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# Primary plant nutrients modulate the reactive oxygen species metabolism and mitigate the impact of cold stress in overseeded perennial ryegrass

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Overseeded perennial ryegrass (*Lolium perenne* L.) turf on dormant bermudagrass (*Cynodon dactylon* Pers. L.) in transitional climatic zones (TCZ) experience a severe reduction in its growth due to cold stress. Primary plant nutrients play an important role in the cold stress tolerance of plants. To better understand the cold stress tolerance of overseeded perennial ryegrass under TCZ, a three-factor and five-level central composite rotatable design (CCRD) with a regression model was used to study the interactive effects of nitrogen (N), phosphorus (P), and potassium (K) fertilization on lipid peroxidation, electrolyte leakage, reactive oxygen species (ROS) production, and their detoxification by the photosynthetic pigments, enzymatic and non-enzymatic antioxidants. The study demonstrated substantial effects of N, P, and K fertilization on ROS production and their detoxification through enzymatic and non-enzymatic pathways in overseeded perennial ryegrass under cold stress. Our results demonstrated that the cold stress significantly enhanced malondialdehyde, electrolyte leakage, and hydrogen peroxide contents, while simultaneously decreasing ROS-scavenging enzymes, antioxidants, and photosynthetic pigments in overseeded perennial ryegrass. However, N, P, and K application mitigated cold stress-provoked adversities by enhancing soluble protein, superoxide dismutase, peroxide dismutase, catalase, and proline contents as compared to the control conditions. Moreover, N, P, and K application enhanced chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids in overseeded perennial ryegrass under cold stress as compared to the control treatments. Collectively, this 2-years study indicated that N, P, and K fertilization mitigated

cold stress by activating enzymatic and non-enzymatic antioxidants defense systems, thereby concluding that efficient nutrient management is the key to enhanced cold stress tolerance of overseeded perennial ryegrass in a transitional climate. These findings revealed that turfgrass management will not only rely on breeding new varieties but also on the development of nutrient management strategies for coping cold stress.

#### KEYWORDS

abiotic stress, antioxidant defense system, *Cynodon dactylon*, *Lolium perenne*, NPK fertilization, oxidative stress

## 1 Introduction

Bermudagrass (*Cynodon dactylon* (L) Pers.) turf is an extensively used warm season turfgrass in sports fields, golf courses, parks, and greenbelts throughout the world (Ihtisham et al., 2020). The optimal temperature for its best growth and performance ranges from 26°C–35°C (Fan et al., 2014). Low temperature severely restricts its growth and performance (Huang et al., 2019) and therefore low temperature is considered a key abiotic factor that confines the use of bermudagrass (Ihtisham et al., 2018). A transitional climatic zone (TCZ) refers to the transitional zone between arid and humid regions that is sensitive to climatic fluctuations (Barker et al., 2019). In China, TCZ is located in marginal areas of the East Asian monsoon zone where the ecosystem has become unstable due to combined effects of climatic change and human activities (Yin et al., 2021; Yin et al., 2022). TCZ possesses both severe cold and hot temperatures in their respective seasons (Christians et al., 2016). As a result, it's difficult for warm-season turfgrass to perform well in winter, and cool-season turfgrass to perform better in summer (Ihtisham et al., 2018). To overcome the winter dormancy of warm-season turfgrasses, overseeding practice with a cool-season turfgrass is commonly practiced (Shi et al., 2014). Perennial ryegrass (*Lolium perenne* L.) is an important cool-season turfgrass that is frequently overseeded on warm-season grasses for the temporary re-establishment of turfgrass cover, color, playability, functionality, and aesthetic value (Xie et al., 2020). The overseeding practice comprises sowing cool-season grass seeds over established dormant warm-season turfgrass (Ozkan and Kir, 2021). Despite a cool-season turfgrass, perennial ryegrass is considered susceptible to utmost cold stress (Hoffman et al., 2010).

Future climatic change predictions are increased summer temperature, higher rainfall, longer growing seasons, and more irregular winter climates (Ostrem et al., 2018; IPCC, 2021). The anticipation and frequency of utmost temperatures (cold and hot) will provoke instability, and the winter short photoperiods with extremely low temperatures in transitional zones will drastically affect cool-season turfgrasses (Uleberg et al., 2014; IPCC, 2021). Cold stress is a major environmental factor that limits crop productivity and poses a serious threat to agricultural

sustainability (El-Sappah and Rather, 2022). Cold stress negatively affects cellular components and metabolism, and imposes stresses of variable severity that depend on the intensity and duration of the stress. Cold stress leads to chlorosis, necrosis, changes in cytoplasm viscosity, membrane damage, and changes in enzyme activities leading to plant death (Atayee and Noori, 2020). Moreover, cold stress disrupts the integrity of intracellular organelles, leading to the loss of compartmentalization, reduction and impairment of photosynthesis, protein assembly, and general metabolic processes (Bera et al., 2022). The effects of cold stress cause great challenges to the utilization of overseeded cool-season turfgrasses globally (Xie et al., 2020). Plants under optimum growing conditions usually maintain reactive oxygen species (ROS) through stable production and scavenging mechanisms. However, during stress conditions, this balance is perturbed (Dahro et al., 2016; Hussain et al., 2016; Sarraf et al., 2020; El-Sappah et al., 2021a). The increased accumulation of ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) under stress causes membrane lipid peroxidation (MDA) and electrolyte leakage (EL), which are effective indicators of oxidative stress (Hu et al., 2018; El-Sappah et al., 2021b). Numerous approaches are being used to acclimate or mitigate the harmful effects of cold stress, such as the application of plant nutrients (macro and micro), plant growth regulators (jasmonic acid, abscisic acid, salicylic acid, brassinosteroids, and gibberellin), the unitization of genetics tools, and plant breeding (Huang et al., 2018; Atayee and Noori, 2020; Faiq and Noori, 2021).

Plant primary nutrients play crucial functions in the growth and development of plants and in improving cold stress tolerance as they are intimately involved in the plant cell organization and metabolic functions. The mineral nutrients are either structural/functional constituents of enzymes or act as activators or regulators of various enzyme activities. The macronutrients such as nitrogen (N) and phosphorus (P) form structural constituents of building block materials, and some of them like potassium (K), calcium (Ca), and magnesium (Mg) are regulators or activators of enzymes (Zhang et al., 2017). Deficiencies of these nutrients impact various physiological or metabolic activities including increased production of (ROS) that cause oxidative stress in plants (Tewari et al., 2021). Mineral nutrients such as N, P, and K play a critical role in temperature stress tolerance and adequate application is vital for

the integrity of plant structure and key physiological processes such as N is a structural part of chlorophyll needed for photosynthesis, P is needed for energy production and storage, is a structural part of nucleic acids, and K is needed for osmotic regulation and activation of enzymes (Waraich et al., 2011; Waraich et al., 2012).

Previous studies of cold stress and nutrient deprivations also showed increased ROS accumulation (Hussain et al., 2016; Huang et al., 2017). Higher plants retain extremely efficient antioxidant defense systems to scavenge excessive ROS against oxidative stress (El-Sappah et al., 2023). Stress-induced damages are alleviated by several enzymatic (superoxide dismutase, SOD; peroxide dismutase, POD; catalase, CAT), and non-enzymatic pathways (Dahro et al., 2016; Hussain et al., 2016). Turfgrass performance is controlled by optimum N, P, and K supply while their deficiency is a key limiting factor. N is a vital element of proteins, amino groups, Rubisco, and chlorophyll that regulates enzymes essential for stress tolerance (Tantray et al., 2020). P is the main part of molecules that are crucial at cellular levels, for example, it is in ADP, ATP, phospholipids, and nucleic acids. Similarly, K is an inevitable part of cellular homeostasis, enzyme catalysis, and osmotic adjustments (Johnson et al., 2022; Zaman et al., 2022).

We conducted a series of experiments on overseeded perennial ryegrass over dormant bermudagrass under a transitional climate. The first experiment was published in 2018 where we studied the morphological, phenotypic, qualitative, and gas-exchange parameters, and optimized the rates of N, P, and K fertilization (N: 30, P: 24, K: 9 and N: 30, P: 27, K: 6 g m<sup>-2</sup> respectively, during two years) for integrated turf performance (Ihtisham et al., 2018). Although many studies documented the role of N, P, K fertilization in alleviating oxidative stress caused by severe cold. Till now, there is very limited research on the combined effects of N, P, and K fertilization on oxidative stress of overseeded perennial ryegrass under cold stress in transitional climates and with the use of central composite design. The ultimate goals of this study were to examine that primary plant nutrients (N, P, and K) play an important role in the cold stress tolerance of overseeded perennial ryegrass under

TCZ. Therefore, this study was performed to assess the interactive effects of N, P, and K fertilization on lipid peroxidation, electrolyte leakage, ROS production, and their detoxification by the photosynthetic pigments, enzymatic and non-enzymatic antioxidants defense of overseeded perennial ryegrass under cold stress conditions in the transitional climate.

## 2 Materials and methods

### 2.1 Study site and experimental operations

The two years study was conducted from autumn 2016 to summer 2018 at Huazhong Agriculture University, Wuhan-China (114°E, 30°N). Weather data for two years were attained from the university weather station (Figure 1). The area has a transitional climatic zone with an average yearly precipitation of 1150–1450 mm and a subtropical monsoon climate. During the two years, the recorded average temperature was 4 – 21°C and 4.5 – 21.5°C, respectively. Moderate snowfall was recorded in December and January during both years. The soil at the experimental site has a loess texture and prior to experimentation, soil samples at the depth of 15 cm were randomly collected for physiochemical properties (Supplementary Figure S1).

Field treatments were arranged in 24 plots of 2m length and 1.5m width. Hybrid bermudagrass [*C. dactylon* (L.) Pers.] “Tifway419” was established from plugs at the start of May each year. A starter dose of N, P, and K fertilization of 10 g m<sup>-2</sup> each was applied at the initiation of new growth to ensure high-quality turf for overseeding. In the autumn of each year, overseeding experimentations were performed during the second week of October. A verticut of 1 cm height was applied to bermudagrass and perennial ryegrass (*Lolium perenne* L.) seeds were overseeded at 25 g m<sup>-2</sup> density, followed by sprinkle irrigation. The plots were fertilized after full germination (fourth week) with combinations of N as Urea (N 46.4%), P as single superphosphate (P<sub>2</sub>O<sub>5</sub> 16–18%), and K<sub>2</sub>O as potassium sulfate (K

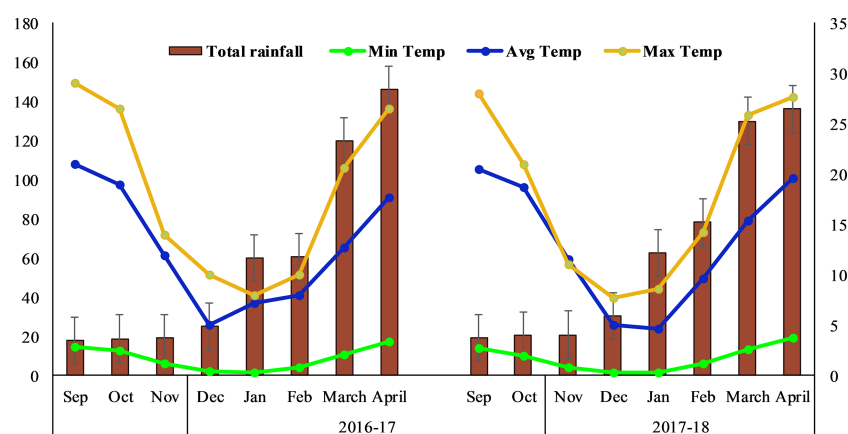


FIGURE 1  
Maximum, minimum, and average temperatures and mean annual rainfall in the study location.

50%) (Table 1). Overseeded perennial ryegrass cover reached <80% after four weeks of fertilization in the treated plots.

## 2.2 Sampling and quantification of biochemical analysis

Following the snowfall in December and January during both years when the snow melted and the average daily minimum temperature was 0 – -5°C, tissue samples (leaves) of fresh overseeded perennial ryegrass were detached and immediately stored at -80°C for biochemical analysis. Soluble protein, SOD, POD, CAT, MDA, and H<sub>2</sub>O<sub>2</sub> were determined according to instructions provided by assay kits manufacturer (Nanjing Jiancheng Bioengineering Institute, China). Proline was determined according to the acid-ninhydrin method, EL by conductivity meter, and chlorophyll *a*, *b*, total chlorophyll, and carotenoids by spectrophotometer.

## 2.3 Crude enzyme extraction and the determination of soluble protein, enzymatic antioxidants, and proline content

For the determination of soluble protein, SOD, POD, CAT, and proline, the crude enzyme was extracted by grounding 0.5 g of tissue samples in an automatic ice-cooled mortar and pestle. The tissue powder was immersed in 4 mL phosphate buffer (150 mM, PH, 7.0), precooled at 4°C and the homogenate was transferred into 10 mL plastic tubes for centrifugation at 10,000 rpm for 15 minutes at 4°C. The resulting supernatant was collected and stored at 4°C.

Soluble protein, SOD, POD, and CAT activities were measured spectrophotometrically and expressed as units mg<sup>-1</sup> protein, according to the manufacturer's instructions. These kits were purchased from the Nanjing Jiancheng Bioengineering Institute, China. The kits used were (A045-2), (A001), (A084-3), and (A007-1) for soluble protein, SOD, POD, and CAT, respectively, and expressed as enzyme units per mg of protein [U mg<sup>-1</sup>(protein)]. Proline content in fresh overseeded ryegrass leaves was appraised spectrophotometrically using the acid-ninhydrin method according to the protocol of Bates et al (1973). By using 4 mL toluene, the reaction mixture was extracted and read at 520 nm. The proline concentration was expressed as micrograms per gram fresh weight (µg g<sup>-1</sup> FW) using a standard curve developed with proline.

## 2.4 Determination of EL, MDA, and H<sub>2</sub>O<sub>2</sub>

EL in the overseeded ryegrass leaves was determined according to the known method of Bates et al (1973). Initial conductivity (C<sub>1</sub>) and final conductivity (C<sub>2</sub>) were measured by a conductivity meter (model: DSS-307, SPSIC, China) and EL was calculated using the formula:

$$EL(\%) = \left(\frac{C_1}{C_2}\right) \times 100 \quad (1)$$

MDA and H<sub>2</sub>O<sub>2</sub> were also determined using the appropriate assay kits from the same company. The manufacturer's instructions were followed, and MDA and H<sub>2</sub>O<sub>2</sub> were expressed as (nmol mg<sup>-1</sup> protein).

## 2.5 Quantification of photosynthetic pigments

The photosynthetic pigments viz. chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (T.Chl), and carotenoids (Car) were extracted with 80% acetone by the previously used known methods (Marcum, 1998).

## 2.6 Experimental design and statistical analysis

The experiments were arranged using a central composite rotatable design (CCRD) for the assessment of interactive effects of N, P, and K fertilization on MDA, EL, electrolyte leakage, and ROS production and their detoxification by photosynthetic pigments, enzymatic, and non-enzymatic antioxidants defense system of overseeded perennial ryegrass under cold stress.

In this design, a total of five levels (0, 6.082, 15, 23.918, and 30 g m<sup>-2</sup>) for each of the three factors (N, P, and K) were used. The CCRD consisted of 2<sup>3</sup> factorial runs with 8-factorial points, 6-axial points, and 9-replications at the center point. There is also a control treatment (N<sub>0</sub>P<sub>0</sub>K<sub>0</sub>), comprising 24 experimental treatments as determined by equation (2).

$$A = 2^b + 2b + d_0 \quad (2)$$

where A=no. of experimental runs, b= no. of factors, and d<sub>0</sub>=no. of center point replications.

Theoretically, replication of the central treatment in central composite designs is very important as it provides an independent estimate of the experimental error and it should be repeated more

TABLE 1 Experimental variables, codes, and coded levels in central composite rotatable design (CCRD).

Experiment variables	Coded symbols	Coded and actual variable levels				
		-1.682	-1	0	1	1.682
Nitrogen (N)	X <sub>1</sub>	0	6.082	15	23.918	30
Phosphorus (P)	X <sub>2</sub>	0	6.082	15	23.918	30
Potassium (K)	X <sub>3</sub>	0	6.082	15	23.918	30

X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, represent coded symbols for N, P, K. -1.682 to 1.682 represent coded and 0, 6.082, 15, 23.918, 30 indicate actual levels of N, P, and K.

than five times. Therefore, in our study, we have repeated the center point treatment nine times to precisely determine value, while the replication of other treatments is not needed (Montgomery, 2001). The total experimental treatments were calculated as (Treatments =  $2^3 + 2 \times 3 + 9 + 1 = 24$ ).

Treatments using various regimes of N, P, and K doses were coded (Table 1) using equation (3):

$$Z_i = z_i - z_i^0 / \Delta z_i \quad (3)$$

Where  $Z_i$  =  $i$ th variable coded value,  $z_i$  =  $i$ th variable real value,  $z_i^0$  =  $i$ th variable real value at the center point, and  $\Delta z_i$  = step change value.

The experimental runs, coded values, and applied doses are shown in (Supplementary Table S1).

The interactive effects of different treatment combinations on biochemical parameters were analyzed with a second-order polynomial equation (4).

$$\begin{aligned} Y = & a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 \\ & + a_7x_1^2 + a_8x_2^2 + a_9x_3^2 \end{aligned} \quad (4)$$

Here,  $Y$  = response variable;  $a_0$  = constant-coefficient;  $a_1, a_2, a_3$  = linear coefficients;  $a_4, a_5, a_6$  = interaction coefficients;  $a_7, a_8, a_9$  = the quadratic coefficients.  $x_1, x_2, x_3$  = N, P, and K coded values.

The interactive effects of  $N \times P$ ,  $N \times K$ , and  $P \times K$  for each parameter were obtained by transforming the data according to equations (5) and (6).

$$X = \frac{x - x_{min}}{x_{max} - x_{min}} \quad (5)$$

$$X = 1 - \frac{x - x_{min}}{x_{max} - x_{min}} \quad (6)$$

Where  $X$  represents the transformed value and  $x$  represents the observed value of each treatment, and  $x_{max}$  and  $x_{min}$  represent maximum and minimum values, respectively.

Equation (5) was used for those indices that were positively correlated with N, P, and K application. However, Equation (6) was used for the indices that were negatively correlated with N, P, and K application, as suggested by previous studies (Li et al., 2019; Roy et al., 2021).

For each variable, an independent linear regression was carried out using the above equation. The SPSS (ver. 16) was utilized to find out the constant, regression coefficient of linear, quadratic, and interaction terms. The significance level of individual and interactive effects of independent variables (N, P, and K) was judged by using an  $F$ -value at  $P < 0.05$ . The coefficient of determination ( $R^2$ ) was used to determine the adequacy of regression equations. For statistical and regression analysis, Microsoft Excel 2018 and SPSS were used, while for correlation analysis and figures, the Origin 2021 software (OriginLab Co., Northampton, MA, USA) was applied.

### 3 Results

We investigated a variety of stress-related biochemical parameters during the two years (2016-2017) and (2017-2018) for

overseeded perennial ryegrass under different fertilization treatment combinations (Tables 2-4) and their regression coefficients with coefficients of determination ( $R^2$ ) for each parameter (Tables 5-7). Pronounced variations in these parameters were detected under cold stress and fertilization.

#### 3.1 Influence of N, P, and K application on MDA, EL, and $H_2O_2$ contents under cold stress

The MDA, EL, and  $H_2O_2$  are important indicators to assess cell membrane integrity and oxidative damage caused by lipid peroxidation. The results indicated that cold stress in the absence of fertilization considerably increased MDA, EL, and  $H_2O_2$  (Table 2), however, N, P, and K fertilization reduced MDA, EL, and  $H_2O_2$  in overseeded perennial ryegrass leaves. For regression equations and  $R^2$ , the data in Table 1 was transformed (Supplementary Tables S2, S3) according to the design of the experiment.

Under the cold stress, N, P, and K application reduced the damage caused by oxidative stress through curtailing the levels of MDA by 68% and 57% as compared to control during the two years (Table 2). The positive values of regression coefficients  $a_1$  (N) and  $a_2$  (P) during the first year and  $a_1$  (N),  $a_2$  (P), and  $a_3$  (K) during the second year indicated amelioration of lipid peroxidation (Table 5).

As compared to the control, fertilization under cold stress decreased EL levels by 13% and 12% respectively during the two studies (Table 2). The regression coefficients,  $a_1$  (N) during both years,  $a_2$  (P), and  $a_3$  (K) during the second and first year respectively showed positive values (Table 5), indicating the mitigation of cell membrane damage by all three nutrients.

Similarly, the production of  $H_2O_2$  in response to fertilization under cold stress was curtailed by 32% and 27% during two years as compared to control (Table 2). Regarding regression coefficients,  $a_1$  (N) and  $a_2$  (P) were positive during both years, while  $a_3$  (K) was positive during the second year (Table 5), suggesting that cold stress-induced  $H_2O_2$  was alleviated by these nutrients.

#### 3.2 Soluble protein, enzymatic antioxidants (SOD, POD, and CAT), and proline content

Soluble protein, SOD, POD, CAT, and proline in perennial ryegrass were leaves significantly increased by N, P, and K fertilization under cold stress (Table 3). Generally, all fertilization treatments alleviated cold-induced adversities by increasing enzymatic and non-enzymatic antioxidant activities as compared to control, with N being the most significant.

N, P, and K treatments combination regulated the activities of soluble protein by 327% and 449%; SOD by 364% and 451%; POD by 340% and 461%; CAT by 488% and 483%; and proline by 77% and 128% under cold stress, respectively, compared with control (Table 3).

The regression coefficients for soluble protein, SOD, POD, and proline were positive for  $a_1$  (N) and  $a_2$  (P), while negative for  $a_3$  (K) during both years (Table 6). These effects were greatly enhanced by



TABLE 2 Cold stress-induced MDA, EL, and H<sub>2</sub>O<sub>2</sub> contents of overseeded perennial ryegrass leave under different treatment combinations of N, P, and K fertilization in 2016-17 and 2017-18.

Treatments	MDA (nmol mg <sup>-1</sup> protein)		EL (%)		H <sub>2</sub> O <sub>2</sub> (nmol mg <sup>-1</sup> protein)	
	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18
N <sub>23,918</sub> P <sub>23,918</sub> K <sub>23,918</sub>	42.59	44.60	82.70	85.45	1.57	1.63
N <sub>23,918</sub> P <sub>23,918</sub> K <sub>6,082</sub>	36.80	36.60	84.31	85.76	1.51	1.53
N <sub>23,918</sub> P <sub>6,082</sub> K <sub>23,918</sub>	41.03	39.07	79.73	85.62	1.51	1.56
N <sub>23,918</sub> P <sub>6,082</sub> K <sub>6,082</sub>	43.69	46.81	83.33	87.58	1.59	1.68
N <sub>6,082</sub> P <sub>23,918</sub> K <sub>23,918</sub>	59.98	59.88	87.79	90.36	1.85	1.88
N <sub>6,082</sub> P <sub>23,918</sub> K <sub>6,082</sub>	45.55	54.96	89.58	87.40	1.69	1.78
N <sub>6,082</sub> P <sub>6,082</sub> K <sub>23,918</sub>	50.78	50.93	89.01	90.62	1.75	1.77
N <sub>6,082</sub> P <sub>6,082</sub> K <sub>6,082</sub>	61.09	57.22	86.16	90.35	1.84	1.84
N <sub>30</sub> P <sub>15</sub> K <sub>15</sub>	39.87	46.06	86.14	88.15	1.58	1.68
N <sub>0</sub> P <sub>15</sub> K <sub>15</sub>	64.36	65.92	91.14	92.33	1.94	1.98
N <sub>15</sub> P <sub>30</sub> K <sub>15</sub>	26.30	42.64	85.70	87.80	1.40	1.63
N <sub>15</sub> P <sub>0</sub> K <sub>15</sub>	47.41	54.35	85.66	85.19	1.66	1.74
N <sub>15</sub> P <sub>15</sub> K <sub>30</sub>	48.37	49.42	85.87	88.47	1.68	1.72
N <sub>15</sub> P <sub>15</sub> K <sub>0</sub>	44.75	54.70	85.69	86.30	1.63	1.76
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	30.27	30.67	85.85	88.81	1.45	1.49
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	29.77	37.06	87.27	82.90	1.46	1.50
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	21.22	45.65	86.88	88.87	1.35	1.68
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	31.58	36.65	83.38	87.59	1.44	1.55
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	29.87	36.85	80.07	89.89	1.37	1.58
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	45.35	31.73	86.38	88.99	1.65	1.51
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	47.21	37.76	87.37	90.17	1.68	1.60
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	25.74	42.74	86.40	86.75	1.40	1.62
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	24.34	30.07	84.29	89.45	1.36	1.49
N <sub>0</sub> P <sub>0</sub> K <sub>0</sub>	67.22	70.14	92.15	93.75	1.99	2.05

N fertilization as compared to P, leading to more than 3.5-fold and 2.5-fold for soluble protein; 4.5-fold and 3.0-fold for SOD; 3.1-fold and 2.8-fold for POD; 3.8-fold and 8.3-fold for proline, respectively, during two years (Table 3). In contrast, CAT activities exhibited positive coefficient values for a<sub>1</sub> (N) and a<sub>3</sub> (K) during both years, while negative for a<sub>2</sub> (P) (Table 6).

### 3.3 Photosynthetic pigments (Chl *a*, Chl *b*, T.Chl, and Car)

In our study, in the absence of N, P, and K supply (especially N and P), the cold stress significantly decreased photosynthetic pigments in perennial ryegrass leaves, however, different treatment combinations of N, P, and K enhanced photosynthetic pigments (Table 4).

Under cold stress, N, P, and K treatment combinations enhanced Chl *a* content by 103% and 89%, Chl *b* content by 233% and 152%,

T.Chl content by 117% and 100%, and Car by 233% and 116%, respectively, during two years as compared with control (Table 4).

Regression coefficients of Chl *a* and Car showed positive values for a<sub>1</sub> (N) during both years and a<sub>2</sub> (P) only during the first year, while Chl *b* showed positive values for a<sub>1</sub> (N) and a<sub>2</sub> (P) whereas negative for a<sub>3</sub> (K) during both years. The effect of N was greater than P, indicating the larger effect of N, while negative values of K showed non-significant effects (Table 7). The positive values of regression coefficients a<sub>1</sub> (N) and a<sub>2</sub> (P), and a<sub>3</sub> (K) in 2016-17 and a<sub>1</sub> (N) and a<sub>2</sub> (P) in 2017-18 indicated significant effects with N being the most effective (Table 7).

### 3.4 Correlation analysis

The current study correlation analysis demonstrated significant results during both years. The ROS (MDA, EL, and H<sub>2</sub>O<sub>2</sub>) were

**TABLE 3** Soluble protein, enzymatic antioxidants activities, and proline contents of overseeded perennial ryegrass leaves under cold stress and different treatment combinations of N, P, and K fertilization in 2016-17 and 2017-18.

Treatments	Soluble Protein (g L <sup>-1</sup> )		SOD (U mg <sup>-1</sup> protein)		POD (U mg <sup>-1</sup> protein)		CAT (U mg <sup>-1</sup> protein)		Proline (μg g <sup>-1</sup> FW)	
	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18
N <sub>23,918</sub> P <sub>23,918</sub> K <sub>23,918</sub>	2.09	1.98	216.96	214.07	110.98	101.03	12.97	12.43	68.19	73.96
N <sub>23,918</sub> P <sub>23,918</sub> K <sub>6,082</sub>	2.22	2.14	225.41	206.17	117.30	104.25	10.07	9.47	72.79	79.06
N <sub>23,918</sub> P <sub>6,082</sub> K <sub>23,918</sub>	1.49	1.64	149.90	193.89	78.99	82.93	20.98	21.12	63.86	65.60
N <sub>23,918</sub> P <sub>6,082</sub> K <sub>6,082</sub>	2.06	1.67	212.50	182.92	108.45	89.06	17.07	20.55	63.99	66.74
N <sub>6,082</sub> P <sub>23,918</sub> K <sub>23,918</sub>	0.91	0.94	80.43	85.71	47.49	47.39	5.74	1.06	57.68	49.40
N <sub>6,082</sub> P <sub>23,918</sub> K <sub>6,082</sub>	1.46	1.15	120.89	117.93	74.68	57.66	2.60	7.92	55.64	52.55
N <sub>6,082</sub> P <sub>6,082</sub> K <sub>23,918</sub>	0.99	0.96	86.36	92.04	51.23	47.84	3.83	7.40	54.70	55.57
N <sub>6,082</sub> P <sub>6,082</sub> K <sub>6,082</sub>	1.51	1.09	134.93	109.96	68.62	53.03	3.41	2.68	52.42	47.08
N <sub>30</sub> P <sub>15</sub> K <sub>15</sub>	1.62	1.56	177.16	170.96	71.64	78.37	19.51	17.36	69.60	55.10
N <sub>0</sub> P <sub>15</sub> K <sub>15</sub>	0.78	0.89	70.74	87.501	37.40	42.39	2.71	1.22	45.23	37.45
N <sub>15</sub> P <sub>30</sub> K <sub>15</sub>	1.30	1.72	136.23	193.53	59.88	83.62	9.95	9.56	48.96	48.89
N <sub>15</sub> P <sub>0</sub> K <sub>15</sub>	0.94	1.15	98.88	114.8	42.07	57.28	19.40	12.33	46.61	52.89
N <sub>15</sub> P <sub>15</sub> K <sub>30</sub>	1.30	1.12	134.65	116.15	64.73	54.86	19.03	19.55	65.91	56.61
N <sub>15</sub> P <sub>15</sub> K <sub>0</sub>	1.12	1.43	114.71	159.48	57.16	70.09	16.85	16.35	75.87	64.56
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.02	1.02	91.22	108.46	52.24	48.79	18.66	20.97	59.70	48.86
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.09	1.77	107.26	195.97	56.13	86.85	20.13	15.09	68.69	49.36
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.23	1.17	112.46	122.88	61.73	57.80	21.24	17.29	75.50	60.03
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	0.96	1.80	75.43	194.35	48.86	84.93	21.92	8.74	55.60	45.77
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.30	1.15	136.70	123.15	65.31	53.65	17.81	13.47	50.87	45.50
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.20	1.17	119.66	130.49	59.28	57.41	19.55	10.78	46.01	38.79
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.90	1.54	182.28	168.63	92.60	79.11	14.67	15.21	54.09	42.11
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.36	1.67	148.58	181.6	64.91	81.00	21.39	11.97	70.27	51.24
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.20	1.69	129.55	181.35	58.42	105.79	17.37	12.69	75.03	38.79
N <sub>0</sub> P <sub>0</sub> K <sub>0</sub>	0.52	0.39	48.55	38.87	26.67	18.85	1.73	1.62	42.95	34.66

positively correlated with each other, while in contrast, enzymatic and non-enzymatic antioxidants (SOD, POD, CAT, proline), soluble protein, and photosynthetic pigments (Chl *a*, Chl *b*, Car, and T.Chl) were positively correlated with each other (Figures 2, 3). At the same time, ROS (MDA, EL, and H<sub>2</sub>O<sub>2</sub>) exhibited negative correlations with soluble protein, SOD, POD, CAT, Chl *a*, Chl *b*, Car, and T.Chl. This shows that soluble protein, SOD, POD, CAT, and photosynthetic pigments (Chl *a*, Chl *b*, Car, and T.Chl) played a major role in scavenging the ROS (MDA, EL, H<sub>2</sub>O<sub>2</sub>) in overseeded perennial ryegrass thereby counteracting the oxidative damage.

## 4 Discussion

Under natural circumstances in the field, plants often experience several types of environmental stresses on a regular basis. These stresses cause morpho-physiological and biochemical

alterations in plants and adversely affect growth and performance (Hasanuzzaman et al., 2020). Extreme low and high temperatures are anticipated to become more frequent (Uleberg et al., 2014; IPCC, 2021) and will cause serious problems for crop production including turfgrasses to perform better specifically in TCZ.

Perennial ryegrass being a cool-season turfgrass and overseeding species is highly sensitive to extremely low temperature, particularly at establishment stages (Guan, 2014; Fan et al., 2020; Xie et al., 2020). Besides cold stress, an optimum supply of macronutrients particularly N, P, and K is imperative for normal plants functioning and coping with stress, while their surfeit or deficiency is detrimental and challenges the survival of plants (Tantray et al., 2020; Kumar et al., 2021; Seleiman et al., 2021). Perennial ryegrass also heavily depends on the optimum supply of N, P, and K for maximum growth, performance, and stress tolerance (Głab et al., 2020; Zanelli et al., 2021). The findings demonstrated that overseeded perennial ryegrass was adversely affected by cold in terms of reduced

**TABLE 4** Chloroplastic pigments of overseeded perennial ryegrass leaves under cold stress and different treatment combinations of N, P, and K fertilization in 2016-17 and 2017-18.

Treatments	Chlorophyll <i>a</i> (mg g <sup>-1</sup> FW)		Chlorophyll <i>b</i> (mg g <sup>-1</sup> FW)		Carotenoids (mg g <sup>-1</sup> FW)		Total Chlorophyll (mg g <sup>-1</sup> FW)	
	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18	2016-17	2017-18
N <sub>23.918</sub> P <sub>23.918</sub> K <sub>23.918</sub>	1.08	1.09	0.43	0.44	0.37	0.36	1.68	1.81
N <sub>23.918</sub> P <sub>23.918</sub> K <sub>6.082</sub>	1.04	1.32	0.45	0.63	0.33	0.50	1.63	2.01
N <sub>23.918</sub> P <sub>6.082</sub> K <sub>23.918</sub>	1.09	1.30	0.45	0.62	0.37	0.49	1.65	1.79
N <sub>23.918</sub> P <sub>6.082</sub> K <sub>6.082</sub>	1.13	1.31	0.48	0.58	0.40	0.47	1.72	1.91
N <sub>6.082</sub> P <sub>23.918</sub> K <sub>23.918</sub>	0.72	0.82	0.27	0.29	0.26	0.27	1.11	1.22
N <sub>6.082</sub> P <sub>23.918</sub> K <sub>6.082</sub>	0.67	0.84	0.24	0.30	0.23	0.31	1.05	1.14
N <sub>6.082</sub> P <sub>6.082</sub> K <sub>23.918</sub>	0.74	0.83	0.27	0.32	0.27	0.28	1.01	1.10
N <sub>6.082</sub> P <sub>6.082</sub> K <sub>6.082</sub>	0.67	0.97	0.24	0.36	0.25	0.31	0.93	1.01
N <sub>30</sub> P <sub>15</sub> K <sub>15</sub>	1.32	1.20	0.62	0.51	0.49	0.42	2.00	1.89
N <sub>0</sub> P <sub>15</sub> K <sub>15</sub>	0.67	0.71	0.27	0.26	0.29	0.27	0.97	1.00
N <sub>15</sub> P <sub>30</sub> K <sub>15</sub>	1.08	1.16	0.43	0.51	0.36	0.45	1.65	1.69
N <sub>15</sub> P <sub>0</sub> K <sub>15</sub>	1.08	0.86	0.41	0.32	0.40	0.27	1.47	1.56
N <sub>15</sub> P <sub>15</sub> K <sub>30</sub>	0.88	0.97	0.33	0.38	0.29	0.34	1.44	1.49
N <sub>15</sub> P <sub>15</sub> K <sub>0</sub>	1.02	1.12	0.39	0.44	0.38	0.39	1.43	1.47
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	0.94	1.29	0.35	0.58	0.31	0.46	1.50	1.63
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.09	1.02	0.43	0.40	0.36	0.34	1.55	1.52
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.14	1.10	0.46	0.43	0.37	0.36	1.63	1.62
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.03	1.07	0.42	0.41	0.40	0.35	1.56	1.55
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.02	1.15	0.40	0.47	0.34	0.44	1.51	1.61
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.17	1.16	0.47	0.49	0.41	0.45	1.64	1.68
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.22	1.28	0.54	0.56	0.44	0.46	1.68	1.70
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.10	1.00	0.43	0.37	0.36	0.35	1.64	1.54
N <sub>15</sub> P <sub>15</sub> K <sub>15</sub>	1.34	1.12	0.70	0.45	0.50	0.36	1.67	1.66
N <sub>0</sub> P <sub>0</sub> K <sub>0</sub>	0.66	0.70	0.21	0.25	0.23	0.25	0.86	0.93

**TABLE 5** Regression equation parameter coefficients ( $Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 + a_7x_1^2 + a_8x_2^2 + a_9x_3^2$ ) for MDA, EL, and H<sub>2</sub>O<sub>2</sub> during 2016-17 and 2017-18.

Y	Year	a <sub>0</sub>	a <sub>1</sub>	a <sub>2</sub>	a <sub>3</sub>	a <sub>4</sub>	a <sub>5</sub>	a <sub>6</sub>	a <sub>7</sub>	a <sub>8</sub>	a <sub>9</sub>	R <sup>2</sup>
MDA	2016-17	0.57	0.15	0.08	-0.02	0.00	0.00	-0.09	-0.17	-0.05	-0.12	0.76**
	2017-18	0.63	0.16	0.03	0.02	0.04	-0.01	-0.09	-0.15	-0.08	-0.11	0.84**
EL	2016-17	0.52	0.18	-0.04	0.02	-0.02	0.06	0.03	-0.07	0.01	0.01	0.58NS
	2017-18	0.53	0.15	0.01	-0.03	-0.02	0.06	-0.05	-0.07	0.06	0.03	0.54NS
H <sub>2</sub> O <sub>2</sub>	2016-17	0.64	0.18	0.06	-0.01	-0.01	0.02	-0.07	-0.16	-0.04	-0.11	0.78**
	2017-18	0.70	0.18	0.03	0.01	0.03	0.01	-0.09	-0.15	-0.06	-0.10	0.87**

\*\*Significance at P < 0.01. \* Significance at P < 0.05. NS, Not Significant.



TABLE 6 Regression equation parameter coefficients ( $Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 + a_7x_1^2 + a_8x_2^2 + a_9x_3^2$ ) for soluble protein, SOD, POD, CAT, and proline during 2016-17 and 2017-18.

Y	Year	a <sub>0</sub>	a <sub>1</sub>	a <sub>2</sub>	a <sub>3</sub>	a <sub>4</sub>	a <sub>5</sub>	a <sub>6</sub>	a <sub>7</sub>	a <sub>8</sub>	a <sub>9</sub>	R <sup>2</sup>
Soluble protein	2016-17	1.35	0.32	0.09	-0.11	0.11	0.05	0.05	0.07	0.04	0.07	0.57NS
	2017-18	1.41	0.32	0.13	-0.08	0.10	0.02	-0.03	-0.05	0.03	-0.03	0.65*
SOD	2016-17	133.17	41.09	8.99	-9.27	12.49	2.25	7.78	7.00	4.74	7.26	0.67*
	2017-18	150.08	38.94	13.00	-7.63	5.22	8.63	-2.17	-7.32	1.50	-4.28	0.66*
POD	2016-17	67.40	16.93	5.35	-4.95	4.81	1.10	1.67	2.93	1.67	5.20	0.54NS
	2017-18	70.66	16.98	5.99	-3.69	3.64	0.76	-0.27	-2.54	1.03	-1.80	0.57NS
CAT	2016-17	14.64	5.40	-2.18	1.03	-2.01	0.41	0.21	-3.84	-2.58	-1.42	0.88**
	2017-18	12.40	5.25	-1.87	0.50	-2.33	0.71	-1.15	-2.16	-1.57	0.91	0.79**
Proline	2016-17	60.75	6.54	1.70	-1.26	0.86	-1.13	-0.59	-0.98	-4.39	3.78	0.54NS
	2017-18	53.31	8.09	0.97	-1.05	2.67	-1.45	-1.95	1.57	3.20	6.63	0.72*

\*\*Significance at  $P < 0.01$ . \* Significance at  $P < 0.05$ . NS, Not Significant.

soluble protein, SOD, POD, CAT, proline, Chl *a*, Chl *b*, Car, and T.Chl contents and higher MDA, EL, and H<sub>2</sub>O<sub>2</sub> concentrations. However, N, P, and K application contributed positively to stress tolerance which indicates the importance of these macronutrients.

#### 4.1 MDA, EL, and H<sub>2</sub>O<sub>2</sub>

Abiotic stresses cause excessive ROS production that causes the oxidation of proteins, lipids, and nucleic acids which ultimately creates oxidative stress (Hasanuzzaman et al., 2019; Imran et al., 2020; Ihtisham et al., 2021). Similarly, in the present study, cold stress triggered the formation of MDA, EL, and H<sub>2</sub>O<sub>2</sub> under control conditions and deprivation of any mineral nutrient particularly N (Table 2). MDA is a product of the peroxidation of lipid membranes in cells and is used as an indicator of oxidative stress in plants. The increased levels of H<sub>2</sub>O<sub>2</sub> coincided with higher MDA rates (Table 2), which is characterized as a biochemical indicator for free radical-mediated damage in plants under stress. N, P, and K play key roles in various biochemical, enzymatic, non-enzymatic, and metabolic activities, as well as serve as the structural

components of many plant compounds and increase plant growth and vigor (Singh et al., 2015; Ma et al., 2022). Our results also illustrated that the application of N, P, and K decreased oxidative stress indicators while on the other hand increasing the activities of enzymatic and non-enzymatic antioxidants such as SOD, POD, CAT, soluble protein, proline, and chloroplastic pigments. Our findings are supported by many earlier studies, that observed increased MDA, EL, and H<sub>2</sub>O<sub>2</sub> production under cold stress (Jan et al., 2018; Fan et al., 2020; Liu et al., 2020; Su et al., 2021; Ma et al., 2022), N deprivation (Tantray et al., 2020), P deprivation (Noor et al., 2021), and K deprivation (Seleiman et al., 2021). Higher accumulation of MDA, EL, and H<sub>2</sub>O<sub>2</sub> under N deprivation as compared with P and K was corroborated by earlier studies (Tewari et al., 2007; Hussain et al., 2016).

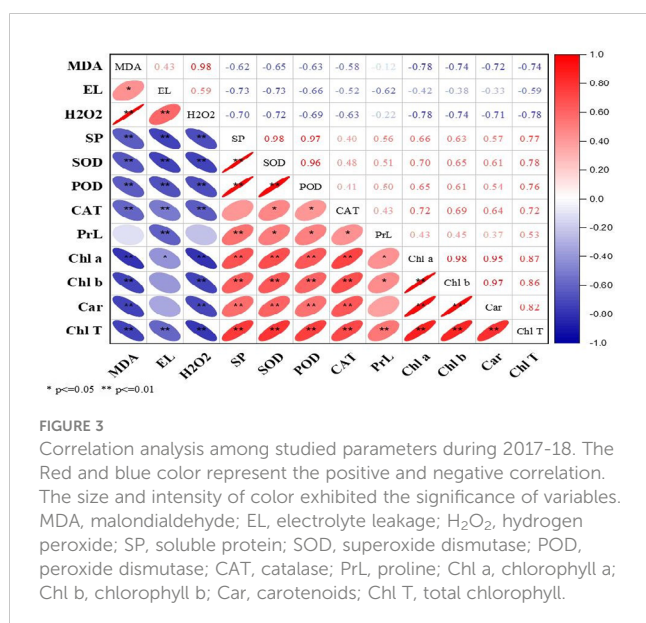
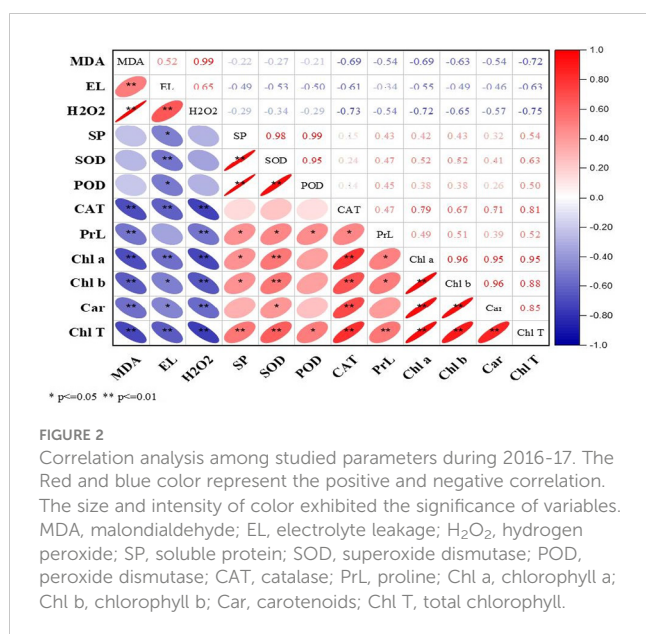
#### 4.2 Soluble protein, SOD, POD, CAT, and proline content

Plants have evolved a complex system of enzymatic and non-enzymatic antioxidants which increases during stress conditions to

TABLE 7 Regression equation parameter coefficients ( $Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1x_2 + a_5x_1x_3 + a_6x_2x_3 + a_7x_1^2 + a_8x_2^2 + a_9x_3^2$ ) for chlorophyll *a*, *b*, total chlorophyll, and carotenoids during 2016-17 and 2017-18.

Y	Year	a <sub>0</sub>	a <sub>1</sub>	a <sub>2</sub>	a <sub>3</sub>	a <sub>4</sub>	a <sub>5</sub>	a <sub>6</sub>	a <sub>7</sub>	a <sub>8</sub>	a <sub>9</sub>	R <sup>2</sup>
Chlorophyll <i>a</i>	2016-17	1.01	0.19	-0.01	-0.01	-0.01	-0.02	0.01	-0.07	-0.04	-0.08	0.81**
	2017-18	1.07	0.18	0.01	-0.05	-0.01	-0.01	-0.01	-0.05	-0.03	-0.02	0.74*
Chlorophyll <i>b</i>	2016-17	0.41	0.10	0.00	-0.01	-0.01	-0.01	0.00	-0.02	-0.03	-0.05	0.68*
	2017-18	0.44	0.10	0.01	-0.02	-0.01	-0.01	-0.03	-0.02	-0.01	-0.01	0.66*
Carotenoids	2016-17	0.36	0.06	-0.01	-0.01	-0.01	-0.01	0.01	-0.01	-0.02	-0.03	0.63NS
	2017-18	0.38	0.07	0.01	-0.02	-0.01	-0.01	-0.02	-0.01	-0.01	-0.01	0.63NS
Total chlorophyll	2016-17	1.48	0.32	0.03	0.01	-0.04	-0.02	0.01	-0.07	-0.04	-0.09	0.94**
	2017-18	1.55	0.33	0.04	-0.01	-0.02	-0.06	-0.01	-0.06	0.00	-0.05	0.95**

\*\*Significance at  $P < 0.01$ . \* Significance at  $P < 0.05$ . NS, Not Significant.



cope with stress-induced adversities. In this study, cold stress aggravated oxidative damage in overseeded perennial ryegrass, while N, P, and K fertilization especially N and P attenuated that damage, which can be attributed to the accumulation of enhanced antioxidants enzymatic activities (Tables 3, 6). Soluble protein and both enzymatic (e.g., SOD, POD, and CAT) and non-enzymatic (e.g., proline) antioxidants played key roles in scavenging the ROS in perennial ryegrass thereby counteracting the oxidative damage. These antioxidant activities are considered to curb the cascades of unrestricted oxidation and defend ROS-induced oxidative damage to the plant cells. The enhanced rates of antioxidants confer capability to the plants to scavenge ROS and resist cold stress (Anjum et al., 2015; Moustafa-Farag et al., 2020; Hasanuzzaman et al., 2021; Imran et al., 2021). Generally, ROS is scavenged by antioxidant enzymes, e.g., proteins deoxidize lipids and nucleic

acids (Mittler, 2002), SOD contributes to catalysis of the dismutation of superoxide (O<sub>2</sub><sup>•-</sup>) (Fahad et al., 2016), whereas the POD and CAT play important roles in scavenging of H<sub>2</sub>O<sub>2</sub> (Sachdev et al., 2021). The role of proline is variable, it acts as a radical scavenger in plants under stress as well as an osmotic agent (Hayat et al., 2012). Increased levels of proline attenuate the damage caused by stress in plant cells by adjusting osmotic potential and reducing water potential (Imran et al., 2020). The elevated antioxidant activities in N, P, and K treated plots particularly N and P correspond to better ROS scavenging ability and protecting cell membrane integrity that conferred tolerance against cold stress. N is an essential part of proteins and amino acids that helps in enzyme regulation, P is the part of important molecules at cellular levels (like, ATP, ADP, NADPH, and nucleicacids), while K is inevitable for plant growth and development, and contribute to enzyme catalysis, charge balance, and osmotic adjustment that eventually attenuated cold stress adversities (Hussain et al., 2016; Kumar et al., 2021). N in the form of nitric oxide is a highly reactive, membrane-permanent free radical with a broad spectrum of regulatory functions in several physiological processes and protects the plant against stress by acting as an antioxidant directly scavenging the ROS generated under cold stress (Wendehenne et al., 2001). K is essential for many physiological processes, such as photosynthesis, translocation of photosynthates into sink organs, maintenance of turgidity, and activation of enzymes under stress conditions (Mengel and Kirkby, 2001). Plants have developed a wide range of adaptive/resistance mechanisms to maintain productivity and ensure survival under a variety of environmental stresses, including cold stress, which affects the fluidity of membrane lipids and thus may alter membrane structure (Marschner, 1995). Under lower amounts of K, cold-induced photo-oxidative damage can be exacerbated causing a decrease in plant growth and yield while adequate K application can provide protection against oxidative damage caused by cold stress (Waraich et al., 2012). The enhanced levels of these enzymatic activities by N, P, and K can also be correlated with decreased levels of MDA, EL, and H<sub>2</sub>O<sub>2</sub> as shown by correlation analysis (Figures 2, 3). Similar results were reported by (Abid et al., 2016; Shao et al., 2020) that N fertilization conferred tolerance to stress conditions by maintaining higher enzymatic and non-enzymatic anti-oxidants and lower ROS generation. Results with increased enzymatic activities under stress conditions in response to N, P, and K are recently reported by Ma et al. (2022). Corresponding results with N fertilization (Chang et al., 2016; Ahanger et al., 2019), P fertilization (Noor et al., 2021), and K fertilization (Seleiman et al., 2021) have been previously reported.

### 4.3 Photosynthetic pigments

Our results revealed that cold stress significantly reduced the photosynthetic pigments in overseeded perennial ryegrass while fertilization on the other hand highly increased photosynthetic pigments (Tables 4, 7). The effects were so obvious that necrosis in the leaves occurred under control conditions and zero N. The enhanced chloroplastic pigments in response to N, P, and K,

particularly N and then P were correlated with increased turf color, total protein, and photosynthesis in previous studies (Ihtisham et al., 2018; Jena and Mohanty, 2020; Ihtisham et al., 2021). N fertilization promotes the accumulation of chlorophyll as it is the major component of chlorophyll (Raza et al., 2022; Roy et al., 2022). Certainly, deficient-N plants show deficient-chlorophyll phenotypes. P and K are not chlorophyll components, the increase recorded in chloroplastic pigments under P might be the result of higher photosynthesis, whereas for K the increase is probably due to secondary mechanisms associated with general growth promotion. The correlation between chloroplastic pigments and photosynthesis can also be justified, as chlorophyll serves as the key light-harvesting pigment for photosystem, and accumulate extremely high levels of photosynthetic proteins, like light-harvesting chlorophyll a/b-binding protein and Rubisco (Ihtisham et al., 2018). N, being a key component of proteins, chlorophyll, and Rubisco affected the whole metabolism of plants during cold stress. Reduced chlorophyll contents in turfgrasses under limited N fertilization and abiotic stress conditions were also previously reported by (Shen et al., 2020; Januškaitienė et al., 2021). The enhanced levels of photosynthetic pigments in response to N, P, and K could also be ascribed to the regulation of enzymatic and non-enzymatic antioxidants defense systems and decreased lipid peroxidation, cell membrane damage, and H<sub>2</sub>O<sub>2</sub> in this study, as confirmed by the correlation analysis. Similarly, previous studies reported that N, P, and K application under stress conditions actively synthesized chlorophyll contents, which was verified by higher chloroplastic pigments, increased antioxidants activities, and lower ROS production (Kozłowska et al., 2021; Saleem et al., 2021; Xu et al., 2021).

## 5 Conclusion

Exogenous applications of N, P, and K are considered an effective way to attenuate plant's cold stress. The results of the present study elucidated that the absence of N, P, and K fertilization as evident from the controlled conditions under cold stress and TCZ triggered lipid peroxidation, cell membrane damage, and excessive hydrogen peroxide accumulation presumably by desynchronizing the ROS-scavenging mechanism in overseeded perennial ryegrass. Nevertheless, N, P, and K application proficiently relieved cold stress provoked inhibitory effects in overseeded perennial ryegrass which could mainly be ascribed to decreased MDA, EL, and H<sub>2</sub>O<sub>2</sub> contents, while increased enzymatic and non-enzymatic antioxidants (soluble protein, SOD, POD, CAT, and proline) and photosynthetic pigments (Chl *a*, Chl *b*, T.Chl, and Car) through elevated ROS scavenging. Thus, our results conclude that NPK application maximized the performance and cold stress tolerance of perennial ryegrass that was overseeded on dormant bermudagrass under a transitional climate. To cope with cold stress, turfgrass management will depend not only on breeding new varieties that are well adapted to cold climates but also on the development of nutrient management strategies, that will contribute to sustainable agriculture.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Author contributions

MI conceived the idea of the experiment and wrote the manuscript. MH provided supervision and technical guidance and revised the manuscript. FZ, NK, and AR contributed to data processing, figures, and tables. MS, SK, AE-S, MA, and MJH helped in statistical analysis, technical guidance, general support, and formatting. JL, XMZ, and XZ provided supervision and funding. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2023.1149832/full#supplementary-material>

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