Cold Plasma and Plasma-Activated Water for Enhancing Growth and Abiotic Stress Resilience in Wheat

By

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List of Abbreviations

Abbreviation	**Explanation**
CP	Cold Plasma
PAW	Plasma-Activated Water
RONS	Reactive Oxygen Nitrogen Species
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species

PEG	Polyethylene Glycol
MDA	Malondialdehyde
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
TBARS	Thiobarbituric Acid Reactive Substances
GABA	Gamma-Aminobutyric Acid
PLP	Pyridoxal Phosphate
NAD^+	Nicotinamide Adenine Dinucleotide (oxidized form)
NADP	Nicotinamide adenine dinucleotide phosphate
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
DBD	Dielectric Barrier Discharge
ORP	Oxidation-Reduction Potential
N_2	Nitrogen
NO	Nitric Oxide
NO_2	Nitrogen Dioxide
NO_2^-	Nitrite Ion
NO_3^-	Nitrate Ion
$ONOO^–$	Peroxynitrite
H_2O_2	Hydrogen Peroxide
O_3	Ozone
O_2^-	Superoxide Radical
OH	Hydroxyl radical
UV	Ultraviolet
CO_2	Carbon dioxide
PS	Photosystem
PGPRs	Plant Growth-Promoting Rhizobacteria
NaCl	Sodium chloride
AR	Argon
He	Helium
O_2	Oxygen
APX	Ascorbate Peroxidase

GPX	Guaiacol Peroxidase
POD	Peroxidase
CAT	Catalase
SOD	Superoxide Dismutase
PAL	Phenylalanine Ammonia-Lyase
RWC	Relative Water Content
FC	Field Capacity
ppm	Parts Per Million
GH	General Hardness
TA	Total Alkalinity
dH_2O	Distilled Water
SEM	Scanning Electron Microscopy
OES	Optical Emission Spectroscopy
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
KG	Kilogram
ml	Millilitre
mg	Milligram
L	Litres
LPM	Litres Per Minute
rpm	Revolutions Per Minute
g	Gram
nmol	Nanomole
mM	Millimolar
W	Wattage
M	Molar
KOH	Potassium hydroxide
CA	Compressed air
nm	Nanometer
µL	Microlitre
mm	Millimetre

µM	Micromolar
G	Gram
°C	Degree Celsius
pH	Potential of Hydrogen
h	Hour
min	Minute
G	Gram
sec	Second
ANOVA	Analysis of Variance
SD	Standard Deviation
SE	Standard Error

ABSTRACT

Climate change poses a major threat to global food security, with drought and heat stress being among the most destructive challenges for cereal crops such as wheat (*Triticum aestivum*). Current mitigation strategies like selective breeding and agronomic management have shown limited success under increasing unpredictable climatic conditions. This study explores cold plasma (CP) and plasma-activated water (PAW) as sustainable, non-chemical technologies to enhance wheat growth and resilience against abiotic stress. Cold plasma is a non-thermal ionized gas with high concentrations of reactive oxygen and nitrogen species (RONS) able to modify seed surface properties, increase metabolism, and promote germination. PAW is a water exposed to plasma discharge with RONS used as bio-stimulants and signalling molecules. The main hypothesis of this study is that treating seed with CP, followed by spray with PAW, promotes germination, growth, tolerance to drought, and drought combined with high-temperature stresses.

The wheat seeds were treated with CP generated using compressed air and nitrogen with 80W and 5LPM flow rate for 10 and 20 minutes. Greenhouse experiments included twelve treatment combinations involving CP-treated and untreated seeds, with either PAW or distilled-water sprays under non-stress, drought, and combined drought + heat stress conditions. Physiological traits such as germination percentage, biomass, and head emergence were measured and analysed. Also, biochemical assays such as Thiobarbituric Acid Reactive Substances (TBARS), Gamma-aminobutyric acid (GABA), and alanine were analyzed to evaluate oxidative and metabolic stress responses.

Cold plasma treatment also brought significant increases in root and shoot growth, especially at 10 minutes of treatment, without any negative effect on germination percentage. Different concentrations of polyethylene glycol (PEG) solution such as 15% and 30% PEG was used to mimic drought stress in wheat seeds. Under combined drought & heat stress (30% PEG & 37ºC) conditions, plasma treatment significantly improved germination percentage in wheat seeds. In greenhouse conditions, PAW reduced growth under optimal conditions but enhanced root and shoot biomass under drought, suggesting an environmental stress dependent biostimulatory effect. The combined CP + PAW treatment increased head dry weight and maintained membrane integrity under combined stress. TBARS results showed reduced

malondialdehyde accumulation, indicating lower oxidative damage, while GABA and alanine assays revealed improved redox regulation and nitrogen balance.

In conclusion, combined effect of CP and PAW enhanced wheat germination, growth, and stress tolerance through physiological and biochemical priming. Based on the obtained results we can conclude that their effects were dose and environmental stress dependent, with CP acting as an efficient seed-priming agent and PAW serving as a secondary stimulant during stress. These findings support the integration of plasma technologies into sustainable agricultural practices to improve crop performance under adverse climatic conditions.

DECLARATION

I, Nivetha Arulkumar certify that this thesis titled “Cold Plasma and Plasma-Activated Water for Enhancing Growth and Abiotic Stress Resilience in Wheat”,

1. Does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university
2. The research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and
3. To the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

NIVETHA ARULKUMAR

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Chapter 1: Introduction

Climate change is one of the biggest and most urgent challenges faced in today's agriculture sector (Malhi et al., 2021; Raza et al., 2019; Verma et al., 2024). Wheat, being a staple crop, is highly susceptible to these climatic change such as heat and drought which hinder yield and agricultural stability (Erenstein et al., 2022; Grote et al., 2021). Abiotic stresses, particularly combined heat and drought simultaneously are crippling for growth, physiological processes, and productivity in wheat (Sato et al., 2024; Ullah et al., 2022; Zahra et al., 2021). Wheat is particularly sensitive during its reproductive and grain-filling phases (Khaeim et al., 2022; Qaseem et al., 2019). The response of plants to combined heat and drought is usually even more devastating compared to single stress impacts, emphasizing the immediate need for revolutionary strategies for crop resilience enhancement (Hussain et al., 2019; Sehgal et al., 2019).

While agronomic methods and breeding programs have resulted in improved growth and yield, increasing frequency of adverse climatic conditions require new, sustainable technologies (Imran et al., 2021; Oyebamiji et al., 2024). Among such novel methods is cold plasma (CP) technology and plasma-activated water (PAW). Cold plasma is an ionized gas formed essentially at room temperature that yields a range of reactive chemical species and has a multiplicity of medical uses. For example, cold plasma is used for surface treatment of medical implants to improve the process of biocompatibility such as in dentistry, in the treatment of chronic wounds, and it holds potential for cancer treatment (Tabares & Junkar, 2021). Previous studies indicate that the use of CP and PAW are increasingly being investigated as an environment-friendly choice in agriculture. This is a relatively new area of study where these technologies are not yet standard practice (Gao et al., 2022; Kocira et al., 2022; Shelar et al., 2022). In a recent study by Lotfy et al. (2019) revealed that wheat seeds treated with CP enhanced the seed germination rate from 49.8% to 93.3% under non-stress conditions, this shows that CP and PAW both can enhance seed germination, when used individually. Also, existing studies shows that CP and PAW enhances seedling vigor, plant growth, and abiotic stress tolerance in cereal crops such as wheat (Jiang et al., 2014; Li et al., 2016).

This study discusses on utilizing plasma treatment and plasma-activated water for improving wheat growth and abiotic stress resistance, including heat and drought. The individual CP treatment of seeds, PAW treatment of seeds, and combined effects of CP and PAW treatment

on seeds are examined. The focus is on reported mechanisms of action, trends and areas of needed research. A review of the literature would help in forming a basis for understanding the role of plasma technologies in sustainable wheat cultivation under intensifying climate stresses.

1.1. Abiotic stress in crop plants

1.1.1 Impact on physiology and yield

Abiotic stresses are environmental non-biological conditions that negatively impact plant growth, development, and productivity, and are a major limitation to global agriculture (Kopecká et al., 2023; Imran et al., 2021). Some of the main types of abiotic stresses focussed on this review includes drought (water shortage), (Ahmad et al., 2018), and heat stress involving temperature extremes that disrupt cellular processes (Ullah et al., 2022). Heat and drought stresses stimulate widespread physiological and biochemical disturbances in plants (Zhang et al., 2022). Drought normally causes osmotic stress that impedes water uptake and lowers cellular turgor necessary for cell growth (Ahmad et al., 2018). Heat and drought commonly lead to stomatal closure and thus restricted CO_2 assimilation and lowered photosynthetic rates; heat can also directly inactivate photosynthetic machinery such as photosystem II (PSII) (Sharma et al., 2020; Ullah et al., 2022). As shown in figure-1.1, the widespread response is overproduction of reactive oxygen species (ROS) that, if antioxidant defence mechanisms are saturated, lead to oxidative harm in membranes, proteins, and DNA (Zhou et al., 2019). Heat and drought during reproductive growth are particularly damaging in wheat and can result in lowered pollen viability, compromised fertilization, shrivelled grains, and major yield loss. Combined stresses usually have synergistic adverse impacts (Ullah et al., 2022).

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Figure-1.1 Abiotic stress decreases crop yield. Environmental abiotic stress agents like heat, cold, drought, salinity, and exposure to heavy metal ions like copper, chromium and cadmium induce plant stress responses like the production of an excess of reactive oxygen species (ROS) and decreased photosynthesis and subsequently reduce plant development and hence crop yield (taken from Godoy et al., 2021).

1.1.2 Existing mitigation strategies

Various methods are being employed against abiotic stress in crops. At a genetic level, stress-tolerant varieties are being developed using conventional breeding for favourable traits, and cutting-edge biotechnological methods such as genetic engineering and marker-assisted selection for improved resilience against stresses (Grigorieva et al., 2023; Imran et al., 2021; Şimşek et al., 2024). Agronomic methods optimize the environment for cultivation, and these include efficient use of water (e.g., irrigation), improving soil health (conservation tillage and addition of organic matter), and modifying cultural operations such as sowing dates in a manner that avoids peak stress phases (Ahmad et al., 2018; Malhi et al., 2021; Oyebamiji et al., 2024). Chemical and biological methods include the use of osmo-protectants, antioxidants, or growth regulators, and biological stimulants such as beneficial microbes (e.g., mycorrhizae, Plant Growth-Promoting Rhizobacteria (PGPRs)) for enhanced nutrient acquisition and tolerance induction (Godoy et al., 2021; Oyebamiji et al., 2024). Though these strategies have led to improvements in growth and in certain cases yield, it is not effective with the increasing frequencies of adverse weather events and associated costs to mitigate these necessitate new and more efficient strategies (Raza et al., 2019).

1.2. Cold plasma technology in agriculture

1.2.1 Overview of Cold Plasma (CP)

Cold plasma (CP), an ionized gas at near-atmospheric pressure and room temperature, is non-thermal; its high-energy electrons coexist in conjunction with cool heavy particles (<40°C), making treatment of heat-sensitive biologicals such as seeds feasible without thermal injury (Bozhanova et al., 2024; Kocira et al., 2022; Misra, 2016; Shelar et al., 2022). CP is generated using different gases and it is a combination of charged particles, neutral/excited species, reactive oxygen species (ROS) (e.g., O_3, ·OH), reactive nitrogen species (RNS) (e.g., NO), UV radiation, and electric fields. CP is gas, power, and design dependent (de Groot et al., 2018; Guo et al., 2021; Los et al., 2019; Lotfy et al., 2019; Shelar et al., 2022). Eco-friendliness and residue-free processing emphasize its agronomic potential (Kocira et al., 2022; Shelar et al., 2022).

1.2.2 Mechanisms of CP action on wheat, cotton, paddy and barley

Cold plasma (CP) benefits the plants by both physical and chemical effects. Physically, CP etches seed coats and increases roughness, hydrophilicity, and micro-cracking, facilitating water/gas exchange (Ahmed et al., 2023; de Groot et al., 2018; Los et al., 2019; Rashid et al., 2021). Chemically, RONS and ROS formed by CP are major mediators. RONS doses in a controlled manner function as signals: breaking dormancy, activating metabolism, and stimulating antioxidant defence responses (Bradu et al., 2020; Sharma et al., 2020; Kocira et al., 2022; Los et al., 2019; Shelar et al., 2022). Nitric oxide (NO), an RNS, affects these processes (Bradu et al., 2020). CP can also deliver nitrogen groups for feeding of seedlings and inactivate seed-borne microorganisms, with UV support for sterilizing processes. These processes synergistically benefit germination, growth, and stress resistance (Feizollahi et al., 2020; Lee et al., 2021; Los et al., 2019).

1.2.3 Applications of CP

1.2.3.1 Seed germination and viability

CP increases seed viability and germination in various crops (e.g., wheat), increasing germination rate from 49.8% to 93.3%, (Lotfy et al., 2019; Bozhanova et al., 2024; de Groot et al., 2018; Ling et al., 2014). Plasma treatment significantly improved wheat germination efficiency to 33.3%, when the germination energy of untreated seeds is 8.24% (Bozhanova et al., 2024), as shown in figure-1.2 in association with changes in the seed coat improving water absorption and RONS stimulation (Shelar et al., 2022). In a study conducted by Ling et al.

(2014), the soybean seed's water absorption capacity was increased by 14.03% compared to the control.

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Figure-1.2 Impact of cold plasma (CP) treatment on seed germination and viability. Production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by cold plasma at low or atmospheric pressure subsequently impacts biochemistry, seed germinating parameters, molecular and physiology processes essential for germination, plant development and sustainability (sourced from Shelar et al., 2022).

1.2.3.2 Early seedling vigor

Cold plasma treatment has been shown to promote enhanced vigor during early seedling growth through enhanced shoot/root growth and biomass (Bozhanova et al., 2024; Ling et al., 2014). In a study conducted by Bozhanova et al. (2024) the wheat seed exhibited 30.18% and 40.42% increased shoot and root growth compared to the control. In Ling et al. (2014) study, the soybean seeds exhibited 13.77% and 21.42% increased shoot and root growth compared to the control. In Lotfy et al. (2019) study, the wheat seed treated with cold plasma has exhibited

64.9% increased seedling weight compared to the untreated seeds. The improvement is attributed to optimal utilization of seed reserves, increased metabolism rate, and enhanced water uptake by 14.03% compared to the untreated seeds (Ling et al., 2014; Li et al., 2016; Los et al., 2019).

1.2.3.3 Abiotic stress tolerance

CP treatment trains seeds/plant for increased abiotic stress tolerance (e.g., drought stress, extreme temperatures) (de Groot et al., 2018; Jinkui et al., 2018; Shelar et al., 2022). For example, in a study conducted by Jinkui et al. (2018) alfalfa seeds were treated with cold plasma and used polyethyleneglycol-6000 (PEG-6000) at 5%, 10% and 15% concentrations which will stimulate drought environment in the seeds. The highest germination percentage observed was 89.7% in the treated seeds compared to 61.7% in the control. In another study conducted by de Groot et al. (2018) with cotton seeds, the seeds treated with cold plasma was exhibiting improved early germination compared to the control, under warm (30ºc) and cold temperature (14ºc). The resulting priming is attributed to RONS-induced antioxidant activation, osmolyte accumulation, and regulated stress-gene expression, enhanced physiological performance (sustained photosynthesis, decreased damage) under stress in soybean and wheat plants (Bozhanova et al., 2024; Kocira et al., 2022; Shelar et al., 2022).

1.3. Plasma-activated water (PAW) in agriculture

1.3.1 What is PAW and how is it produced?

Plasma-activated water (PAW) is plasma-treated water enriched with reactive chemical species (Guo et al., 2021; Zhou et al., 2020). It is created by subjecting water (distilled water, tap water) to cold plasma discharge (e.g., dielectric barrier discharges, plasma jets) in contact or by bubbling plasma-generating gas (air, nitrogen, etc.) through it (Bradu et al., 2020; Gao et al., 2022; Zhou et al., 2020). This injects energy and reactive species into the water and changes its pH, conductivity and oxidation-reduction potential (ORP), but not its temperature significantly (Gao et al., 2022; Guo et al., 2021).

1.3.2 Reactive species in PAW and their role

PAW properties originate from short- and long-lived dissolved reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bradu et al., 2020; Zhou et al., 2020). The major ROS are short-lived hydroxyl radicals (OH) and superoxide anions (O_2^-), and long-lived hydrogen

peroxide (H_2O_2) and ozone (O_3) (Guo et al., 2021; Zhou et al., 2020). RNS from air/nitrogen plasma are particularly nitrogen dioxide (NO_2), nitric oxide (NO), and their aqueous products including nitrate (NO_3^-), nitrite (NO_2^-), and peroxynitrite ($ONOO^-$) (Bradu et al., 2020; Gao et al., 2022). These RONS represent PAW main bioactive agents stimulating plant physiology and exerting antimicrobial activity (Bafoil et al., 2018; Guo et al., 2021). Their concentration and composition vary according to generation conditions and PAW storage conditions (Zhou et al., 2020).

1.3.3 Mechanisms of action

PAW affects plants through dissolved RONS as signalling compounds or direct effectors. Nitrates and nitrites are a source of nitrogen (Bradu et al., 2020; Zhou et al., 2020). Low concentrations of H_2O_2 and NO regulates hormone routes, stimulating antioxidant defense mechanisms, and regulating growth and stress-response gene expression; NO also induces breaking of dormancy in seeds (Bafoil et al., 2018; Bradu et al., 2020; Guo et al., 2021). The acidity of PAW from nitric/nitrous acids has an effect on seed permeability through the seedcoat and nutrient access (Zhou et al., 2020). These cumulative effects promote enhanced metabolic activity and improved physiological condition of plants.

1.3.4 Applications of PAW

1.3.4.1 Seed germination and growth

PAW induces seed germination and further plant growth. Soaking or irrigating seeds in PAW enhances percentage of germination, rate of germination, and vigour of the seedlings (Bafoil et al., 2018; Guo et al., 2021; Rashid et al., 2021; Zhou et al., 2020). For example., in a study conducted by Rashid et al. (2021), rice plant irrigated with PAW showed 16.67% increase in grain yield compared to control. These results occur because RONS break dormancy, activate enzymes, and release nitrogenous compounds (Bradu et al., 2020). The foliar use of PAW can also promote growth by providing nutrients and activating metabolism (Rashid et al., 2021).

1.3.4.2 Stress resistance (Heat/Drought)

PAW treatment can increase abiotic stress tolerance in plants such as drought and possibly heat, although individual combined heat/drought studies in wheat under PAW are not as comprehensive in sources available as for CP. RONS in PAW being H_2O_2, NO are capable of acting as priming agents for activating defense mechanisms in plants prior to stress (Guo et al., 2021). Priming allows for an increased stress response involving increased antioxidant enzymes, accumulation of compatible solutes (e.g., proline), improved cellular homeostasis,

and consequently reducing stress damage and facilitating growth under stress conditions (Mahanta et al., 2022).

1.4. Combined cold plasma and PAW treatment

1.4.1 Rationale for combined use

Synergistic combination of subsequent PAW treatment after CP seed treatment provides an efficient approach for leveraging additive or synergistic effects during distinct developmental phases in different plants. CP acts directly on the seed by altering its surface for increased water uptake, minimizing microbial load, and infusing an immediate dose of reactive species for enhanced germination and early vigor in seedlings (Los et al., 2019; Shelar et al., 2022). Subsequent PAW treatment through irrigation or foliar spray can contribute supplemental support during growing plant development. PAW provides continuous release of nitrogenous compounds and reactive oxygen and nitrogen species (RONS), which may enhance nutrient uptake, promote additional growth, and even stress resistance through priming or systemic responses (Bradu et al., 2020; Guo et al., 2021; Rashid et al., 2021). The dual approach seeks enhanced seed establishment and supplemental plant support.

1.4.2 Current evidence

Current studies involving combined CP seed treatment and PAW use, while limited in number, report positive results. One illustrative study on paddy (rice) showed that seeds treated with air plasma under low pressure and then subjected to foliar PAW post-treatment during vegetative growth showed marked enhancements in growth and yield traits compared to separate treatments or controls. The combined treatment resulted in enhanced rates of seed germination, increased plant height, increased tillers, increased grain yield, and enhanced grain quality in aspects related to soluble protein and sugar content (Rashid et al., 2021). The researchers attributed these synergistic benefits to enhanced water imbibition in seeds induced by CP combined with nutrient and biostimulant impacts of foliar PAW treatment (Rashid et al., 2021). Even though this is direct evidence for rice, the recorded individual benefits of CP for different seeds, including soybean (Ling et al., 2014), and PAW's general growth stimulation effects (Bafoil et al., 2018), all indicate a strong possibility of similar benefits for different crops, which needs to be further examined.

1.4.3 Knowledge gaps

Although promising in its preliminary findings, major gaps in understanding combined CP and PAW use remain unexplored. Investigations have largely been conducted on small numbers of crops, and particular effects of combined treatments on major staples like wheat under abiotic stresses like heat and drought are unknown. A comprehensive investigation is necessary in order to optimize parameters for both PAW and CP seed treatment (e.g., plasma type, composition of gas involved, duration of treatment) and for PAW use (e.g., RONS concentration, timing of application, frequency of use, method of application is irrigation vs. foliar) in order to realize maximal synergistic effects for certain crop species (Rashid et al., 2021). Long-term effects on subsequent plant growth, yield, and product quality under various field conditions and soils must also be accurately assessed. A more profound understanding of the underlying molecular and physiological processes of synergism, particularly of how CP seed pre-treatment can prep plants for enhanced response under stress by PAW, is essential. The economic feasibility and scalability of these dual plasma technologies for practical use in agriculture must also be properly examined.

1.5. Methodological advances and research design

1.5.1 CP and PAW treatment parameters

Research in agricultural use of CP and PAW requires accurate control and reporting of treatment conditions, as these have significant impacts on results. These include for CP treatment of seeds such as type of plasma source equipment (e.g., Dielectric Barrier Discharge (DBD), plasma jet), type of operating gas composition (e.g., air, N_2, Ar, He, or combinations), gas flow rate, applied voltage or power, treatment duration, and source-to-seed plasma source distance (Bozhanova et al., 2024; Ling et al., 2014; Lotfy et al., 2019). Likewise for PAW generation, plasma generation conditions (gas type, power level, treatment duration of water), amount of water treated, type of water (e.g., distilled, tap) are important (Guo et al., 2021; Zhou et al., 2020). Physicochemical properties of PAW (ORP, pH, conductivity, concentrations of major RONS such as H_2O_2, NO_2^-, NO_3^-) must also be analyzed after activation and preferably also prior to use, as they have time-dependent stability (Gao et al., 2022). Standard reporting of these conditions is required for comparability and reproducibility between studies.

1.5.2 Experimental approaches

Research designs in this fields normally consist of controlled laboratory, and greenhouse experiments. Laboratory research can emphasize seed-stage responses through determination of germination percentage, rate, and vigor indices (e.g., shoot/root growth and seedling dry matter) under optimal or induced stress conditions (e.g., through PEG for drought, NaCl for salinity, or temperature chambers for heat/cold stress) (de Groot et al., 2018; Jinkui et al., 2018). Pot greenhouse experimentation enables measurement of longer-term impacts on plant growth, physiological processes, and initial stress response under relatively more managed environmental conditions compared to field conditions (Li et al., 2016). Field trials, although resource-demanding and challenging in execution, are indispensable for testing practical effectiveness of CP and PAW treatments on crop yield and performance under realistic agronomic conditions and fluctuating environments (Rashid et al., 2021). Experimental designs must include proper controls (untreated seeds/plants, water controls for PAW tests) and replication for sound statistical analysis (Ling et al., 2014).

Table-1.1: Summary of experimental approaches and results of cold plasma treatment on seed germination, plant growth and abiotic stress tolerance in varying plant species

Author	Seed	Power (wattage)	Treatment Duration	Carrier gas	Temperature during treatment	Stress/Normal	Result
Li Ling, et al.	Soybean	80 W	15 seconds	Helium	~25°C	Normal (Light incubator at 25°C) for 7 days	Improved soybean seed germination, seedling growth, water uptake, seed reserve utilization, and soluble sugar and protein content
Jiang Jiafeng, et al.	Wheat	80 W	15 seconds	Helium	~25°C	Normal (Light incubator at 25°C) for 7 days	Improved wheat seed germination, plant growth (height, root length, weight), physiological traits (chlorophyll content), and increased wheat yield by 5.89%
Gerard J. J. B. deGroot, et al.	Cotton	38 kVpp	27 minutes	Air	14°C and 30°C	Cold stress and water stress	Improved water absorption and seed germination of cotton seeds, enhanced chilling tolerance, and maintained these benefits for months after treatment
Ling Li, et al.	Peanut	120 W	15 seconds	Helium	~25°C	Normal (incubator in dark at 25°C)	Improved peanut seed germination, seedling growth, plant development, and final peanut yield by 10% compared to untreated controls
Li Ling, et al.	Rapeseed	100 W	15 seconds	Helium	~25°C	Only drought stress (15% PEG 6000 solution to mimic water deficit); Temperature normal (Light incubator at 25°C)	Improved oilseed rape seed germination, seedling growth, and drought stress resistance by enhancing antioxidant enzyme activities, increasing soluble sugar and protein contents, improving wettability, and reducing oxidative damage.
Agata Los, et al.	Wheat	80 kV	30-60 seconds	Atmospheric air	22–25°C	Normal laboratory conditions	Improved wheat seed germination rate, seedling growth, and water uptake by modifying seed surface chemistry, but longer treatments (180 s) had negative effects.
Violeta Bozhanova, et al.	Durum wheat	12 W microwave power at 2.45 GHz	Microwave plasma torch for 20 seconds / underwater discharge for 5 minutes	Argon	24°C	Only Drought stress (1 M sucrose solution to mimic drought stress)	Improved germination energy, germination rate, seedling growth, and osmotic stress tolerance.
Jinkui FENG, et al.	Alfalfa seeds	40 W	15 seconds	Air and Helium mixture	20°C (Incubator)	Only drought stress (PEG 6000)	Improved germination potential, germination rate, seedling height, root length, and vigor index

1.5.3 Analytical tools

Plasma sources, treated materials, and plant responses are characterized by a variety of analytical tools. The presence of excited species within plasma during CP generation is identified by optical emission spectroscopy (OES) (Lotfy et al., 2019). Surface changes in seeds induced by CP are examined through X-ray photoelectron spectroscopy for chemical composition on surfaces, scanning electron microscopy for morphology and contact angle analysis for wettability (Ahmed et al., 2023; Los et al., 2019). Chemical probes, spectrophotometric techniques, and ion chromatography are applied for RONS quantification in PAW (Bradu et al., 2020; Zhou et al., 2020). Physiological responses in plants are analyzed using techniques for measurement of photosynthetic parameters (e.g., chlorophyll fluorescence, gas exchange), oxidative stress indicators (MDA content), antioxidant enzyme activity (spectrophotometric assays), and osmolyte concentrations (e.g., proline assays) (Carillo & Gibon, 2011). PCR, RNA sequencing, and proteomics studies can determine gene expression and protein profile changes.

1.6. Critical analysis of trends, gaps, and debates

1.6.1 Oxidative stress and reactive oxygen species (ROS)

Exposure of plants to abiotic stresses including heat and drought, or even exogenous stimulants like plasma-activated water (PAW) and cold plasma (CP), tends to disrupt the balance between generation and scavenging of reactive oxygen species (ROS) (Hussain et al., 2019; Zhou et al., 2019). ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and superoxide radicals (O_2^-) are byproducts of aerobic metabolism but under conditions of stress lead to oxidative stress due to their excessive accumulation (Raja et al., 2020). Oxidative stress can harm important cellular constituents including lipids (membrane peroxidation, commonly determined through malondialdehyde - MDA content), proteins and nucleic acids and thus disrupt normal cellular processes and general plant health (Hussain et al., 2019; Sharma et al., 2020). Yet, under controlled and low concentrations, ROS induced by PAW or CP can additionally play a signalling role initiating protective responses in plants (Bafoil et al., 2018; Shelar et al., 2022).

1.6.2 Antioxidant enzyme activities: CAT, SOD, PAL, POD

To neutralize oxidative stress, plants have an intricate antioxidant protection system consisting of enzymatic and non-enzymatic elements. The major antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and different peroxidases (POD, e.g., ascorbate peroxidase – APX, guaiacol peroxidase - GPX) (Hussain et al., 2019; Zhou et al., 2019). SOD catalyzes superoxide radicals' dismutation into H_2O_2 and O_2, whereas CAT and peroxidases detoxify H_2O_2 into water and oxygen (Raja et al., 2020). Research involving CP and PAW treatments, as well as abiotic stress reaction studies, in most cases provides evidence for modulated activity of these enzymes.

1.6.3 Gamma-Aminobutyric Acid (GABA) and alanine accumulation

Plants accumulate osmolytes or compatible solutes during stress conditions that aid in osmotic regulation, protect cellular components, and scavenge ROS. One of the well-studied metabolites involved in such adaptive responses is alanine, which plays a vital role in maintaining redox and nitrogen balance under hypoxic or energy-limited conditions. The conversion of pyruvate to alanine via alanine dehydrogenase serves as a key step in nitrogen recycling and metabolic stabilization when carbon flow or respiration is restricted (Allaway et al., 2000; Li et al., 2001). Enhanced alanine aminotransferase activity has been linked to improved nitrogen use efficiency and stress adaptability in cereals such as rice and wheat (Tiong et al., 2021). A non-protein amino acid, gamma-aminobutyric acid (GABA), also accumulates in plants quickly under different abiotic and biotic stresses and plays a role in stress tolerance through mechanisms involving regulation of pH, carbon/nitrogen metabolism, and ROS scavenging. Applications of treatments like CP or PAW may affect the content of these compounds and hence play a role in improved stress tolerance (Shelar et al., 2022).

1.6.4 Overall Plant Health and Productivity Metrics

The final measure of profitable physiological and biochemical responses induced by either CP/PAW treatments or constitutive stress tolerance mechanisms is expressed in enhanced overall plant health and productivity, e.g., wheat biomass increased by 87% after 28 days of PAW treatment, rice treated with CP & PAW showed 16.67% increase in grain yield (Guo et al., 2021; Gao et al., 2022). Some of these key indicators are increased germination ratings and seedling vigor, increased efficiency in photosynthesis (e.g., chlorophyll concentration, gas exchange characteristics such as stomatal conductance, net photosynthesis rate, and rate of transpiration), and enhanced water status (e.g., relative water content - RWC) in conditions of

stress (Ahmad et al., 2018; Sharma et al., 2020; Ullah et al., 2022). These ultimately are expressed in enhanced growth parameters including leaf area, plant height, dry and fresh weight of biomass accumulated, and most importantly, economic yield (e.g., grains per plant, fruit numbers, size) and quality (Li et al., 2016; Rashid et al., 2021). Research involving CP and PAW commonly demonstrates remarkable increases in these productivity indicators, implying induced physiological and biochemical changes are successful in expressing enhanced performance in plants under adverse environment conditions (Jinkui et al., 2018; Ling et al., 2014).

1.6.5 Trends, gaps, and debates

CP and PAW are rising quickly as environmentally friendly agriculture technologies for improving plant growth, stress resistance, and seed germination (Guo et al., 2021; Shelar et al., 2022). One main trend is treating RONS as the principal drivers of plasma's biological effects, functioning either as signaling compounds or direct stimulants (Bradu et al., 2020; Kocira et al., 2022). Many lab studies document stimulatory effects in different model plants and crops, including wheat (Bafoil et al., 2018; Ling et al., 2014).

However, significant gaps remain large, preventing large-scale field adoption. Major obstacles are converting findings from the lab to stable and economically viable field deployment, protocol standardization due to plasma device and treatment parameter differences as well as response variability in plants (Bozhanova et al., 2024; Zhou et al., 2020) and elucidating fully the molecular mechanisms of CP/PAW activity under single conditions, particularly under combined heat/drought stress in wheat. Long-term persistence of CP-induced seed improvements and optimal delivery of PAW's reactive species must also be extensively researched (Ahmed et al., 2023; Guo et al., 2021).

Several debates today revolve around finding optimal parameter for plasma treatment (optimizing RONS effects) and the relative significance of physical versus chemical effects of CP on seeds (Los et al., 2019). Can or do CP/PAW replace or just complement conventional stress-management methods, especially for extreme combined stresses under climate change conditions? Detailed studies are necessary (Sato et al., 2024). Additional studies of combined treatments of CP and PAW need to be conducted (Rashid et al., 2021), as do life-cycle assessments in order to validate these methods.

Although cold plasma (CP) and plasma-activated water (PAW) are both promising agents to enhance crop tolerance to abiotic stresses, there are certain research gaps which has to be

addressed. The variability of plasma parameters (e.g., gas type, power, exposure time) has to be optimized and standardize the treatment protocols of CP (Bozhanova et al., 2024; Shelar et al., 2022). The determination of optimal "doses" of CP to apply to different crops is complicated (Ling et al., 2014). The molecular mechanisms responsible for CP action, especially the role of RONS in response to stresses also need to be explored (Kocira et al., 2022; Los et al., 2019). Long-term impacts, scalability, cost-effectiveness, as well as transition from lab to farm are also research gaps to be addressed (Ahmed et al., 2023; de Groot et al., 2018). Likewise, the short lifespan of RONS in PAW poses challenges and thus requires timely and precise application (Guo et al., 2021). Standardization of the generation of PAW and the identification of optimal RONS levels to be used in crops are required, in particular in combined drought and heat shock in wheat. Scalable and affordable on-farm systems of PAW generation are also still in the development stages (Gao et al., 2022). Finally, synergistic action of CP and PAW has been unexplained and requires focused research (Rashid et al., 2021).

1.8 Research objectives and hypothesis

1.8.1 Hypothesis

The combined application of cold plasma (CP) and plasma-activated water (PAW) treatment in wheat will improve the seed germination, plant growth and tolerance to drought & heat stress.

1.8.2 Research objective

1. To determine the optimal parameters for CP generated from air and nitrogen gas treatments such as gas type and exposure duration to improve the seed germination and seed viability.
2. To compare the results of plant growth and biomass between the CP and PAW treated seeds and untreated seeds under heat and drought stress in greenhouse conditions.
3. To investigate the effect of CP & PAW treatments on stress markers such as malondialdehyde (MDA), gamma aminobutyric acid (GABA), and Alanine.

Chapter 2: Materials and Methodology

2.1 Materials and reagents

Scepter wheat (*Triticum aestivum L.)* seeds of uniform size were used for all experiments. The materials included a dielectric barrier discharge (DBD) cold plasma treatment unit with adjustable flow rate and wattage controls, compressed air and nitrogen gas cylinders (99.9% purity), Petri dishes, Whatman No. 1 filter papers, polyethylene glycol (PEG 6000, Sigma-Aldrich), distilled water, 70% ethanol, micropipettes with sterile tips, measuring cylinders, beakers, funnels, forceps, tweezers, thermostatic incubator (37 °C), plastic tubs, field soil and sand mixture (3:1 ratio), weighing balance (0.01 g precision), polyethylene trays, watering cans, Silwet 77 surfactant, rulers, scissors, labelling tags, paper bags, hot-air oven (60 °C). Additional laboratory materials included 2 mL Eppendorf tubes, metal grinding beads, vortex mixer, CLARIOstar Plate Reader (BMG LABTECH, Australia), Nunclon Delta Surface 96-well plate (Thermo Scientific fisher) and ice boxes. All reagents were supplied by Sigma-Aldrich (USA) unless stated otherwise. PEG 6000 solutions (15% and 30% w/v in distilled water), trichloroacetic acid (TCA), Thiobarbituric acid (TBA), malondialdehyde (MDA) standards (0, 1, 3, 4 nmol), pyridoxal phosphate (PLP), $NADP^+$, α-ketoglutarate, GABase enzyme mix, γ-aminobutyric acid (GABA) standards (0, 10, 50, 100 mM), 100% methanol (Chem-Supply, Australia), alanine dehydrogenase, NAD^+, alanine standards (0, 50, 100, 250, 500, 750 mM), and all other chemicals and reagents of analytical grade obtained from standard suppliers.

2.2. Methods

2.2.1 Treatment parameters

2.2.1.1 Identifying optimal treatment parameters

To determine the optimal setting comprising of treatment duration, flow rate, and wattage for cold plasma (CP) treatment. Wheat seeds were treated with CP for time periods such as 15 seconds, 1 minute, 10 minutes, and 20 minutes, flow rate of 5 Liters Per Minute (LPM) and wattage of 80 W. Non-treated seeds were used as the control. After treatment, seeds were placed on petri dishes containing moist Whatman filter paper on the surface in two conditions, one with the use of distilled water, and another with the use of a 15% polyethylene glycol (PEG) solution in water to mimic drought stress. Each of the treatment parameter, including the control, was performed in three replicates, where ten seeds were sown in every petri plate. For the subsequent seven days the seeds were constantly monitored, watered for the control and

used the PEG solution for the seeds subjected to the drought stress conditions. On the seventh day after the treatment, the percentage of germination, root and shoot length was assessed to determine the optimal time of treatment. The 10-minute and 20-minute time intervals of treatment, 5 LPM flow rate, and 80 W wattage produced better results.

2.2.1.2 Evaluation of Carrier Gas and Stress Conditions

To further examine the effects of varying carrier gases in the CP treatment. Seeds were treated with CP employing both compressed air and nitrogen as the carrier gas, both for the optimally identified 10-minute and 20-minute durations. These treated seeds were then germinated in the presence of complete set of stress conditions, such as 15% PEG-induced drought, 30% PEG-induced drought, 37°C temperature-induced heat stress, combined 15% PEG + 37°C stress, and combined 30% PEG + 37°C stress. Untreated seeds were used under all experimental conditions, as control. Each experiment was replicated three times, with ten seeds per replicate. For the following seven days the seeds were monitored regularly. On day 7, root length, shoot length, and germination percentage were recorded and computed in order to determine the effectiveness of the CP treatment on seeds under different conditions. Data also helped to determine the optimal treatment duration.

2.2.2 Greenhouse experiment

A controlled environment greenhouse experiment was conducted to assess the combined effects of cold plasma (CP) seed treatment and plasma-activated water (PAW) foliar sprays on wheat growth, biomass, and physiological responses under non-stress, drought, and combined drought and heat stress conditions. For this aim, CP and PAW generated using compressed air (CA) were only used, as air plasma generation is more feasible and cost-effective for potential field scale applications compared to nitrogen plasma. The PAW utilized here consisted of 10 ppm (or 10 mg/L) of peroxide, 250 mg/L of nitrate (NO_3^-), and 5 mg/L of nitrite (NO_2^-) and with no measurable chlorine, total hardness (GH) or total alkalinity (TA). PAW was generated with distilled water in a beaker exposed to the cold plasma gas discharge for 30 minutes. The PAW was used on the plants within 2-3 hours of production.

Bulk wheat seeds were treated with cold plasma using compressed air as a carrier gas. The treatment was carried out at 80 W power of discharge with a 5 LPM (Liter Per Minute) gas flow rate for 10 minutes to achieve uniform exposure of the entire set of the seeds in the chamber. Untreated seeds and distilled water spray were used as control. After treatment, both

treated as well as control seeds were planted in plastic tubs with a uniform mixture of field soil collected from upper mid north region of South Australia and sand. Each tub was split into four sections and each section with 5 seeds or each tub with 20 seeds. Each tub had 35 kg dry soil, or 42 kg soil watered to 100 % field capacity (FC). To achieve different moisture regimes, 80 % FC was determined to be 40.6 kg, and 40 % FC was 37.8 kg. These weights were maintained during the experiment by repeated weighing and watering to weight.

The greenhouse was maintained at an average temperature of 17 °C for control as it is optimal for wheat growth. The experiment included 12 treatments as mentioned in Table 2, i.e., two seed treatments (CP-treated and untreated), two foliar sprays (PAW and distilled water), and three environmental conditions (no stress, drought stress, and combined drought + heat stress).

Table-2.1: Summary of treatment combinations used in the greenhouse experiment

Treatment No.	Seed Type	Stress Condition	Temperature (°C)	Field Capacity (%)	Spray Type
1	Untreated	Control	17	80	PAW
2					dH_2O
3		Drought	17	40	PAW
4					dH_2O
5		Drought + heat	37	40	PAW
6					dH_2O
7	Treated (CP)	Control	17	80	PAW
8					dH_2O
9		Drought	17	40	PAW
10					dH_2O
11		Drought + heat	37	40	PAW
12					dH_2O

All the tubs with the treatment combinations as mentioned in table-2 were set up in randomized complete block designs to reduce environmental variation within the greenhouse. The plants were held at 80 % FC during the first four weeks to achieve uniform germination and establishment. At week 4, the designated drought and combined stress treatments had the soil moisture maintained at 40 % FC to induce drought stress. From week 5, foliar sprays of PAW or distilled water were used twice weekly. The sprays had 0.03 % Silwet L-77 silicone surfactant to improve leaf adhesion. The calibration of the electrical hand sprayer showed that a 10-second spray resulted in 33.78 ml of solution; thus, a 12-second spray released about 40 ml – 40.5 ml per tub, about 2 ml per plant per applications or 4 mL per plant per week.

During week 10, the wheat plants were at Feekes growth stage 10.5 (equivalent to Zadoks 50–59) at which the inflorescence completely emerged out of the flag leaf sheath and was visible on all stems. In the greenhouse conditions of this experiment, this occurred at the 10th week after sowing, which was the start of the reproductive period as flowering and grain growth begins and it is a significant period for evaluating stress-response mechanisms.

After exposure to respective stress conditions, plants were sampled (after 72 h of heat stress exposure at 37º C) for further analyses. Five replicates were utilized to conduct biochemical assays such as GABA, alanine, and TBARS analyses, and five replicates to conduct biomass measurements for shoots, roots, and heads. Fresh weights and dry weights were obtained to determine growth responses as well as physiological adaptation to the various CP and PAW treatment combinations in the greenhouse conditions.

2.2.3 Enzyme and chemical analysis

The biochemical measurements were carried out for oxidative stress markers of wheat plants treated with CP and PAW when exposed to different levels of stress. The measurements involved three major physiological indicators: γ-aminobutyric acid (GABA), alanine, and Thiobarbituric acid reactive substances (TBARS) indicative of lipid peroxidation. Five biological replicates (5 wheat plants) per treatment were used for each analysis and two technical replicates (duplicates of each biological sample) were performed for each biological replicate to ensure accuracy and reproducibility. These assays were applied to quantify the responses of wheat plants to treatments applied and if the treatments had an effect on the ability to mitigate oxidative stress and metabolic adaptation.

At the heading time (72 hours following heat-stress treatment), leaves were collected in the pre-cooled 10 ml microcentrifuge tubes that contained a small sterile metal grinding bead and instantly snap-frozen in liquid nitrogen and stored at –80 °C until further analysis. The tube with the frozen leaf samples and the steel beads were vortexed briefly to grind the tissue finely. The 40-70 mg of powdered leaf sample was taken into two sterile pre-weighed Eppendorf tubes, one for the TBARS assay and the other for the GABA and alanine assays. In all treatment groups, five biological replicate were analyzed in duplicate to achieve precision and reproducibility. The data was normalised to the weight of the tissue used in the experiment.

2.2.3.1 Thiobarbituric Acid Reactive Substances (TBARS) assay

Lipid peroxidation levels were measured with the thiobarbituric acid reactive substances (TBARS) assay with minor modification (Singh et al., 2011; Hodges et al., 1998). About 40–70 mg of the frozen powdered material was homogenised in 1 mL of 5 % (w/v) trichloroacetic acid (TCA) and centrifuged at maximum speed for 15 minutes. From the resulting solution, 400 µL of supernatant was transferred into two different tubes: one with 200 µL of 20 % TCA with 0.5 % (w/v) thiobarbituric acid (TBA) and the second one with 200 µL of 20 % TCA without TBA in the blank. Both the preparations were kept at 96 °C for 30 minutes in heating blocks, then instantly cooled in ice and centrifuged at 9,500 g for 10 minutes. Then 100 µL of supernatants were in a 96-well plate in duplicate and the absorbance measured at 440, 532, and 600 nm.

A standard curve was created with malondialdehyde (MDA) concentrations of 0, 1, 3, and 4 nmol made from malondialdehyde tetrabutylammonium salt. The MDA equivalents were computed with the following formulas:

1) [(Abs 532+TBA) - (Abs 600+TBA) - (Abs 532-TBA - Abs600-TBA)] = A

2) [(Abs 440+TBA - Abs 600+TBA) 0.0571] = B

3) MDA equivalents (nmol · ml-1) = ((A-B)/157 000) 106

The principle of TBARS assay involves the interaction of MDA which is a by-product of lipid peroxidation and TBA in acidic and high temperature conditions. This reaction forms a pink MDA-TBA complex that has a high absorption at 532 nm. The intensity of absorbance is directly proportional to the concentration of MDA which indicates the degree of lipid peroxidation which is the level of the oxidative stress in the plant tissue. The final lipid peroxidation values were expressed in nmol MDA g^{-1} fresh weight and reflected the extent of oxidative damage to lipids under each treatment.

2.2.3.2 GABA Enzyme Assays

The quantification of γ-aminobutyric acid (GABA) and alanine was done by enzyme-coupled spectrophotometric assays. Extraction involved the treatment of about 50 mg of the powdered frozen tissue in 400 µL of 100 % methanol. This mixture was incubated at 25 °C for 10 minutes and evaporated to dryness by the SpeedVac concentrator. The dried tissue was resuspended in 500 µL of 70 mM lanthanum chloride and subjected to 100 rpm shaking for 15 minutes and then centrifuged at 12,000 rpm for 5 minutes. Aliquot of 400 µL of supernatant was transferred

into new tube containing 160 µL of 1 M KOH and subjected to 100 rpm shaking for 5 minutes and then centrifuged at 12000 rpm for 5 mins. The resulting supernatant was transferred into new tube and was stored at –80 °C until further analysis.

The GABA assay reaction mixture contained final concentrations of 900 µL of 0.5 M potassium pyrophosphate buffer (pH 9.0), 19.8 µL of 2-mercaptoethanol, 1.5 ml of 20 mM α-ketoglutarate, and 750 µL of 10 mM NADP for 60 samples. To each well of a 96-well microplate, 45.2 µL sample extract was combined with 52.8 µL of reaction mixture, and baseline absorbance at 340 nm for three cycles (120 s each) at 25 °C was measured. The reaction started with the addition of 2 µL of GABase enzyme (5 U/ml) and absorbance at 340 nm for 30 cycles was measured to assess NADPH formation. The principle of the GABA assay is the enzyme transformation of GABA to succinic semialdehyde by GABA transaminase in the presence of NADP+, and the subsequent formation of NADPH as the by-product. The NADPH formation is stoichiometric with the GABA content, which is measured by the increase in the absorbance at 340 nm using spectrophotometer. This indicates the relative concentration of GABA in each sample. The concentration of GABA was determined with the help of a standard curve made with 0, 10, 50, and 100 µM GABA solutions.

2.2.3.3 Alanine Enzyme Assays

The alanine assay was performed using the same extract prepared for GABA enzyme assay. The assay involved the mixing of 10 µL sample extract with 187 µL master mix comprising final concentration of 10.8 ml of 0.05 M sodium carbonate buffer at pH 10 and 420 µL of 30-mM NAD+ for 60 samples. Pre-reaction reading was performed at 340 nm for three cycles and were followed by the addition of 3 µL alanine dehydrogenase (0.1 U μL^{-1}) in order to start the reaction. The absorbance was then measured at 340 nm for 30 cycles at 25 °C. The alanine assay is based on the enzymatic conversion of L-alanine to pyruvate by alanine dehydrogenase, with the reduction of NAD^+ to NADH. The increase in absorbance at 340 nm corresponds to NADH formation and reflects the alanine content in the sample, indicating metabolic activity and stress-related amino acid accumulation. The concentrations of alanine were determined using standard curve made with 0, 50, 100, 250, 500, and 750 µM alanine solutions. All the absorbance data were processed in Microsoft Excel and GraphPad Prism v10.2 (blank subtraction, standard curve fit, and determination of concentration). Results of the finals for GABA and alanine were given in µmol g^{-1} fresh weight, which indicates their relative accumulation in CP and PAW treatment under various stress conditions.

Chapter 3: Results

Results chapter reports the experimental findings from the application of cold plasma technology, PAW and their combined effects on the scepter wheat plants (*Triticum aestivum*) to enhance its abiotic stress tolerance. There are the results in four major sections, each of them covering a distinct research objective.

SECTION-1

Section-1 covers first part of Aim 1, exploring the optimal treatment duration of cold plasma seed treatment and its impact on germination and initial seedling morphology. This encompasses a comparison of germination percentage, root and shoot elongation under ideal control conditions and under osmotic stress conditions.

3.1.1 Germination percentage of seedlings

The percentage germination (Figure-3.1.1) was found to be consistently high in all the treatments in the situations of non-stress and drought induced by 15% PEG and were found to be 90-100%. There was no significant difference in the effect of plasma treated seeds and untreated seeds on germination, so exposure of cold plasma did not have any adverse effect on germination under moderate stress. These findings indicate that seed germination is not negatively or positively influenced by cold plasma treatment under moderate drought stress.

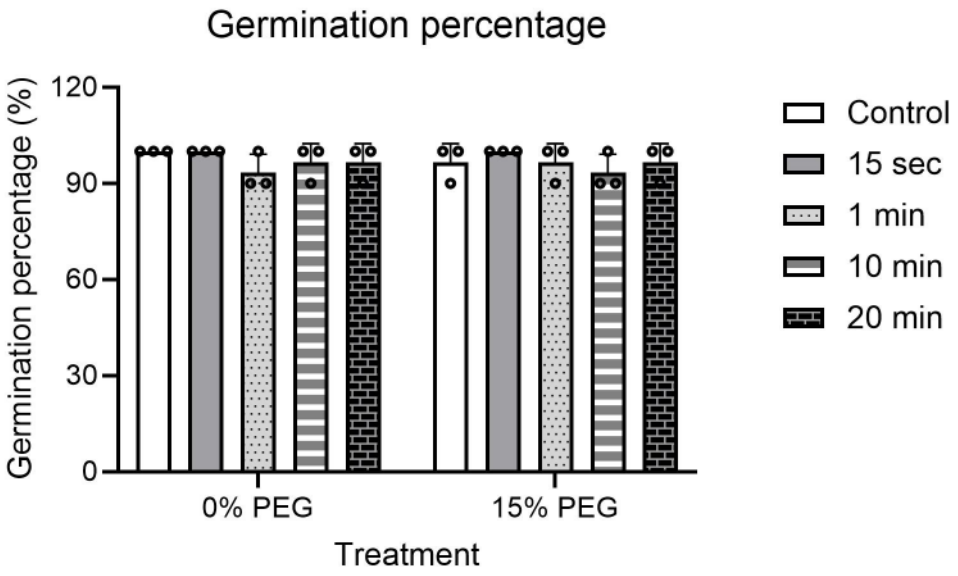

Figure-3.1.1 Germination percentage (%) of wheat seeds treated with compressed air (CA) plasma at different time duration (15 sec, 1 min, 10 min, 20 min) under 15% PEG induced water stress and non-stress conditions. Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and germination percentage measured after 7 days. Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.1.2 Root length of seedlings

CP treated seeds under non-stress conditions exhibited a significant difference in root length (Figure-3.1.2) as compared to the untreated control with the most root elongation observed in the 10 minutes treatment (151 mm). This indicates that exposure to cold plasma had a positive effect on early root development, which may have been caused by the increase of seed surface reactivity and uptake of water. On the contrary, the root length in general was less in all treatments under 15% PEG-induced drought stress indicating the inhibitory effect of the osmotic stress on root growth. But plasma treated groups with shorter time of plasma exposure such as 15 sec and 1 min of seed treatment had low root length (average of 55.3 mm and 64.3 mm) than the untreated control (average of 73.3 mm). However, the seed groups subjected to 10 and 20 min had notable greater root lengths (average of 80 mm and 80.6 mm) as compared

to the non-treated control (average of 73.3 mm), but it is not a significant difference, which suggests better stress tolerance and the adaptive root growth. These findings have indicated that 10-minute optimal plasma exposure can induce root growth in ideal conditions and partially benefit under drought induced stress conditions.

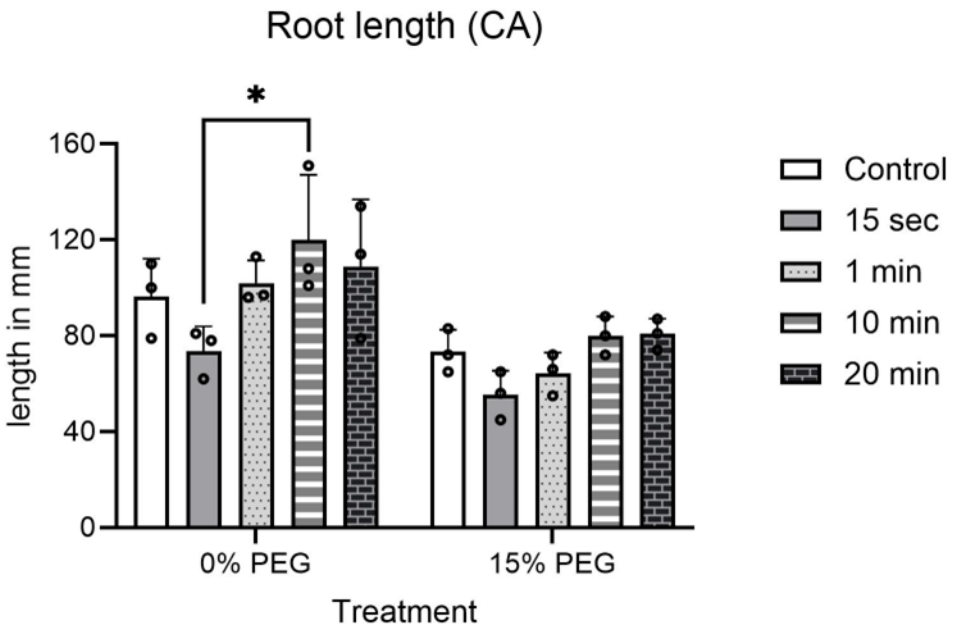

Figure-3.1.2 Root length (mm) of wheat seeds treated with compressed air plasma (CA) at different time durations (15 sec, 1 min, 10 min, 20 min) under non-stress conditions and 15% PEG induced water stress conditions. Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and root lengths measured after 7 days. Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.1.3 Shoot length of seedlings

Shoot length was relatively uniform at non-stress conditions with no significant difference (Figure-3.1.3) between all the duration of plasma treatment and the untreated control. However, the plasma treated particularly the 10 min treatment (average of 80.3 mm) had notable difference in the shoot length with untreated control seed groups (average of 67 mm). Also, the reduction in shoot length of 20 min treatment (mean of 70.6 mm) indicates that the over

exposure of cold plasma to seeds under the optimum conditions may inhibit the shoot growth. However, shoot length was significantly shortened in all groups at 15% PEG-induced drought stress, indicating that the osmotic stress has a strong inhibitory impact on the shoot elongation of wheat. In spite of this, the shoot length of the plasma-treated seeds was significantly increased over the untreated seeds (average of 14 mm). Seeds with the treatment of 10-minute (average of 28.3 mm) and treatment of 20-minute (average of 31 mm) exhibited the most significant difference in shoot length compared to the untreated control. This shows that exposure to cold plasma increased shoot growth resilience under drought stress, which could be as a result of increasing seed vigor, water uptake, and drought-responsive physiological mechanisms. Overall, plasma treatment, especially 10 minutes treatment was able to effectively mitigate drought-induced reduction in shoot growth under both the ideal and PEG-induced drought conditions.

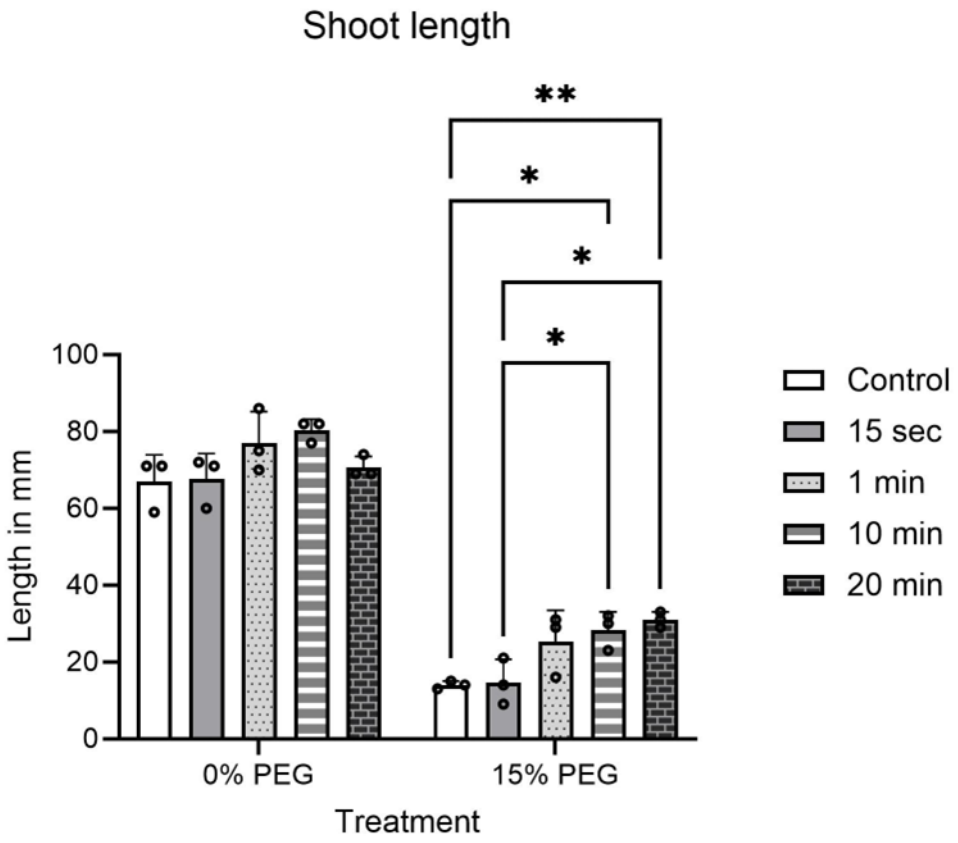

Figure-3.1.3 Shoot length (mm) of wheat seeds treated with compressed air (CA) plasma at different time duration (15 sec, 1 min, 10 min, 20 min) under non-stress conditions and 15% PEG induced water stress conditions. Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes,

incubated as per the experimental conditions and shoot lengths measured after 7 days. Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

SECTION-2

This second section deals with second part of Aim 2, exploring the impact of cold plasma treatment using different carrier gases compressed air and nitrogen under different stress conditions including non-stress conditions (control), drought, and combined drought + heat stress conditions.

3.2.1 Germination percentage of seeds treated with CP generated from compressed air (CA)

Under combined water stress (30% PEG) + heat stress (37° C), seed treatment with the CA cold plasma increased the germination percentage significantly at 10 min and 20 min treatments in comparison to the control untreated seeds, where the 20 min treatment showed the highest effect (F). Whereas, under non-stress condition (Figure-3.2.1) (A), the germination percentage did not show variation among untreated and treated seeds. Similarly, under 37 °C (heat) stress (B), moderate water stress (15% PEG) (C), and under the presence of moderate (15%) PEG stress in combination with 37° C heat stress (D), the use of plasma did not show significant differences in germination from control untreated seeds. At 30% PEG with severe osmotic stress, no significant differences were detected between untreated and treated seeds (E).

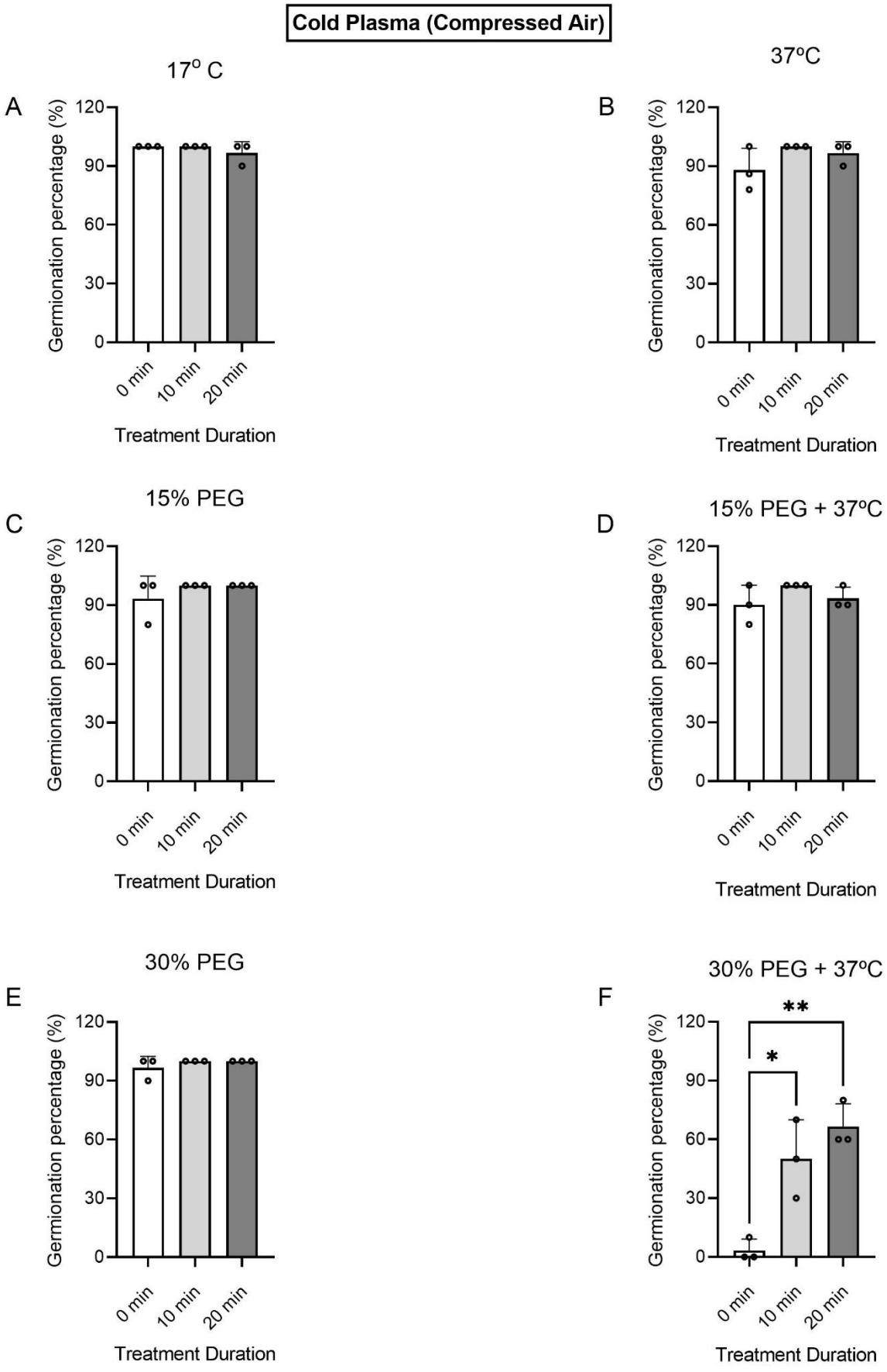
Cold Plasma (Compressed Air)
17° C
37°C
15% PEG
15% PEG + 37°C
30% PEG
30% PEG + 37°C
A
B
C
D
E
F
Germionation percentage (%)
120
90
60
30
0
0 min
10 min
20 min
Treatment Duration
*
**

Figure-3.2.1 Germination percentage (%) of wheat seeds treated with cold plasma generated from Compressed air (CA). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and germination percentage was calculated after 7 days. A) Germination percentage (%) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Germination percentage (%) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Germination percentage (%) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.2.2 Root length of wheat treated with CP generated from CA

CA cold plasma treated at 10 minutes duration had notable influence on root elongation under non-stress conditions (Figure-3.2.2) (A) and moderate stress conditions (15% PEG) (C) compared to untreated seeds. At 30% PEG induced severe osmotic stress, 20 min CA plasma treatment significantly enhanced root elongation in relation to untreated controls (E). In contrast, root elongation declined markedly under heat stress alone (B) and (15% & 30%) PEG + heat dual-stress (D, F) in both treated and untreated seeds. Generally, there were beneficial effects of CA cold plasma treatment on wheat seeds under stress compared to the untreated seeds but in case of heat stress alone or in combined heat and drought stress inhibited root elongation across all treatment cases.

Cold Plasma (Compressed Air)

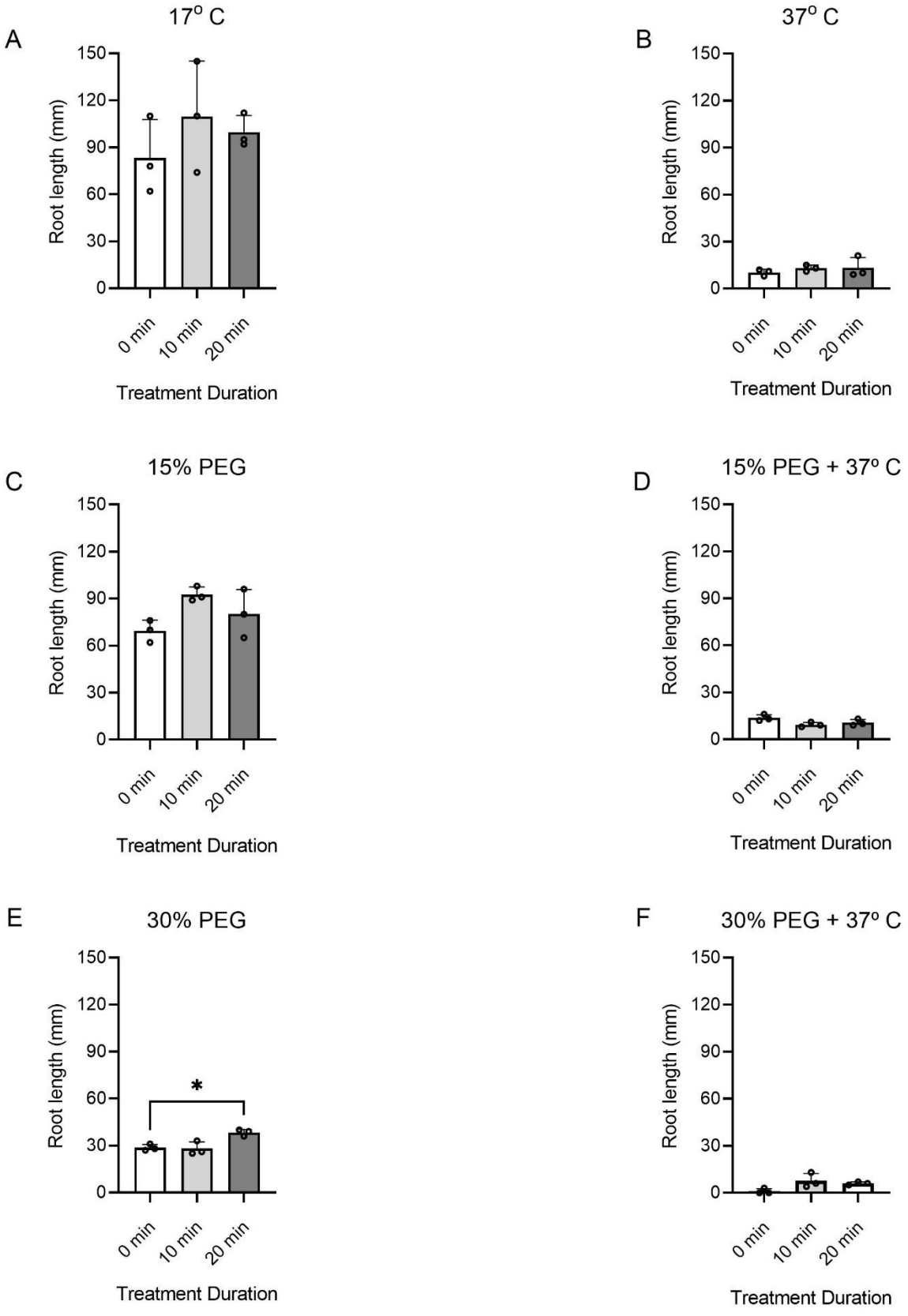

Figure-3.2.2 Root length (mm) of wheat seeds treated with cold plasma generated from Compressed air (CA). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and root lengths measured after 7 days. A) Root length (mm) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Root length (mm) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Root length (mm) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.2.3 Shoot length of wheat treated with CP generated from CA

CA cold plasma treatment at 20 min (Figure-3.2.3) (C) duration significantly enhanced the shoot elongation under moderate osmotic stress (15% PEG) in comparison to untreated seeds. Similarly, under 15% PEG + heat stress (37º C), 10 min duration of the CA plasma treatment enhanced shoot development in comparison to untreated seeds (D). In contrast, severe osmotic stress (30% PEG) suppressed the shoot elongation completely irrespective of plasma treatment (E). Under 30% PEG + heat severe stress combination, 10 min and 20 min duration of the CA plasma treatments enhanced the shoot elongation significantly in comparison to untreated seedlings (F). No differences were observed under non-stress (A) or under heat (37º C) stress alone (B). Overall, the CA cold plasma treatment showed a positive effect on shoot elongation under moderate osmotic stress (15% PEG) and combination stress regimes, where the 10 min duration showed the best effectiveness, whereas severe osmotic stress (30% PEG) alone, heat stress at 37º C, and non-stress conditions (control) significantly inhibited seedling shoot development in all cases.

Cold Plasma (Compressed Air)

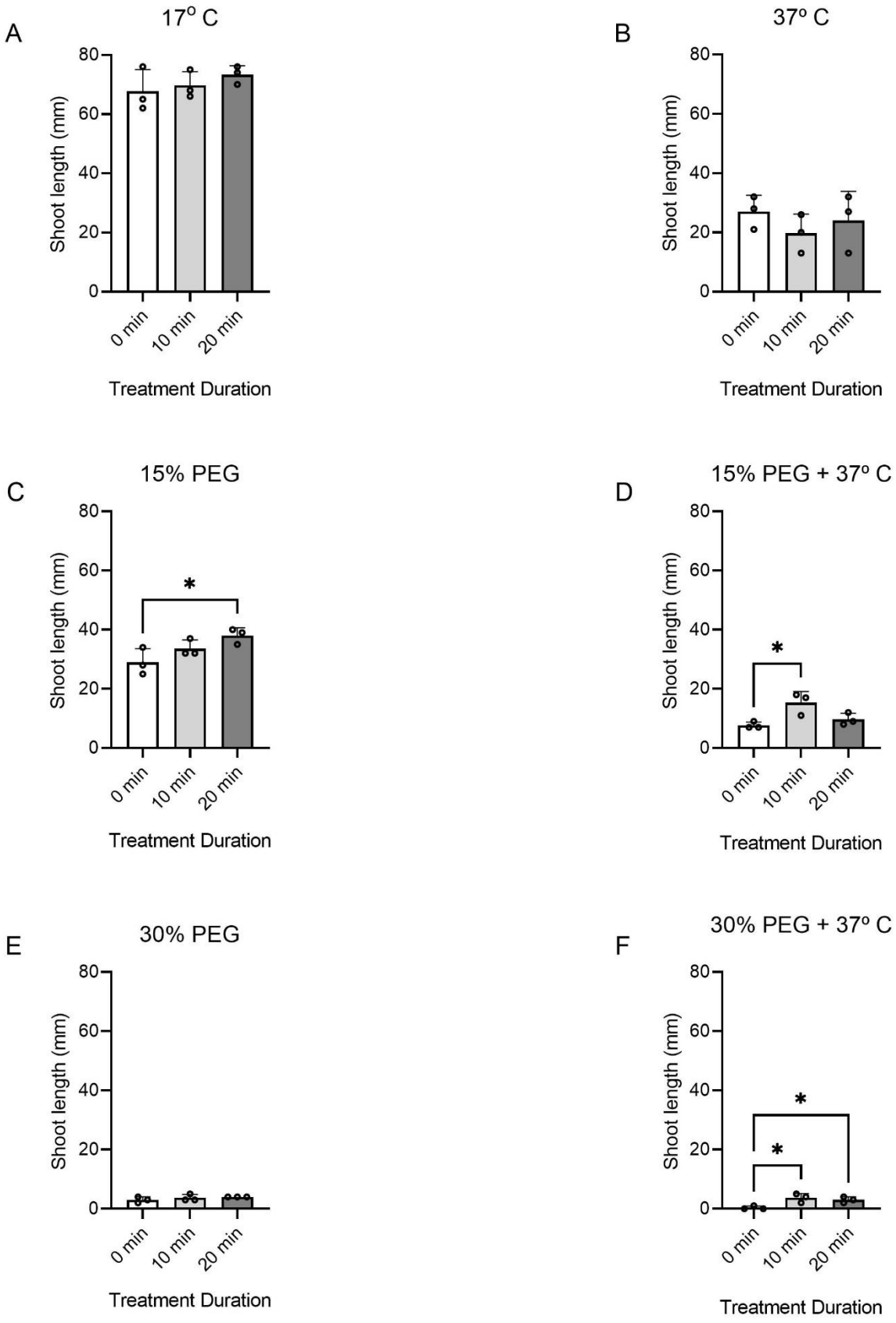

Figure-3.2.3 Shoot length (mm) of wheat seeds treated with cold plasma generated from Compressed air (CA). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and shoot lengths measured after 7 days. A) Shoot length (mm) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Shoot length (mm) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Shoot length (mm) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.2.4 Germination percentage of wheat treated with CP generated from nitrogen (N2)

Under the combined severe stress (30% PEG + 37° C) (F), N_2 plasma significantly enhanced germination, with the 20-min exposure significantly greater than the control, and the 10-min exposure also showed a positive trend compared to the control. Also, under non-stress conditions (Figure-3.2.4) (A), germination remained ~100% with no difference among untreated and N_2-treated seeds (10 or 20 min). Heat stress at 37 °C (B) resulted in equal germination among control (untreated seeds) and 20-min treated seeds, whereas the 10-minute treated seeds showed a slight improvement in germination percentage compared to the other two treatments (control and 20-min treatment). Moderate osmotic stress (15% PEG) (C) had greater mean germination in plasma-treated seeds, but the variations were not significant. Combined 15% PEG water stress + 37° C heat stress (D) showed better germination percentage in 10 and 20-minutes N2 cold plasma treatment compared to untreated seeds (control). Severe osmotic stress (30% PEG) alone (E) did have a slight impact on germination percentage of treated seeds (10 & 20 min) compared to untreated seeds, but no significant difference was observed. change germination among treatments.

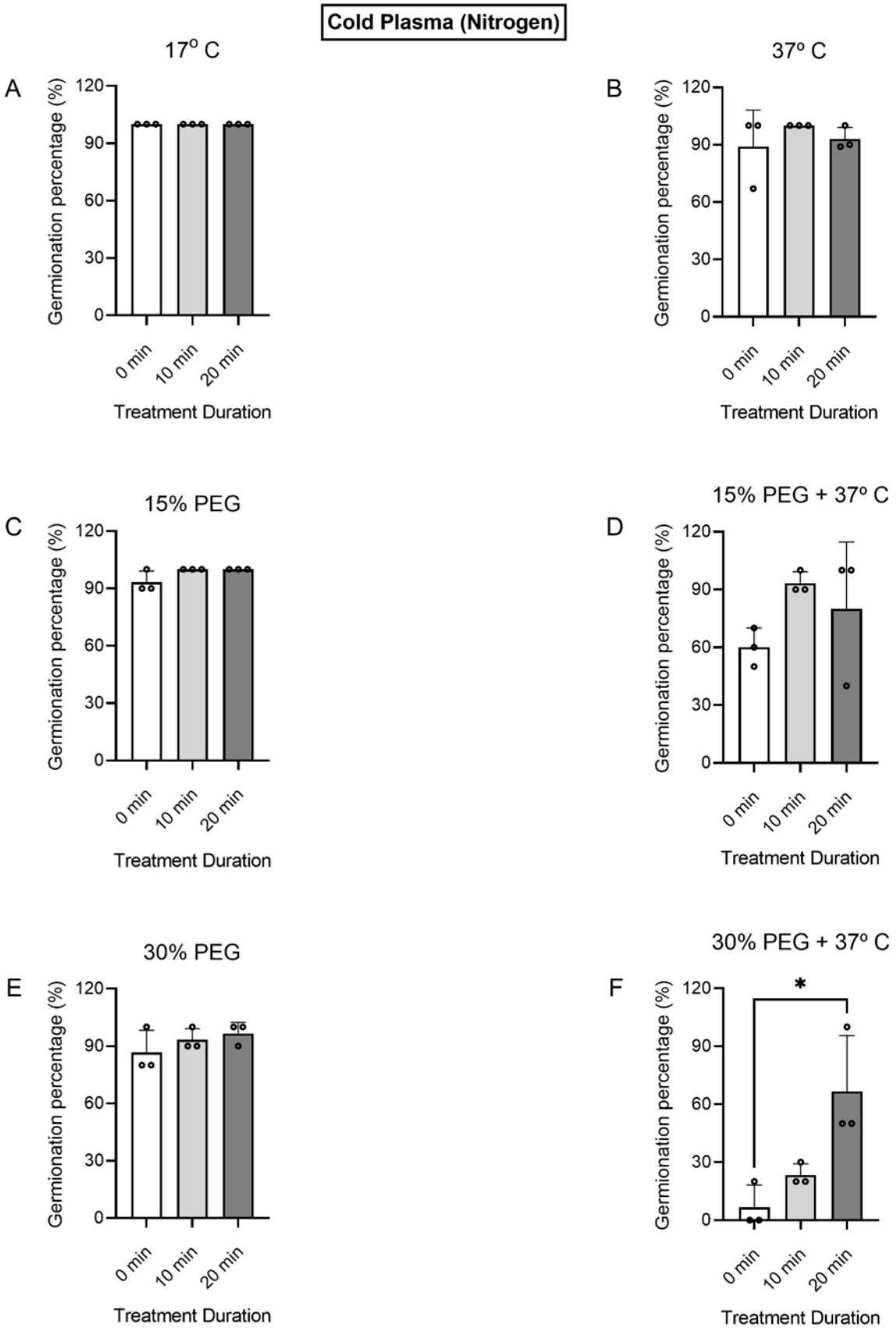
Cold Plasma (Nitrogen)
17° C
37° C
A
B
C
D
E
F
15% PEG
15% PEG + 37° C
30% PEG
30% PEG + 37° C
Germionation percentage (%)
120
90
60
30
0
0 min
10 min
20 min
Treatment Duration
*

Figure-3.2.4 Germination percentage (%) of wheat seeds treated with cold plasma generated from Nitrogen (N2). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and germination percentage was calculated after 7 days. A) Germination percentage (%) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Germination percentage (%) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Germination percentage (%) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.2.5 Root length of wheat treated with CP generated from nitrogen (N_2)

Under non-stress conditions (A) (Figure-3.2.5), even though N_2 plasma treatment had an impact on root length, there was no significant impact on root length relative to control, untreated seeds. Under heat stress at 37 °C (B), root elongation was intensely suppressed in treated and control seeds, and no alleviation from plasma treatment was apparent. At moderate osmotic stress (15% PEG), plasma treatment, both 10-minute and 20-minute duration significantly increased root length relative to untreated controls, especially the 10-minute treatment duration (C). Under combined 15% PEG + 37º C heat stress (D), plasma treatment resulted in an average recovery of root elongation, but still less than the moderate water stress alone (15% PEG). At severe osmotic stress (30% PEG), there was a small but significant increase in root length in 20 min plasma-treated seeds relative to control seeds (E). In comparison, combined severe stress (30% PEG + 37 ºC heat) suppressed root elongation entirely across all treatments (F).

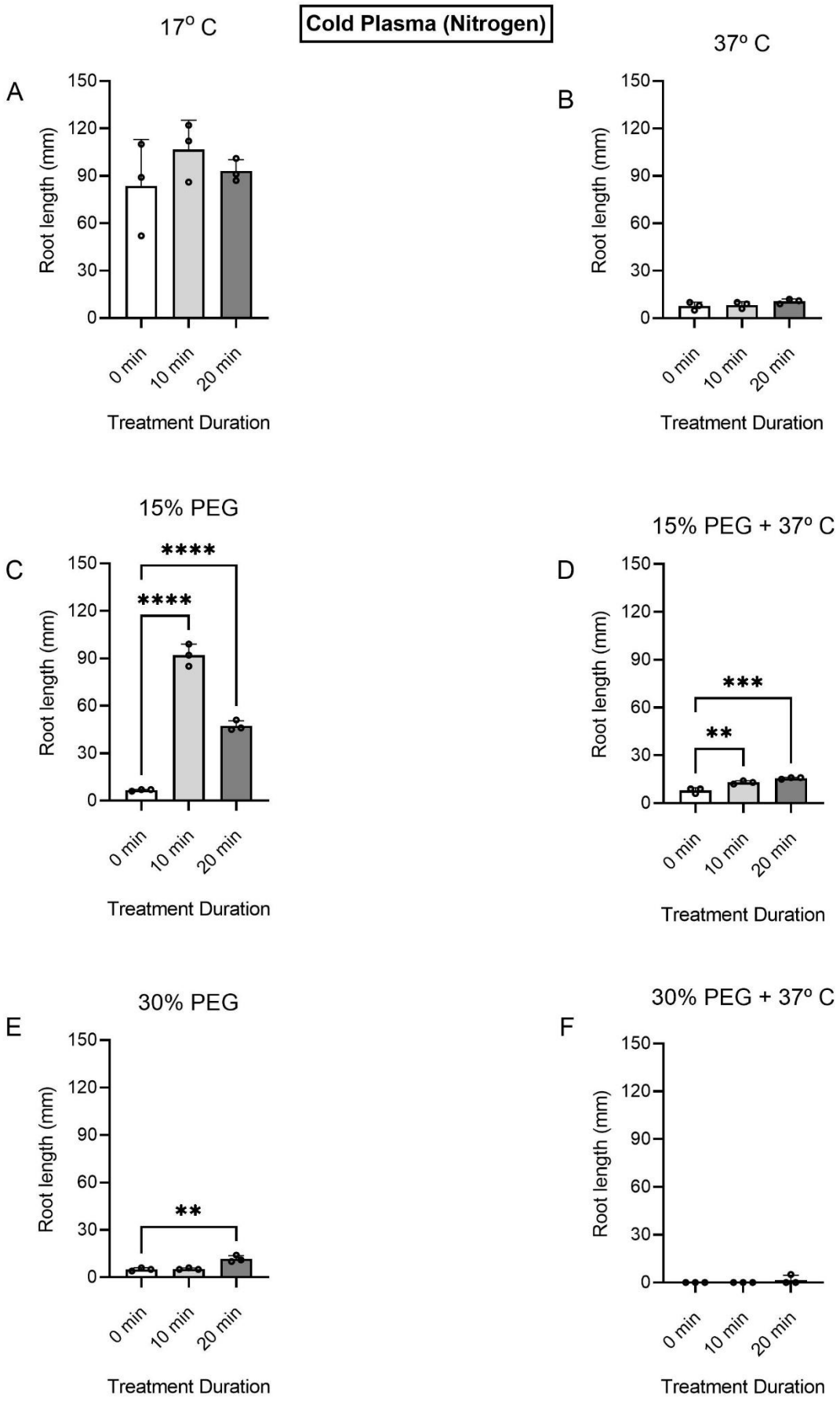
Cold Plasma (Nitrogen)
A
17° C
B
37° C
C
15% PEG
D
15% PEG + 37° C
E
30% PEG
F
30% PEG + 37° C
Root length (mm)
0
30
60
90
120
150
0 min
10 min
20 min
Treatment Duration
****
****
**
***
**

Figure-3.2.5 Root length (mm) of wheat seeds treated with cold plasma generated from Nitrogen (N2). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and root lengths measured after 7 days. A) Root length (mm) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Root length (mm) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Root length (mm) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

3.2.6 Shoot length of wheat treated with CP generated from nitrogen (N_2)

Under non-stress (A) (Figure-3.2.6), N_2 plasma (10 or 20 min) did not significantly impact the shoot length compared with the control, untreated seeds. (B) Under heat stress at 37° C, shoot length significantly improved after N_2 plasma exposure at both 10- and 20-minute treatment duration in comparison with control, untreated seeds. (C) Under osmotic stress of 15% PEG, shoot length significantly improved through N_2 plasma treatment, with the best response being that of the 10-min exposure over control, untreated seeds. (D) Under 15% PEG + 37 °C combined stress, shoots were short, and plasma exposure provided slight positive impact but no obvious benefit. (E) Severe osmotic stress, 30% PEG, reduced shoot growth across all treatments. (F) Severe combined stress, 30% PEG + 37 °C, eliminated shoot elongation regardless of plasma exposure.

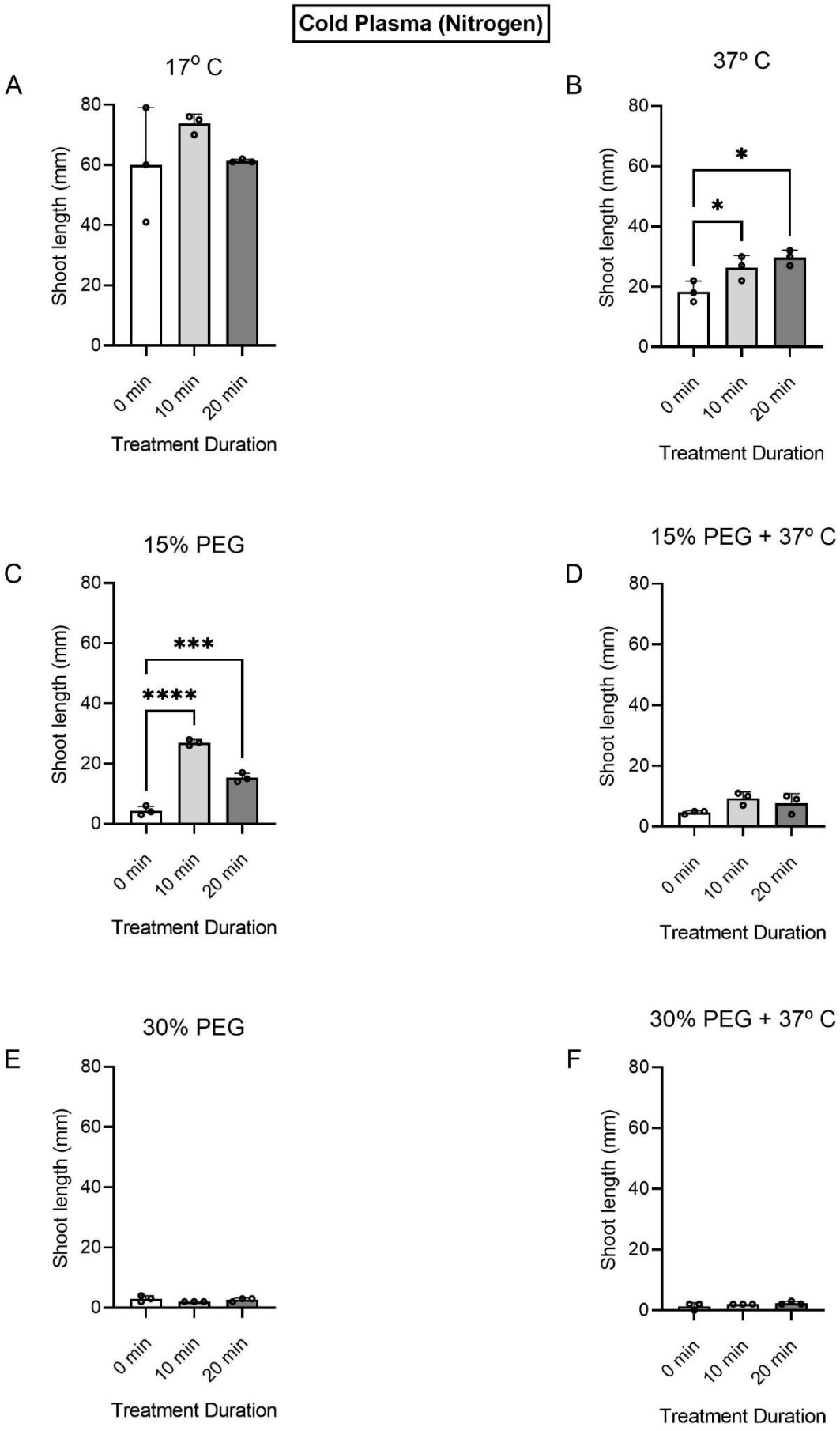
Cold Plasma (Nitrogen)
A
17° C
B
37° C
C
15% PEG
D
15% PEG + 37° C
E
30% PEG
F
30% PEG + 37° C
Shoot length (mm)
0
20
40
60
80
0 min
10 min
20 min
Treatment Duration
*
*
***
****

Figure-3.2.6 Shoot length (mm) of wheat seeds treated with cold plasma generated from Nitrogen (N2). Ten seeds per replicate (three biological replicates) were either treated for 0, 10 or 20 min, plated on moist filter papers in petri dishes, incubated as per the experimental conditions and shoot lengths measured after 7 days. A) Shoot length (mm) of seedlings from untreated and treated seeds in non-stress conditions. (B) Seedlings subjected to heat stress at 37 °C with and without plasma treatment. (C) Seedlings with and without plasma treatment under water stress induced by 15% PEG. (D) Shoot length (mm) of the untreated and plasma treated seeds subjected to combined water stress (15% PEG) and heat stress (37 °C). (E) Seedlings subjected to 30% PEG induced water stress in untreated and plasma treated seedlings. (F) Shoot length (mm) of the untreated and plasma treated seeds subjected to combined water stress (30% PEG) and heat stress (37 °C). Graphs were created using GraphPad Prism v10.2. One-way Anova was used to test for statistical significance with differences at the $p < 0.05$ considered significant.

SECTION-3

Section-3 consists of the results of aim-2, effects of combined treatment of CP treated seeds, and application of Plasma-Activated Water (PAW) as foliar spray. Its effects on the growth and biomass accumulation of aged wheat plants during the heading stage. This analysis has been done using control, drought and heat + drought stress conditions.

3.3.1 Root weight of wheat at the heading stage

The application of PAW foliar spray had a significant inhibitory influence on root growth under control or non-stress control conditions (Figure-3.3.1) irrespective of the initial seed treatment. In plants from untreated seeds, PAW significantly lowered root fresh weight ($p<0.001$) (A) and dry weight ($p<0.01$) (C) under control conditions. There was a comparable significant reduction in root fresh ($p<0.05$) (B) and dry biomass ($p<0.05$) (D) in the control treatment for plants sowed from treated seeds. However, this inhibitory influence was overturned under abiotic stress. Particularly under drought stress, the PAW application increased root dry weight (C) significantly ($p<0.05$) in the plants grown from untreated seeds and it showed a notable increase in root dry weight in plants from the treated seeds (D). For the treated seeds, PAW's

adverse influence was fully nullified under combined drought-heat stress (D) where no significant variation relative to controls was noted. This means that while PAW is not beneficial under optimal conditions, it has a beneficial effect when the plants are under abiotic stress. Also, the decrease in dry weight compared to the fresh weight indicates the water retention ability of the plants.

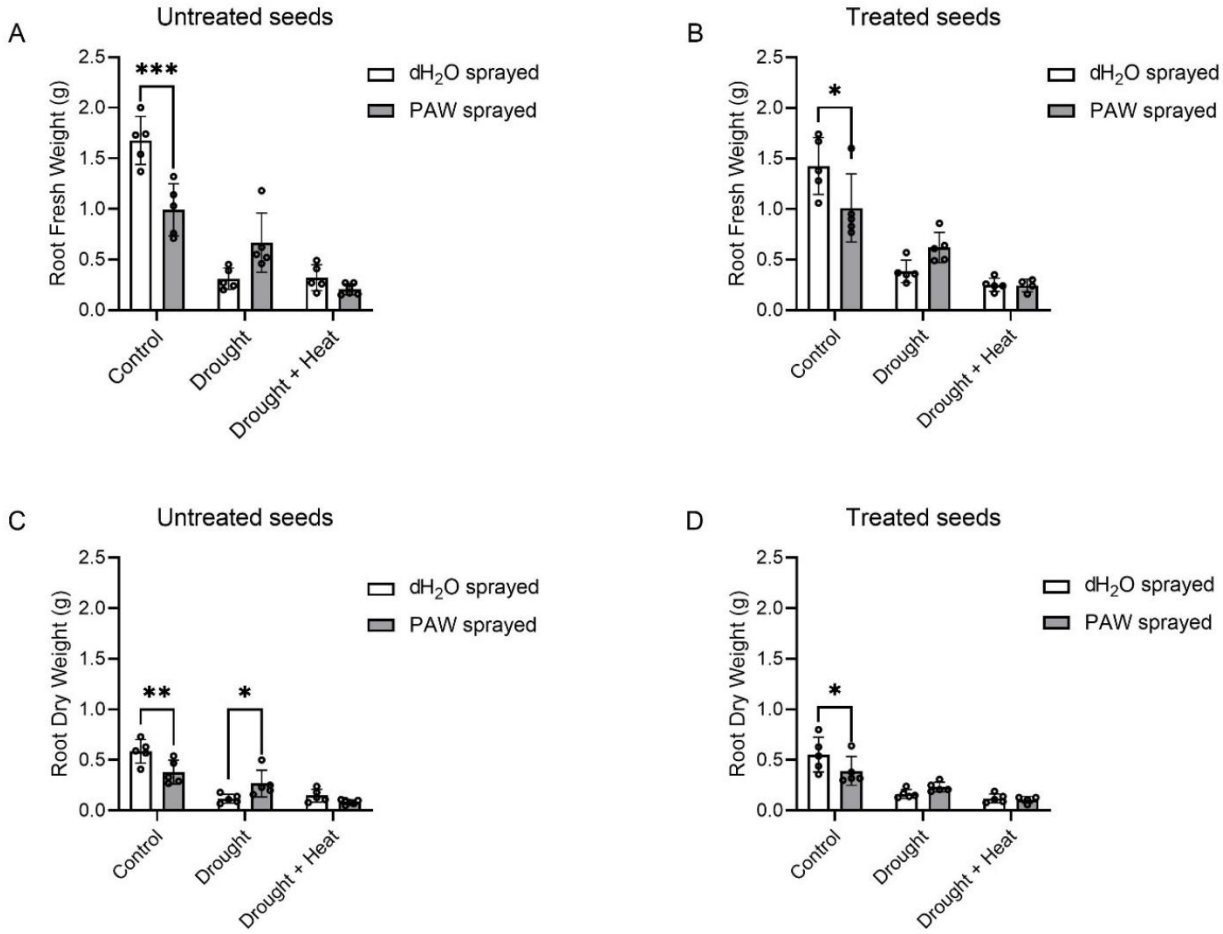

Figure-3.3.1 Root weight (g) of wheat at heading stage treated with cold plasma generated using compressed air. Five biological replications were measured for each treatment. The treatment was carried out at 80 W power of discharge with a 5 LPM (Liter Per Minute) gas flow rate for 10 minutes**.** Plants in tubs grew under greenhouse conditions, and PAW or distilled water (dH_2O) were sprayed during growth. PAW was generated using plasma bubble reactor at 5 LPM flow rate for 30 minutes. Plants were subjected to various stress conditions such as no stress condition (control), drought, or combined drought and heat conditions. Roots were

harvested at the heading stage and weighed instantly for fresh weight analysis and then oven dried for dry weight analysis. (A) Root fresh weight (g) of plants germinated in untreated seeds sprayed with dH_2O or PAW under control (normal), drought (D), or combined drought and heat (D + H) conditions. (B) Root fresh weight (g) of plants germinated in plasma-cold-treated seeds subjected to the same spray and stress regimes. (C) Root dry weight (g) of plants germinated in untreated seeds subjected to similar stress conditions. (D) Root dry weight (g) of plants germinated from cold plasma treated seeds sprayed with dH_2O or PAW under different stress conditions. One-way ANOVA used for determining the data statistical significance wherein $p < 0.05$ used for significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Graphs plotted with GraphPad Prism v10.2.

3.3.2 Shoot weight of wheat at the heading stage

The effect of PAW foliar spray on shoot biomass was significantly conditional and dependent on the seed priming treatment and the imposition of abiotic stress (Figure-3.3.2). For the plants that developed from non-treated seeds, PAW application showed a marginal and slightly adverse impact significantly reducing shoot dry weight (C) under control conditions but failing to show a notable impact when the controls were subjected to combined drought heat stress. Whereas under drought alone stress condition, untreated seeds (A & C) sprayed with PAW showed notably improved biomass compared to the control dH_2O spray. For the treated seeds, PAW application to plants showed a distinct adverse effect under control conditions, significantly reducing both shoot fresh (B) and dry weight (D). Notably, this impact was fully reversed when the controls were exposed to drought stress such that the PAW spray showed a significant benefit compared to the controls that were sprayed with water by enhancing the shoot fresh (B) and dry weight (D). This indicates that for the primed seed, PAW foliar spray does not increase vegetative growth under favourable control conditions, but it acts as a biostimulator when the condition is drought stress such that it improves the shoot biomass (Figure-3.3.2). Under severe combined drought and heat stress, no notable difference was observed in plants grown from both the untreated (C) and treated seeds (D). Also, the decrease in dry weight compared to the fresh weight indicates the water retention ability of the plants.

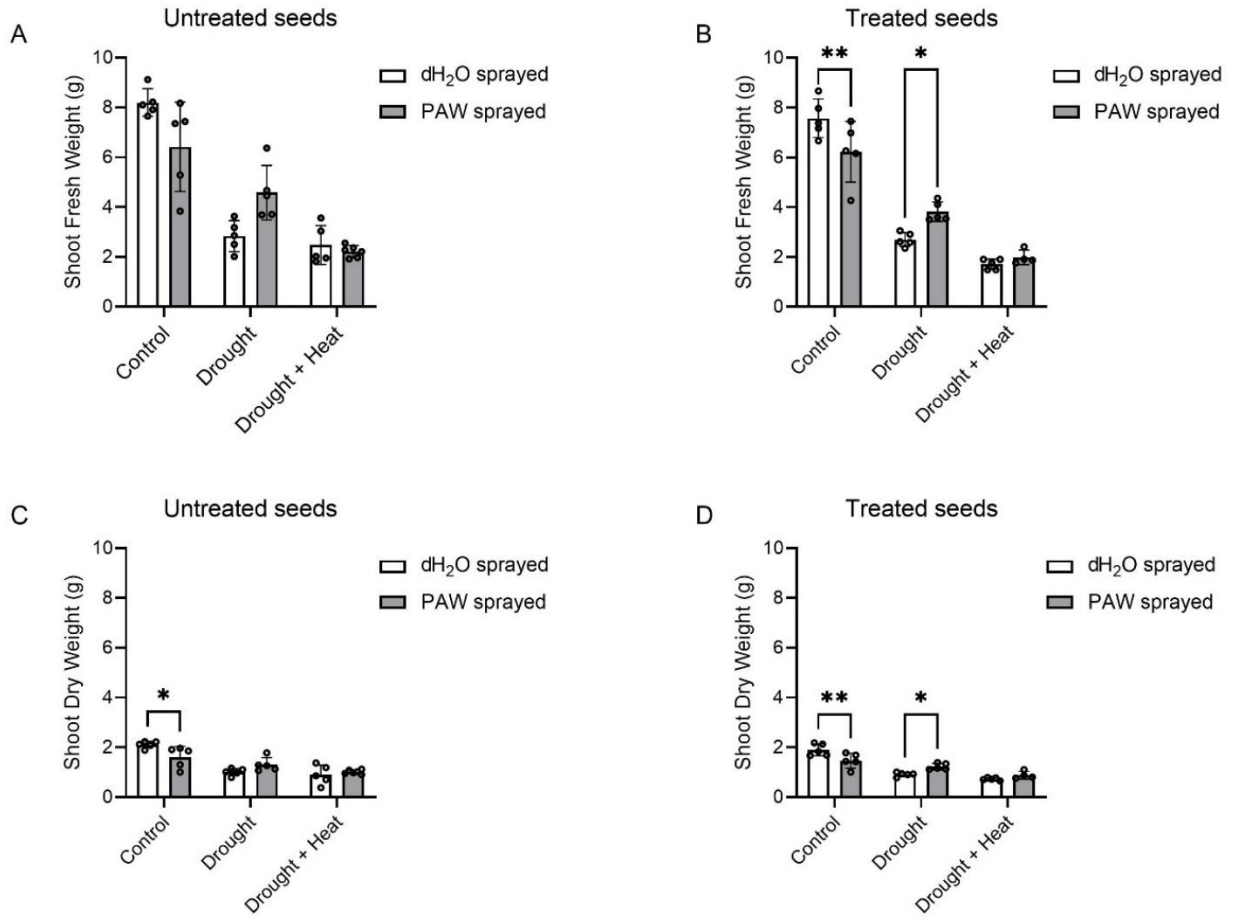

Figure-3.3.2 Shoot weight of wheat crop at heading stage treated with cold plasma generated using compressed air. Five biological replications were measured for each treatment. The treatment was carried out at 80 W power of discharge with a 5 LPM (Liter Per Minute) gas flow rate for 10 minutes. Plants in tubs grew under greenhouse conditions, and plasma-activated water (PAW) or distilled water (dH_2O) were sprayed during growth. Plants were subjected to various stress conditions such as no stress condition (control), drought, or combined drought and heat conditions. Shoots were harvested at the heading stage and weighed instantly for fresh weight analysis and then oven dried for dry weight analysis. (A) Shoot fresh weight (g) of plants germinated in untreated seeds sprayed with dH_2O or PAW under control (normal), drought (D), or combined drought and heat (D + H) conditions. (B) Shoot fresh weight (g) of plants germinated in plasma-cold-treated seeds subjected to the same spray and stress regimes. (C) Shoot dry weight (g) of plants germinated in untreated seeds subjected to similar stress conditions. (D) Shoot dry weight (g) of plants germinated from cold plasma treated seeds sprayed with dH_2O or PAW under different stress conditions. One-way ANOVA

used for determining the data statistical significance wherein $p < 0.05$ used for significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Graphs plotted with GraphPad Prism v10.2.

3.3.3 Head emergence of wheat

Table-3.3 Head emergence of wheat at week 10 under different environmental conditions

Conditions	Foliar spray	Seed Treatment	No. of. Head Emergence (Week-10)					
			Day-1	Day-2	Day-3	Day-4	Day-5	Day-6
Control	PAW	Untreated	0	3	7	9	10	10
		CP treated	0	1	5	7	9	10
	dH2O	Untreated	0	3	6	7	10	10
		CP treated	0	1	3	6	8	10
Drought	PAW	Untreated	1	4	8	10	10	10
		CP treated	2	4	8	9	10	10
	dH2O	Untreated	5	7	10	10	10	10
		CP treated	4	6	9	10	10	10
Drought + Heat	PAW	Untreated	4	7	9	10	10	10
		CP treated	5	8	10	10	10	10
	dH2O	Untreated	4	7	9	10	10	10
		CP treated	5	7	10	10	10	10

Differences in the head emergence were also observed depending on the various stress conditions as shown in table-3.3. There was an overall delay in head emergence under control conditions as compared to wheat subjected to drought and combined drought + heat stress. By day 4 in week 10, nearly all the plants in drought and combined stress treatment had developed heads, whereas wheat in the control group partially emerged, which indicates the delay in plant development. In the control, cold plasma (CP)-treated seed wheat had a delayed head emergence compared to the plants with untreated seed with no notable difference between PAW- and water-sprayed plants. In drought alone condition, the head was emerging a little bit earlier in the plants from untreated seeds than CP-treated ones, but the difference was not significant. Also, water-sprayed plants recorded a minor earlier head emergence compared to PAW-sprayed plants under drought alone condition. Head emergence was earlier in general under combined drought and heat stress, than in the control and drought conditions. Nevertheless, there were no notable or significant differences in plants of CP-treated and untreated seeds or PAW- and water-sprayed. This observation indicates that the stress conditions of drought and heat stress could have accelerated the growth from vegetative to reproductive phase, as an adaptive physiological response to environmental stress.

3.3.4 Head weight of wheat at the heading stage

Biomass of the reproductive structures was measured at the stage of heading as the fresh and dry weight of the heads and the results were shown in Figure-3.3.3. As head dry weight is the most accurate measure of true biomass accumulation, it was the primary focus of this analysis. There was an overall trend in the fresh weight (A & B), where both the drought alone and the combined drought and heat stress decreased overall biomass of the head as compared to the non-stress control. Although there was a significant decrease in fresh weight of PAW sprayed plants with untreated seeds in control conditions ($p<0.05$) (A), in the case of head dry weight, no significant differences were observed in both the untreated fresh and dry weight group (A & C). Although no significant differences were observed in the control or moderate drought alone stress conditions in the treated group (B & D) the greatest result of this analysis was found in the head dry weight of the plants grown out of plasma-treated seeds followed by PAW spray (D). The combined cold plasma treatment of seeds and the subsequent treatment of the established plants with PAW showed a significantly greater head dry weight ($p<0.05$) (D) in the most severe combined drought and heat stress condition when compared to dH_2O sprayed plants. This shows that there is a positive interaction between the pre-treatment of the seed and the foliar PAW spray, which increases accumulation of reproductive biomass in adverse abiotic stress.

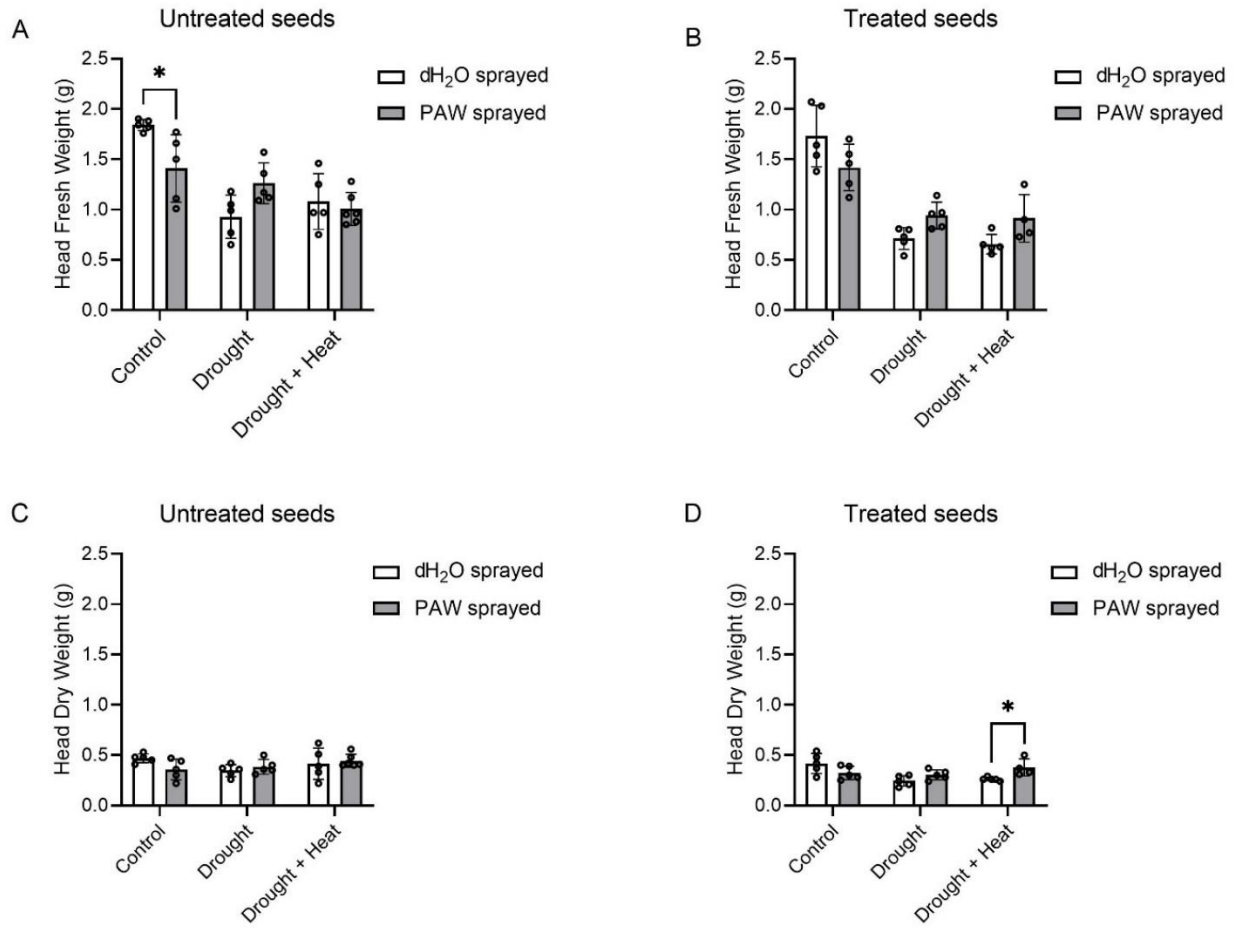

Figure-3.3.3 Head weight (g) of wheat crop at heading stage treated with cold plasma generated using compressed air. Five biological replications were measured for each treatment. The treatment was carried out at 80 W power of discharge with a 5 LPM (Liter Per Minute) gas flow rate for 10 minutes. Plants in tubs grew under greenhouse conditions, and plasma-activated water (PAW) or distilled water (dH_2O) were sprayed during growth. Plants were subjected to various stress conditions such as no stress condition (control), drought, or combined drought and heat conditions. Heads were harvested at the heading stage and weighed instantly for fresh weight analysis and then oven dried for dry weight analysis. (A) Head fresh weight (g) of plants germinated in untreated seeds sprayed with dH_2O or PAW under control (normal), drought (D), or combined drought and heat (D + H) conditions. (B) Head fresh weight (g) of plants germinated in plasma-cold-treated seeds subjected to the same spray and stress regimes. (C) Head dry weight (g) of plants germinated in untreated seeds subjected to similar stress conditions. (D) Head dry weight (g) of plants germinated from cold plasma treated seeds sprayed with dH_2O or PAW under different stress conditions. One-way ANOVA used for

determining the data statistical significance wherein $p < 0.05$ used for significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Graphs plotted with GraphPad Prism v10.2.

SECTION-4

The fourth section will show the results for Aim 3 that explores the biochemical mechanisms underlying the physiological responses detected. This is a quantitation of the oxidative stress markers (Thiobarbituric Acid Reactive Substances assay) and major stress-associated metabolites (Gamma-aminobutyric acid and Alanine assays).

3.4.1 Measurement of oxidative stress using TBARS assay

In order to determine the presence of oxidative stress through lipid peroxidation, Thiobarbituic Acid Reactive Substances (TBARS) assay was conducted. The findings from figure-3.4.1 indicated that there was a general trend in which the measure of Malondialdehyde (MDA) concentration rises with the intensity of abiotic stress across all groups. In the non-stress control conditions, the PAW spray led to lesser MDA content compared to the dH_2O spray in both the untreated (A) and treated seed samples (B). In drought alone condition, the trend of overall MDA levels were found to be less in the treated seed group (B) as compared to the untreated seed group (A). In the plants from untreated seed groups (A), the PAW foliar spray exhibited a similar level of MDA concentration effect compared to the dH_2O sprayed control plants. Whereas, in plants from treated seed groups (B), the PAW sprayed plants exhibited increased MDA concentration compared to the dH_2O sprayed control plants. Lastly, this trend was reversed in the combined drought and heat stress depending on seed treatment, in the untreated seed group (A) PAW spray showed higher MDA concentration whilst in the treated seed group (B) PAW spray produced a lesser MDA concentration than their respective dH_2O sprayed controls. Overall, these findings suggest that the combined application of cold plasma seed treatment and PAW foliar spray were effective in decreasing the oxidative stress and tolerance to stress in wheat under combined drought and heat stress conditions but no significant differences were observed due to the variability in the replicates.

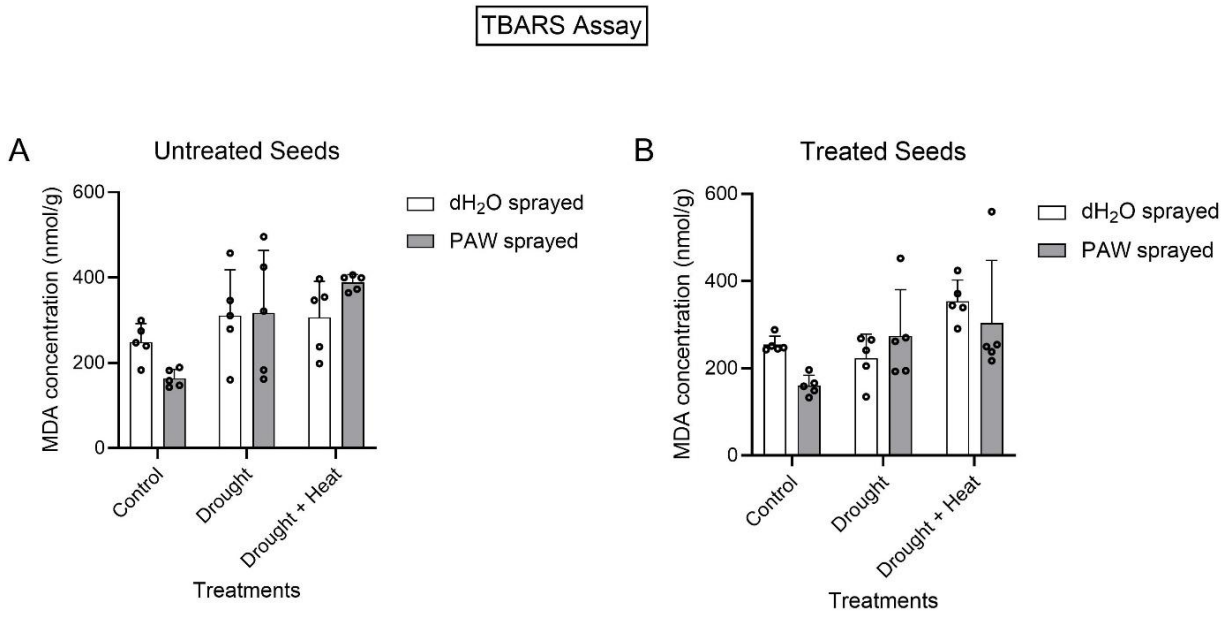

Figure-3.4.1 Malondialdehyde (MDA) concentration (TBARS assay) in wheat plants sampled from untreated (control) and cold plasma treated seeds under different stress conditions. Thiobarbituric acid reactive substances (TBARS) assay was performed on leaf samples collected from greenhouse grown wheat plants at the heading stage in order to quantify the degree of lipid peroxidation and membrane damage under different treatments. (A) Malondialdehyde (MDA) content of plants resulting from untreated seeds (control) were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. (B) MDA content of plants resulting from cold plasma (generated using compressed air) treated seeds were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. Five biological replications were measured for each treatment. Absorbance was read at 440nm, 532 nm and 600 nm wavelength to quantify MDA concentration. Mean ± SE (n = 5) data were presented. Two-way ANOVA was used tocalculate significance between treatments with $p < 0.05$ regarded as significant. Graphs were created in GraphPad Prism v10.2.

3.4.2 Measurement of GABA concentrations

The quantification of Gamma-aminobutyric acid (GABA) was measured on the leaf samples to analyse the plant's GABA concentration as shown in Figure-3.4.2. It was found that the PAW

spray had particular effects on the plants based on the effect of seed primary cold plasma treatment and stress conditions. At non-stress control conditions, PAW sprayed plants exhibited low mean GABA concentration compared to the control dH_2O sprayed plants in both the untreated (A) and treated (B) seed groups. This trend of foliar sprays was reversed under drought stress alone. Under this condition, the mean absorbance in PAW sprayed plants was higher than the control dH_2O sprayed plants irrespective of its primary cold plasma seed treatment. Also, when the two groups were compared, it was observed that the overall GABA concentration were reduced notably in the treated seed group (B) as compared to the untreated seed group (A). Lastly, as combined drought and heat stress was applied the impact of the foliar spray was also comparable with the control non-stress condition. Under these both conditions, PAW sprayed plants exhibited a lower mean absorbance than the dH_2O sprayed controls (A & B). Overall, under drought alone and combined drought and heat conditions, the GABA concentration was notably low in treated seeds (B) compared to the untreated seed groups (A) in both the PAW and dH_2O sprayed plant groups. Overall, the PAW sprayed plants exhibited low GABA concentration compared to dH_2O sprayed plants under non-stress control and combined drought and heat condition, irrespective of its primary cold plasma seed treatment (A & B). Whereas, it is the opposite outcome under drought alone stress conditions, the PAW sprayed plants exhibited high concentration of GABA compared to the dH_2O sprayed control plants under both the untreated and treated seed groups (A & B) but no significant differences were observed due to the variability in the replicates.

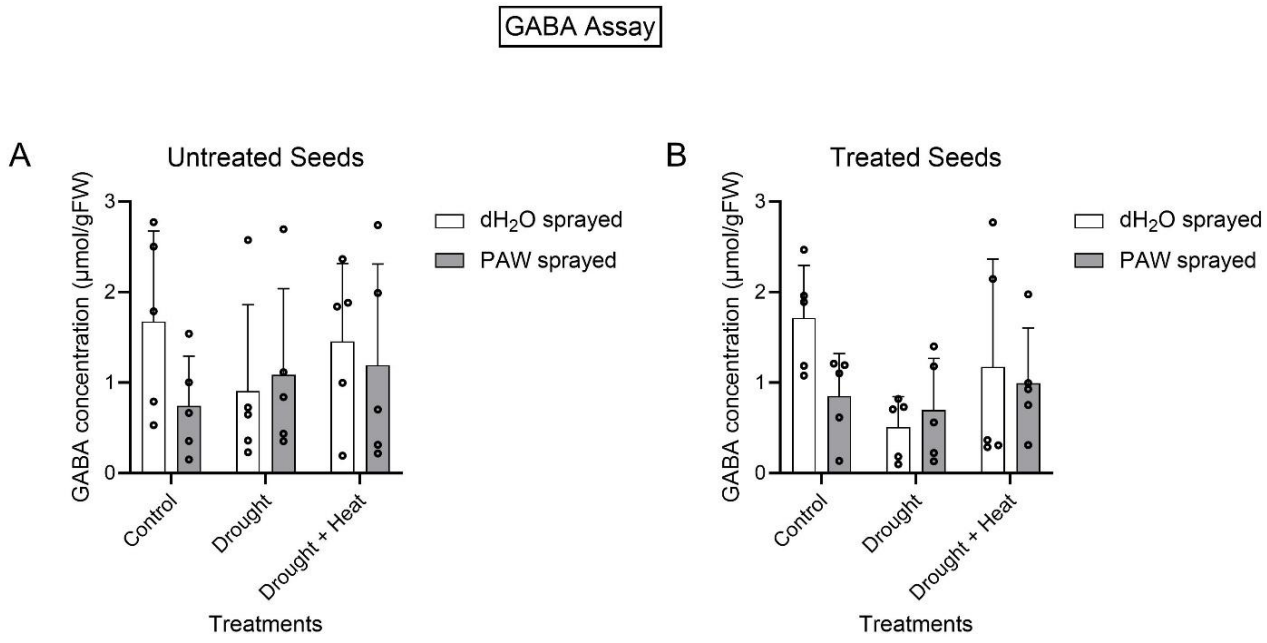

Figure-3.4.2 GABA concentrations in wheat plants sampled from untreated (control) and cold plasma treated seeds under different stress conditions. Leaves were collected from greenhouse grown wheat plants at the heading phase in order to quantify the content of γ-aminobutyric acid (GABA) via colorimetric assay. (A) GABA concentration of plants resulting from untreated seeds (control) were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. (B) GABA concentration of plants resulting from cold plasma (generated using compressed air) treated seeds were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. Five biological replications were measured for each treatment. Absorbance was read at the 340 nm wavelength to quantify GABA concentration. Mean ± SE (n = 5) data were presented. Two-way ANOVA was used in calculating statistics where $p < 0.05$ was regarded as significant. Graphs were drawn with the help of GraphPad Prism v10.2.

3.4.3 Measurement of alanine concentrations

The accumulation of alanine was measured to further analyse the plant's metabolic reaction to stress. This analysis, as illustrated in Figure-3.4.3, demonstrated that the alanine concentration was at a low basal level under both the non-stress control and the moderate drought conditions, and also no significant differences were found in both the untreated and the treated seed group

(A & B). However, the combined drought and heat stress showed a notable rise in alanine level compared to the non-stress and drought alone stress conditions. In such an extreme stressful environment, the effect of PAW spray was totally opposite based on the primary cold plasma treatment of the seed. In plants from untreated seed group (A), the alanine concentration of the PAW sprayed plants was lower than the dH_2O sprayed plants under combined drought and heat stress conditions. On the other hand, the PAW sprayed group had a greater mean absorbance of alanine compared to its dH_2O spray control in the case of the cold plasma treated seeds (B). Therefore, the maximum accumulation of alanine was only evident in the presence of intense combined stress, and the influence of PAW foliar spray was opposite to the effect of the initial cold plasma seed treatment.

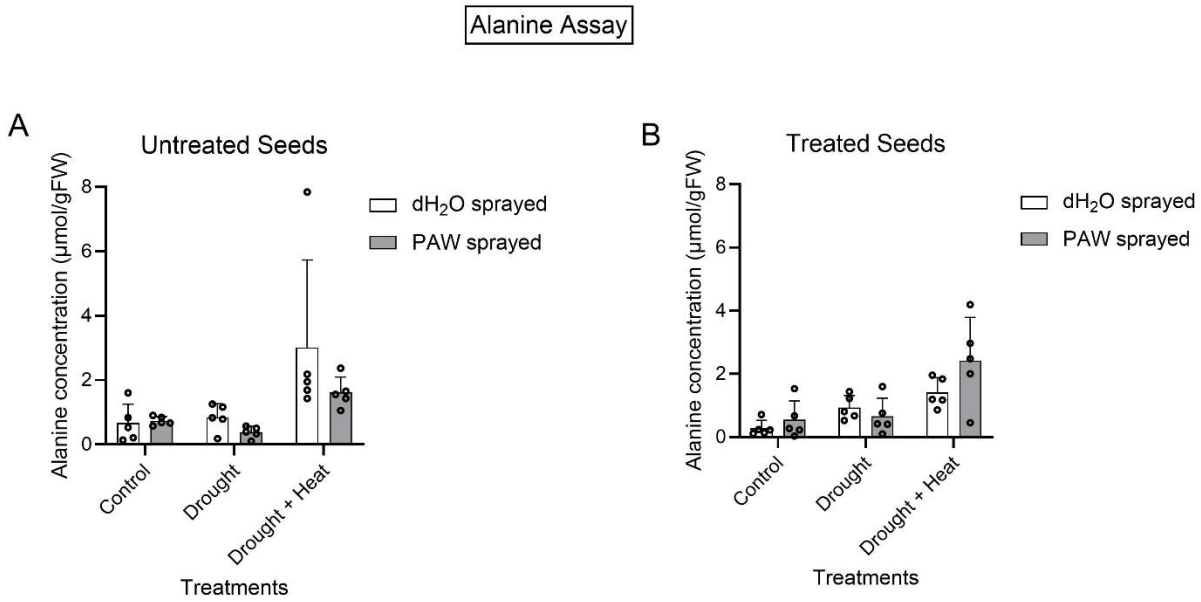

Figure-3.4.3 Alanine concentrations in wheat plants sampled from untreated (control) and cold plasma treated seeds under different stress conditions. Leaves were collected from greenhouse grown wheat plants at the heading phase in order to quantify the concentrations of alanine via colorimetric assay. (A) Alanine content of plants resulting from untreated seeds (control) were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. (B) Alanine content of plants resulting from cold plasma (generated using compressed air) treated seeds were sprayed with either plasma-activated water (PAW) or distilled water (dH_2O) under different stress conditions such as non-stress (control), drought, and combined drought and heat stress conditions. Five biological replications were measured for each treatment. Absorbance was read at the 340 nm wavelength to quantify alanine

concentration. Mean ± SE (n = 5) data were presented. Two-way ANOVA was used in calculating statistics where $p < 0.05$ was regarded as significant. Graphs were drawn with the help of GraphPad Prism v10.2.

Chapter 4: Discussions

4.1 Overview of results

The current study explored the potential of cold plasma (CP) and plasma-activated water (PAW) in enhancing the germination, growth, and stress tolerance of wheat (*Triticum aestivum*) under drought and heat stress. The experiments were designed to cover three main objectives, (1) to identify the optimal CP conditions using air and N_2 as working gases; (2) to elucidate biomass accumulation by treated and control plants in greenhouse conditions; and (3) to assess the biochemical stress markers, i.e., thiobarbituric acid reactive substances (TBARS), gamma-aminobutyric acid (GABA), and alanine. In general, the findings validated that CP seed treatment, particularly at 80 W for 10 and 20 minutes at 5 L min^{-1} air flow, significantly enhanced root and shoot elongation in both regular and moderate osmotic stress conditions. Although both treatments were effective, the 10-minute treatment was very consistent in enhancing root and shoot length compared to the 20-minute treatment. The combined action of CP priming seeds and foliar spray of PAW improved the biomass of the head under combined drought and heat stress. The biochemical assays indicated that CP and PAW treatment showed a trend of minimizing the oxidative stress induced damage and regulated the stress associated metabolites. These findings collectively support the proposal that CP and PAW can enhance seed performance and stress tolerance in wheat, in particular under adverse environmental conditions.

4.2 Influence of CP on germination and early seedling growth

The findings of the current research confirm that CP treatment has an essential role in the enhancement of seed germination and early seedling establishment in wheat under combined drought and heat stress. Increased root and shoot lengths of the CP treated seeds (Figure- 3.1.2, 3.1.3, 3.2.2, 3.2.3, 3.2.5 and 3.2.6) agree with the previous literature in various crops such as wheat, tomato and *Arabidopsis thaliana* in which exposure to plasma was shown to enhance water uptake, permeability of the seed coat, and activation of the metabolic enzyme needed for

germination (Los et al., 2019; Bozhanova et al., 2024; Li et al., 2016). The physical etching of the seed's outer surface by RONS enhances hydrophilicity, which enables faster water imbibition and improved germination efficiency. Scanning electron microscopic studies have confirmed microstructural changes in plasma treated Bambara, chilli, papaya and cotton seeds, resulting in enhanced diffusion of oxygen and gas exchange (Ahmed et al., 2023; de Groot et al., 2018).

In the current study, the optimal treatment duration was the 10-minute plasma treatment that led to significant increases in root (Figure-3.1.2) and shoot (Figure-3.1.3) elongation under non-stress control and 15% PEG-induced drought stress. However, exposure times of 20 minutes did not provide a consistent positive results (Figure-3.1.2), underlining the dose dependency of plasma action. There was a similar report from Ling et al. (2014) in soybean, where a 15 sec helium cold plasma exposure at 80 W enhanced germination and vigor indices and Lotfy et al. (2019) in wheat, where a 4-minute nitrogen plasma treatment increased germination by more than 40% but longer exposure reduced vigor by over oxidation. The occurrence of RONS in CP creates transient redox signals that induce germination associated gene expression, abscisic acid and antioxidant responses that pre-condition the seed for upcoming stress (Bradu et al., 2020; Kocira et al., 2022). Also, the higher value of root length recorded under moderate drought stress (15% PEG) in the CP treated (using nitrogen gas), seeds as shown in Figure-3.2.5 (C), suggests that the plasma priming had turned on the defensive mechanisms, providing an enhancement in osmotic adjustment and cell extension potential.

However, in heat stress (37 °C) and combined drought and heat stress (30% PEG + 37 °C) conditions, the positive action of plasma was limited. This trends indicate that although CP priming enhances early stress preparedness, the induced physiological tolerance has thresholds beyond which excessive abiotic stress inhibits metabolic recovery. Similar findings were seen in barley and alfalfa, where the seeds primed by plasma exhibited enhanced germination under moderate stress levels, but not under extreme stress conditions (Jinkui et al., 2018). Nitrogen based plasma treatment yielded better shoot extension under heat stress, likely because of increased synthesis of nitric oxide free radicals, which are the signalling molecules that regulate heat-shock protein production and uphold cell stability during temperature changes (Zhou et al., 2020). Overall, these findings verify that CP functions as an efficient seed priming agent that stimulates germination and early vigor under mild to moderate stress conditions yet has restricted action under intense abiotic stress levels.

4.3 Physiological responses to CP exposure and PAW under greenhouse conditions

The greenhouse experiment provided deeper understanding of the role of PAW in later developmental plant growth. The findings showed a context dependent response which is under non-stress conditions, PAW tended to inhibit root (figure-3.3.1) and shoot biomass (figure-3.3.2), while under drought stress, the application of PAW significantly enhanced biomass accumulation (figure-3.3.1 C & figure-3.3.2 B, D). Under combined drought and heat stress, there was no notable difference between any treatment groups such as the treated, untreated, PAW and the dH_2O sprayed plants (figure-3.3.1 & figure-3.3.2). The reduced growth under control conditions can be explained by the acid nature and high oxidation-reduction potential of PAW, which in unstressed plants can provoke mild oxidation stress (Guo et al., 2021). However, under drought, the same trait turns beneficial, as low doses of hydrogen peroxide and nitric oxide function as signalling molecules that activate antioxidant defences and osmoprotectant synthesis (Bradu et al., 2020; Gao et al., 2022).

The combined action of CP seed treatment and PAW foliar spray under the current study, specifically in the enhancement of head dry weight (Figure-3.3.3 D) under combined heat and drought stress, indicates a double-phase priming action. Cold plasma priming at the seed stage, establishes metabolic activation and antioxidant responses, while PAW maintains and enhances the same defense throughout the plant's vegetative and reproductive stages. Comparable combined effect results were documented by Rashid et al. (2021) in rice, where CP seed priming and subsequent PAW foliar spraying enhanced plant height, tillering, and grain output by approximately 17%. The nitrogenous substances in PAW also add nutrients in supplementation, thereby supporting plant development and reproductive biomass accumulation. The combined CP and PAW treatment establishes an overall mechanism that underlines physiological stability, especially under drought stress.

4.4 Head emergence and reproductive development

The emergence of the head and the synchrony (table-3.3) indicate the ability of the plant to change from vegetative phase to reproductive phase of growth under different stress levels. In this experiment, the wheat plants which was subjected to drought and combined drought + heat stress had an earlier head emergence than the control where the head emergence of few plants was delayed until day 6 in week ten (table-3.3). This stress-induced acceleration is indicative of an adaptive change in phenology that helps the plant to reach its reproductive phase before the resources are depleted (Sehgal et al., 2018; Sato et al., 2024). Similar findings were reported

that simultaneous heat and drought stress accelerates heading in wheat to reduce the duration of exposure of the sensitive floral tissues to extended stress (Ullah et al., 2022). Redox modulation and hormonal balance are connected to the plasma treatment, specifically, regulation of abscisic acid and ethylene signaling that ensure developmental stability in the case of stress (Kocira et al., 2022). Therefore, stress increased early emergence of head as a survival mechanism. The combined CP and PAW treatments did not notably delay or accelerate the head emergence compared to the untreated control. However, under combined drought and heat stress, combined CP and PAW treatment contributed to more synchronized and uniform head emergence across plants. Cold plasma and PAW would have promoted consistency in head development in the treatment, as both stress increased synchrony and stability in heading (Bradu et al., 2020; Feizollahi et al., 2020). This improved synchrony may contribute to sustained fertility and better head biomass observed under combined drought and heat stress conditions (figure-3.3.3 D).

4.5 Oxidative stress mitigation

The TBARS assay revealed the effects of CP and PAW on oxidative stress. The malondialdehyde (MDA) contents escalated gradually with increasing severity of stress, validating the enhancement of ROS accumulation and membrane damage by drought and heat (Raja et al., 2020; Sharma et al., 2020). Separate trends were exhibited among treatments. In non-stress environments (Figure-3.4.1), PAW spray exhibited notably reduced MDA concentration in plants grown from both CP-treated (B) and untreated seeds (A) compared to distilled water (dH_2O) controls, reflecting reduced lipid peroxidation and improved membrane stability. Under drought conditions, PAW and dH_2O exhibited comparable levels of TBARS in untreated plants, while in CP treated plants, PAW showed a moderate rise in MDA. This suggests that the additional reactive species from PAW exceeded the redox tolerance in the already primed tissues, briefly increasing the oxidative stress (Zhou et al., 2019; Shelar et al., 2022). In combined drought and heat stress, the trend was reversed, PAW increased MDA in plants from untreated seeds (Figure-3.4.1 A), while reducing it in plants from CP-treated seeds (Figure-3.4.1 B). This indicates that CP priming reduced lipid peroxidation under combined heat and drought stress, which caused less membrane damage compared to the plants from untreated seeds. Similar results were reported by Li et al. (2016), that plasma treatments minimized oxidative damage in stressed plants.

Overall, these results indicate that the effects of CP and PAW on oxidative stress depends on the environmental conditions. In combined drought and heat stress, combined CP and PAW

treatment exhibited the decreased MDA concentration, which suggests that there were less membrane damage and increased tolerance to the stress. However, under drought stress, PAW application slightly increased MDA in plants from CP-treated seeds, suggesting that the reactive species input may transiently heighten oxidative stress. Thus, the response appears condition-dependent, with beneficial effects when reactive species remain within the plant's tolerance range.

4.6 GABA and Alanine accumulation as stress markers

The alterations in the levels of gamma-aminobutyric acid (GABA) and alanine provided the biochemical confirmation of stress mitigation mechanisms influenced by CP and PAW treatments. GABA, being a recognized non-protein amino acid, gets accumulated quickly in abiotically stressed plants and acts as a signalling molecule in maintaining intracellular pH, redox homeostasis, and in carbon and nitrogen metabolism regulation (Carillo & Gibon, 2011; Ghosh et al., 2022). In the current experiment, in the case of drought stress, GABA accumulation was reduced in CP treated plants as compared to untreated controls, indicating that the plasma priming has lowered the intensity of stress perception by increasing the antioxidant base state. This aligns with previous findings that showed the pre-activation in defense mechanisms by the application of primers generally resulted in the reduced stress responses (Mahanta et al., 2022).

Alanine levels exhibited elevation under combined heat and drought stress, especially in combined cold plasma (CP) and plasma-activated water (PAW) treated plants. This elevated alanine accumulation implies alanine shunt pathway activation which plays a key role in maintaining redox homeostasis and nitrogen balance when respiration is limited during stress. The enzyme alanine dehydrogenase converts pyruvate to alanine, while allowing for nitrogen cycling and energy conservation when under hypoxic or energy restricted conditions (Allaway et al., 2000; Day et al., 2001). Similarly, increased alanine aminotransferase expression in cereal crops such as rice, wheat, and barley is linked with improved nitrogen use efficiency and adaptive metabolic flexibility when under environmental stress (Tiong et al., 2021). Therefore, elevated alanine accumulation in CP as well as PAW treated plants suggests enhanced metabolic adaptation that would enable them to sustain carbon–nitrogen flux and redox stability when under extreme stress thus supporting improved resistance and biomass preservation under stressful conditions.

4.7 Integrating CP and PAW effects within wheat stress physiology

The combined activity of CP and PAW is consistent with current understanding of stress physiology in wheat under the challenging climatic conditions. The combined stress of drought and heat is especially destructive because it disrupts photosynthesis, and decreases grain filling, frequently leading to yield loss more than 50% (Sehgal et al., 2018; Qaseem et al., 2019; Ullah et al., 2022). The current results suggests that CP and PAW collectively enhance multiple level stress tolerance in plants such as physiological (growth and biomass accumulation), biochemical (reduction in lipid peroxidation and modulation of stress metabolites) and developmental (improved synchrony in head emergence) responses. In the seed stage, CP enhances the properties of the seed's surface and enhances early vigor and germination. In later stages, PAW likely sustained RNS signalling, contributing to improved physiological stability (Figure-3.3.1 C, Figure-3.3.2 D, Figure-3.3.3 D) under prolonged stress (Zhou et al., 2020; Gao et al., 2022). The significant rise in the head's dry weight indicate that the combined CP and PAW treatment improved tolerance and maintenance of plant growth under combined drought and heat stress. Therefore, the plasma technology offers a hopeful method to build multi-level stress tolerance by an integrated chemical and physical priming.

4.8 Limitations

Although the current study has good evidence of the stress mitigation potential of CP and PAW in wheat, there are few limitations that must be considered. Plasma treatment is very sensitive to factors like power, gas flow, and seed moisture and only one optimized parameters was closely investigated. The future studies must investigate a broader parameter matrix so as to establish the minimum stress mitigation exposure and avoid possible over-oxidation due to excessive plasma exposure. The chemical composition of PAW was characterized in only a limited way. Although peroxide, nitrate, nitrite and chlorine concentrations, total hardness (GH) or total alkalinity (TA) were assessed, other crucial factors like pH, oxidation-reduction potential were not assessed. Although, the generated PAW was used within 2-3 hours, time dependent stability was not monitored and the activity of the reactive species drops rapidly following production, the differences in the storage period of the PAW can have affected treatment effectiveness (Guo et al., 2021; Zhou et al., 2020). The greenhouse conditions for the replication of the drought as well as the heat stress also cannot accurately capture the field variability in the temperature, soil heterogeneity, and the vapor pressure deficit (Sato et al., 2024). Also, the biochemical studies were limited to TBARS, GABA, and alanine analysis due to time constraint. The inclusion of the proline, glutamate and enzymatic antioxidants can give

a much broader scope of the stress physiology. Finally, due to the variability in the replicates no significant difference were observed in the biochemical assays.

4.9 Conclusions and future directions

The overall hypothesis that combined CP and PAW treatment increases wheat germination, seedling growth, water and heat stress tolerance can be regarded as partly accepted. CP treatment significantly increased germination and seedling vigor under stressful conditions which satisfied the first aim. The combined CP and PAW treatment improved biomass accumulation under drought and combined stress, satisfying the second aim. The third aim was also fulfilled, as CP and PAW influenced oxidative and metabolic stress markers, suggesting biochemical priming. However, the treatments were less effective under extreme stress conditions, and PAW inhibited growth under control conditions, showing that their advantages are treatment dependent. Therefore, CP and PAW can only be seen as mostly adaptive priming agents, not as overall stimulants of growth, where they offer the greatest benefit under stressful environmental conditions.

Future research needs to focus on plasma parameters and PAW chemistry to enhance reproducibility and effectiveness. Experimental design approaches with combination of different factors to identify the interaction between wattage, gas species, and exposure time to achieve the best treatment effects. Comprehensive measurement of the reactive species composition, pH, and oxidation-reduction potential of PAW over time will establish its stability and guide application duration. Addition of other biochemical and molecular indicators such as SOD, CAT, APX, and proline concentration, along with gene expression evaluation of stress-responsive pathways, would strengthen mechanistic interpretations. Multilocational field trials in various agroclimatic regions are required to confirm the findings in the laboratory and greenhouse under actual farm conditions. Also, cost benefit analyses will establish the economic viability of adopting CP and PAW systems in commercial scale production of wheat. Combination of plasma treatment with sustainable agricultural practices like microbial inoculants or organic manuring may enhance overall plant resilience and soil health.

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Appendices

Appendix-1: Germination percentage (%) of wheat treated with plasma at different time durations under different stress levels

Germination percentage (%)			
Treatment Duration	Replicates	0% PEG	15% PEG
0 sec (Untreated)	1	100%	100%
	2	100%	100%
	3	100%	90%
15 sec treated	1	100%	100%
	2	100%	100%
	3	100%	100%
1 min treated	1	90%	90%
	2	100%	100%
	3	90%	100%
10 min treated	1	100%	90%
	2	90%	100%
	3	100%	90%
20 min treated	1	100%	100%
	2	90%	90%
	3	100%	100%

Appendix-2: Shoot length (mm) of wheat treated with plasma at different time durations under different stress levels

Shoot length (mm)			
Treatment Duration	Replicates	0% PEG	15% PEG
0 sec (Untreated)	1	71	13
	2	71	14
	3	59	15
15 sec treated	1	72	14
	2	71	21
	3	60	9
1 min treated	1	75	31
	2	70	29
	3	86	16
10 min treated	1	82	23
	2	82	30
	3	77	32
20 min treated	1	69	33
	2	69	31
	3	74	29

Appendix-3: Root length (mm) of wheat treated with plasma at different time durations under different stress levels

Root length (mm)			
Treatment Duration	Replicates	0% PEG	15% PEG
0 sec (Untreated)	1	110	72
	2	100	83
	3	79	65
15 sec treated	1	81	65
	2	62	56
	3	78	45
1 min treated	1	96	72
	2	97	55
	3	113	66
10 min treated	1	151	88
	2	108	72
	3	101	80
20 min treated	1	114	81
	2	79	74
	3	134	87

Appendix-4: Germination percentage (%) of wheat treated with plasma generated using compressed air (CA) at different time durations under different stress levels

Germination percentage (%)				
Treatments (CA)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG (Contol)	1	100	100	90
	2	100	100	100
	3	100	100	100
17º C + 15% PEG	1	100	100	100
	2	80	100	100
	3	100	100	100
17º C + 30% PEG	1	100	100	100
	2	90	100	100
	3	100	100	100
37º C + 0% PEG	1	86	100	100
	2	78	100	100
	3	100	100	90
37º C + 15% PEG	1	90	100	90
	2	100	100	100
	3	80	100	90
37º C + 30% PEG	1	10	30	80
	2	0	70	60
	3	0	50	60

Appendix-5: Root length (mm) of wheat treated with plasma generated using compressed air (CA) at different time durations under different stress levels

Root length (mm)				
Treatments (CA)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG (Contol)	1	78	74	112
	2	110	110	92
	3	62	145	95
17º C + 15% PEG	1	70	89	80
	2	62	98	65
	3	76	91	96
17º C + 30% PEG	1	31	33	36
	2	28	25	40
	3	27	26	39
37º C + 0% PEG	1	8	13	10
	2	12	11	9
	3	11	15	21
37º C + 15% PEG	1	16	9	10
	2	13	8	9
	3	12	11	13
37º C + 30% PEG	1	3	4	5
	2	0	13	6
	3	0	6	7

Appendix-6: Shoot length (mm) of wheat treated with plasma generated using compressed air (CA) at different time durations under different stress levels

Shoot length (mm)				
Treatments (CA)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG (Contol)	1	76	75	76
	2	62	68	70
	3	65	66	74
17º C + 15% PEG	1	25	32	35
	2	28	37	40
	3	34	32	39
17º C + 30% PEG	1	4	3	4
	2	2	5	4
	3	3	3	4
37º C + 0% PEG	1	28	26	32
	2	21	20	27
	3	32	13	13
37º C + 15% PEG	1	7	11	12
	2	7	18	8
	3	9	17	9
37º C + 30% PEG	1	1	2	3
	2	0	5	2
	3	0	4	4

Appendix-7: Germination percentage (%) of wheat treated with plasma generated using compressed air (N_2) at different time durations under different stress levels

Germination percentage (%)				
Treatments (N2)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG	1	100	100	100
	2	100	100	100
	3	100	100	100
17º C + 15% PEG	1	90	100	100
	2	100	100	100
	3	90	100	100
17º C + 30% PEG	1	80	90	100
	2	80	90	90
	3	100	100	100
37º C + 0% PEG	1	100	100	89
	2	100	100	90
	3	67	100	100
37º C + 15% PEG	1	70	90	100
	2	60	90	100
	3	50	100	40
37º C + 30% PEG	1	0	20	100
	2	0	20	50
	3	20	30	50

Appendix-8: Root length (mm) of wheat treated with plasma generated using compressed air (N_2) at different time durations under different stress levels

Root length (mm)				
Treatments (N2)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG (Contol)	1	89	86	101
	2	110	112	87
	3	52	122	91
17º C + 15% PEG	1	7	85	45
	2	6	99	51
	3	7	92	46
17º C + 30% PEG	1	4	5	14
	2	6	6	11
	3	5	5	10
37º C + 0% PEG	1	10	6	9
	2	8	9	12
	3	5	10	11
37º C + 15% PEG	1	9	12	15
	2	6	13	16
	3	9	14	16
37º C + 30% PEG	1	0	0	5
	2	0	0	0
	3	0	0	0

Appendix-9: Shoot length (mm) of wheat treated with plasma generated using compressed air (N_2) at different time durations under different stress levels

Shoot length (mm)				
Treatments (N2)	Replicates	Treatment duration		
		0 min	10 min	20 min
17º C + 0% PEG (Contol)	1	60	70	61
	2	79	75	62
	3	41	76	61
17º C + 15% PEG	1	6	26	15
	2	3	28	14
	3	4	27	17
17º C + 30% PEG	1	2	2	3
	2	3	2	2
	3	4	2	3
37º C + 0% PEG	1	22	22	27
	2	18	27	32
	3	15	30	30
37º C + 15% PEG	1	5	10	10
	2	4	11	9
	3	5	7	4
37º C + 30% PEG	1	2	2	3
	2	2	2	2
	3	0	2	2

Appendix-10: Root biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Root weight (g) - PAW sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	1.03	0.29
	2	0.71	0.27
	3	1.32	0.54
	4	1.14	0.47
	5	0.76	0.33
CP-treated seed [Control]	1	0.83	0.32
	2	0.95	0.38
	3	0.9	0.32
	4	1.6	0.64
	5	0.77	0.3
Untreated seed [Drought]	1	0.52	0.16
	2	1.18	0.5
	3	0.62	0.25
	4	0.55	0.23
	5	0.46	0.2
CP-treated seed [Drought]	1	0.61	0.25
	2	0.5	0.19
	3	0.49	0.21
	4	0.64	0.21
	5	0.86	0.31
Untreated seed [Drought + Heat]	1	0.22	0.1
	2	0.16	0.08
	3	0.27	0.1
	4	0.15	0.05
	5	0.27	0.11
	6	0.17	0.07
CP-treated seed [Drought + Heat]	1	0.16	0.06
	2	0.28	0.13
	3	0.24	0.11
	4	0.3	0.13

Appendix-11: Root biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of dH_2O as foliar spray on plants under different stress levels at greenhouse conditions.

Root weight (g) - dH2O sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	1.37	0.41
	2	2	0.73
	3	1.54	0.59
	4	1.73	0.63
	5	1.74	0.57
CP-treated seed [Control]	1	1.06	0.36
	2	1.38	0.44
	3	1.67	0.63
	4	1.28	0.54
	5	1.74	0.8
Untreated seed [Drought]	1	0.27	0.11
	2	0.45	0.18
	3	0.2	0.07
	4	0.24	0.09
	5	0.39	0.14
CP-treated seed [Drought]	1	0.36	0.15
	2	0.35	0.13
	3	0.57	0.24
	4	0.37	0.17
	5	0.27	0.14
Untreated seed [Drought + Heat]	1	0.26	0.13
	2	0.27	0.11
	3	0.41	0.18
	4	0.49	0.24
	5	0.17	0.08
CP-treated seed [Drought + Heat]	1	0.26	0.14
	2	0.24	0.11
	3	0.24	0.08
	4	0.17	0.09
	5	0.35	0.19

Appendix-12: Shoot biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Shoot weight (g) - PAW sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	7.35	1.85
	2	3.84	1
	3	7.44	1.91
	4	8.17	1.96
	5	5.29	1.3
CP-treated seed [Control]	1	4.27	1
	2	6.99	1.69
	3	7.45	1.78
	4	6.26	1.44
	5	6.16	1.42
Untreated seed [Drought]	1	3.69	1.07
	2	6.37	1.78
	3	4.45	1.26
	4	4.67	1.29
	5	3.7	1.13
CP-treated seed [Drought]	1	4.36	1.39
	2	3.5	1.11
	3	3.59	1.16
	4	4.11	1.34
	5	3.54	1.13
Untreated seed [Drought + Heat]	1	2.36	1.12
	2	2.28	1.02
	3	2.55	1.08
	4	1.97	0.9
	5	2.21	0.98
	6	1.92	0.95
CP-treated seed [Drought + Heat]	1	1.91	0.8
	2	1.76	0.76
	3	1.87	0.87
	4	2.41	1.1

Appendix-13: Shoot biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of dH_2O as foliar spray on plants under different stress levels at greenhouse conditions.

Shoot weight (g) - dH2O sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	7.65	1.89
	2	8.22	2.23
	3	9.12	2.22
	4	8.1	2.08
	5	7.91	2.12
CP-treated seed [Control]	1	7.38	1.83
	2	6.67	1.69
	3	8.67	2.18
	4	7.17	1.67
	5	7.97	2.13
Untreated seed [Drought]	1	2.85	1.01
	2	3.63	1.16
	3	2.01	0.79
	4	2.5	0.97
	5	3.18	1.1
CP-treated seed [Drought]	1	2.59	0.9
	2	2.91	1
	3	3.05	0.93
	4	2.61	0.94
	5	2.35	0.78
Untreated seed [Drought + Heat]	1	1.95	0.38
	2	1.81	0.7
	3	3.56	1.37
	4	3.04	1.19
	5	2.02	0.85
CP-treated seed [Drought + Heat]	1	1.9	0.77
	2	1.49	0.65
	3	1.77	0.72
	4	1.49	0.7
	5	1.9	0.79

Appendix-14: Head biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Head weight (g) - PAW sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	1.5	0.36
	2	1.01	0.22
	3	1.77	0.44
	4	1.66	0.47
	5	1.11	0.3
CP-treated seed [Control]	1	1.12	0.25
	2	1.55	0.39
	3	1.7	0.4
	4	1.46	0.28
	5	1.26	0.3
Untreated seed [Drought]	1	1.12	0.35
	2	1.57	0.5
	3	1.36	0.4
	4	1.17	0.36
	5	1.09	0.31
CP-treated seed [Drought]	1	0.96	0.33
	2	0.81	0.24
	3	0.83	0.28
	4	1.14	0.37
	5	0.97	0.3
Untreated seed [Drought + Heat]	1	1.28	0.56
	2	0.88	0.41
	3	1.12	0.48
	4	0.95	0.4
	5	0.96	0.42
	6	0.85	0.4
CP-treated seed [Drought + Heat]	1	0.77	0.33
	2	0.73	0.31
	3	0.9	0.37
	4	1.25	0.5

Appendix-15: Head biomass (g) of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of dH_2O as foliar spray on plants under different stress levels at greenhouse conditions.

Head weight (g) - dH2O sprayed			
Treatments	Replicates	Fresh	Dry
Untreated seed [Control]	1	1.76	0.41
	2	1.88	0.73
	3	1.82	0.59
	4	1.9	0.63
	5	1.84	0.57
CP-treated seed [Control]	1	1.64	0.36
	2	1.54	0.44
	3	2.07	0.63
	4	1.38	0.54
	5	2.03	0.8
Untreated seed [Drought]	1	0.99	0.11
	2	1.05	0.18
	3	0.65	0.07
	4	0.77	0.09
	5	1.18	0.14
CP-treated seed [Drought]	1	0.68	0.15
	2	0.8	0.13
	3	0.73	0.24
	4	0.81	0.17
	5	0.54	0.14
Untreated seed [Drought + Heat]	1	0.97	0.13
	2	0.75	0.11
	3	1.46	0.18
	4	1.25	0.24
	5	0.97	0.08
CP-treated seed [Drought + Heat]	1	0.63	0.14
	2	0.65	0.11
	3	0.82	0.08
	4	0.56	0.09
	5	0.62	0.19

Appendix-16: Malondialdehyde (MDA) concentration of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Treatments	Replicates	MDA (nmol/g)	
		PAW	dH2O
Untreated seed [Control]	1	158.039	274.709
	2	182.203	247.493
	3	147.210	299.217
	4	142.891	183.179
	5	189.352	239.921
CP-treated seed [Control]	1	132.874	247.174
	2	148.675	242.604
	3	165.543	251.188
	4	158.563	288.142
	5	196.014	244.990
Untreated seed [Drought]	1	424.648	346.374
	2	183.021	457.164
	3	161.987	310.921
	4	321.078	160.157
	5	495.772	279.227
CP-treated seed [Drought]	1	192.837	268.171
	2	270.155	241.439
	3	194.120	205.023
	4	452.304	265.250
	5	261.691	134.948
Untreated seed [Drought + Heat]	1	399.671	198.028
	2	406.085	354.054
	3	364.101	237.867
	4	399.385	396.668
	5	372.831	346.746
CP-treated seed [Drought + Heat]	1	237.824	339.022
	2	254.163	371.133
	3	249.525	290.889
	4	217.053	424.080
	5	559.465	344.019

Appendix-17: GABA concentration of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Treatments	Replicates	GABA (umoles/g)	
		PAW	dH2O
Untreated seed [Control]	1	1.004	0.791
	2	0.152	0.530
	3	0.355	1.788
	4	0.667	2.773
	5	1.541	2.505
CP-treated seed [Control]	1	1.211	1.187
	2	1.104	1.079
	3	0.137	2.468
	4	0.615	1.890
	5	1.194	1.960
Untreated seed [Drought]	1	0.353	0.649
	2	0.842	2.575
	3	1.116	0.231
	4	0.435	0.361
	5	2.696	0.725
CP-treated seed [Drought]	1	0.223	0.183
	2	1.181	0.730
	3	1.402	0.707
	4	0.561	0.820
	5	0.130	0.099
Untreated seed [Drought + Heat]	1	1.990	0.998
	2	2.741	2.366
	3	0.703	0.193
	4	0.314	1.884
	5	0.219	1.840
CP-treated seed [Drought + Heat]	1	0.928	0.308
	2	1.976	0.365
	3	0.753	0.289
	4	0.996	2.770
	5	0.311	2.146

Appendix-18: Alanine content of wheat subjected to combined treatment of seed treated with CP generated using compressed air (CA) and use of PAW as foliar spray on plants under different stress levels at greenhouse conditions.

Treatments	Replicates	Alanine (umoles/g)	
		PAW	dH2O
Untreated seed [Control]	1	0.905	0.528
	2	0.683	1.606
	3	0.574	0.138
	4	0.841	0.838
	5	0.667	0.209
CP-treated seed [Control]	1	0.278	0.245
	2	0.036	0.114
	3	0.675	0.136
	4	1.535	0.714
	5	0.231	0.233
Untreated seed [Drought]	1	0.500	1.255
	2	0.100	0.791
	3	0.571	0.828
	4	0.390	1.164
	5	0.339	0.182
CP-treated seed [Drought]	1	0.763	0.809
	2	0.100	0.532
	3	0.426	1.221
	4	0.409	0.678
	5	1.604	1.444
Untreated seed [Drought + Heat]	1	1.431	1.693
	2	1.055	7.841
	3	1.555	1.949
	4	1.660	1.433
	5	2.368	2.183
CP-treated seed [Drought + Heat]	1	0.456	1.197
	2	2.972	1.843
	3	2.484	0.864
	4	4.195	1.178
	5	2.010	1.962

Appendix-19: Standard curve of T-bar assay

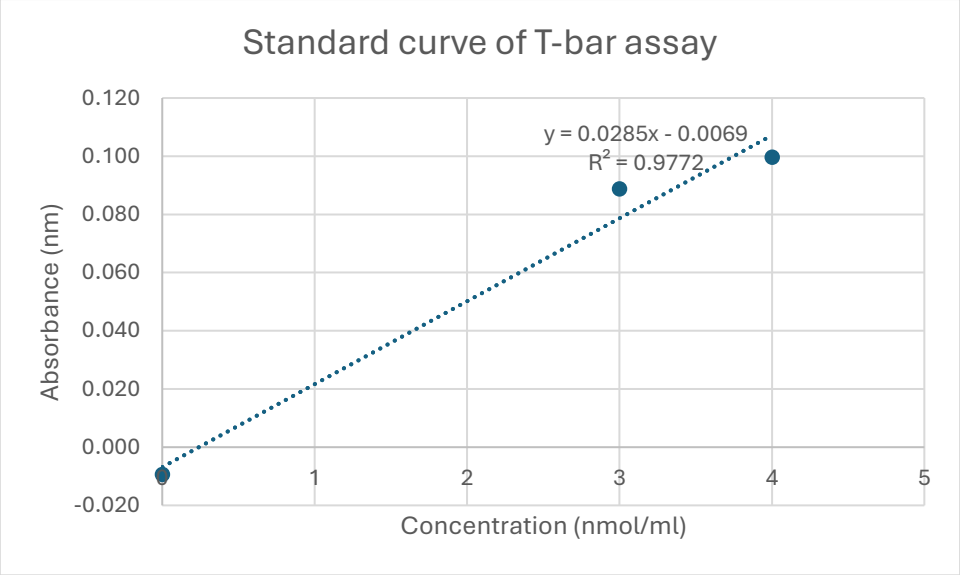

Figure-1A standard curve of T-bar assay

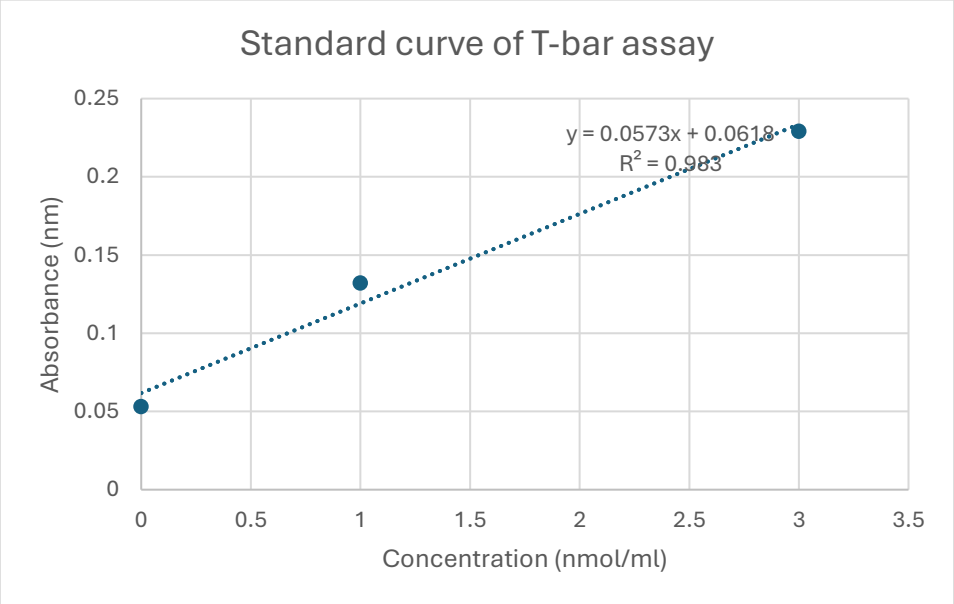

Figure-2A standard curve of T-bar assay

Appendix-20: Standard curve of GABA assay

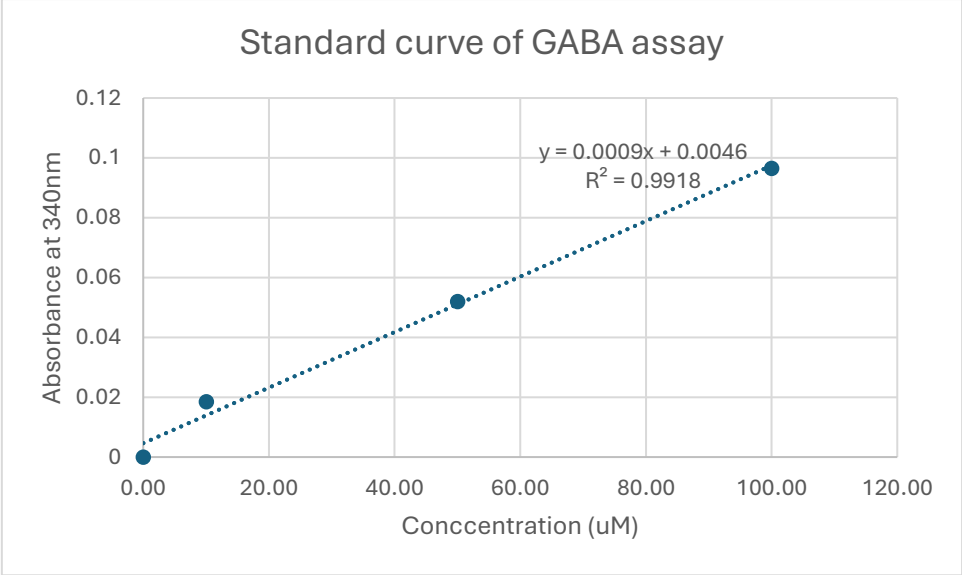

Figure-3A standard curve of GABA assay

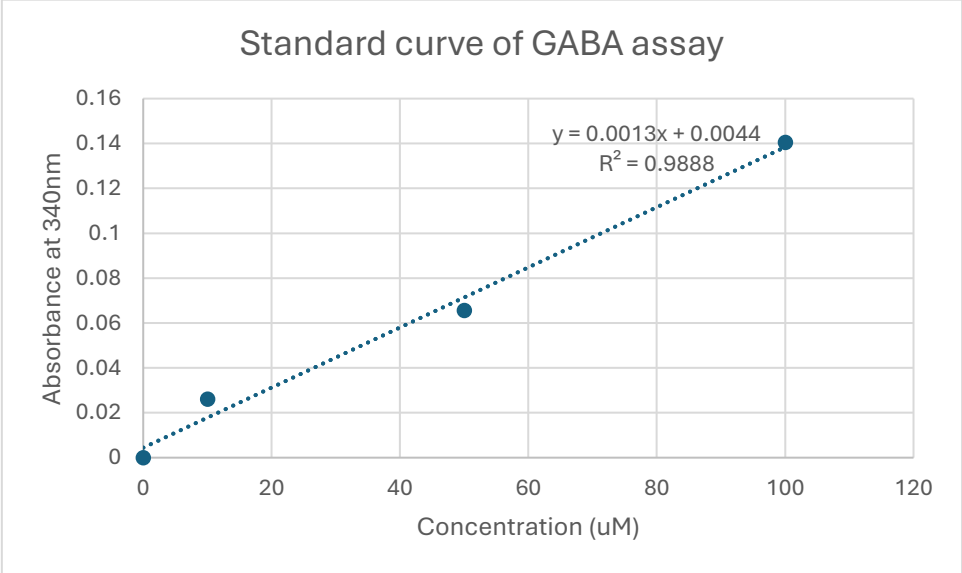

Figure-4A Standard curve of GABA assay

Appendix-21: Standard curve of Alanine assay

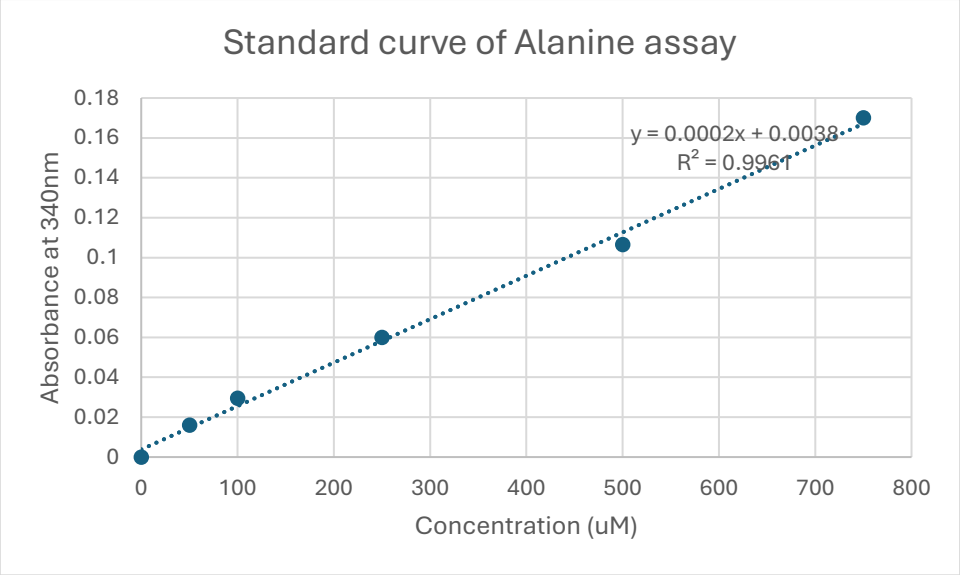

Figure-5A Standard curve of Alanine assay

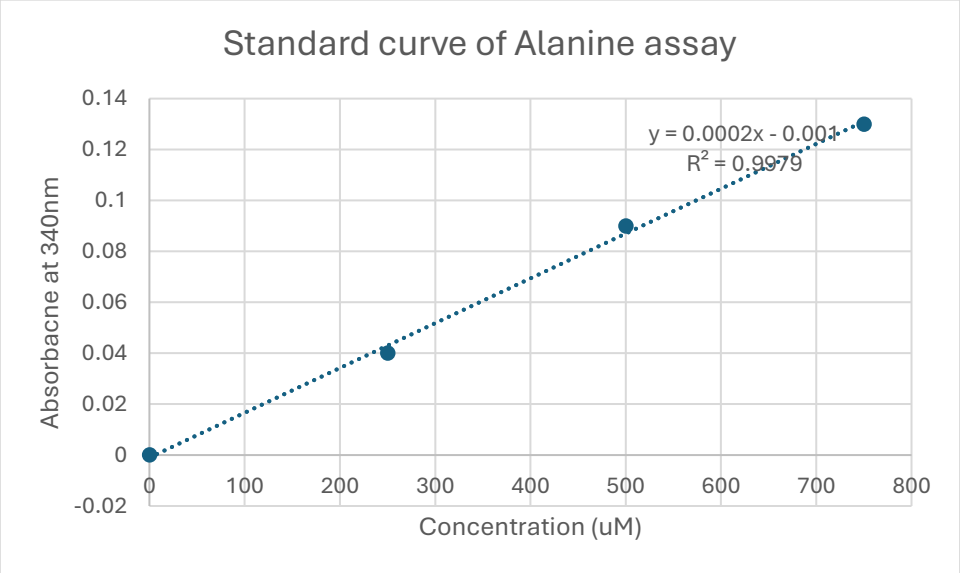

Figure-6A Standard curve of Alanine assay