carbohydrate contents, and MDA level. A significant decline in seed germination percentage was recorded at a 20% drought level, which indicated that 20% of soil water holding capacity is the threshold value of water availability for normal germination after 14 days. Seedling fresh weight, dry weight, and plant height were significantly reduced with a positive correlation as water availability was decreased. There was a significant decrease with a positive correlation in Chl *a* and Chl *b* contents in response to 7 days and 14 days of drought. GABA shunt metabolites were significantly increased with a negative correlation as water availability decreased. Pea seedlings showed a significant increase in protein content as drought stress was increased. Total carbohydrate levels increased significantly when the amount of water availability decreased. MDA content increased slightly but significantly after 7 days and sharply after 14 days under all water stress levels. The maximum increase in MDA content was observed at 20% and 10% water levels. Overall, the significant increases in GABA, protein and carbohydrate contents were to cope with the physiological impact of drought stress on *Pisum sativum* L. seedlings by maintaining cellular osmotic adjustment, protecting plants from oxidative stress, balancing carbon and nitrogen (C:N) metabolism, and maintaining cell metabolic homeostasis and cell turgor. The results presented in this study indicated that severe (less than 40% water content of the holding capacity) and long-term drought stress should be avoided during the germination stage to ensure proper seedling growth and metabolism in *Pisum sativum* L.

Keywords: drought stress; GABA: gamma aminobutyric acid; metabolism; pea; *Pisum sativum* L.; seedling growth; water deficit

1. Introduction

Pea (*Pisum sativum* L.) was used by Mendel to lay the foundation of modern genetics [1]. It is one of the major food legumes that can grow in different regions and rich in proteins, vitamins, minerals, carbohydrates, and seed oil [2]. Pea is predominantly a self-pollinated crop with limited variation in the number of flowers per node [3]. Most garden pea germplasm/varieties lines have either one or two flowers per node [4]. The

agro-ecological importance of pea is connected with its capability to form symbiotic nitrogen fixation with rhizobial bacteria that promotes and enhances seed germination, seedling vigor and emergence, root and shoot growth, plant stand and biomass, and seed weight [5].

Drought is a major abiotic stress that affects plant growth and yield [6]. Plant species and genotype adaptation and level of tolerance vary according to the type of drought stress (meteorological, agricultural, hydrological, or socioeconomic drought) [7]. Drought limits the productivity of many crops, especially during the seedling stage [8]. Water scarcity negatively affects vegetative growth by direct influence on its metabolic sink strength and fruit composition [9]. It causes reduced quality and quantity of the crop yield, growth rate, leaf expansion, stem elongation [8,10], stomatal conductance [11], and grain filling) [12]. Increasing the level of CO_2 in plants mitigates the impact of drought stress in many legume species [13].

Physiological, biochemical, and morphological processes in plants can occur as a result of drought stress causing changes in the expression of genes that lead to alteration in protein production [8]. In addition, respiration, photosynthesis, enzyme activities, and mineral nutrition, Redox (oxidation/reduction) homeostasis, and chloroplast metabolism are influenced by drought [12,14]. Analysis of proteomics data in plant leaves showed that many drought-responsive proteins that are involved in osmotic regulation, cell structure modulation, ROS scavenging, drought signal transduction, as well as carbohydrate metabolism, were upregulated under drought stress [15].

Drought stress reduces the quality and yield of many crops. Extended periods of water deficit results in a reduction in plant growth, photosynthesis efficiency and initiates a series of actions to maintain plant survival. These actions include regulation of stomatal conductance, osmotic adjustment, cell turgor maintenance, and protection of cellular membranes, enzymes, and macromolecules from oxidative damage [16]. During drought stress, plants close their stomata which leads to plasma membrane damage; consequently, the internal CO_2 concentration will be decreased, and excessive generation of reactive oxygen species will be increased, leading to a reduction in the photosynthetic rate and plant growth [17,18]. Water deficiency in maize caused a reduction in seedling survival rate, acceleration of the post-pollination embryo abortion rate, and ultimately, yield loss by postponing silking through the increase in the anthesis to the silking interval that leads to a reduction in ear and kernel number per plant [19–22]. Furthermore, water scarcity decreased photosynthetic efficiency, chlorophyll content, and CO_2 exchange in maize seedlings [21,23].

Drought stress decreased total barley (*Hordeum vulgare* L.) grain yield through a drop in tillers, spikes, and grains number per individual plant [24]. In soybean, drought stress decreased branch seed and total crop yields [25]. Cell elongation in higher plants can be inhibited through water movement interruption to the surrounding elongating cells from the xylem under severe water deficiency [26].

The development of candidate drought-tolerant associated genes is based on precise screening for germplasm and breeding materials in limited water environments using bioinformatics [27]. Various studies on some plant genes subjected to drought stress described the biochemical pathways that are involved in drought acclimation. Proteins and metabolites that were involved in protective mechanisms against drought conditions were identified. These mechanisms include detoxification enzymes, redox status regulation, signaling pathways, protein folding and degradation, photosynthesis stability, and primary metabolism [18]. In addition, drought and low water content reduction lead to an increase in sugar and amino acid concentrations in plants [28].

GABA is a four-carbon non-protein amino acid that increases in plant tissues under stress [29]. The GABA shunt has a functional role in biotic and abiotic stress in plants through the improvement in the antioxidant activity to restrict ROS species production, plant cell signaling, and metabolism under stress [29,30]. The GABA shunt pathway is composed of three enzymatic reactions. GABA is largely produced through glutamate decarboxylation that is catalyzed by glutamate decarboxylase enzyme, then GABA is catabolized

inside the mitochondrial matrix to succinic semialdehyde (SSA) by GABA transaminase enzyme. Then, succinate is produced from SSA oxidization by the mitochondrial succinic semialdehyde dehydrogenase enzyme or reduced to gamma-hydroxybutyrate (GHB) [30].

GABA is made from glutamate by glutamate decarboxylase that is present in symbiotic rhizobia bacteroids [31]. Furthermore, GABA was shown to be the second most plentiful amino acid in detached pea (*Pisum sativum* L.) nodules through the nuclear magnetic resonance analysis, which, in turn, reflects the vital role of GABA in amino acid cycling during bacteroid metabolism [31]. Data showed that large amounts of GABA is accumulated in root nodules, while GABA that is newly made appeared to be limited to pea nodules that are metabolically active [32]. Bound forms of GABA accumulated in nodules of many legume species, reaching 20% of the total nitrogen content [32].

Drought stress could increase the activation of GABA transporters (ProTs and AAP$_3$) that regulate the entry of GABA across the cell membrane. The entrance of GABA decreased drought leaf wilting and improved membrane stability via the reduction in oxidative damage in plants [33]. Endogenous GABA enhanced drought tolerance through the inhibition of lipid peroxidation and photosynthesis [33]. In addition, GABA stabilized the intracellular pH during drought stress and provided a source of nitrogen and carbon during the Krebs cycle and carbon–nitrogen metabolism in the maturation of green peas [34]. Drought stress directly influences metabolic sink strength during vegetative growth, which triggers an imbalance in redox homeostasis that affects overall plant growth and development.

In this study, the effects of drought stress on the physiological and biochemical characterization of the GABA shunt pathway in green pea (*Pisum sativum* L.) were investigated. Characterization of seeds germination pattern, seedling growth (plant height, fresh weight, dry weight, and chlorophyll content), oxidative damage (Malondialdehyde level), GABA shunt metabolite levels (GABA, Glutamate and Alanine), total proteins, and total carbohydrate levels were determined.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The green pea seeds (*Pisum sativum* L.) that were used in this study were harvested in 2018 and obtained from local pea growers in Irbid/Jordan. All experiments were performed in the laboratory using a plant growth medium. The growth medium was a mixture of peat moss and perlite growth soil with a ratio of (2:1) wt/wt, later referred to as "soil" in a pot (7 g). Seeds were surface disinfected with 6% (*v/v*) sodium hypochlorite for 10 min and then washed five times with sterile distilled water. Surface sterilized seeds were planted in soil. Tap water was used to irrigate the seeds at a full water holding capacity level (80–100%) for two weeks until the appearance of seedlings) [35]. The two-week-old seedlings were then subjected to drought treatments according to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% separately for 7 days and another set for 14 days. Plants were grown at 25 °C under continuous cool white fluorescent lamp illumination (40 μmol m^{-2} s^{-1}). For assays and parameters determinations, three replicates of 6 pea (*Pisum sativum* L.) seeds for each replicate were used.

2.2. Seed Sensitivity to Drought Stress Assay

Three replicates of 15 pea (*Pisum sativum* L.) surface-sterilized seeds were placed on three filter papers as artificial growth surface in sealed Petri dishes and irrigated with tap water in drought treatments according to full water holding capacity levels: 80%, 60%, 40%, 20%, and 10% separately for 14 days and allowed to grow at 25 °C. Radicle emergence from the seeds was recorded daily for 14 days. Drought treatment effect on seed germination was calculated.

2.3. Seedling Physiological Growth Parameters Assay

After each drought treatment for the 7 and 14 days separately, plant height (cm), fresh weight (g), and dry weight (g) were determined. Shoot fresh weight (g) was determined

by directly weighing the collected seedlings' shoot tissues. Shoot dry weight (g) was determined following weighing the seedlings' shoot tissues after oven drying at 70 °C for 48 h.

2.4. Chlorophyll Content Determination

After each drought treatment for the 7 and 14 days separately, fresh shoots tissues were harvested. Chlorophyll pigments (Chl *a* and Chl *b*) extraction and determination were performed according to Jeffrey et al. protocol [36] with the following adjustments: 300 mg fresh leaves mixed with one ml of ice-cold 90% acetone were ground in an Eppendorf tube using a micro centrifuge tube pestle to prepare the chlorophyll extract. The extracted liquid was placed in a new Eppendorf tube and centrifuged at $11,000 \times g$ for 5 min. The supernatant was used for the determination of both chlorophyll pigments. The resulted supernatant absorbance was measured spectrophotometrically at 647 nm, 664 nm, and 750 nm wavelengths. Equation and extension coefficients [36] were used to calculate the concentration of Chl *a* and Chl *b* (μg/mL). The average of three replicates for each treatment was calculated.

2.5. Metabolites Extraction

After each drought treatment for the 7 and 14 days separately, fresh shoot tissues were harvested. GABA shunt metabolites were extracted and determined according to Zhang and Bown [37] with the following adjustments: 500 mg of fresh shoot tissues were ground in an Eppendorf tube using a micro centrifuge tube pestle until a fine powder was obtained, and then placed in 1.5 mL Eppendorf tubes and vortexed with 400 μL methanol for 10 min. Methanol from samples was removed by overnight evaporation at room temperature. To each tube, 500 μL of 70 mM lanthanum chloride was added and vortexed for 15 min. Then tubes were centrifuged at $12,400 \times g$ for 5 min. One hundred and sixty microliters of 1 M KOH was added to the collected supernatants and vortexed for 10 min. Tubes were centrifuged at $12,400 \times g$ for 5 min. The resulted supernatant (metabolites extract) was used for the determination of GABA shunt metabolites (GABA, alanine, and glutamate). The average of three replicates was used for each drought treatment.

2.6. GABA (γ-Aminobutyric Acid) Level Determination

The protocol of Zhang and Bown [37] was used for GABA determination with the following adjustments: 50 μL of metabolites extract, 14 μL of 4 mM NADP$^+$, 19 μL of 0.5 M potassium pyrophosphate, pH (8.6), 10 μL of (2 u/μL) GABASE enzyme (GABASE enzyme (Sigma-Aldrich Corp., St. Louis, MO, USA) was suspended in 0.1 M potassium pyrophosphate, pH (7.2), containing 12.5% Glycerol and 5 mM β-mercaptoethanol), and 10 μL of α-ketoglutarate were mixed in an Eppendorf tube to prepare the reaction mixture. The change in absorbance at 340 nm after the addition of α-ketoglutarate was measured after 90 min incubation at 25 °C using a microplate reader. The GABA level (nmol/mg FW) was determined using the NADPH standard curve. For each drought treatment, the average of three replicates was used.

2.7. Alanine Level Determination

The Bergmeyer protocol [38] was used for alanine determination with the following adjustments: 180 μL of 0.05 M Na-carbonate buffer, pH (10), 7 μL of 30 mM β-NAD$^+$, 10 μL of sample extract, and 1 μL of 0.3 u/μL alanine dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA) enzyme suspension were mixed in an Eppendorf tube to prepare the reaction mixture. The change in absorbance at 340 nm after the addition of alanine dehydrogenase was measured after 60 min incubation at 25 °C using a microplate reader. The alanine level (nmol/mg FW) was determined using the NADH standard curve. For each drought treatment, the average of three replicates was used.

2.8. Glutamate Level Determination

The Bergmeyer protocol [38] was used for glutamate determination with the following adjustments: 180 μL of 0.1 M Tris-HCl pH (8.3), 8 μL of 7.5 mM β-NAD$^+$, 10 μL of sample extract, and 1 μL of 0.8 u/μL glutamate dehydrogenase enzyme suspension (Sigma-Aldrich, St. Louis, MO, USA) were mixed in an Eppendorf tube to prepare the deamination reaction mixture. The change in absorbance at 340 nm after the addition of glutamate dehydrogenase was measured after 60 min incubation at 25 °C using a microplate reader. The glutamate level (nmol/mg FW) was determined using the NADH standard curve. For each drought treatment, the average of three replicates was used.

2.9. Total Protein Content Determination

After each drought treatment for the 7 and 14 days separately, fresh shoot tissues were harvested. Total protein content was determined using a SMART BCA Protein Assay Kit (Intron Biotechnology, Gangnam-gu, Seoul, Korea) according to the manufacturer's instructions. Five hundred milligrams of fresh shoots tissues mixed with 100 μL of distilled water were ground in an Eppendorf tube using a micro centrifuge tube pestle. Fifty microliters of the extracted liquid was obtained and mixed well with 1 mL of the kit working solution. All tubes were incubated at 37 °C for 30 min and then kept at room temperature for 5 min. The absorbance of the samples was measured at 562 nm using a microplate reader. The BSA standard curve was used to determine the total protein concentration (μg/mL). For each drought treatment, the average of three replicates was used.

2.10. Total Carbohydrates Content Determination

After each drought treatment for the 7 and 14 days separately, fresh shoot tissues were harvested. Total carbohydrates content was determined using the Total Carbohydrate Quantification Assay Kit (catalog number: ab155891, Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Fifty milligrams of fresh shoot tissues mixed with 200 μL ice-cold kit assay buffer was ground in an Eppendorf tube using a micro centrifuge tube pestle. Tubes were then centrifuged at 12,400× g for 5 min. The resulted supernatant was collected. In a 96-well microplate, 30 μL of collected supernatant from each sample and the kit standard solution wells were mixed with 150 μL of concentrated H$_2$SO$_4$. The microplate was mixed well for 5 min on a shaker and then incubated at 90 °C for 15 min. The microplate was cooled at room temperature for 15 min. Thirty microliters of the kit developer solution was added to all treated samples and kit standard solution wells, mixed on a shaker at room temperature for 15 min, and measured OD at 490 nm. A glucose standard curve was used to determine the total carbohydrate concentration (μg/mg FW). For each drought treatment, the average of three replicates was used.

2.11. Oxidative Damage Assay

After each drought treatment for the 7 and 14 days separately, fresh shoot tissues were harvested. Lipid peroxidation as malondialdehyde (MDA) equivalent was determined by estimating the TBA reactive substances (TBARS) as described by Heath and Packer [39] with some adjustments: 20 mg of fresh shoots tissues was mixed with 0.25 mL of 0.5% (*w/v*) thiobarbituric acid in 20% (*w/v*) trichloroacetic acid and 0.25 mL of 175 mM NaCl in 50 mM Tris-HCl (pH 8) and ground in an Eppendorf tube using a micro centrifuge tube pestle. Tubes were placed in a water bath at 90 °C for 25 min. Tubes were then centrifuged at 12,400× g for 20 min and the supernatant was collected. A microplate reader was used to measure the absorbance of the supernatant at 532 nm. A standard curve of MDA was used to calculate the MDA level (nmol/mg FW). For each drought treatment, the average of three replicates was used.

2.12. Experimental Design and Data Analysis

A completely randomized design (CRD) was used for all experiments in this study. All treatments and assays were replicated three times. For all assays parameters, mean

and standard deviation (SD) values were determined. Results were expressed as mean. Data analysis was performed by one-way analysis of variance (ANOVA) using the Least Significant Difference (LSD) multiple comparison tests on the means using the SPSS version 16.0 software. For all data analyses, *p*-value $\leq$ 0.05–0.01 was considered significant, and *p*-value < 0.01 was considered highly significant.

3. Results and Discussion

3.1. Seed Sensitivity to Drought Stress

Germination percentage of *Pisum sativum* L. was affected significantly (*p* < 0.05) by drought treatments to all saturation levels (80%, 60%, 40%, 20%, and 10%) in pea seeds for 14 days. In general, the germination percentage significantly decreased with a positive correlation as water content decreased (Table 1). The highest seed germination percentage was observed at the 80% saturation level (*p* < 0.05). A significant decline was recorded at 40% water saturation level. After 14 days, the germination percentage was 100, 80, and 30 at 60, 40, and 20% soil water saturation levels, respectively. No germination was observed at 10% drought treatment point (Table 1). These results indicated that the 40% water saturation level is the threshold value for normal pea seed germination after 14 days (*p* = 0.0001, *r* = 0.929).

Table 1. Germination percentage of pea (*Pisum sativum* L.) in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C. Means followed by different letters are significantly different (*p* $\leq$ 0.05) by LSD; *r* = correlation coefficient.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 8	Day 10	Day 12	Day 14
80%	0.0	40.0 [a]	59.0 [a]	75.0 [a]	75.0 [a]	95.0 [a]	100.0 [a]	100.0 [a]	100.0 [a]	100.0 [a]
60%	0.0	30.0 [b]	60.0 [a]	72.5 [a]	75.0 [a]	85.0 [b]	100.0 [a]	100.0 [a]	100.0 [a]	100.0 [a]
40%	0.0	12.5 [c]	45.0 [b]	60.0 [b]	65.0 [b]	70.0 [c]	75.0 [b]	75.0 [b]	80.0 [b]	80.0 [b]
20%	0.0	0.0 [d]	0.0 [c]	7.5 [c]	15.0 [c]	18.0 [d]	20.0 [c]	25.0 [c]	30.0 [c]	30.0 [c]
10%	0.0	0.0 [d]	0.0 [c]	0.0 [cd]	0.0 [d]	0.0 [e]	0.0 [d]	0.0 [d]	0.0 [d]	0.0 [d]
r *		0.987	0.924	0.929	0.913	0.953	0.942	0.942	0.929	0.929

* means α < 0.05.

Seed germination is a critical stage for plant survival. Drought stress greatly affects seed germination, but the response intensity and harmful effects of stress depend on the plant species [40]. A decrease in the germination of *Eremosparton songoricum* was observed under drought stress [40]. The results of the current study indicated that as water saturation decreased, a significant drop in the germination percentage in pea (*Pisum sativum* L.) was observed. Similar to our finding, Liu et al.'s [41] study revealed that seed germination of two maize cultivars (Liansheng15 cultivar and Zhengdan 958 cultivar) was reduced under drought stress. Increased water deficit minimized absorption of water by seeds, therefore preventing their germination [42]. However, it has been shown that failure of the emergence of the root was due to a reduction in water level between the soil and the seeds [43]. In addition, a reduced water gradient affected enzymatic reactions, which caused a delay in seed germination [44].

Muscolo et al. [45] found significant differences between two lentil cultivars, *Ustica* and *Pantelleria*, collected from Sicilian islands subjected to drought stress with a remarkable decrease and delay in seed germination. Inhibition of seed germination was directly related to energy production through respiration, enzyme and hormonal activity, reserve mobilization, and dilution of the protoplasm to increase metabolism for successful embryonic growth [45]. Water availability is a limiting factor for non-dormant seed germination. It also affected the speed, uniformity, and percentage of seed emergence [46]. Dornbos et al. [47] showed that the germination percentage was decreased during the seed filling stage in soybean (*Glycine max* L.c.v Merr.) under severe drought stress. Inhibition of starch catabolism under drought and salt stress decreased seed germination [48,49]. AL-Quraan et al. [50] showed a significant decrease in seed germination of five wheat cultivars (*Triticum aes-*

tivum L.) under salt and osmotic stress. Furthermore, AL-Quraan et al. [51] reported that cold, heat, salt, and osmotic stresses significantly reduced seed germination in two lentil (*Lens culinaris* Medik) cultivars. The results of this study showed that reduction in water content had a severe impact on seed germination and early seedling growth in pea (*Pisum sativum* L.) that might be caused by a reduction in water absorption by seeds during metabolic processes and enzymatic activity in germinating pea seeds.

3.2. Seedling Physiological Growth in Response to Drought Stress

Seedling fresh weight, dry weight, and seedling height of *Pisum sativum* L. were measured in response to drought treatments according to soil water holding capacity levels of 80%, 60%, 40%, 20%, and 10% for 7 and 14 days, separately. Seedling fresh weight and seedlings height were significantly reduced with a positive correlation as water availability decreased (Table 2). The seedling fresh weight (p = 0.001, 0.05, r = 0.957, 0.979) and seedling height (p = 0.001, 0.001, r = 0.867, 0.992) were reduced significantly after 7 and 14 days, respectively. Dry weight reduction was not significant under all drought treatment after 7 (p = 0.096) and 14 days (p = 0.228), respectively.

Table 2. Seedling height (cm), fresh weight, and dry weight (gm), and water content (%) of pea (*Pisum sativum* L.) in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Means followed by different letters are significantly different ($p \leq 0.05$) by LSD; r = correlation coefficient.

Treatment	Day 7			Day 14		
	Seedlings Height (cm)	Fresh Weight (gm)	Dry Weight (gm)	Seedlings Height (cm)	Fresh Weight (gm)	Dry Weight (gm)
80%	21.0 [a]	0.51 [a]	0.051 [a]	25.5 [a]	0.57 [a]	0.052 [a]
60%	15.0 [b]	0.355 [b]	0.046 [a]	14.5 [b]	0.415 [a]	0.046 [a]
40%	10.4 [bcd]	0.25 [c]	0.035 [a]	13.5 [bc]	0.3 [ab]	0.04 [a]
20%	6.36 [d]	0.185 [cd]	0.031 [ab]	11.5 [bd]	0.205 [ac]	0.031 [a]
10%	5.4 [de]	0.2 [ce]	0.03 [ac]	11.5 [bd]	0.21 [ad]	0.03 [a]
r *	0.992	0.957	0.979	0.867	0.979	0.995

* means $\alpha \leq 0.05$.

In agreement with our study, Embiale et al. [52] reported that unavailability of water resulted in a significant decline in pea's basal diameter increment, area, width, and expansion length of leaf, number of branches and leaves, and total seedlings length. Plant cells save water by avoiding active growth. Stomatal closure, diminished leaf water potential, turgor loss, reduction in cell enlargement and growth [53], and inhibition of shoot and root growth [54] are common plant responses due to water stress. In general, reduction in plant biomass is positively linked with prolonged water deficiency and inhibition of cell expansion per cell growth due to low turgor pressure [55]. Reduction in plant dry weight under water stress could be due to imbalanced stomatal conductance that leads to a reduction in carbon assimilation per unit leaf area and low biomass production [56,57]. Drought stress was associated with reduced cellular division and elongation during germination, causing a reduction in root length [58].

Khorasaninejad et al. [59] reported a reduction in shoot fresh and dry weight, root dry weight, internodes, and internodes length of peppermint (*Mentha piperita* L.) under drought stress. A decrease in root and shoot fresh and dry weights and shoot length was observed in sensitive bean genotypes under water stress [60]. Zhang et al. [61] reported that the length and width of maize leaves on seedlings were shorter under water stress due to a reduction in chlorophyll content. A reduction in water content decreased plant height, stem diameter, stem and root biomass, and total biomass of *Populus nigra* (poli) [62]. In agreement with our study, Riad et al. [63] reported that decreasing water content resulted in a reduction in plant growth parameters (root length, root, and leaves fresh and dry

weights, and plant length), biomass accumulation, and vegetative growth of green peas as compared with 100% water availability.

3.3. The Effect of Drought on Chlorophyll Content

Table 3 shows that the chlorophyll content were significantly decreased after 7 and 14 days as water content decreased. *Pisum sativum* L. seedlings possessed the highest total Chl *a* and Chl *b* contents at 80% water saturation level (p = 0.020, 0.003) with positive correlation (r = 0.938, 0.986) after 7 and 14 days, respectively. There was a significant decrease with a positive correlation in Chl *a* and Chl *b* contents in response to 7 days and 14 days of drought treatments.

Table 3. Chlorophyll content (Chl *a*, Chl *b*, total chlorophyll) (μg/mL) of pea (*Pisum sativum* L.) seedlings in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Means followed by different letters are significantly different ($p \leq 0.05$) by LSD; r = correlation coefficient.

	Day 7			**Day 14**		
Treatment	**Chl *a***	**Chl *b***	**Total**	**Chl *a***	**Chl *b***	**Total**
80%	7.670 [a]	5.350 [a]	13.020 [a]	10.470 [a]	9.950 [a]	20.420 [a]
60%	3.552 [b]	3.870 [b]	7.422 [b]	8.200 [b]	8.220 [a]	16.420 [b]
40%	2.500 [c]	1.420 [c]	3.920 [c]	6.460 [c]	7.040 [ab]	13.500 [c]
20%	2.300 [cd]	1.220 [cd]	3.520 [cd]	5.740 [cd]	5.380 [bc]	11.120 [d]
10%	1.140 [e]	0.699 [e]	1.839 [e]	3.210 [e]	4.870 [bd]	8.080 [e]
r *	0.913	0.963	0.938	0.975	0.998	0.986

* means α < 0.05.

Chlorophyll is a major chloroplast component and has a positive relationship with photosynthetic rate. Our results agreed with Nyachiro et al.'s [64] study, which found a significant reduction in Chl *a* and Chl *b* caused by drought stress in six wheat (*Triticum aestivum* L.) cultivars. Shinde and Thakur [65] reported that drought stress significantly reduced Chl *a* and Chl *b* contents in three varieties of chickpeas during vegetative growth or anthesis. Our data indicated that decreased water availability caused a significant decrease in Chl *a* and Chl *b* contents in *Pisum sativum* L. after 7 and 14 days. This reduction in total chlorophyll content leads to a reduction in photosynthesis and plant growth which was correlated with a significant reduction in seedling growth under all water stress levels. Depending on the duration and severity of drought stress, plant genotypes, and environmental conditions, a reduction in Chl *a* and *b* contents has been reported in other plant species [66,67]. Previous studies on *Triticum aestivum* and *Zea mays* [68], *Gossypium hirsutum* [69], and *Tectona grandis* [70] found that the content of photosynthetic pigments reduced as water availability decreased. It has been reported that metabolic imbalance occurred as a result of a decrease in chlorophyll synthesis and an increase in chlorophyll degradation under drought stress [71–73].

Nitrogen is a component of the chlorophyll structure in plant tissues. Under stress conditions, a decline in chlorophyll content was associated with changes in nitrogen metabolism as a result of proline synthesis to maintain osmotic adjustment [74]. Low nitrogen levels reduced photosynthetic rates due to a reduction in chlorophyll synthesis [75]. Severe water stress decreased stomatal conductance, transpiration, and photosynthetic rate [56,76]. As a drought response mechanism, chloroplasts reduced light absorption by decreasing the chlorophyll content [77]. Studies revealed that the inhibition of photosynthesis efficiency as a result of an imbalance between light capture and usage under water shortage enhances oxidative stress. Furthermore, under water stress, stomatal closure and variation in photosynthetic metabolism lead to a reduction in CO_2 availability and directly impacted the photosynthetic rate [78]. Under severe drought conditions, chlorophyll content in wheat seedlings was reduced as a result of the increased activity of chlorophillase and peroxidase enzymes [79]. In response to drought stress, chlorophyll content was

reduced in the vegetative and flowering stages of the *Avena* species [80]. Alberte et al. [81] reported a reduction in Chl *a/b* contents occurred in lamellar content in response to water deficit. Patro et al. [82] and Ghotbi-Ravandi et al. [83] reported that stomatal closure due to water deficit is a major factor inhibiting photosynthesis in *Arabidopsis thaliana* and barley (*Hordeum vulgare* L.) seedlings, respectively. Moreover, drought stress significantly decreased the chlorophyll content in *Pisum sativum* L. seedlings [84] and three wheat varieties (*Triticum aestivum* L.) [85]. The results of our study indicated that a water imbalance due to drought stress caused a reduction in chlorophyll content that might be attributed to the photosynthetic apparatus activity disturbance.

3.4. GABA Shunt Activation in Response to Drought Stress

The GABA shunt metabolites increased significantly as a result of drought stress caused by different water saturation levels (Table 4). There were significant differences in GABA, alanine, and glutamate content at 80%, 60%, 40%, 20%, and 10% soil water content between the 7- and 14-day treatments. In general, there was a significant increase in GABA, alanine, and glutamate (p = 0.001, 0.0001, 0.002, respectively) with a negative correlation (r = −0.987, −0.968, −0.978, respectively) with water availability after the 7-day treatment. A significant increase was also found in GABA, alanine, and glutamate (p = 0.0001, 0.003, 0.870, respectively) with a negative correlation (r = −0.906, −0.989, −0.939, respectively) after the 14-day treatment. The increase in GABA content was about 4 and 5 times higher at the 20% and 10% water levels, respectively, after the 14-day treatment when compared with GABA content at the same water levels after the 7 day treatment. The increase in GABA levels was accompanied by a slight but significant ($p \leq$ 0.01–0.05) increase in the levels of alanine and glutamate after 7- and 14-day treatments under all water saturation levels.

Table 4. Levels of GABA shunt metabolites (GABA, alanine, and glutamate) of pea (*Pisum sativum* L.) seedlings in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Metabolite levels were calculated as nmol/mgFW. GABA (γ-Aminobutyric acid), Ala (alanine), Glu (glutamate). Means followed by different letters are significantly different ($p \leq$ 0.05) by LSD; r = correlation coefficient.

|	Day 7			Day 14		
Treatment	**GABA**	**Ala**	**Glu**	**GABA**	**Ala**	**Glu**
80%	3.966 [a]	0.185 [a]	0.166 [a]	22.291 [a]	0.535 [a]	0.429 [a]
60%	6.999 [b]	0.237 [b]	0.198 [b]	25.211 [a]	0.603 [a]	0.446 [a]
40%	8.170 [bc]	0.268 [c]	0.221 [bc]	25.648 [ab]	0.666 [a]	0.584 [a]
20%	10.106 [cd]	0.275 [cd]	0.228 [bd]	40.973 [c]	0.797 [ab]	0.590 [a]
10%	12.111 [de]	0.302 [e]	0.252 [be]	49.969 [d]	0.838 [bc]	0.722 [a]
r *	−0.987	−0.968	−0.978	−0.906	−0.989	−0.939

* means α < 0.05.

The GABA shunt has been associated with physiological responses, such as cytosolic pH regulation [86], nitrogen metabolism, and carbon fluxes into the Krebs cycle [87], protection against ROS production [88], and osmoregulation and signaling [89]. The GABA shunt is also involved in carbon and nitrogen metabolism, maintenance of cell membrane integrity [90], and minimizing the negative effects of abiotic stresses on plant metabolism [91].

Many studies reported that various stresses caused an increase in endogenous GABA accumulation in plants [92]. The current study showed a significant increase in GABA content as a result of a reduction in water availability to ease the metabolic damage in pea seedlings during vegetative growth. Studies showed that GABA effectively decreased leaf wilting and improved membrane stability induced by drought stress in perennial ryegrass (*Lolium perenne*) [93], black pepper [94], and white clover (*Trifolium repens*) [33], which confirmed the beneficial effect of GABA in protecting plants from oxidative stress. Insufficient availability of oxygen resulted in significant suppression of melon seedling

growth. However, seedling growth was significantly improved when exogenous GABA was applied [95]. Several amino acid syntheses are regulated by the TCA cycle [96]. Glutamate, as a precursor amino acid of GABA production, is produced from the α-ketoglutaric acid (an intermediate of the TCA cycle) transamination [90]. Glutamate metabolic pathway activation via the TCA cycle was confirmed by GABA production and defense of creeping bentgrass against drought stress [97]. An increase in endogenous GABA levels due to an increase in glutamate content occurred in white clover in response to drought stress [33]. Increased content of glutamic acid and GABA were found in six winter wheat genotypes due to drought stress [98]. In addition, alanine was increased at the beginning of drought stress due to an increase in glutamate levels [99]. In this study, the increase in GABA content as water availability decreased mitigated the damaging effects of drought stress on pea (*Pisum sativum* L.) seedlings by supplying enough carbon/nitrogen source to the TCA cycle and amino acid synthesis.

3.5. The Effect of Drought on Seedling Proteins Level

In general, the total protein level in *Pisum sativum* L. seedlings increased with increasing water deficit. A significant difference was found in the total protein level of pea seedlings between the 7-day ($p = 0.0001$, $r = -0.984$) and 14-day ($p = 0.0001$, $r = -0.982$) treatments (Figure 1). The total protein level was 2 to 3 times higher after 7-day treatments when compared with the 14-day treatments under all water saturation levels. The significant increase in protein content after 7 days indicated that *Pisum sativum* L. seedlings could accumulate protein to lower the osmotic potential to maintain cellular structures and metabolic stability under drought stress. However, the steady-state level of protein content after 14 days indicated the ability of pea seedlings to tolerate prolonged water deficit by keeping stable protein metabolism to adjust cellular osmolarity and provide protection from further dehydration damage.
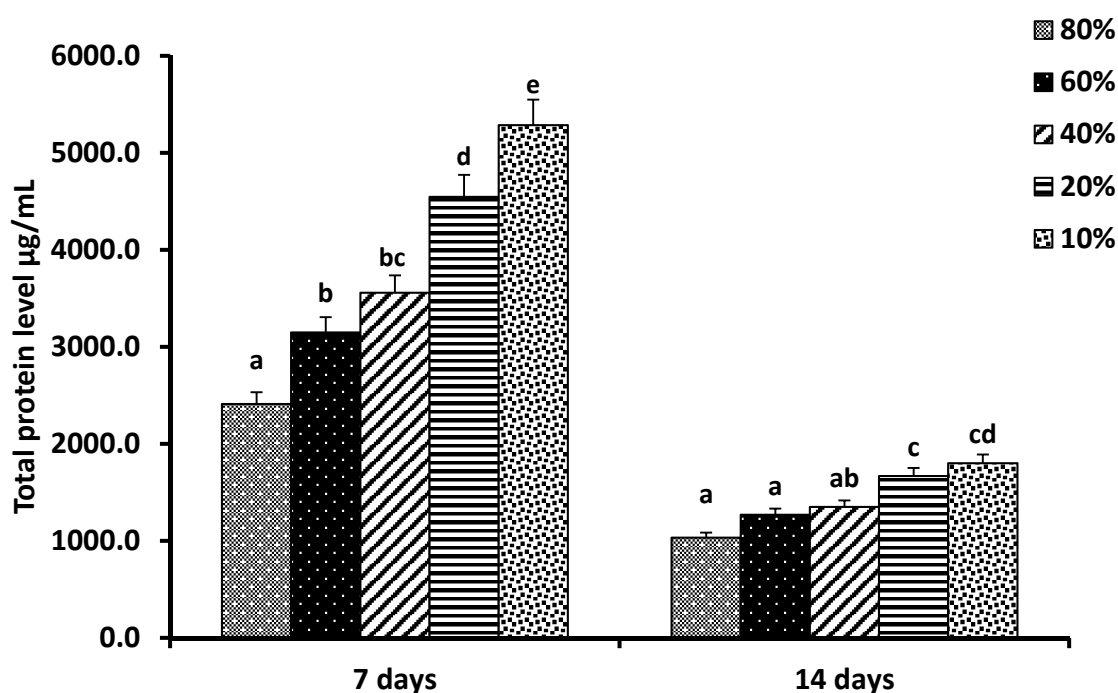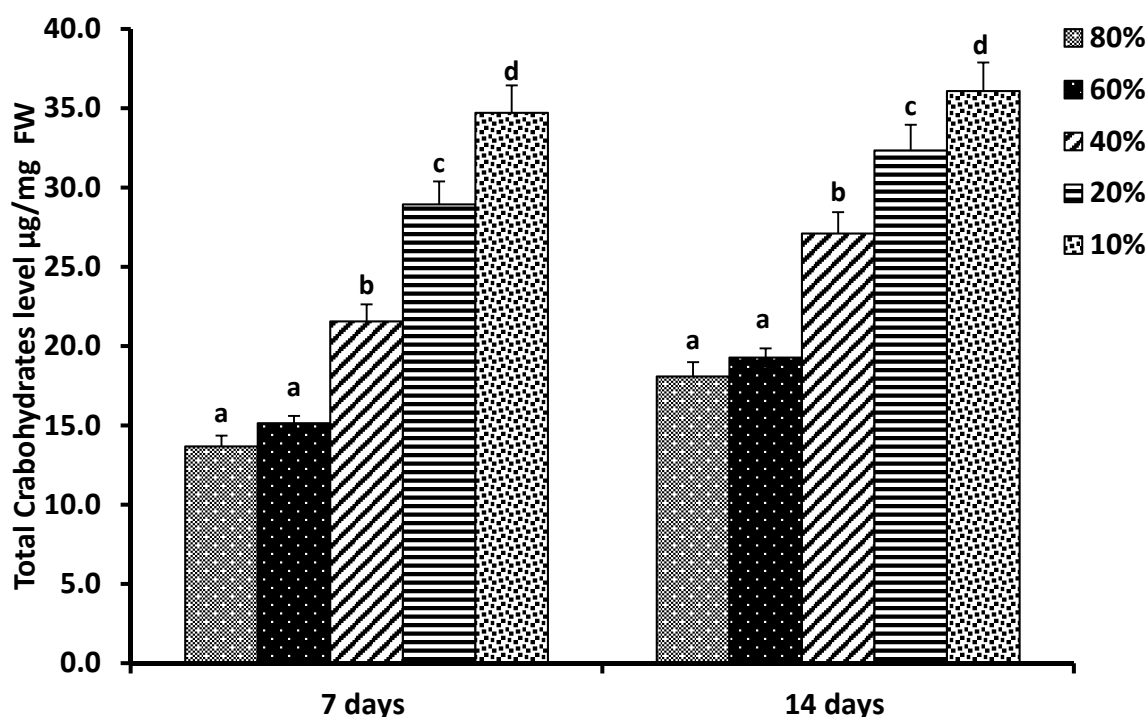

Figure 1. Total proteins level of pea (*Pisum sativum* L.) seedlings in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Protein levels were calculated as μg/mL. Means (columns) with different letter scripts are significantly different ($p \leq 0.05$) by LSD.

Under environmental stress factors, plants stimulated specific changes in protein synthesis [100]. In agreement with our study, Guttieri et al. [101] reported increased protein concentration under water deficit due to higher rates of nitrogen accumulation in hard wheat (*Triticum aestivum* L.) grains. Ozturk and Aydin [102] also reported an increase in protein and gluten content in winter wheat cultivars grain in response to drought stress when compared with the fully irrigated treatment. During a combination of heat and drought stress, glutamine, ornithine, tyrosine, valine, and tryptophan were accumulated in *Arabidopsis thaliana* and purslane plants to maintain cellular osmotic adjustment and keep leaf turgor in response to such stress combination [103,104]. Drought stress induced the accumulation of protein by enhancing the protein biosynthesis in wheat and barley [105,106]. However, a reserve of available substrates for protein synthesis could be associated with amino acid accumulation to facilitate quick retrieval of osmotic adjustment and plant metabolism in response to water deficiency [107]. In a study that used three Australian bread wheat (*Triticum aestivum* L.) cultivars, Ford et al. [108] suggested that the cultivar RAC875 had the highest capacity to withstand drought stress by increasing the cellular protein synthesis. Although there was an increase in total protein levels which were involved in ROS scavenging, they observed a decrease in proteins involved in the Calvin cycle and photosynthesis. In contrast to our results, Gołębiowska et al. [109] observed a decrease in the number of differential proteins in leaves of winter barley subjected to drought stress for three weeks. Mohammadkhani and Heidar [110] reported an initial increase followed by a decrease in protein concentration in maize varieties (*Zea mays* L.) subjected to drought stress. The initial increase in total soluble proteins might be due to the expression of new proteins involved in stress adaptation. However, the decrease in protein concentration might be due to a severe reduction in photosynthesis. Under water stress, proline amino acid accumulated in plant cells prevents cellular oxidation through scavenge ROS [111] and regulates plant osmotic pressure for efficient water absorption [112]. Free proline level was reported to be increased under water deficit in wheat [113,114]. The amount of proline was increased significantly in ginger (*Zingiber officinale*) in response to a reduction in water availability and prolonged duration of drought stress [112].

3.6. The Effect of Drought on Seedling Carbohydrates Content

In the current study, data showed that the total carbohydrate level in *Pisum sativum* L. seedlings increased as water availability decreased (Figure 2)., the total carbohydrate level increased significantly ($p = 0.0001$) with a negative correlation ($r = -0.970$) in response to drought stress for 7 days. Similar results were obtained under drought stress for 14 days ($p = 0.0001$, $r = -0.0980$).
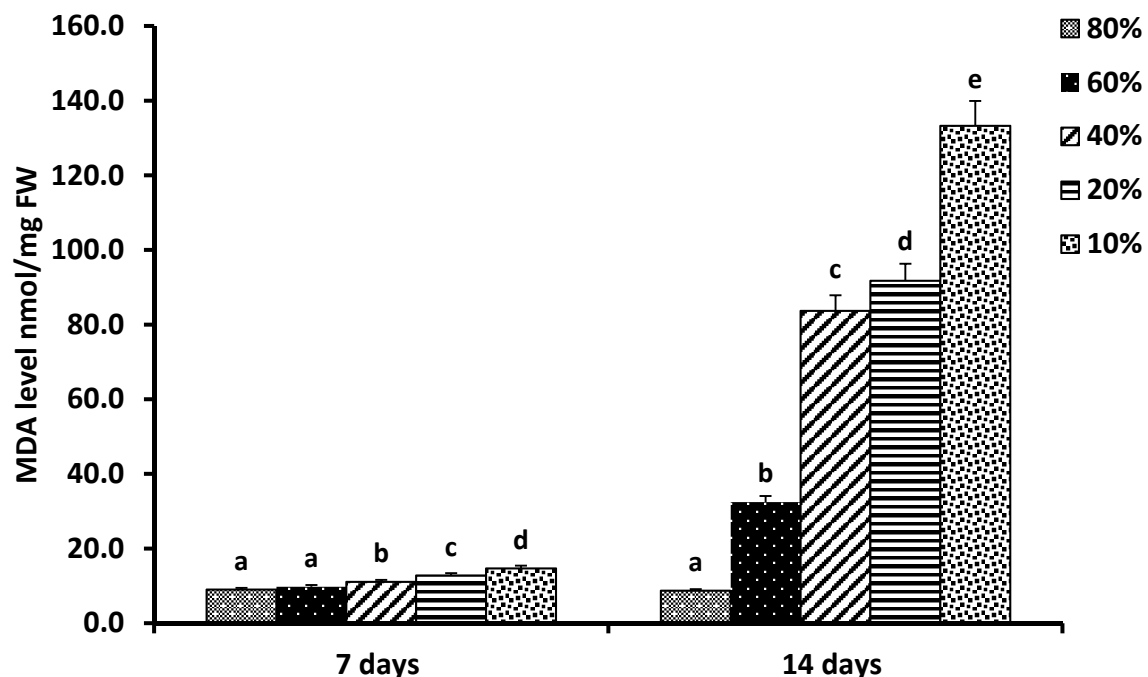
Figure 2. Total carbohydrates level of pea (*Pisum sativum* L.) seedlings in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. Total carbohydrate level was calculated as μg/mg FW. Means (columns) with different letter scripts are significantly different ($p \leq 0.05$) by LSD.

Water stress induces carbohydrate accumulation and osmolytes synthesis to maintain the water potential of plants [115,116]. Soluble sugar levels in plants also increased in response to drought stress. Plants respond to water shortage by balancing their potential osmotic proportion with the external environment by increasing their soluble sugars at the cellular level, reducing activities in roots, reducing the metabolism of carbohydrates as a result of severe pressure, and reducing the transfer of sugars in rinsing vessels [117]. In agreement with our study, an increase in total soluble sugars in durum (*Triticum durum* L.) wheat [118] and oligosaccharides in two sesame cultivars [119] were reported. In contrast, Akinci and Losel [120] reported a decrease in total sugar in cucumber cultivars under water stress. However, a major reserve of carbohydrates was detected in the leaves of cucumber seedlings. Under drought stress, sugar accumulation might be due to the fact that sucrose content increased because the enzyme activities involved in sucrose breakdown were diminished [121]. Furthermore, sugars protect the cells during drought by two physiological mechanisms. The first mechanism involves the hydroxyl group of sugars, which substitute the water to maintain hydrophilic interactions and hydrogen bonding in membranes and proteins during dehydration to prevent protein denaturation [122]. The second mechanism involves sugar vitrification in dehydrated cells through the formation of a biological glass in the cytoplasm to reduce water permeability [123]. Mohammadkhani and Heidari [110] reported an increase in soluble sugar concentrations as a result of starch degradation in two maize (*Zea mays* L.) cultivars. Soluble sugars accumulated only in roots of eucalyptus trees under drought stress [124]. Regier et al. [62] reported an increase in soluble sugar concentrations in the roots of two divergent clones of *Populus nigra* to maintain osmotic adjustment under water deficit. Soluble sugars were also reported to be increased and participated in plant metabolic regulation and stress signaling in response to osmotic stress [125]. Additionally, increased soluble sugars may act as osmoprotectants to stabilize the membranes and sustain cell turgor in response to water deficit [126]. Li and Li [127] showed an increase in the glucose, fructose, and sucrose content in micro-propagated apple plants (*Malus domestica* Borkh) in response to water stress. It is concluded

from these previous studies that fluctuations of sugar concentrations in different plants under drought stress might be due to variations in CO_2 assimilation, partitioning of carbon source-sink, activity of enzymes involved in sucrose–starch partitioning, and inhibition of Calvin cycle enzymes.

3.7. Oxidative Damage in Response to Drought Stress

The accumulation of MDA, which is a byproduct of membrane lipids' oxidative damage, can be used as a marker for oxidative stress [128]. In this study, the MDA levels in the pea seedlings were determined to evaluate the lipid peroxidation in response to drought stress. Figure 3 shows that the malondialdehyde (MDA) level of pea (*Pisum sativum* L.) seedlings was slightly but significantly increased after 7 days ($p = 0.0001$, $r = -0.967$). However, the MDA level was increased sharply and significantly under all water stress levels reaching the highest level (140 nmol/mgFW at 10% water saturation level) ($p = 0.0001$, $r = -0.975$) after 14 days. Our results showed that the pea seedlings suffered minor damage under mild (60% water saturation level) drought stress, but severe lipid peroxidation and oxidative damage of the cell membrane occurred with increased (40%, 20%, and 10% water saturation levels) water deficit (Figure 3).

Figure 3. Malondialdehyde (MDA) level of pea (*Pisum sativum* L.) seedlings in response to soil water holding capacity levels: 80%, 60%, 40%, 20%, and 10% grown under continuous light at 25 °C for 7 days and 14 days, separately. The MDA level was calculated as nmol/mg FW. Means (columns) with different letter scripts are significantly different ($p \leq 0.05$) by LSD.

Many abiotic stresses lead to the accumulation of MDA in plant tissues due to lipid peroxidation of cellular membranes and overproduction of ROS [39]. Severe water stress caused agitation and instability in the metabolic processes in the mitochondria and chloroplasts, leading to high ROS production [129]. ROS resulting from oxidative stress are toxic and highly reactive molecules that can damage cellular macromolecules and subcellular structures [130,131]. The levels of MDA and H_2O_2 are indicators of the free radical reactions occurring in plant-stressed tissues [132]. Similar to our findings, Pandey et al. [133] reported that the MDA level was increased in *Avena* species leaves under drought stress, increasing membrane leakage. In addition, Zlatev et al. [134] reported an increase in membrane damage and ROS production in three bean (*Phaseolus vulgaris* L.) cultivars as a result of water deficit. Similarly, Tatar and Gevrek [135] reported an increase in the

level of MDA in wheat as the duration of drought stress increased. Leaves of growing carrots (*Daucus carota* L.) accumulated high levels of MDA content under water deficit stress [91]. Morabito and Guerrier [136] reported that oxidative stress occurred 12 h after the application of drought stress. However, after a longer period of drought stress, an antioxidants defense system was initiated. Abid et al. [137] showed that a higher MDA concentration in wheat (*Triticum aestivum* L.) was associated with higher H_2O_2 content and greater rate of $O_2^{\bullet-}$ generation. Hernandez et al. [138] reported that salinity stress in plant tissues could lead to cell membrane rupture due to the accumulation of ROS and lipid peroxidation.

3.8. Correlation between GABA Level and All Physiological and Metabolic Parameters

Under all water treatment levels used in this study, the GABA level was negatively correlated with seed germination, seedlings height, fresh and dry weight, and chlorophyll content (Table 5). On the other hand, GABA level was positively correlated with protein and carbohydrate contents and MDA level under all water treatments. The elevated level of GABA metabolites (GABA, alanine, and glutamate), total proteins, and total carbohydrates content might be involved in cellular osmotic adjustment, protecting plants from oxidative stress, balancing of carbon and nitrogen (C:N) metabolism, and maintaining cell metabolic homeostasis and cell turgor under water stress [9,22].

Table 5. Correlation analysis between the GABA level and all physiological and metabolic parameters that were measured in this study under drought stress treatments after 7 and 14 days, separately of pea (*Pisum sativum* L.) seedlings. G% = germination percentage; *r* = correlation coefficient.

	Drought Treatments Duration	
r	7 Days	14 Days
GABA vs. G%	−0.897	−0.989
GABA vs. Seedling height	−0.886	−0.861
GABA vs. Fresh weight	−0.937	−0.812
GABA vs. Dry weight	−0.941	−0.911
GABA vs. Chl *a*	−0.936	−0.894
GABA vs. Chl *b*	−0.935	−0.901
GABA vs. Protein	0.988	0.956
GABA vs. Carbohydrates	0.955	0.923
GABA vs. MDA	0.967	0.877

4. Conclusions

Our study showed that water deficit had a suppressive effect on *Pisum sativum* L. growth and metabolism. Drought stress significantly inhibited seed germination, decreased fresh and dry weight and plant height due to a reduction in water absorption by seeds during metabolic processes and enzymatic activities in germinating pea seeds and early seedling growth. In addition, water deficit caused a significant decrease in chlorophyll pigments that might be attributed to disturbances in the activity of the photosynthetic apparatus. Pea seedlings suffered minor cellular damage under mild drought stress. However, the oxidative damage and ROS production were more severe with increased duration and levels of water deficit. The increase in GABA content as water availability decreased mitigated the damaging effects of drought stress on pea (*Pisum sativum* L.) seedlings by supplying enough carbon/nitrogen sources to the TCA cycle and amino acid synthesis. The significant increase in protein content might lower the osmotic potential to maintain cellular structures and metabolic stability under drought stress. Pea seedlings tolerate prolonged water deficit by keeping stable protein metabolism to adjust cellular osmolarity and to provide protection from further dehydration damage. Water deficit induced carbohydrate accumulation to maintain water potential and osmotic adjustment via the activation of osmolytes synthesis. Overall, the significant increases in GABA, protein and carbohydrate contents were to cope with the physiological impact of drought stress on

Pisum sativum L. seedlings by maintaining cellular osmotic adjustment, protecting plants from oxidative stress, balancing carbon and nitrogen (C:N) metabolism and maintaining cell metabolic homeostasis and cell turgor. According to data presented in the current study, a sufficient water supply is vital for normal growth and metabolism in pea seedlings. Severe (less than 40% water content of the holding capacity) and long-term drought stress should be avoided during the germination stage to ensure proper seedling growth.

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