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Impact of high tropospheric ozone and nitrogen concentration on total protein and chlorophyll content of *Pinus roxburghii*

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Abstract

Pinus roxburghii (*P. roxburghii*) is an important ecological and industrial conifer of the Himalayas. Plant boom is incredibly impacted by way of environmental stresses, in particular high ranges of nitrogen (N) and ozone (O3). This work aims to explore how these factors affect the total protein content and chlorophyll levels of *P. roxburghii*. Three consortiums were inoculated with two-year-old *P. roxburghii* seedlings. The stresses of 100 kg N h^{-1} and 100 ppb O3 were applied for 1 month to study their effect on chlorophyll level and total protein content. To evaluate their potential mitigating effects, the fungal consortium presented promising outcomes for the chosen plant species. The elevated metabolic activities and photosynthesis rate were determined by improved total protein content and high chlorophyll level (p<zero.05). The highest observed protein content (30.57548 µg/ml) occurred under combined O3 and N stress with consortium 1 (C1+O3+N). On the other hand, consortium 2 under nitrogen stress (C2+N3) alone resulted in the lowest protein content (14.537733 µg/ml). In addition, the high photosynthesis rate was determined by enhanced chlorophyll content, and C2-treated under O3 species showed high chlorophyll content C2 had the highest Chlorophyll a levels under O3 stress. C2+O3 had the biggest impact on Chlorophyll a content, while chlorophyll b increased the most. Both C1 and C2 enhanced *P. roxburghii* stress resistance. Further research is needed to clarify the mechanisms underlying those interactions and perceive the particular fungal lines answerable for the found consequences.

Keywords: Ozone, Nitrogen, Chlorophyll Content, Phenylmethylsulfonyl fluoride, *Pinus roxburghii.*
Abbreviations: *P. roxburghii*_ Pinus roxburghii; O3_Ozone; N_Nitrogen; C1_Consortium 1; C2_Consortium 2; C3_Consortium 3; Ppb_Parts per billion; ppm_Parts per million; HEPES_4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS_3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT_Dithiothreitol; BSA_Bovine serum albumin.

Introduction

P. roxburghii, typically referred to as Chir pine, is an ecologically and economically important tree species local to the Himalayan place spanning Pakistan, India, Nepal, and Bhutan (Kaushik et al., 2013). It grows predominantly at elevations of a 1000-2000 m inside the essential Himalayas (Singh and Bisht 1992). *P. roxburghii* is a large conifer which can reach heights of 55 m with a trunk diameter of up to 2 m. The tree possesses needle-like leaves prepared in fascicles of 3 and a taproot device with radial ground roots (Kumar et al., 2022). Similar to its high-quality use for wood, *P. roxburghii* affords numerous non-timber wooded area products and ecosystem services. domestically, it is hired in traditional

medicinal tablets to deal with skin illnesses, gastrointestinal problems, and snake bites (Naudiyal and Schmerbeck 2017). Extracts from the bark, needles, and resin have exhibited antioxidant properties (Kaushik et al., 2013; Salem et al., 2014). The species additionally contributes to carbon sequestration, slope stabilization, and biodiversity conservation in the Himalayan forests (Singh 2007).

Stress in plants refers to any outside biotic or abiotic element that negatively affects physiological features and boom (Izuta and Nakaji 2003). Common environmental stresses consist of salinity, temperature extremes, flooding, sunlight, ozone, and detrimental soil nutrient stages (De Oliveira 2019). Tropospheric ozone is an effective air pollutant shaped by way of photochemical reactions among nitrogen oxides (nox), volatile natural compounds (vocs), and different precursors emitted from vehicular exhaust, power flora, and industries (Casper et al.,). Each nitrogen deficiency and extra can disrupt plant metabolism, with deficiencies limiting growth and excess causing phytotoxic outcomes (Khan et al., 2016).

O3 is a highly reactive molecule that contributes to greenhouse gases and acts as a subordinate air pollutant (Lelieveld et al., 2016). Depending on where it occurs in the atmosphere, ozone affects life on Earth in either good or bad ways as O3 is a strong oxidizing agent with a high oxidizing potential (±2.07eV) capable of interacting with almost every biomacromolecule, including nucleic acids, lipids, carbohydrates and proteins (Faoro and Iriti 2009). In the same way, Nitrogen occurs in soil both inorganic and organic form. N in organic form is made available to plants by its mineralization to NH4+ and then its subsequent nitrification of $NO3^+$ (Whalen et al., 2013). For 9 nitrogen assimilation, acquisition and partitioning plant adapted and evolved diverse strategies such as organic and inorganic N from the soil, transportation of organic N from so-called source organs (e.g., roots and leaves) that are newly synthesized to developing flowers, seeds, and leaves (to sink) are essential to the plant's physiology and integral to the development of plant (Tegeder and Masclaux-Daubresse 2018). High ozone and nitrogen are a severe air pollution problem, principally enhanced by urbanization and industrialization in most parts of the world (IPCC 2014), predominantly in Asia, Western Europe and in the USA (Pachauri et al., 2014).

In plant stress research, Cell components are frequently extracted using methods like homogenization and grinding, which can change cellular integrity while providing information about biochemical markers (Hopkins 1991). On the other hand, techniques that do not cause damage to plants, such as chlorophyll fluorescence imaging, offer physiological data in real time without damaging them (Valcke 2021). Disruptive approaches produce thorough analysis, but they might not fully represent in vivo situations. A thorough understanding of plant stress responses can be ensured by striking a balance between these strategies, which are essential for correct data collecting. Recognizing disruption, defending its necessity, and stressing appropriate methodology can all help to lessen its effects (Shulaev et al., 2008).

Damages from high O3 and N exposure on pine plants depend on the species of pine plant along with the age of the pine tree and duration of exposure etc. It affects the water system of pine plants which adversely affects pine photosynthetic carbon assimilation. Extreme level of ozone and nitrogen inside the troposphere acts as phytotoxicants that bring about soil acidification and eutrophication in jungle systems, decomposing nutrient status such as deficiency of Phosphate (PO4), excessive accumulation of Mn and Mg in foliage, and augmented sensitivity to famine, cold and gassy air pollutants. Extreme amassing of NH4by reason of agricultural doings leads to plant injury (Miller et al., 2010).

Elevated ozone and nitrogen concentrations have been shown to damage photosynthetic pigments like chlorophyll, decrease photosynthetic rates, induce cell death, and alter protein content and enzyme activities in various plant species (Tiwari 2017). High ozone and nitrogen concentration in the troposphere is the most important factor of forest reduction as well as ecosystem disturbance globally (Ollinger et al., 2002). *P. roxburghii* is a long tree that constitutes a major portion of the jungle and plays a considerable role in sustaining the ecosystem, however in Pakistan, there exists very limited information regarding the impact of high ozone and nitrogen concentration on *P. roxburghii*. Therefore, the impacts on the total protein content and chlorophyll content of *P. roxburghii*, need to be explored.

Results

Total protein analysis

Protein standard curve

In the current research focused on the total protein content of *P. roxburghii* while it was subjected to combined ozone and nitrogen stress. Several consortiums labeled as 1, 2, and 3 were used in a comprehensive method to explore this investigation's complexities. The well-renowned Bradford assay was used to accurately measure the total protein level, and the results were represented in terms of micrograms per milliliter (µg/ml).

Eight distinct dilutions of pure protein, bovine serum albumin, were used to create this popular curve through a series of complicated experiments. Every dilution was cautiously organized and rigorously tested, resulting in a dependable reference curve that allowed for the appropriate estimation of protein content material in *P. roxburghii* samples. This medical method enabled the take a look at the consequences of combined ozone and nitrogen stress on the full protein content material of *P. roxburghii* with the highest degree of precision and validity as proven in Figure 1.

A vast finding about the total protein content of *P. roxburghii* samples emerged from our experiment. It is particularly surprising that the sample with the label C1+N+O3 had the best overall protein content material ever determined. comparing this dimension to the managed sample, which became grown underneath traditional, stress-loose settings, found that it was over two times as huge. Contrarily, the sample such as C2+N3 showed the bottom level of total protein.

There may be a discernable series to the exchange in total protein content, supplying intriguing information on how the experimental settings affected the consequences. It is possible to express this increase in protein content in the following manner:

C2+N3<C3+N1<C3+N+O3<C1+O3<C2+N2+O1<C1+N2+O3<C1+N+O1<C2+O3<C1+N+O3.Notably, this order strikingly matches

Table 1. Dilutions for BSA Standard Curve. The volume of protein standard (µl) and dH2O (µl) was compared with the Final Concentration of Protein (mg/ml).

Volume of protein standard (µl)	Volume of dH2O (µl)	Final Concentration of Protein (mg/ml)
0	50	0
2.5	47.5	0.025
5	45	0.05
10	40	0.1
20	30	0.2
30	20	0.3
40	10	0.4
50	0	0.5

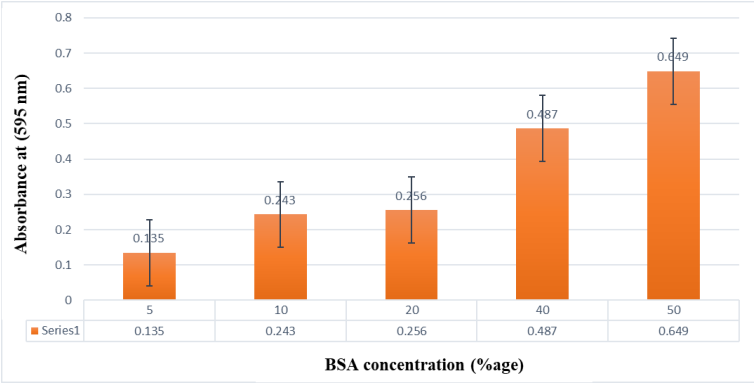

Figure 1. BSA standard curve of total protein analysis. x-axis shows BSA concentration (µg/mL), y-axis: Absorbance at 595 nm, Line: Linear regression line fitted to the data and Points: Absorbance values for each BSA standard concentration.

the change in the Bradford reagent's hue that can be seen. The color of the reagent changes from brown to an intense shade of blue as proteins in the samples attach to the reagent molecules, which is a clear change. The relationship between protein content and the change in the reagent's color further emphasizes the significance of these findings in our experiment and offers important information about how the samples under study react to various stressors as shown in Figure 2.

Plant chlorophyll analysis

Chlorophyll "a" analysis

The assessment of the chlorophyll concentration in *P. roxburghii* under the combined influence of ozone and nitrogen stress, in conjunction with the application of various consortiums denoted as 1, 2, and 3, was a special focus of our extensive experiment. To do this, an exact measurement using the tried-and-true Acetone method was made, and the results were precisely reported in terms of micrograms per milliliter (µg/ml).

The effects of these studies revealed charming variances in chlorophyll contents that genuinely confirmed distinctions from the manipulated organization. Chlorophyll content varies appreciably, displaying a huge range of outcomes where, in a few instances, the content becomes higher than the manage degrees and, in different instances, it shows a decline below the manage baseline. This change in chlorophyll contents highlights the complex interaction of variables gift within the experimental setup.

The analysis of chlorophyll content material, which discovered unexpected patterns, is of unique interest. The C2+O3 pattern had the finest attention of chlorophyll of all the samples

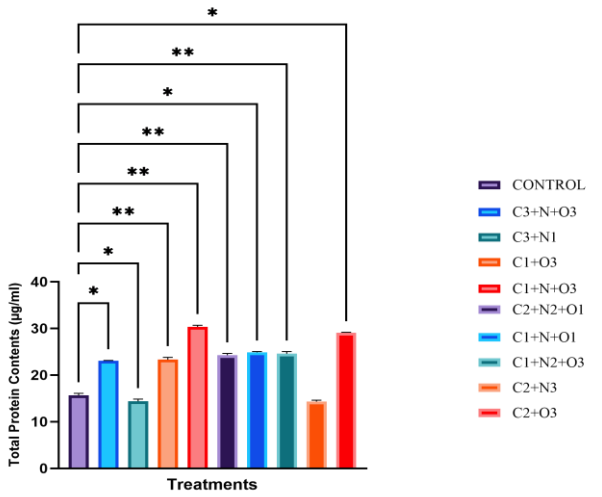

Figure 2. Effect of ozone and nitrogen stress on total protein content in *P. roxburghii*. Mean total protein content (µg/ml) is shown for each treatment group (Control, Ozone, Nitrogen). Error bars represent the standard error of the mean. Significant differences between treatments were determined using (one-way ANOVA, $p < 0.05$).

examined. This sample confirmed a particularly extended concentration of chlorophyll a, demonstrating a strong response to the carried-out strain situations. In assessment, the C2+N3 pattern had the lowest chlorophyll level, which indicated a considerable decrease from the manipulated institution.

These consequences spotlight the complex hyperlink between the stressors and chlorophyll content and supply mild

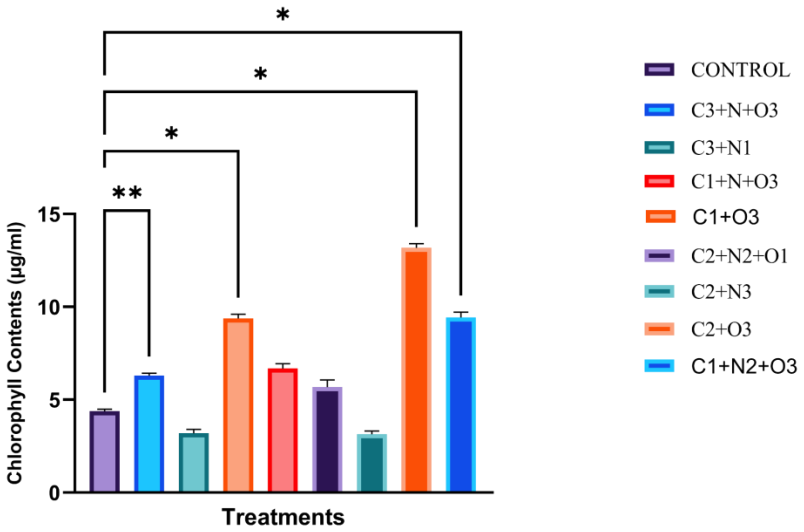

Figure 3. Effect of ozone and nitrogen stress on chlorophyll a content in *P. roxburghii*. Error bars represent standard deviation. Individual replicate data points are indicated by symbols. Significant differences between treatments were determined using (one-way ANOVA, $p < 0.05$).

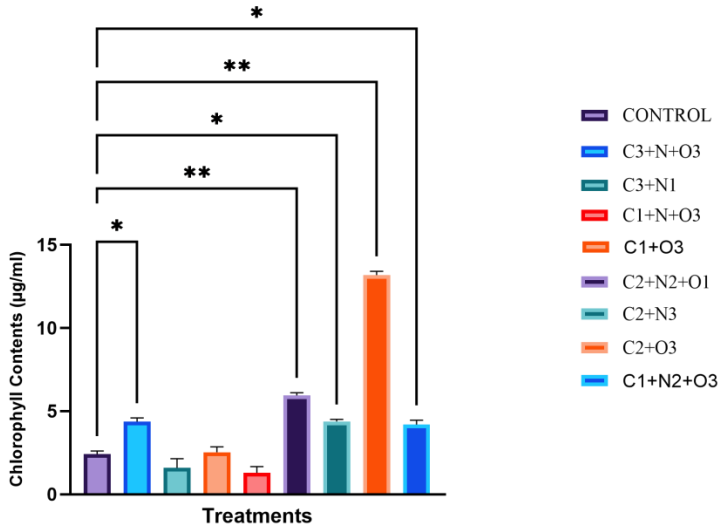

Figure 4. Effect of ozone and nitrogen stress on chlorophyll b content in *P. roxburghii*. Significant differences between treatments were determined using (one-way ANOVA, $p < 0.05$).

dynamic responses of *P. roxburghii* to the mixed pressures of ozone and nitrogen. Such results are essential for comprehending how this plant species reacts physiologically to environmental stimuli and could have wider ramifications for plant technological know-how and environmental research. The growth of chlorophyll content is in the order together with;

$C_2+N_3<C_3+N_1<C_2+N_2+O_1<C_3+N+O_3<C_1+N+O_3<C_1+O_3<C_1+N_{2+}O_3<C_2+O$ as shown in Figure 3.

Chlorophyll 'b' analysis

We analyzed the chlorophyll b content of *P. roxburghii* in our experiment under the blended effect of ozone and nitrogen stress. We additionally delivered exceptional consortiums, precise 1, 2, and 3, to the experimental setup. We used the trusted Acetone technique to measure the amount of chlorophyll b and expressed the outcomes in micrograms consistent with milliliter (µg/ml). The findings of this examination revealed hanging variances in chlorophyll concentration, indicating significant departures from the managed institution. those deviations were especially exquisite considering that they showed a wide variety of outcomes, some of which included an increase in the chlorophyll content that was almost two times greater than those visible in the manage pattern.

The chlorophyll content of the *P. roxburghii* samples is numerous depending on the strain situations. In some cases, the chlorophyll degree become reduced below that of the manipulated sample, at the same time as in different instances, it increases considerably. The C2+O3 sample had the best chlorophyll b content material, at the same time as the C2+N3 sample had the lowest. This indicates that *P. roxburghii*

has an extraordinary response to mixed ozone and nitrogen pressure at unique tiers of nitrogen stress.
The following is the observed order of increasing chlorophyll b content: C2+N3 < C3+N1 < C3+N+O3 < C1+N2+O3 < C1+O3 < C1+N+O3 < C2+N2+O1 < C2+O3. Those findings shed light on the complex interplay between *P. roxburghii* and the mixed stresses of nitrogen and ozone. They also highlight the plant's capability for environmental edition and reaction, which may have wider ramifications for research on plant physiology and ecology as shown in Figure 4.

Discussion

In our study, we used a destructive approach to observe unique intracellular compounds crucial for understanding plant responses to blended ozone and nitrogen pressure. While non-destructive techniques offer valuable insights, our focus on protein expression and chlorophyll content necessitated the use of methods allowing direct analysis of these components. Despite acknowledging the disruptive nature of grinding plant samples, our optimized extraction protocol aimed to minimize damage and preserve the integrity of the proteins under investigation. Nitrogen and ozone are required by the plants in balanced quantities. However, high ozone and nitrogen stress are considered major air pollutants. The results obtained by (Nouroozi et al., 2015) confirmed, that providing high ozone as well as nitrogen concentrations, which brings about widespread reductions in total protein content in pine plants. (Ahmad et al., 2009) showed that high ozone and nitrogen stress curtailed chlorophyll a and Chl b content and Watanabe (Watanabe et al., 2011), for the 2-year-old Quercus serrata seedlings, observed that Chl a showed greater sensitivity to combine nitrogen and ozone stress than Chl b. However, in this study, combined ozone and nitrogen stress along with diverse consortiums i.e. 1, 2, and 3 were supplied, and the treatments were: C1+N2+O3, C3+N+O3, C1+O3, C1+O3+N, C2+N2+O1, C1+N+O1, C2+N3, C2+O3, C3+N1.
The results obtained by quantifying protein content using Bradford's assay implied that the highest amount of total protein content was found in the C_1+NO_3 sample as compared to the control sample which showed the least protein content while the lowest was observed in C_2+N_3 which means that consortium 1 better tolerate the combined stress of ozone and Nitrogen and leads to an increase in protein content while consortium 2 under Nitrogen stress could not tolerate stress and resulted in a decrease in total protein content. The order of protein content in our treatments was observed to be; $C_2+N_3<C_3+N_1<C_3+N+O_3<C_1+O_3<C_2+N_2+O_1<C_1+N_2+O_3<C_1+N+O_1<C_2+O_3<C_1+N+O_3$.
High tropospheric ozone and nitrogen stress influence plant chlorophyll content in diverse means as these stresses cause visible leaf injury, vandalize photosynthetic pigments and peroxidate the lipid content of the thylakoid membrane. Moreover, (Ahmad et al., 2009) showed that ozone and nitrogen stress curtailed chlorophyll a plus Chl b content. The results obtained by providing treatments such as N60 and AA+120; significantly reduced the ratio of chlorophyll a as well as chlorophyll b in *Toona sinensis* leaves and lessened chlorophyll a/b value which means it accelerated aging in the leaf. On the other hand, contrary to the O_3 stress effect, N application in this experiment significantly increased the amount of chlorophyll a, and chlorophyll b in a class of *Toona sinensis* leaves (Lin et al., 2019). However, in our study, combined ozone and nitrogen stress along with diverse consortiums i.e. 1, 2, and 3 were supplied, and the treatments were: $C1+N2+O_3$, C_3+N+O_3, $C1+O_3$, C_1+O_3+N, $C_2+N_2+O_1$, C_1+N+O_1, C_2+N_3, C_2+O_3, C_3+N_1. Additionally, in this study, combined ozone and nitrogen stress along with different consortiums were provided and results obtained by analyzing chlorophyll quantity using the Acetone method implied that the highest content of chlorophyll a was observed in the sample C_2+O_3 while the lowest content was observed in C_2+N_3 which shows that consortium 2 better tolerate combine Ozone and Nitrogen stress while consortium 2 cannot tolerate Nitrogen stress. The increase of chlorophyll content is in the order such as; $C_2+N_3<C_3+N_1<<C_3+N+O_3<C_1+N+O_3<$ $C1+N_2+O_3<C_1+O_3<$ $C_2+N_2+O1<C_2+O_3$.
In the case of chlorophyll b the highest content was observed in the sample C_2+O_3 while the lowest content was observed in C_2+N_3 which shows that consortium 2 better tolerates combine ozone and nitrogen stress while consortium 2 cannot tolerate individual nitrogen stress. The increase of chlorophyll b content is in the order such as; $C_2+N_3<C_3+N_1<<C_3+N+O_3<C_1+N_2+O_3<$ $C_1+O_3<$ $C_1+N+O_3<$ C_2+N_2+ $O1<C_2+O_3$.
Finally, our result demonstrated that, under combine ozone and nitrogen stress, chlorophyll a content was more enlarged as compared to chlorophyll b content. This findings are also consistent with the conclusions of (Watanabe et al., 2011) for the 2 year-old *Quercus serrata* seedlings, where it was observed that Chl a showed greater sensitivity to combine nitrogen and ozone stress than Chl b.
Chlorophyll a and b analyses revealed significant variability between treatments. The highest chlorophyll concentration in the C2+O3 sample indicates this treatment promoted photosynthetic capacity the most. Conversely, the lowest levels in C2+N3 imply damaging effects. A similar pattern was seen for chlorophyll b. though chlorophyll a content was more enlarged as compared to chlorophyll b content.

Materials and Methods

Plant materials
Two-year-old *P. roxburghii* seedlings were obtained from the Khyber Pakhtunkhwa Forest Department in Abbottabad, as part of the Billion Tree Afforestation project (Green Growth Initiative). These seedlings were carefully nurtured for a month in the Biotic & Abiotic Stress in Transcriptomic & Proteomics Lab at COMSATS University Islamabad, Abbottabad Campus, during which they received regular watering with tap water. All the experimentation was done in three replicates in separate pots. Seedlings were watered with deionized water as necessary.

Extraction of total protein
100 mg of *P. roxburghii* was grounded finely and then homogenate was made using extraction buffer which comprises HEPES (100 mM, pH 6.8), NaCl (150 mM), CHAPS (0.1% W/V), glycerol (10% V/V), DTT (10 mM) and CaCl2 (50 mM) in ice-cold produced by ice maker machine. These plant samples were centrifuged at 16,000 rpm for 20 minutes (4°C) (Jamil et al., 2024). In our study, we introduced combined ozone and nitrogen stress along with diverse consortiums

labeled 1, 2, and 3. The treatments included C1+N2+O3 (consortium 1 + nitrogen + oxygen), C3+N+O3 (consortium 3 + nitrogen + oxygen), C1+O3 (consortium 1+ Ozone), C1+O3+N (consortium 1 + Ozone+ nitrogen), C2+N2+O1 (consortium 2 + nitrogen 2 + oxygen), C1+N+O1 (consortium 1 + nitrogen 2 + oxygen), C2+N3 (consortium 2 + nitrogen 3), C2+O3 (consortium 2+ Ozone), C3+N1 (consortium 3 + nitrogen 1). After subjecting the plant samples to these treatments, we collected supernatant containing soluble proteins, which were subsequently used for protein assays to quantify their levels (Bradford, 1976).

Ozone treatment

Every day, from 12:30 pm to 3 pm, O3 was sprayed for three hours. OTCs measuring 1.525 m in diameter and 3.048 m in height were used in open-top chamber tests. Two pipes—one an ozone pipe and the other a centrifugal air blower—were linked to an ozone generator through the boxes. The air blower was running continuously, and the ozone generator was programmed to generate ozone in 4 seconds, with a 7-second time delay to reach 100 ppb or 0.1 ppm. OTCs are aluminum rectangular frames covered in a transparent film. They contain many small holes (diameter of 10 mm at intervals of 10 cm) through the pipe. Ozone is distributed via a pipe that is 80 cm above the canopy and is powered by a centrifugal blower to emit a mixture of air and ozone that is forced upward. High-voltage electric discharge was used to create O3 from oxygen. The ozone content of OTCs is controlled by flowmeters. Using an O3 analyzer (ZA-XM-E-O3 Pump Priming Ozone Monitor), the OTCs' O3 concentrations were measured. One OTC for each treatment and one OTC for control were arranged in three lines, two meters apart. Ozone was measured externally at a distance of 1.5 meters and through three of the chamber's holes. The first hole starts from an 80cm canopy. The 03 concentration was measured twice an hour using an 03 analyzer (Li et al., 2017).

Nitrogen treatment

Ammonium nitrate (NH4NO3), a N stressor, was applied to the seedlings. In this experimental work, the high N stress treatment was administered at 0 kg N ha-1 (C) and 100 kg N ha-1 (T), or 0 mg l-1 and 145 mg l-1, respectively. Every experiment was carried out in three distinct pots with duplicates. The deionized water was used to irrigate the seedlings, when required (Jamil et al., 2020).

Quantification of total protein

Bradford's assay was performed for the quantification of protein present in plant extracts. The main principle of this assay is that protein molecules under acidic conditions bind to Coomassie dye which changes color from brown to blue. Under acidic conditions, protein molecules bind to Coomassie dye which results in brown to blue change in color (Nouroozi et al., 2015).

Chemical and materials

The following chemicals and materials for Bradford Assay were Bradford reagent, Bovine serum albumin (BSA); protein standard and Microplate and microplate reader.

Bradford composition

In 95% ethanol (50ml) add Coomassie Brilliant Blue G-250 (100 mg) followed by the addition of 85% phosphoric acid (100 ml). Set the final volume to 1000 ml after the complete dissolving of dye. Filter it before use.

Bovine serum albumin (BSA); protein standard

At first, a standard solution of (0.5 mg/ml) Bovine Serum Albumin was prepared. Then we prepared a few dilutions of this standard (Giner-Chavez et al., 1997).

Microplate and microplate reader

The 96-well microplate was incubated for about 5-10 minutes. Microplate reader set at 595 nm (Noble 2014).

Procedure of experiment

Step 1. A different range of standard solutions are prepared as we 1 mg/ml aliquot of protein standard such as 0.05 to 1 mg/ml. We used 50 µl of total volume for this standard curve assay.

Step 2. 1.5ml of Bradford reagent is added to the microreader plate wells. Both samples and standard curve were loaded in triplicates shown in Table 1

Step 3. 50ul of sample or standard was added to the wells in sequence by pipetting each sample and reagent. In order to avoid cross-contamination new pipette is used for each sample.

Step 4. Microreader plate was incubated for 5-10 minutes at ambient temperature.

Step 5. Readings are obtained at 595nm using a Microreader plate

Step 6. Using the R^2 value of our standard dilutions' absorbance when plotted in a graph, we calculated the protein content of our samples (Nouroozi et al., 2015).

Chlorophyll analysis

Sample collection

Leaves were amassed from *P. roxburghii* and brought to the Photosynthetic lab E Block COMSAT for further studies. Leaves were covered in aluminum foil and kept in the freezer where they were freeze and dried, thusly can be used for the extraction of chlorophyll.

Chlorophyll extraction

For extraction of chlorophyll Frozen, frozen, or fresh samples are used. These samples were weighed (2.5mg) using an Analytical weighing balance. 2ml acetone was added to each sample and ground each sample with a pestle and mortar to extract Chlorophyll. A solution containing chlorophyll extract was then filtered to 15ml falcon tube using single layer bandage cloth. Maximize the sample solution up to 10ml by addition of further acetone. After that centrifugation of the samples was done for 10 minutes at 2500 rpm in 4^{O}c. and then samples were transferred to new falcon tubes and 5 dilutions (10ml) of each sample were made.

Spectrophotometer measurements

A double-beam spectrophotometer was used for analyzing chlorophyll content by checking absorbance at 662nm and

646nm. Around 3ml from each dilution and sample were put in a Quartz Cuvette and measured absorbance at 662nm and 646nm.

Calculation of chlorophyll content

We use a recent formula published by (Ritchie 2006) for the calculation of chlorophyll content.

Chl a (µg/mL) = (12.7A×662-2.69A×646) ×10/1000×150

Chl b (µg/mL) = (22.9×A646-4.68×A662) ×10/1000×150

Statistical analysis

The experiment was conducted with at least three repetitions of each treatment. Every result was displayed as a mean with standard error bars, utilizing the GraphPad Prism 9. By using analysis of variance (ANOVA) (St and Wold 1989), it was possible to compare the differences between the various treatments. A $P \leq 0.05$, the difference was considered significant.

Conclusions

High ozone and nitrogen concentrations lead to the production of ROS species which negatively influences the chlorophyll content and total protein content of plants. In the present study, high ozone and nitrogen concentrations were applied, also different consortiums i.e., 1, 2, and 3 were provided, that were isolated from the pines rhizosphere. The results verified that these consortiums have a significant impact on the growth and stress tolerance ability of the *P. roxburghii*. In addition, the total protein content was highly increased when consortium 1 was given under the combined stress of ozone and nitrogen(C1+N+O) as consortium 1 better tolerated the combined stress of ozone and nitrogen while consortium 2 against nitrogen stress could not tolerate it and resulted in decline of total protein content. In the case of chlorophyll content consortium 2 better tolerates ozone (C2+O) as compared to the combined effect of both stresses. In general, elevated ozone and nitrogen stresses increased chlorophyll content in *P. roxburghii*; however, Chlorophyll content was more ameliorated as compared to chlorophyll b content. On the other hand, consortium 2 could not bear solo nitrogen stress (C2+N) and turn down chlorophyll quantity in *P. roxburghii*. Lastly, it is recommended that consortium 1, 2, and 3 compositions should be evaluated genetically, to find out the central characteristic in these consortiums that are primarily involved in managing stress.

Authors' contribution

Khan, S masterfully executed experiments and summarized results, while **Alam, O** expertly interpreted findings, managed references, and refined the manuscript. **Khan WU, Khan NK, Sardar A, Jabeen P** and **Ullah, A** revised the manuscript and finalized it.

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CONFLICT OF INTEREST: Authors declare no conflict of Interest.

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