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Research Article

Potential role of nitrogen supplementation in alleviating flooding stress

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Abstract

Flooding is one of the most damaging abiotic stresses, affecting seventeen million square kilometers of land surface per year, and it is expected to increase in severity in many parts of the world with climate change. Therefore, understanding the mechanisms by which plants cope with flooding stress is important for the development of new flood-tolerant cultivars. The aim of this study was to investigate the effects of different nitrogen doses [no-nitrogen(N_0), 100 kg ha^{-1} (N_{10}), and 200 kg ha^{-1} (N_{20})], on the physiological responses of plants under flooding stress. In this context, spinach plants were subjected to flooding stress, and several physiological, biochemical, and nutritional parameters were investigated. The results showed that flooding stress caused a decrease in aboveground fresh and dry weight of spinach, while chlorophyll a (Cl a), b (Cl b), and total chlorophyll (TCl), as well as carotenoid, protein, and proline contents, increased. In addition, the uptake of the macronutrients N, P, K, and Mg increased during flooding stress. N application under flooding stress alleviated its negative effects and suppressed the induction of H2O2 through increased proline biosynthesis. Similarly, Cl a, Cl b, and TCl levels minimized the negative effects of flooding stress. In conclusion, different N doses improved spinach plant parameters and alleviated the effects of flooding stress, with N10 application in particular N10 improved biomass by 22% under flooding producing significant results in suppressing the detrimental effects of stress.

Keywords: antioxidant enzyme; flooding stress; nitrogen; macronutrients; PCA; prolin

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Introduction

When soil water content in the surface layer is 20% higher than the carrying capacity of the field, water is released from the soil's surface (Orcutt and Nilsen, 2000; Aggarwal *et al.*, 2006). This is known as flooding, an abiotic stress that affects large areas of the world. Although its variability depends on time and frequency, flooding negatively alters soil structure (Unger *et al.*, 2009; Tang *et al.*, 2023; Zhang *et al.*, 2023) and plant nutrient dynamics in ecosystems (Glazebrook and Robertson, 1999; Jackson and Colmer, 2005; Zhou *et al.*, 2020). Flooding stress is a result of inappropriate irrigation practices, global warming, other anthropogenic factors, and natural processes that vary depending on plant metabolism, growth form, and ecogeographic

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distribution, and is considered a major problem for agricultural cultivation (Rao and Li, 2003; Schaffer *et al*., 2006; Irfan *et al*., 2010; Pan *et al*., 2021).

During periods of extreme rainfall, water can accumulate in the soil, with the result that roots in the soil remain surrounded by water (Colmer and Voesenek, 2009; Kaur *et al.*, 2020). Gas diffusion under flooding has a key role in plant survival, as oxygen diffusion occurs more slowly (approximately 104-fold) under water than in the open air (Panda *et al.*, 2008; Liu *et al.*, 2023). This creates hypoxic (low-oxygen) and anoxic (no-oxygen) conditions around plant tissues during flooding (Hasanuzzaman *et al.*, 2012; Zahra *et al.*, 2021; Basit *et al.*, 2024). Among the clearly visible symptoms of flooding in plants are yellowing of the lower leaves, and death (Loreti *et al*., 2016). Premature senescence of leaves and delayed shoot growth in flooded plants are known to result from inhibition of nitrogen (N) uptake and consequent N redistribution within the shoot (Drew and Sisworo, 1977). During flooding events, partial or complete inundation of the soil or shoots severely inhibits gas diffusion by preventing the entry of CO_2 necessary for photosynthesis (Ponnamperuma, 1984; Panda *et al.*, 2008; Kaur *et al.*, 2020). The metabolic and physiological effects of flooding on plants (Kozlowski, 1984; Gibbs and Greenway, 2003; Zhang *et al.*, 2021) are mainly due to the disruption of aerobic mitochondrial root respiration (Kögel-Knabner *et al.*, 2010; Yamauchi *et al.*, 2017; Qi *et al.*, 2019) and subsequent oxygen deprivation (Sasidharan *et al.*, 2018; Martínez-Alcántara *et al.*, 2012). The resulting low oxygen levels in the root system can limit the cultivation of many species, resulting in plant damage or death (Drew, 1997; Winkel *et al.*, 2013; Patel *et al.*, 2014; Yamauchi *et al.*, 2017).

It is known that various reactive oxygen species (ROS), such as superoxide molecules (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), and nitrous oxide (NO) (Lamattina *et al.*, 2003; Pandey *et al.*, 2017) are formed in plants due to flooding stress (Dat *et al.*, 2000; Bailey-Serres and Voesenek, 2008; Lal *et al.*, 2019). Moreover, the excessive production of ROS has been found to cause disruptions in many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation and even mutation (Halliwell, 2006; Foyer, 2018). In order to neutralize the toxicity of ROS, plants under flooding stress rely on antioxidant enzyme systems (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), etc.) and metabolites (ascorbate, glutathione, proline, betaine, etc.), as well as abscisic acid. These enzymatic antioxidants, metabolites, and hormone are known to be essential in plant defense mechanisms under abiotic stress (Taiz and Zeiger, 2002; Apel and Hirt, 2004; Choudhury *et al.*, 2017; Singh *et al.*, 2019).

Spinach (*Spinacia oleracea* L.), a member of the Amaranthaceae family (Caparrotta *et al.*, 2019), is a leafy vegetable (Muchate *et al.*, 2018; Caparrotta *et al.*, 2019) that has attracted much attention in recent years, with an increasing cultivation area globally. Spinach is known as an excellent food source worldwide (Zheng *et al.*, 2021) due to its high nutritional value and health benefits, which derive from its contents of many important vitamins and minerals (Nishihara *et al.*, 2001; Thomas *et al.*, 2019). Widespread flooding stress due to excessive rainfall or anthropogenic factors has prompted efforts to minimize its negative effects and develop appropriate management practices for the cultivation of nutrient-rich vegetables such as spinach (Seymen, 2021). Nitrogen is the essential nutrient for growth and development in plants and plays a critical role in many vital processes such as chlorophyll biosynthesis, amino acid and protein synthesis, and nucleic acid metabolism (Muhammad *et. al.*, 2022). Spinach is a cool-season vegetable grown mainly under short-day conditions, i.e., from autumn to spring. Given the growing season, it's clear that flooding significantly limits productivity in spinach farming. In addition, an unfavorable winter climate, characterized by relatively low temperatures combined with prolonged rainfall, increases the risk of N leaching. The ability of spinach to use N efficiently due to its root system, and the need for farmers to achieve adequate yields and products of appropriate quality, make it particularly difficult to make the right decisions about the dose, timing, and type of N-fertilizer applications to spinach (Lawlor, 2002; Canali *et al.*, 2014; Overeem, 2015; Navarrete, 2016). In this context, there is little information on the interactions between the two limiting factors of flooding stress and N dose in relation to crop production. The effects of abiotic stress factors such as drought (Seymen *et al.*, 2024), salinity (Yavuz *et al.*, 2022), low/high temperature (Chitwood *et al.*, 2016) and UV radiation (Kasım and Kasım, 2016) on spinach have been identified in previous studies. However, no comprehensive studies have been conducted on the negative effects of flooding stress on spinach and the mitigation of these effects by different doses of N application. Therefore, the aim of this study was to evaluate the morphophysiological and biochemical responses related to growth regulation in spinach under N application during the vegetative growth period, and under flooding stress during the harvest period.

Materials and Methods

Experimental area

The study was conducted in the research greenhouse of the Department of Horticulture, Faculty of Agriculture, Selçuk University, Konya, Türkiye. Climatic parameters such as temperature and relative humidity were measured and recorded on an hourly basis using a portable automatic weather station (Davis Vantage Pro 2) located inside the greenhouse. During the study period, the temperature inside the greenhouse varied between 1.3-39.4 °C, and the highest daily average temperature was 18 °C. The relative humidity ranged from 32.1-69.5%. There were no climatic problems limiting the growth of spinach. To ensure uniformity of temperature and humidity, natural ventilation was provided through side windows and roof vents, which allowed sufficient air circulation. Spinach is sensitive to prolonged oxygen deficiency in the root zone. Therefore, clay loam soil, with its adequate water-holding capacity and favorable drainage properties, was selected to evaluate the responses of spinach plants under such conditions. The soil used in the study had an organic matter content of 3.05%, pH 7.98, EC 1.278 μS/cm, and 6.7% lime.

Seeding, irrigation, and test subjects

A total of 12.5 kg of soil was evenly distributed among each pot. The plant material used was a baby-leaf spinach variety (*Spinacia oleracea* L. cv. 'Red Kitten RZ F1', Rijk Zwaan®)that can be grown for 4 seasons. The study was designed using a factorial experimental design in randomized plots with three replicates, three pots per replicate, and four plants per pot. It evaluated the effectiveness of three different nitrogen (N) doses (N_0 control, N_{10}, and N_{20}) under two irrigation scenarios: full irrigation (FI) and flooding stress (FS). The N doses were calculated according to the amount of pure N applied per hectare. For the 0 control (no N), 100 kg ha^{-1}, and 200 kg ha^{-1} of N, the N was dissolved in pure water and applied to the pots. Approximately 1.5 liters of irrigation water was given to the seedlings in each pot. To ensure the soil in the pots reached field capacity, an additional 1.6 L of irrigation water was applied five days after planting the seedlings. Observations were made until flood stress application, and each pot was weighed using digital scales. When the water-holding capacity of the control group decreased by approximately 50%, irrigation water was applied to all treatment groups to reach field capacity. Irrigation water was applied 11 times until the flood stress application (110 days). Approximately 110 days after sowing, the drainage channels of the pots in the I150 application groups, where flood stress was applied, were closed. Then, irrigation water was applied so that it would accumulate on the soil surface. In the I150 application group, flood stress was applied for seven days.

Growth parameters

After the plants were harvested, the aerial parts were cut and the roots were cleaned prior to determining the fresh plant weight (g) and fresh root weight (g), respectively. The fresh aerial and belowground parts were dried in an oven at 72 °C for 72 h, after which the aerial dry weight (g) and the belowground dry weight (g) were determined (Seymen, 2021).

Physiological parameters

After harvesting the plants, the fresh weight of 1 cm^2 disks taken from leaf samples was measured and recorded after saturating them with pure water to determine their turgor weight (TW). The samples were then dried in an 80 °C oven for 48 hours and their dry weights were determined. Leaf water content was calculated according to Sanchez *et al.* (2004) and Demiral and Türkan (2005). Additionally, three leaf discs, each measuring 1 cm^2, were taken and passed through triple-distilled water in glass tubes. After this process, 10 ml of water was added and the closed tubes were shaken at 25 °C for 24 hours in a shaker. Immediately afterwards, electrical conductivity (EC_1) was measured. The same samples were then autoclaved at 120 °C for 20 minutes, cooled to 25 °C and the EC_2 measurement taken again. The average malondialdehyde content in the leaf samples was calculated as described by Lutts *et al.* (1996) and expressed as membrane damage (%).

Biochemical parameters

To determine the carotenoid and chlorophyll a and b contents in 0.2-gram leaf samples taken at harvest, the samples were ground in 10 mL of acetone and centrifuged. These samples were then read as 2 mL by spectrophotometry at 470, 652 and 663 nm, respectively. Chlorophyll a and b contents were determined according to the formula by Lichtenthaler and Buschmann (2001), and carotenoid content according to the formula by Jasper (Witham *et al.*, 1971).

For the determination of protein content, 0.5 g of plant samples from each treatment were cut into small pieces, ground in a mortar and pestle in 10 times the volume of 0.05 M phosphate buffer (pH: 6.5), and centrifuged at 15,000 rpm for 20 min. 0.2 mL of the supernatant was taken, 3 ml of Bradford dye was added, and the sample was read at 595 nm (Bradford, 1976). To determine proline content, 0.1 g of fresh leaf sample was digested with 3% sulfosalicylic acid. After centrifugation at 15,000 rpm for 10 min, 2 mL of the filtered samples was taken, to which 2 mL glacial acetic acid and 2 mL acid-ninhydrin were added, and the resulting mixture kept for 1 h in a hot water bath set to 100 °C. Acid-ninhydrin was prepared from ninhydrin, acetic acid, and orthophosphoric acid. After the samples were removed from the hot water bath and kept in an ice bath for 10 min, 4 mL of toluene was added to each tube and mixed by vortexing for 30 sec. The phase remaining at the top was then pipetted and read at 520 nm (Bates *et al.*, 1973), while the transmittance of the mixture was read at 390 nm. Malondialdehyde (MDA), a measure of lipid peroxidation, was determined according to Madhava and Sresty (2000). The leaves were soaked in 3 mL of trichloroacetic acid (0.1% TCA). The extract was centrifuged at 10,000 rpm for 10 min and mL of the superficial residue was added to 4 mL of a mixture of 20% TCA and 0.5% TBAA. The mixture was heated at 95 °C for 30 min, then cooled on ice and centrifuged at 10,000 rpm for 5 min. The transmittance of the surface residue was measured spectrophotometrically at 532 and 600 nm. To determine the concentration of H_2O_2, leaf tissue was placed in 5 mL of 0.1% TCA in an ice bath and centrifuged at 12,000 rpm for 15 min. 0.5 mL of the superficial residue was added to a mixture of 0.5 mL phosphate buffer at pH 7.0 and 1 mL 1M KI (Velikova *et al.*, 2000). Finally, enzyme changes were determined using the method described by Angelini and Federico (1989) to quantify the contents of SOD, peroxidase (POX), and CAT in the samples. Plant SOD content was determined by a spectrophotometric method based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Agarwal and Pandey, 2004); this measurement was based on the color change of NBT at 560 nm. CAT content in plant extracts was determined by spectrophotometric absorbance at 240 nm according to the method described by Havir and McHale (1987). The POX content in the samples was calculated according to the method described by Chance and Maehly (1955), who calculated the rate of increase of absorbance at 470 nm in the same extracts.

Plant nutrient analysis

Leaf analysis was used to determine the nutritional status of the plants. For this purpose, leaf samples were dried in an oven at 72 °C for 48 h. After drying, approximately 1 g of each sampled was weighed (accuracy:

0.001 g) and ground in a mill with a sieve diameter of 0.5 mm for macro- and micronutrient (N, P, S, K, Ca, Na, B, Cl, Mo, Mg, Fe, Mn, Cu, and B) analyses. The reagents used were used in the preparation of calibration standards, and purified water was used in all processing and purification steps. Standard solutions were prepared at eight different concentrations for each element. Instrument calibration was performed based on these solutions. Internal standards were automatically added during the analytical procedures of the ICP-OES instrument. All samples were analyzed in triplicate, and elemental concentrations were determined using the prepared standards to a ± 0.5-3% accuracy. The prepared samples were read by inductively coupled plasma spectrophotometry (ICP-OES) (Skujins, 1998).

Data analysis

The morphophysiological and biochemical changes resulting from plant growth and enzymatic changes under different N doses and flooding stress conditions were analyzed, and significance was determined at 1% and 5% levels in the SPSS-22 program. Principal Component Analysis (PCA) in JMP-14 was used to identify the parameters that showed significant changes instead of many parameters in the study. To investigate the relationships between the traits measured under the control and flooding stress, a score plot and a flooding plot were constructed between the first and second principal components. Additionally, we used the Mahalanobis distance to identify outliers in our research data. It calculates the distance between two points by calculating the covariance matrix from the data without tracking or recording other points. The distance obtained in this way is expressed as standard deviations and is a data-dependent value (Şavkan *et al.*, 2024; Şavkan *et al.*, 2025).

Results and Discussion

Effects of flooding stress on growth and physiological parameters

The statistical analysis revealed that N applications of different doses had significant effects on growth parameters under flooding stress (Table 1). For example, increasing N dosage increased the RWC (%) of spinach plants (Table 1), while the application of N_{20} caused a statistically significant increase in the flooding stress (FS) groups compared to those under full-irrigation (FI) conditions. Seymen (2021) stated that flooding stress increased the amount of RWC in crops harvested at different times of flooding stress application. In addition, the application of plant biostimulants such as N has been found to stimulate leaf growth, especially in plants with vegetative parts, and this situation is known to be related to the turgor state of the leaf and triggers various molecular and physiological processes (Khan *et al.*, 2017; Rouphael *et al.*, 2018; Caruso *et al.*, 2019; Coussement *et al.*, 2021).

In the present study, MD (%) values showed a decreasing trend in all interactions except FI × N_{20} (Table 1). The reason for this is that O_2^- radicals are synthesized under stress conditions and appear as membrane damage. Nitrogen application protects membrane stability while limiting ROS accumulation by stimulating antioxidant enzyme activities, but it has been reported that high doses of N (N_{2O}) may exacerbate MD (%) by increasing oxidative metabolism. On the other hand, as a result of increasing N doses applied to reduce the FS effect, it was observed that N_{10} had significant impacts on leaf fresh weight, root fresh weight, leaf dry weight, and root dry weight compared to the control group. When the interaction between stress conditions and N doses was analyzed, the differences in leaf fresh weight, root fresh weight, and leaf dry weight were found to be statistically significant and the best results were obtained from the FI × N_0 and FI × N_{10} treatments, which were in the same group. Flooding stress caused a decrease in all agronomic parameters, as shown in Table 1, but when the interaction was analyzed, it was found that N_{10} had a positive effect on parameters under flooding stress conditions. Early responses to FS have been reported to include changes in plant water use, nutrient uptake, and dry weight, with reduced root water uptake (Beutler *et al.*, 2014; Kim *et al.*, 2019). Under FS, plant phytology and catabolism are disrupted, and stomatal conductance, gas permeability, and CO_2 metabolism are restricted. Reduced CO_2 uptake into the leaf leads to leaf wilting through reduced transpiration and reduced dry matter accumulation, resulting in a decrease in Cl content, which in turn leads to a decrease in leaf fresh weight followed by a decrease in leaf dry weight (Ashraf, 2012; Ren *et al.*, 2014), but N applications have been shown to positively modify the effect of flooding stress. Di Mola *et al.* (2019) reported that different biostimulants improved plant growth in greenhouse-grown baby lettuce, especially when optimal N fertilization was applied to the leaves. However, the literature describing reduced flood susceptibility in N-supplemented plants is quite limited (Mugnai *et al.*, 2012; Gupta and Igamberdiev, 2016; Kal *et al.*, 2023).

Table 1. Effect on aboveground fresh weight, belowground fresh weight, aboveground dry weight, belowground dry weight, leaf relative water content, and membrane damage of increasing nitrogen doses applied to spinach under flooding stress conditions

Treatments	**AFW (g)**	**BFW (g)**	**ADW (g)**	**BDW (g)**	**RWC (%)**	**MD (%)**
Irrigation (I)						
FI	57.1^{a}	2.85^{a}	5.50^{a}	0.31	67.0	28.8^{a}
FS	39.5^{b}	2.04^{b}	4.74^{b}	0.35	60.3	22.9^{b}
Azot (N)						
N$_{0}$	48.8^{b}	2.51^{a}	5.69^{a}	0.23^{b}	63.1^{b}	31.1^{a}
N$_{10}$	53.2^{a}	2.69^{a}	5.76^{a}	0.36^{a}	56.0^{b}	23.7^{b}
N$_{20}$	42.8^{c}	2.14^{b}	3.92^{b}	0.41^{a}	71.7^{a}	22.9^{b}
IxN						
FI N$_{0}$	66.1^{a}	3.31^{a}	6.92^{a}	0.17	36.0	36.0
FI N$_{10}$	65.9^{a}	3.29^{a}	6.02^{b}	0.39	22.9	22.9
FI N$_{20}$	39.2^{c}	1.96^{bc}	3.56^{c}	0.38	27.6	27.6
FS N$_{0}$	31.4^{d}	1.71^{c}	4.45^{d}	0.28	26.2	26.2
FS N$_{10}$	40.5^{c}	2.10^{b}	5.49^{c}	0.34	24.4	24.4
FS N$_{20}$	46.5^{b}	2.32^{b}	4.29^{d}	0.43	18.2	18.2
Significance						
Irrigation(I)	**	**	**	ns	ns	*
Azot (N)	**	**	**	**	**	*
IXN	**	**	**	ns	ns	ns

NS: not significant; *, P < 0.05; **, P < 0.01; CV, the coefficient of variation; lowercase letters indicate significant differences between treatments (P < 0.05) according to Duncan's test. AFW: aboveground fresh weight; BFW: belowground fresh weight; ADW: aboveground dry weight; BDW: belowground dry weight; RWC: leaf relative water content; MD: membrane damage

Effects of flooding stress on biochemical parameters

The statistical analyses showed that FS had significant effects on biochemical contents in N-treated spinach seedlings (Table 2 and Table 3). As shown in Table 2, Cl a, Cl b, and TCl levels increased significantly with the effect of flooding stress (Table 2). N doses applied to reduce the effect of flooding stress showed the best efficiency for both Cl a, Cl b, and TCl content in the FI × N$_{10}$ treatment (Table 2). While the FS × N10 interaction resulted in non-significant fluctuations in chlorophyll content, the increase observed in the FS × N20 interaction indicates a potential role of N in promoting photosynthetic activity. Indeed, the rate of leaf CO$_2$ assimilation (photosynthesis) has been found to increase significantly in spinach (Evans and Terashima, 1988) and soybean (Lee, 2022) leaves with increasing N content. In our study, N doses applied to spinach under flooding stress conditions were found to contribute significantly to chlorophyll content. The carotenoid content increased in parallel with the chlorophyll content in response to stress, and the obtained values for these factors suggested that plants under N$_{10}$ application best tolerated the effect of flooding stress. As shown in Table 2, the photosynthesis activity values under the N20 treatment were lower than those observed with N10. This outcome has been reported to be associated with excessive nitrogen application leading to oxidative stress, disruption of metabolic balance, or insufficient nitrogen uptake by roots under flooding conditions (Ren *et al.*, 2017; Aslam *et al.*, 2023).When the carotenoid content was examined in the interaction between stress conditions and N doses, the effect of flooding stress was low, while high carotenoid content was obtained in the FI × N_{10} treatment (Table 2).

Table 2. Effects of increasing nitrogen application rates on chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, protein, and proline in spinach under flooding stress

Treatments	**Cl a (mg g^{-1})**	**Cl b (mg g^{-1})**	**TCL (mg g^{-1})**	**CT (mg g^{-}1)**	**PT (mg g^{-1})**	**PL (mg g^{-1})**
Irrigation (I)						
FI	$18,0^{b}$	$5,97^{b}$	$28,1^{b}$	$4,61^{b}$	$70,5^{a}$	$15,3^{b}$
FS	$19,2^{a}$	$6,83^{a}$	$30,3^{a}$	$5,26^{a}$	$37,3^{b}$	$61,0^{a}$
Azot (N)						
N_0	$14,2^{c}$	$4,94^{c}$	$22,2^{c}$	$3,86^{c}$	$33,9^{b}$	$17,1^{c}$
N_{10}	$21,9^{a}$	$7,51^{a}$	$34,6^{a}$	$5,92^{a}$	$63,8^{a}$	$66,7^{a}$
N_{20}	$19,7^{b}$	$6,75^{b}$	$30,8^{b}$	$5,02^{b}$	$64,1^{a}$	$30,7^{b}$
IxN						
FI N_0	$10,3^{d}$	$3,29^{e}$	$15,7^{e}$	$2,61^{e}$	$52,6^{bc}$	$10,3^{c}$
FI N_{10}	$27,2^{a}$	$9,10^{a}$	$43,0^{a}$	$7,16^{a}$	$67,8^{b}$	$17,9^{c}$
FI N_{20}	$16,5^{c}$	$5,53^{d}$	$25,5^{d}$	$4,05^{d}$	$91,2^{a}$	$17,8^{c}$
FS N_0	$18,1^{c}$	$6,60^{c}$	$28,7^{c}$	$5,11^{c}$	$15,2^{d}$	$23,8^{c}$
FS N_{10}	$16,7^{c}$	$5,91^{d}$	$26,2^{cd}$	$4,69^{c}$	$59,8^{b}$	$115,6^{a}$
FS N_{20}	$22,9^{b}$	$7,98^{b}$	$36,1^{b}$	$5,98^{b}$	$37,0^{c}$	$43,6^{b}$
Significance						
Irrigation(I)	*	**	*	**	**	**
Azot (N)	**	**	**	**	**	**
IxN	**	**	**	**	**	**

NS: not significant; *, $P < 0.05$; **, $P < 0.01$; CV, the coefficient of variation; lowercase letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's test. Cl a: chlorophyll a; Cl b: chlorophyll b; TCl: total chlorophyll; CT: carotenoid; PT: protein; PL: proline

As shown in Table 3, MDA content in spinach seedlings did not show statistically significant differences between N treatments and FI × N interaction groups. This indicates that increased ROS production, along with the effectiveness of the antioxidant defense system and the preservation of membrane structure, also indicated that plants have the capacity to limit oxidative damage and maintain homeostatic balance under flooding and N stress conditions (Qi *et al.* 2019; Hasanuzzaman *et al.*, 2020). On the other hand, statistically significant differences were found between the N treatment and interaction groups in terms of H_2O_2 content. Compared to the control group (N_0 = 1.08 μmolg^{-}1), both N_{10} and N_{20} appeared to suppress H_2O_2 production, but did not have the same effect on more advanced oxidative damage indicators such as MDA. This suggests that while N is decisive in regulating ROS levels, its effect on suppressing advanced oxidative damage is limited (Gill and Tuteja, 2010; Thiruvengadam *et al.*, 2024).When the interaction groups were analyzed, FS × N_{10} was the group that suppressed H_2O_2 production under flooding stress. Flooding stress-induced H_2O_2 accumulation leads to lipid peroxidation in biomembranes, preventing their selective property. H_2O_2 is thus known to cause ROS formation by disrupting metabolic processes that prevent normal plant growth and development under stress, and N application has been observed to alleviate its accumulation (Walter *et al.*, 2004; Da Cruz *et al.*, 2013; Yadav and Hemantaranjan, 2017; Radmann *et al.*, 2018).

Table 3. Effects of increasing nitrogen application rates in spinach under flooding stress on peroxidase, catalase, superoxide dismutase, malondialdehyde, and H_2O_2

Treatments	POX (EU g^{-1})	CAT (EU g^{-1})	SOD (EU g^{-1})	MDA (nmol mL^{-1})	H_2O_2 (µmol g^{-1})
Irrigation (I)					
FI	3760^{b}	11502^{b}	2011	$3{,}51^{b}$	$0{,}84^{b}$
FS	6929^{a}	15211^{a}	1836	$3{,}79^{a}$	$1{,}08^{a}$
Azot (N)					
N_0	5853^{a}	12503^{b}	1629^{b}	3,73	$1{,}08^{a}$
N_{10}	5963^{a}	16330^{a}	2132^{a}	3,59	$0{,}86^{c}$
N_{20}	4217^{b}	11237^{b}	2008^{a}	3,63	$0{,}93^{b}$
IxN					
FI N_0	3520^{d}	5660^{d}	1416^{c}	3,57	$0{,}95^{c}$
FI N_{10}	5136^{c}	14727^{b}	2577^{a}	3,53	$0{,}78^{d}$
FI N_{20}	2624^{d}	14120^{b}	2039^{b}	3,42	$0{,}78^{d}$
FS N_0	8187^{a}	19347^{a}	1843^{b}	3,89	$1{,}21^{a}$
FS N_{10}	6789^{b}	17933^{a}	1686^{bc}	3,66	$0{,}94^{c}$
FS N_{20}	5811^{c}	8353^{c}	1978^{b}	3,83	$1{,}08^{b}$
Significance					
Irrigation(I)	**	**	ns	**	**
Azot (N)	**	**	**	ns	**
IXN	**	**	**	ns	*

NS: not significant; *, $P < 0.05$; **, $P < 0.01$; CV, the coefficient of variation; lowercase letters indicate significant differences between treatments ($P < 0.05$) according to Duncan's test. POX: peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; H_2O_2: hydrogen peroxide

When antioxidant enzyme systems were analyzed (Table 3), flooding stress was found to have caused an 84.2% increase in POX activity from 3760 to 6929, and a 32% increase in CAT activity from 11502 to 15211.With N application, POX activity remained at a similar level at 5963 in N_{10} compared to the control group (N_0 = 5853), but decreased to 4217 in N_{20}, decreasing by 27.9%. Similarly, CAT activity increased from 12503 in N_0 to 6330 in N_{10} (a 30.7% increase), and decreased to 11237 in N_{20}, decreasing by 10.1%. According to the Duncan test, FIxN10(5136) and FSxN20(5811) were placed in similar statistical groups, indicating that stress-induced POX production was neutralized by N application. The CAT value was found to have increased 2.5-fold in the FI × N_0 and FI × N_{20} interactions, while decreasing significantly in the FS × N_{10} and FS × N_{20} interactions. This shows that the effect of N on the CAT content of spinach plants under flooding stress was non-significant (Table 3). Increasing N doses increased the SOD activity of spinach plants under flooding stress (Table 3).

When the interactions between flooding stress and N doses were analyzed, SOD activity was measured as 2039 in FI × N_{20} and 1978 in FS × N_{20}, and they were statistically in the same group. Since the difference between these values was only around 3%, flooding stress did not individually increase SOD amounts in these two groups. In plant metabolism, N has a critical role in processes such as continuous detoxification and the scavenging of free radicals. Low N leads to faster degradation of H_2O_2, which enhances plants' primary defense mechanisms, illustrating their survival methods under certain stress conditions (Aydinoglu and Akgul, 2019; Kapoor *et al.*, 2019; Çimrin *et al.*, 2020). Similar antioxidant-content results to ours were obtained in previous studies (Fan *et al.*, 2014; Radmann *et al.*, 2018; Seymen, 2021; Kıymacı *et al.*, 2024).

Proline, which was 15.3 mg g^{-1} under FI conditions, increased to 61.0 mg g^{-1} under FS conditions, an approximately 4-fold (298%) increase. When I × N interactions were analyzed by Duncan's test, FIxN_0, FIxN_{10}, FIxN_{20}, and FSxN_0 were in the same group, while FSxN_{10} group increased proline content by 115.6 mg g^{-1} compared to the FIxN_0 group (10.3 mg g^{-1}), resulting in an approximately 11-fold (1022%) increase in proline content. This indicates that proline actively scavenges free O_2 radicals that accumulate under flooding stress, with its synthesis increasing rapidly (Barickman *et al.*, 2019; Meena *et al.*, 2019; Guan *et al.*, 2021; Rehman *et al.*, 2021). These findings indicate that N application interacts with ROS and plays a protective role against flood stress, reducing its negative effects. This is also reflected in the growth parameters in Table 1. Leaf dry weight was higher in the N_{10} treatment than in the N_0 treatment at 5.76 g, while root dry weight showed one of the highest values at 0.36 g. Furthermore, the rate of membrane damage was determined to be 23.7% in the N_{10} treatment and 31.1% in the N_0 treatment. These findings indicate that the optimum nitrogen dose (N_{10}) reduces oxidative stress by increasing proline accumulation, maintains membrane integrity, and supports growth parameters despite flooding stress (Kaur and Asthir, 2015).

PCA of morphophysiological and *biochemical measures*

The agronomic and biochemical parameters of spinach under flooding stress conditions and different N doses were subjected to PCA (Table 4). The first two components of the PCA (PC1 and PC2) explained 66.73% of the total variance, suggesting that the data obtained from the experiment can be strongly explained by PCA. Previous studies have indicated that the first two components should explain more than half of the total variance for PCA to be effective (Yavuz *et al.*, 2022; Seymen *et al.*, 2024).

Table 4. Principal component analysis (PCA) results of agronomic, biochemical, and elemental-nutrient contents of increasing nitrogen doses applied to spinach under flooding stress conditions

Agronomic, biochemical PCA				**Elemental-nutrient PCA**			
Items	**PC1**	**PC2**	**PC3**	**Elements**	**PC1**	**PC2**	**PC3**
Eigenvalue	6.10	4.57	2.60	Eigenvalue	7.80	3.25	1.07
Percentage of variance	38.14	28.58	16.27	Percentage of variance	60.06	25.03	8.24
Cumulative variance	38.14	66.73	83.01	Cumulative variance	60.06	85.10	93.34
Eigenvectors							
AFW	-0.202	0.265	0.397	N	-0.251	0.362	-0.107
UFW	-0.201	0.248	0.421	B	-0.274	0.213	0.359
ADW	-0.215	0.020	0.505	Ca	0.223	-0.201	-0.557
UDW	0.302	0.241	-0.180	Cu	0.038	-0.378	0.637
RWC	-0.112	0.171	-0.358	Fe	0.295	-0.304	-0.093
MD	-0.370	-0.103	0.008	K	0.273	0.355	0.052
Cl a	0.317	0.261	0.151	Mg	0.294	0.312	0.021
Cl b	0.345	0.213	0.140	Mn	0.336	-0.114	0.119
CT	0.340	0.207	0.187	Na	0.338	-0.044	0.227
POX	0.279	-0.284	0.223	P	0.201	0.432	-0.043
CAT	0.243	-0.104	-0.112	Pb	0.301	-0.107	0.012
SOD	0.233	0.356	0.033	S	0.355	0.056	-0.005
MDA	0.220	-0.323	0.170	Zn	0.272	0.325	0.250
PT	-0.139	0.335	-0.258	-	-	-	-
PL	0.136	-0.154	0.010	-	-	-	-
H_2O_2	0.128	-0.389	0.102	-	-	-	-

AFW: aboveground fresh weight; BFW: belowground fresh weight; ADW: aboveground dry weight; BDW: belowground dry weight; RWC: leaf relative water content; MD: membrane damage; Cl a: chlorophyll a; Cl b: chlorophyll b; TCl: total chlorophyll; CT: carotenoid; POX: peroxidase; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; PT: protein; PL: proline; HO: hydrogen peroxidase. Boldface parameters showed significant differences in PCA

PC1 explained 38.14% of the variance under control conditions, and BDW, Cl a, Cl b, and CT were the parameters explained positively, while MD was the parameter explained negatively. On the other hand, PC2 explained 28.58% of the variance, with SOD and PT being the parameters explained positively, and MDA and HO explained negatively (Table 4). When the PCA result was evaluated together, the parameters that explained the effect of flooding stress (PC1) with different N doses in spinach were PL, CAT, POX, MDA, and HO. However, the parameters AFW, ADW, BFW, BDW, RWC, and PT, which showed their importance in the control conditions (PC3), emerged as important selection criteria. The results of the analysis of nutrient contents showed that the first component explained 60.06% of the total variance and the positively explained elements were Mn, Na, Pb, and S. The second component explained 25.03% of the total variance, and the positively explained elements were N, K, Mg, P, and Zn, while Cu and Fe were negatively explained. The interaction between nutrients under flooding stress was explained by PCA (Álvarez-Robles *et al.*, 2021).

Loading plots were generated using PC1 and PC2 components (Figure 1A, 1B). In loading plots, if the angle between two vectors is less than 90, there is a positive correlation, if it is greater than 90, there is a negative correlation, and if it is equal to 90, there is no significant relationship between the two variables (Yavuz *et al.*, 2020; Seymen, 2021). In light of this information, when the loading plots were analyzed, a high positive correlation was found between Cl a, Cl b, and CT, while a high negative correlation was found between these parameters and MD (Figure 1A). When the loading plots of minerals in spinach leaves were analyzed (Figure 1B), high positive correlations were found between Mn, Pb, Na, P, K, Mg, and Zn. The PCA revealed a positive correlation between fresh weight and dry weight, while these two parameters showed a negative correlation with MDA, POX, and Pl. Similar results were reported by Seymen (2021), who also studied FS stress in spinach. In addition, a positive relationship was reported between plant growth parameters in lettuce under drought stress conditions (Yavuz *et al.*, 2021).

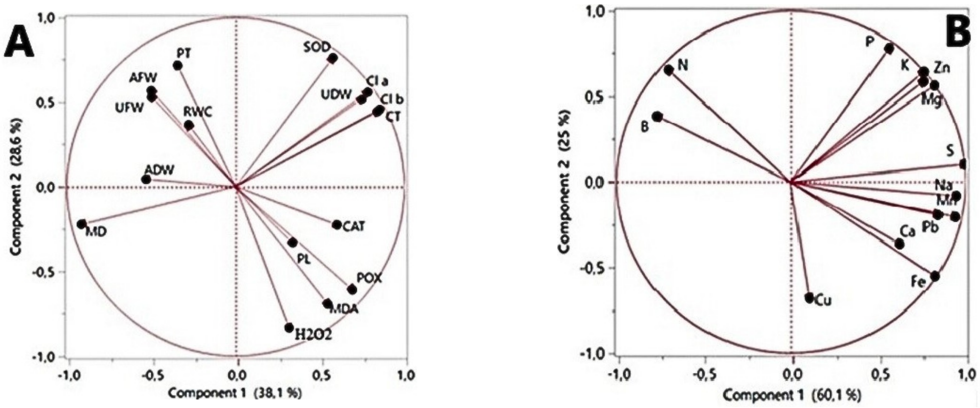

Figure 1. Loading plot of PC1 and PC2 as a result of principal component analysis,: agronomic and biochemical characteristics(A); nutrient elements (B)

Similarly, a score plot graph was constructed using PC1 and PC2 components to examine the relationship between treatments (Figure 2A, 2B). Increased N doses are positively correlated with PC2, while lower N doses are negatively correlated. This suggests that PC2 reflects changes related to nitrogen application levels. When analyzing the parameters in the positive region of PC1, it was observed that the pigment contents including CT, Cl a, and Cl b-as well as the PT and CAT contents, increased with increasing N dose. This shows that increasing the nitrogen dose application improves the plant's photosynthetic capacity and antioxidant defense system. Similarly, N doses caused an increase in S and Na contents. Therefore, PC1 biologically represents the direct effects of nitrogen fertilization on photosynthetic pigments, antioxidant enzyme activities, and element uptake. The second component placed FS and FI treatments in the positive range, while stress treatments were in the negative range.

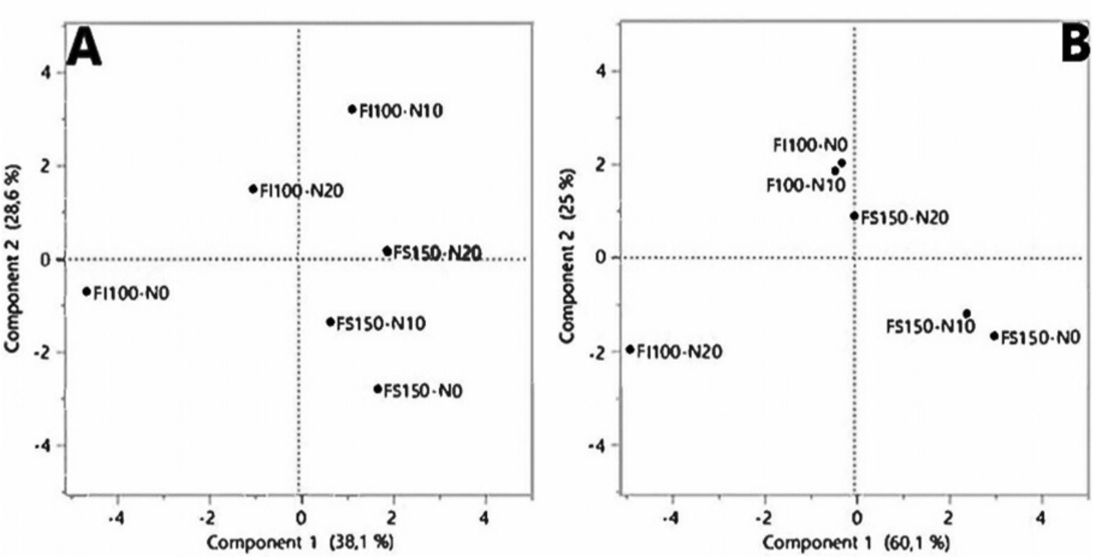

Fig 2. Score plot of PC1 and PC2 as a result of principal component analysis agronomic and biochemical characteristics(A); nutrient elements (B)

Many studies have shown that applied N increases chlorophyll content (Taş, 2021). This is consistent with the positive charges of chlorophyll and antioxidant enzymes on PC1 and PC2. Men *et al.* (2020) reported that N doses applied under flooding stress conditions increased SOD, CAT, and POX contents as well as chlorophyll content in *Brassica napus*. Chlorophyll is an important pigment that increases the rate of photosynthetic activity. N is the main component of chloroplasts, and its deficiency therefore has a negative effect on photosynthetic activity (Guo *et al.*, 2007). Many studies have shown that flooding stress reduces chlorophyll content (Yordanova and Popova, 2001; Zhou, 2007).

Conclusions

In this study, the morphophysiological, biochemical, and mineral contents of spinach plants under flooding stress, as well as the role of different N doses (N_0, N_{10}, N_{20}) on stress tolerance, were evaluated. The results showed statistically significant differences in the parameters of spinach under flooding stress conditions. In this context, N_{10} treatment showed physiological- and biochemical-stress-alleviating effects during flooding stress in spinach plants, with this dose showing strong positive influences on most of the agronomic, physiological, and biochemical parameters during flooding stress. The application of N under flooding stress alleviated the effects of stress by increasing proline biosynthesis and suppressing the induction of H_2O_2. Another important result was that the uptake of the macronutrients N, P, K, and Mg, which play a fundamental role in several plant processes, increased during flooding stress. Increased N doses were also observed to increase the uptake of elemental S and Na. All these results indicate that the greatest risk in spinach cultivation under flood stress is loss of photosynthesis and yield reduction, but that plant stress tolerance can be increased with appropriate nitrogen management, drainage improvements, and nutrient supplementation.

Authors' Contributions

Conceptualization: Y. Dal-Canbar; Data curation: Y. Dal-Canbar; Formal analysis: Y. Dal-Canbar; Funding acquisition: Y. Dal-Canbar; Investigation: Y. Dal-Canbar; Methodology: Y. Dal-Canbar; Project administration: Y. Dal-Canbar; Resources: Y. Dal-Canbar; Software: Y. Dal-Canbar; Supervision: Y. Dal-Canbar; Validation: Y. Dal-Canbar; Visualization: Y. Dal-Canbar; Roles/Writing - original draft: Y. Dal-Canbar; and Writing - review & editing: Y. Dal-Canbar.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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