Biofortification of rice (*Oryza sativa* L.) with zinc through nutripriming

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the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

By

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

15 Ascorbate content (A), Glutathione content (B) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

16 Total phenolics (A), Flavonoids (B) and Anthocyanin content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

17 Superoxide content (A), H_2O_2 content (B) and MDA content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

18 Electrolyte leakage (A) and Membrane stability index (B) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

19 Total soluble sugars (A), Total free amino acids (B) and Total protein content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

20 Principal component analysis (PCA) plot representing distribution of treatments (A) and PCA variable biplot (B) representing the relationship between physiological and biochemical traits and treatment effects in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

21 The correlation matrix heatmaps of Annapoorna (A), Kumkumashali (B) and both (C) subjected to various priming treatments with $ZnNO_3$ showing the values of the Pearson correlation coefficient between parameters.

22 Principal component analysis (PCA) biplot representing effect of various $ZnNO_3$ priming treatments on grain Zn, phytic acid, phytic acid: Zn molar ratio and bioavailable Zn (A), correlation matrix (B) showing the relationships among grain phytic acid (PA), PA:Zn molar ratio, total Zn, and bioavailable Zn content studied in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

23 Boxplot for grain Zn content, phytic acid content, phytic acid to Zn molar ratio and bioavailable Zn content in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$

24 Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Plant height (A), Primary root length (B), Crown root number (C)

25 Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Root dry weight (A), Shoot dry weight (B), Number of tillers per plant (C)

26 Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Number of reproductive tillers per plant (A), Panicle length (B), Number of grains per panicle (C)

27	Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, 100 grain weight (A), Harvest index (B)
28	Zn content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
29	Zn content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
30	Fe content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
31	Fe content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
32	Cu content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
33	Cu content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
34	Mn content in various tissues root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
35	Mn content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions
36	Image of melt curves of genes in qRT-PCR analysis
37	Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in root upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions
38	Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in node I upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions
39	Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in flag leaf upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions

40	Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in panicle upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions
41	Principal Component Analysis (PCA) biplot (A) depicting the distribution of treatments (control, seed priming, foliar spray, seed priming + foliar spray) under Zn deficient and sufficient conditions based on gene expression, yield traits, and elemental content. PCA biplot (B) showing the correlation between gene expression, yield traits, and elemental content under Zn deficient and sufficient conditions
42	Principal component analysis (PCA) biplot of rice traits under various treatments involving priming memory and repriming (A), Correlation matrix (B) of rice traits under various treatments

Abstract

Zinc (Zn) is an essential trace element needed by all living organisms for their proper growth, development and reproduction. Deficiency of Zn in human diets constitutes a significant nutritional issue. Biofortification of rice presents a viable and economical approach to enhance Zn intake, especially among populations that primarily consume rice. This study aimed to biofortify rice through nutripriming with Zn compounds ($ZnSO_4$ and $ZnNO_3$) to increase grain Zn content without compromising the yield. A preliminary evaluation of 30 rice genotypes, comprising 15 landraces and 15 elite varieties, was performed to determine their grain Zn content, as well as levels of other micronutrients, phenolics, amylose, anthocyanins, and flavonoids. Based on these observations, four hybrid elite varieties, viz. Annapoorna, Jyothi, Ponmani, and Uma, and four landraces such as Adukkan, Gandhakashala, Kumkumashali, and Mullankayama were selected for further biofortification studies. In these varieties, standardisation of priming concentration and duration for the priming agents $ZnSO_4$ and $ZnNO_3$ were analysed for seed priming and seedling priming. The priming dosage and duration with the priming agents $ZnSO_4$ and $ZnNO_3$ was fixed as 0.5M, 18 h. The dosage of seedling priming was also standardized at 0.5%. Seed and seedling priming in these rice varieties showed an enhancement in growth, yield, and grain Zn content, and the effect was more pronounced with combined seed and seedling priming treatments. The increase in grain Zn content and yield was greater with $ZnNO_3$ priming compared to $ZnSO_4$, leading to the selection of $ZnNO_3$ for further studies. The two rice varieties, Annapoorna and Kumkumashali, were chosen for additional analysis as they demonstrated a more pronounced response to the priming treatments. Priming with $ZnNO_3$ increased photosynthesis, as evident from the enhanced pigment content, photosystem activities, chlorophyll stability index, Chl *a* fluorescence parameters, and leaf gas exchange parameters. The antioxidative function of the primed plants was improved, as indicated by the activities of superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase. Consequently, lipid peroxidation was lower, the levels of superoxide and H_2O_2 were reduced, and decreased membrane leakage, alongside increased membrane stability, was observed in primed plants

compared to the non-primed control plants. Thus, by enhancing the photochemistry and antioxidant network, the growth and yield of the rice plants were improved in the primed plants. Priming at the seed and seedling stages, along with the additional foliar application of $ZnNO_3$ during critical reproductive stages such as booting, flowering, and the milky stages, enhanced the Zn content, reduced the phytate levels, and increased the bioavailability of Zn in rice grains. Among all the parameters analysed, Kumkumashali performed better than Annapoorna; thus, further analysis was conducted on this plant. Further studies aim to analyse the performance of rice growth, biomass, yield, and grain Zn content, alongside the expression patterns of membrane transporters, such as zinc-regulated iron-regulated transporter-like proteins (ZIP) and heavy metal ATPases (HMA) family genes (*OsZIP 1* to *10* and *OsHMA2*), in root, node I, flag leaf, and panicle under both Zn deficient and sufficient conditions, employing the techniques of seed priming and foliar spray at reproductive stages (booting, flowering, and milky stages). Under Zn deficient condition, the Zn treatments significantly enhanced yield and grain Zn content, including Zn content in various rice tissues, to levels comparable to those observed under the Zn sufficient condition. The various $ZnNO_3$ treatments induced the expression of several transporter genes, such as *OsZIP2*, *OsZIP8* and *OsHMA2*, particularly under Zn deficient conditions, indicating a crucial role for these transporters in improving Zn uptake, transport, and remobilization in rice. This research illustrated the impacts of seed priming with $ZnNO_3$, highlighting its capacity to enhance agronomic and yield characteristics, along with increasing grain Zn content in subsequent generation of rice. The carryover effects into the subsequent generation indicate that priming imprints could provide a viable approach for enhancing crop nutrition across various growing cycles. Re-priming further amplifies these advantages, suggesting its capacity to support biofortification initiatives in areas experiencing chronic Zn deficiencies. The outcomes of this research are promising for broader adoption in sustainable agricultural practices, paving the way for nutritionally enriched rice production.

Keywords: Biofortification, Foliar Spray, Hidden Hunger, HMA Seed Priming, ZIP.

സംഗ്രഹം

എല്ലാ ജീവജാലങ്ങൾക്കും അവയുടെ ശരിയായ വളർച്ചയ്ക്കും വികാസത്തിനും പുനരുൽപ്പാദനത്തിനും ആവശ്യമായ ഒരു അവശ്യ മൂലകമാണ് സിങ്ക്. മനുഷ്യരുടെ ഭക്ഷണത്തിൽ സിങ്കിന്റെ കുറവ് ഒരു പ്രധാന പോഷകാഹാര പ്രശ്നമാണ്. ബയോഫോർട്ടിഫിക്കേഷൻ എന്ന സുസ്ഥിര രീതിയിലൂടെ അരിയിൽ അടങ്ങിയിരിക്കുന്ന സിങ്ക് വർദ്ധിപ്പിക്കാൻ കഴിയും. സിങ്ക് സംയുക്തങ്ങൾ പ്രൈമിംഗ് ഏജന്റുകളായി ഉപയോഗിച്ച് ബയോഫോട്ടിഫിക്കേഷൻ രീതിയിലൂടെ ധാന്യത്തിന്റെ സിങ്ക് ഉള്ളടക്കം വർദ്ധിപ്പിക്കാൻ ഈ പഠനം ലക്ഷ്യമിടുന്നു. ധാന്യത്തിലെ സിങ്കിന്റെ അളവ്, മറ്റ് അവശ്യ ധാതുക്കൾ, ചില ന്യൂട്രാസ്യൂട്ടിക്കലുകൾ എന്നിവയുടെ അടിസ്ഥാനത്തിൽ 15 ലാൻഡ് റേസുകളും 15 എലൈറ്റ് ഇനങ്ങളും ഉൾപ്പെടുന്ന 30 അരി ഇനങ്ങളിൽ പ്രാഥമിക പരിശോധന നടത്തി. ഈ നിരീക്ഷണങ്ങളുടെ അടിസ്ഥാനത്തിൽ, നാല് എലൈറ്റ് ഇനങ്ങളായ അന്നപൂർണ, ജ്യോതി, പൊൻമണി, ഉമ, കൂടാതെ നാല് ലാൻഡ് റേസുകളായ അടുക്കൻ, ഗന്ധകശാല, കുംകുമശാലി, മുള്ളംകയമ തുടങ്ങിയവ കൂടുതൽ പഠനത്തിനായി തിരഞ്ഞെടുത്തു. ഈ ഇനങ്ങളിൽ വിത്ത് പ്രൈമിംഗിനും തൈകൾ പ്രൈമിംഗിനുമായി തിരഞ്ഞെടുത്ത പ്രൈമിംഗ് ഏജന്റുകളായ സിങ്ക് സൾഫേറ്റ്, സിങ്ക് നൈട്രേറ്റ് എന്നിവയുടെ വിത്ത് പ്രൈമിങ്ങിന് സാന്ദ്രതയും, ദൈർഘ്യവും, 0.5 മോളാർ, 18 മണിക്കൂറുമായും, തൈകൾ പ്രൈമിംഗിനുമായി 0.5 ശതമാനവും സ്റ്റാൻഡേർഡൈസ് ചെയ്തു. ഈ ട്രീട്മെന്റുകൾ ധാന്യ വിളവും ധാന്യത്തിലെ സിങ്കിന്റെ അളവും തിരഞ്ഞെടുത്ത നെല്ലിനങ്ങളിൽ വർദ്ധിപ്പിച്ചു. സിങ്ക് സൽഫേറ്റിനെ താരതമ്യം ചെയ്യുമ്പോൾ സിങ്ക് നൈട്രേറ്റ് പ്രൈമിങ് ട്രീറ്റ്മെന്റുകൾ കൂടുതൽ ഫലം ഉളവാക്കി. തിരഞ്ഞെടുത്ത 8 നെല്ലിനങ്ങൾ താരതമ്യം ചെയ്യുമ്പോൾ അന്നപൂർണയും കുംകുമശാലിയും മികച്ച പ്രതികരണം കാണിച്ചു. ആയതിനാൽ തുടർപഠനങ്ങളിൽ സിങ്ക് നൈട്രേറ്റും, നെല്ലിനങ്ങളായ അന്നപൂർണ കുംകുമശാലി എന്നിവയെ തിരഞ്ഞെടുക്കാൻ കാരണമായി. ഈ അരി ഇനങ്ങളിൽ പ്രകാശസംശ്ലേഷണം, ആന്റിഓക്സിഡന്റ് സിസ്റ്റം, ഓക്സിഡേറ്റീവ് സ്ട്രെസ്, മെറ്റബോളൈറ്റുകൾ എന്നിവയിൽ സിങ്ക് നൈട്രേറ്റിന്റെ സ്വാധീനം പഠിച്ചു.സിങ്ക് നൈട്രേറ്റ് ഉപയോഗിച്ചുള്ള വിവിധ പ്രൈമിംഗ് രീതികൾ പ്രൈമഡ് സസ്യങ്ങളിലെ ഫോട്ടോസിന്തസിസ്, ആന്റിഓക്സിഡന്റ് സിസ്റ്റം, മെറ്റബോളിറ്റുകൾ എന്നിവ മെച്ചപ്പെടുത്തുകയും അവയിലെ ഓക്സിഡേറ്റീവ് സമ്മർദ്ദം

കുറയ്ക്കുകയും ചെയ്തതായി ശ്രദ്ധിക്കപ്പെട്ടു. വിത്ത്, തൈകൾ എന്നിവയുടെ പ്രൈമിംഗിന് പുറമേ, പ്രത്യുൽപാദനത്തിന്റെ നിർണായക ഘട്ടങ്ങളിൽ (ബൂട്ടിംഗ്, പൂവിടൽ, പാൽ ഘട്ടങ്ങൾ) സിങ്ക് നൈട്രേറ്റ് ഉപയോഗിച്ച് അനുബന്ധ ഫോളിയർ സ്പ്രേ നടത്തി. വിവിധ സിങ്ക് ആപ്ലിക്കേഷൻ രീതികൾ ധാന്യ സിങ്കിന്റെ അളവും ജൈവ ലഭ്യമായ സിങ്കും വർദ്ധിപ്പിച്ചതായും ഫൈറ്റിക് ആസിഡിന്റെയും ഫൈറ്റിക് ആസിഡ് സിങ്ക് മോളാർ അനുപാതത്തിലും കുറവുള്ളവയാകുന്നതായതും ഈ പഠനം വെളിപ്പെടുത്തി. മേൽപറഞ്ഞ പഠനങ്ങളിൽ അന്നപൂർണയെയേക്കാൾ കുംകുമശാലി മികച്ച പ്രകടനം കാഴ്ചവെച്ചതിനാൽ കൂടുതൽ പഠനങ്ങൾക്കായി കുംകുമശാലിയെ തിരഞ്ഞെടുത്തു. വിളവ്, ധാന്യ സിങ്കിന്റെ അളവ്, സിങ്ക് നിയന്ത്രിത ട്രാൻസ്പോർട്ടറുകളുടെ ഡിഫറൻഷ്യൽ എക്സ്പ്രഷൻ പാറ്റേൺ, എന്നിവ സിങ്ക് കുറവുള്ള സാഹചര്യത്തിലും സിങ്ക് കൂടുതലുള്ള സാഹചര്യത്തിലും വിവിധ പ്രൈമിംഗ് രീതികൾ എങ്ങനെ ബാധിക്കുന്നു എന്നുള്ളവ കൂടുതൽ പഠനങ്ങൾ ലക്ഷ്യമിടുന്നു. സിങ്ക് ട്രീട്മെന്റുകൾ വിവിധ അരി കോശങ്ങളിലെ (റൂട്ട്, നോഡ് I, ഫ്ലാഗ് ലീഫ്, പാനിക്കിൾ) സിങ്കിന്റെ അളവ് വർധിപ്പിക്കുന്നതായും, കൂടാതെ ഈ സസ്യങ്ങളിൽ ധാന്യ വിളവ് കൂടുന്നതായും കണ്ടെത്തി. വിവിധ സിങ്ക് നൈട്രേറ്റ് ട്രീറ്റ്മെന്റ് *OsZIP*, *OsHMA* പ്രത്യേകിച്ച് *OsZIP2*, *8*, *10*, *OsHMA2* എന്നിവയുടെ എക്സ്പ്രഷൻ പ്രൈമഡ് പ്ലാന്റുകളിൽ നിയന്ത്രിച്ചു. വ്യക്തിഗത ട്രീട്മെന്റുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ പഠിച്ച എല്ലാ പാരാമീറ്ററുകളിലും, സംയോജിത ആപ്പ്ളിക്കേഷനിൽ സിങ്ക് നൈട്രേറ്റ് മികച്ച ഫലങ്ങൾ നൽകി. പ്രൈമിംഗ് ട്രീറ്റ്മെന്റിന്റെ ഈ പ്രഭാവം തുടർന്നുള്ള തലമുറയിലും പ്രകടമാകുന്നതായും റീ-പ്രൈമിംഗ് അതിനെ കൂടുതൽ മെച്ചപ്പെടുത്തുന്നതായും കണ്ടെത്തി. ഈ ഗവേഷണത്തിന്റെ ഫലങ്ങൾ പോഷക സമൃഷ്ടമായ നെല്ല് ഉൽപാദനത്തിന് വഴിയൊരുക്കുന്ന സുസ്ഥിര കാർഷിക രീതികൾ സ്വീകരിക്കുന്നതിനുള്ള വാശാനമാണ്.

സൂചകപദങ്ങൾ: ബയോഫോർട്ടിഫിക്കേഷൻ, പോഷക അപര്യപ്തതാ, വിത്ത് പ്രൈമിംഗ്, ഫോളിയർ സ്പ്രേ, സിപ്പ്, എച്ച്എംഎ.

ABBREVIATIONS

ABS/CSo	-	Absorption flux per cross section
APX	-	Ascorbate peroxidase
BSA	-	Bovine serum albumin
CAT	-	Catalase
CSI	-	Chlorophyll stability index
DCMU	-	3 (3,4dichlorophenyl) -1, 1-dimethyl urea
DCPIP	-	2, 6, Dicholrophenolindophenol
DTNB	-	5 , 5-dithio-bis(2-nitrobenzoic acid)
EDTA	-	Ethylenediamine tetra-acetic acid
EL%	-	Electrolyte leakage %
Fm	-	Maximum Chl *a* fluorescence
Fo	-	Initial Chl *a* fluorescence
Fv	-	Variable Chl *a* fluorescence
Fv/Fo	-	The ratio of photochemical to non photochemical quantum efficiencies
GPOX	-	Guaiacol peroxidase
HMA		Heavy metal ATPase
H_2O_2	-	Hydrogen peroxide
HEPES	-	(N-(2-Hydroxyethyl) piperazine-N-(2- ethane sulphonic acid)
MDA	-	Malondialdehyde
mM	-	Millimolar
MV	-	Methyl viologen
NADH	-	Nicotinamide adenine dinucleotide
NaN_3	-	Sodium azide

NBT	-	Nitro blue tetrazolium
NPQ	-	Non-photochemical quenching
$O_2^{\bullet -}$	-	Superoxide
^oC	-	Degree Celsius
PA		Phytic acid
PBQ	-	Para-benzoquinone
PC1	-	Principal component 1
PC2	-	Principal component 2
PCA	-	Principal component analysis
PI(abs)	-	Performance index on absorption basis
PS I	-	Photosystem I
PSII	-	Photosystem II
r	-	Pearson correlation coefficient
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
ZIP	-	Zinc regulated Iron regulated transporter like protein
Zn	-	Zinc
$ZnNO_3$	-	Zinc nitrate
$ZnSO_4$	-	Zinc sulphate

INTRODUCTION

Zinc (Zn) is the 30th element in the periodic table. The German Andreas Sigismund Marggraf identified the purified metallic form in 1746 (Cleave and Crans, 2019). The necessity of Zn for biological processes was originally recognized by Raulin when it was seen that the common bread mold (*Aspergillus niger*) could not proliferate without Zn (Raulin, 1905). Later, Keilin and Mann identified Zn as a cofactor in the enzyme carbonic anhydrase in erythrocytes. This enzyme plays a crucial role in transporting carbon dioxide in the blood (Riordan, 1976; Natasha et al., 2022). Aside from its catalytic function, it also plays a role in gene expression and sustains the structure of nucleic acids, proteins and the integrity of membranes and organelles. It have vital function in the immune system function (Vallee and Auld, 1990; Suganya et al., 2020a) and also act as a second messenger (Yamasaki et al., 2007; Chen et al., 2024). Additionally, it can control epigenetic pathways (Yusuf et al., 2021). Zn is a redox-active element in the field of chemistry. However, in the realm of biology, it consistently exists as Zn(II) and its redox characteristics are not significant. However, they can provide redox activity focused on ligands at Zn coordination sites by interacting with cysteine's sulphur in biological proteins. The binding sites of Zn possess a high affinity and specificity, enabling regulation in redox biology (Krężel and Maret, 2016; Maret and Blower, 2022). Hence Zn have plethora of essential functions in the entire domains of life.

Zn is the second most prevalent metal and divalent cation in humans. It is essential for various cell functions, such as cell division, differentiation, cell growth, cellular transport, wound cure, insulin production and release, blood pressure regulation and function of endocrine system. It is very essential for the metabolism of thyroid hormone as it plays role in the synthesis of thyroid releasing hormone and thyroid stimulating hormone, thus insufficiency in Zn lead to hypothyroidism. Over 300 human enzymes require Zn as a cofactor or functional component for their activity. Zn is crucial for maintaining the tertiary structure of various proteins, particularly transcription factors (TFs), including Zn finger TFs, and is also essential

for gene expression. Therefore, it is essential for the physiological and biological functions of the human body (Gibson, 2012). The highest concentration of Zn is observed in the testes, muscle, liver, bones, and brain. It is also involved in the formation of central nervous system. It is highly prevalent in the synaptic vesicles and it performs crucial functions in the processes of learning and memory. The total Zn content in the body is roughly 1.5 g in females and 2.5 g in males. Serum or plasma Zn concentrations are commonly utilised in clinical practice to evaluate Zn status. In healthy individuals, serum or plasma Zn levels range from 80 to 120 mcg/dL. Serum Zn concentrations below 70 mcg/dL in females and 74 mcg/dL in males signify insufficient Zn status (Ryu and Aydemir, 2020). The recommended dietary allowance of Zn is 8 and 11 mg/day for women and men respectively, while for new-borns, infants, pregnant and lactating women, the daily requirement is higher. The recommended dietary requirement of Zn for babies is 3 to 5 mg per day, and for children aged 1 to 10 years, it is 10 mg per day. The dietary allowance for breastfeeding women is 16 to 19 mg/day (Marschner, 1995; Singh and Prasad, 2014). A reduction in dietary Zn results in diminished serum Zn levels and exhibits corresponding clinical signs of insufficiency. The risk of age-related macular degeneration, eventually causing vision loss is also high under Zn deficiency. Imbalance in the Zn levels is crucial for progression of cardiovascular illnesses, cancer, diabetics, obesity, skin disorder, mental disease, neurodegenerative diseases and aging (Chasapis et al., 2020). Furthermore, an excessive depletion of Zn can lead to gastrointestinal or urinary tract illnesses and bowel diseases. The deficiency also contributes to the compromise of immune system, thus people become more prone to pathogens, and hence infections from microorganisms like bacteria and viruses (Chasapis et al., 2012). Zn deficiency in humans may result in blindness, cognitive impairments, reduced intelligence, growth retardation, and infertility. Newborns, infants, children, pregnant and lactating women, and older adults constitute high-risk populations for Zn deficiency. The Zn deficiency in humans is due to low Zn intake, inadequate absorption of Zn due to health issues or pathological conditions, and increased requirements during different growth stages (Hussain et al., 2022).

The maximum acceptable amount of consumption is 40 mg/day in adults. Zn is not retained in the body, hence a daily dose of Zn is necessary to maintain optimal levels and support its various functions (Cakmak et al., 2017). Presently, Zn insufficiency affects approximately 17% of the worldwide population and is accountable for 4% of global infant illness and death (Chasapis et al., 2012, 2020). The Zn deficiency can also cause, a rare autosomal recessive skin disease called acrodermatitis enteropathica. In this case, SLC39A4 gene on chromosome 8, which codes for ZIP4, a transporter for Zn is mutated (Stiles et al., 2024). It is asserted that protein-rich foods are abundant in Zn, but carbohydrate-rich foods are deficient in Zn. The meat source possesses a high Zn level, ranging from 0.40 to 6.77 mg per 100 g. Animal derived foods are the primary sources of Zn in the human diet. The oyster, red meat and liver are also abundant in Zn. Cereals, legumes and potatoes possess Zn with low bioavailability (Kaur et al., 2014). Seeds, including pumpkin, hemp, chia, and nuts such as cashew, hazelnut, and peanut, are also abundant in Zn (Dodevska et al., 2022). However, these rich sources of Zn are not accessible to impoverished population, due to its high cost. Zn deficiency results in widespread malnutrition, often referred to as hidden hunger, on a global scale. Approximately 2 million individuals globally are impacted by Zn malnutrition. This deficiency is exacerbated in developing and underdeveloped countries, where the majority of the population relies on staple foods for their daily caloric intake (Stangoulis and Knez, 2022). Plant micronutrient levels and availability are crucial for crop health and at the same time significantly affect the absorption of Zn in human diets. Zn deficiency in humans causes more than 400,000 deaths per year (Stanton et al., 2022).

In 2004, the International Zn Nutrition Consultative Group (IZiNCG) classified India as a high-risk nation for Zn deficiency due to above 25% dietary Zn inadequacy. However, there are no meaningful national estimates available. Consequently, Pullakhandam et al. (2021) sought to assess the national and state-level prevalence of low serum Zn concentrations (SZCs) in Indian children based on the nationally representative comprehensive national nutrition survey. The national frequency of low SZC among preschool (17%) and school-age children (16%) was below 20%, the threshold suggesting a public health concern; nonetheless, variances

existed by state and socioeconomic position. The comprehensive national nutrition survey (2016-18) results by the national health mission indicate plasma Zn deficiency among children under 4 years (19%), girls (28.4%), and boys (35%) in India. Approximately 5 to 55% of the population across various states were impacted by Zn deficiency (Khokhar et al., 2024). The correlation between soil Zn content and health outcomes, including stunting in children and height in women, was evaluated by Morton et al. (2023). They discovered that an increase in bioavailable Zn could diminish stunting in children and promote growth in height for women.

Zn is also an essential plant nutrient. Importance of Zn in plants was initially demonstrated in maize in 1915. In 1940, Skoog reported that severe Zn shortage symptoms in tomato plants included decreased stem elongation. Severe Zn deficiency causes root apex necrosis, while mild Zn deficiency leads to uneven chlorosis between veins, shortened internodes, epinasty and reduced leaf size. In rice, the common symptoms include elevated plant death rates, reduced growth, leaf discolouration, and delayed blooming (Younas et al., 2023). The primary source of Zn for plants is soil. Zn in soil originates from the weathering of rocks. In sedimentary rocks, its concentrations vary from 80 to 120 mg/kg, in argillaceous sediments and shales, 15 to 30 mg/kg in sandstones and 10 to 25 mg/kg in limestones and dolomites. In magmatic rocks, the values range from 40 to 120 mg/kg. Zn in agricultural soils is predominantly irregularly distributed, with concentrations varying from 10 to 300 mg/kg (Noulas et al., 2018). Zn insufficiency is prevalent in crops, especially in alkaline soils with low Zn levels. The ideal concentration of Zn in leaves is 30 to 100 ppm. Concentrations over 100 to 700 ppm can induce Zn toxicity; nevertheless, Zn hyperaccumulators can sequester over 3000 ppm in their above-ground tissues (Stanton et al., 2022).

Approximately half of cultivated soils all over the globe is deficient in Zn (Alloway, 2009). Nearly all rice-producing countries have reported losses in rice production due to Zn deficiency. A reduction in yield of approximately 10 to 60% may occur due to Zn deficiency, with severe deficiency potentially leading to plant

death and stand loss (Coffin and Slaton, 2020). In 2021, Shukla and colleagues conducted an analysis of phytoavailable Zn in Indian soil, utilising 242,827 soil samples collected from agricultural fields across 615 districts in 28 states of India. It was determined that 51.2% of the soil samples exhibited a deficiency in Zn. In several states, including Andhra Pradesh, Assam, Bihar, Chhattisgarh, Goa, Gujarat, Karnataka, Madhya Pradesh, Maharashtra, Odisha, Rajasthan, Telangana, and Uttar Pradesh, the deficiency in Zn exceeded 50%. Approximately 15% of soil in Kerala exhibited a deficiency in Zn (Shukla et al., 2021).

During green revolution, the major concern was to feed the growing population and to eradicate hunger from the globe. Hence, the scientists and policy makers prime aim was to increase the yield of crop plants like rice. As a result, unintentionally the nutritional traits were left behind, causing a major issue called micronutrient malnutrition or hidden hunger. One of the major cause for this issue is the enhanced dependence on staple food crops such as cereals like rice and wheat. Rice serves as the primary nutritional source for approximately 50% of the global population. Rice is inherently low in bioavailable Zn. The cultivation of rice in Zn deficient regions further diminishes the Zn content in the grain, adversely affecting the marginalized population and contributing to Zn malnutrition (Lowe et al., 2024). There are various methods for alleviating Zn malnutrition. It includes diet diversification, food fortification and biofortification. The former two methods are not cost effective and sustainable in developing countries. The best way to diminish Zn malnutrition is biofortification (Veena and Puthur, 2022).

Biofortification enhances the nutrient content of edible crop parts by increasing the availability of essential elements and vitamins, especially in the staple food crops. This can be achieved through plant breeding, agronomic methods and transgenic technologies (Bouis and Saltzman, 2017). Agronomic biofortification can be implemented through various methods, including soil fertilization, seed priming, and foliar spraying. It offers greater advantages over genetic manipulations that utilize diverse breeding strategies and recombinant DNA technologies. The latter two techniques require significant time investment, and moreover, the transgenic

technique faces ethical clearance challenges, hindering its release in many countries (Garg et al., 2018).

In 1991, CGIAR (Consultative Group on International Agricultural Research) began research on the development of "micronutrient-dense" staple crops under the initiative of addressing global micronutrient insufficiency concerns expressed by the international nutrition community. CGIAR initiated the harvestplus challenge programme in 2003 as a worldwide initiative to create biofortified staple crops including wheat, rice, maize, cassava, etc., using plant breeding techniques (Rao et al., 2020). By 2024, they had successfully introduced 450 biofortified cultivars across 12 crops in 41 nations (Bouis et al., 2024). The Food and Agricultural Organisation (FAO) of the United Nations (UN) also considers biofortification as a good tool to combat micronutrient deficiency. The World Health Organisation (WHO) views biofortification as a sustainable strategy to combat micronutrient malnutrition and recommends its inclusion in every country's food and nutrition programs (Bouis et al., 2024).

The United Nations (UN) introduced a sustainable development framework consisting of 17 goals, known as the sustainable development goals (SDGs), with the aim of achieving them by 2030. The objectives aim to enhance prosperity while ensuring environmental protection. The second goal is to eliminate hunger, aiming to achieve food security through enhanced nutrition and sustainable agricultural practices. Hunger and micronutrient malnutrition, often referred to as hidden hunger, can impair an individual's energy status, increasing susceptibility to diseases. Goal 3 of the SDGs aims to ensure good health and well-being for all age groups. The COVID-19 pandemic and its subsequent effects have disrupted the existing health system. A medical emergency can similarly impact both affluent and impoverished nations, potentially resulting in poverty and bankruptcy (https://www.un.org/sustainabledevelopment/). The COVID-19 pandemic, a highly transmissible respiratory illness, has significantly affected global socioeconomic conditions. Various risk factors, such as poor nutrition and underlying non-communicable diseases, weaken the immune system, increasing vulnerability to severe infections

that can lead to pneumonia-related mortality. Zn supplementation is commonly used alongside other therapeutic agents in COVID-19 treatment due to its role in enhancing antiviral immunity. Zn^{2+} ions help inhibit RNA-dependent RNA polymerase, limiting viral replication, while Zn finger antiviral proteins bind to and degrade viral RNA within host cells, aiding in the body's defense against the virus (Rahman and Idid, 2021).

Biofortification aims to augment the nutrient content in crops while preserving desirable features favoured by consumers and farmers. This is acknowledged as a nutrition-sensitive agriculture solution capable of mitigating vitamin and mineral deficiencies (Talsma and Pachón, 2017). Iron (Fe) biofortification of beans, cowpea, and pearl millet; Zn biofortification of maize, rice, and wheat and pro-vitamin A carotenoid biofortification of cassava, maize, rice, and sweet potato are presently in progress and at various developmental phases (Bouis et al., 2011, 2024). The biological process by which biofortified crops enhance nutritional status is straightforward: biofortified crops possess a higher nutrient density compared to non biofortified types (Saltzman et al., 2013). Consequently, presuming comparable micronutrient bioavailability and retention, post-cooking, processing, and storage, individuals will ingest and assimilate a greater quantity of micronutrients from biofortified crops than from an equivalent amount of non biofortified crops (Rosado et al., 2009; Frano et al., 2014; Moura et al., 2015).

Provitamin A biofortified orange sweet potato has helped to mitigate vitamin A insufficiency in children in Mozambique (Low et al., 2007; Hotz et al., 2012a), Uganda (Hotz et al., 2012b), and South Africa (Jaarsveld et al., 2005). The outcomes for Fe biofortified crops are encouraging, Fe biofortified rice enhanced the Fe in reproductive-age women in the Philippines (Haas et al., 2005), Fe biofortified pearl millet was found to be augmenting Fe levels and rectifying Fe deficiency in school children in India (Finkelstein et al., 2015), and Fe biofortified beans elevated Fe in women of Rwanda (Haas et al., 2016).

Two primary behavioural concerns pertain to biofortification: one affecting the farmer and the other the consumer. Agriculturalists seek to cultivate novel

varieties that exhibit agronomic superiority over existing ones, such as cultivars that have enhanced drought resistance, increased yield, or reduced disease susceptibility (Pfeiffer and McClafferty, 2007). Crops exhibiting enhanced micronutrient concentration but equivalent or inferior agronomic performance will not be embraced or adopted by farmers (Bouis et al., 2011). Consequently, biofortified varieties must be agronomically comparable or ideally superior to the less nutrient-dense market and traditional types with which they will contend. The protracted process of developing biofortified crops, has led to a focus on accelerating selective plant breeding activities (Birol et al., 2015). Biofortified crops are often assumed transgenic or genetically modified organisms. While biofortification initiatives through international programs mostly focus on cultivating nutritionally enriched crops by traditional breeding techniques, there is an increasing inclination towards utilising genetic modification to improve both staple and non-staple crops (Saltzman et al., 2013). Consequently, consumer education and an effective regulatory framework to evaluate the advantages and disadvantages of genetically modified crops will be essential to enhance consumer confidence in biofortified crops created by genetic alteration prior to their introduction in local markets.

Nonetheless, this is evolving due to the implementation of cost-effective and efficient laboratory tests to identify promising varieties, as biofortification is increasingly acknowledged as a sustainable strategy to be integrated into various national programs on food and nutrition security in countries like Colombia, Kenya, Nicaragua, Nigeria, and Panama. The emphasis is transitioning to enhancing seed systems, generating demand and supply, dissemination, and marketing as components of the biofortification package. Global initiatives are in progress to stimulate demand for high-yield biofortified crops and to produce nutritious food products from these crops, ensuring that non-farmers and urban customers can also reap the benefits (Talsma and Pachón, 2017). Biofortification of staple food crops, such as rice, serves as an appropriate intervention for addressing Zn malnutrition and its associated consequences in developing countries like India (Senguttuvel et al., 2023).

Rice (*Oryza sativa* L.) is a staple food that sustains a large segment of the world's population. However, the challenge of sustaining and enhancing rice yield is hindered by shifting environmental conditions and increasing demands. Reducing the adverse effects of environmental change and biotic stressors on rice is crucial for enhancing and stabilizing rice yield (Rezvi et al., 2023). Enhancing the photosynthetic efficiency of rice plants is essential for achieving higher crop yields under the current circumstances. Zn is a vital transition trace element required by all living organisms for optimal growth, development, and reproduction. This element plays a crucial role in all six classes of enzymes. It is vital for key physiological processes, including photosynthesis, antioxidant defence, mitigation of free radical damage, and the preservation of membrane structure and functional integrity. It also contribute to gene expression and protein synthesis. And also essential for increasing crop yields and enhancing pollen fertility (Blakeney et al., 2020). Additionally, Zn is essential for detoxifying reactive oxygen species (ROS). It simultaneously activates genes involved in antioxidant defence, including ascorbate peroxidase (APX) and glutathione reductase (GR). Moreover, Zn is a key component for the proper functioning of the enzyme superoxide dismutase (Cu/Zn SOD), which plays a critical role in neutralizing oxidative stress (Cakmak, 2000). Zn deficiency disrupts cell homeostasis, resulting in increased free radicals and subsequent growth inhibition. Therefore, providing an adequate amount of Zn enables the plant to mitigate these adverse effects, resulting in improved growth (Clemens, 2022). The adaptation of seed priming and foliar spray with Zn compounds is hypothesized to enhance the photosynthetic efficiency of rice plants and mitigate oxidative stress, thereby improving yield and nutritional status (Hassan et al., 2024).

Cereals, such as rice, are highly susceptible to Zn deficiency. Rice cultivated in low phytoavailable Zn conditions experiences diminished productivity and yields nutrient deficient grains. An 80% reduction in grain Zn content was observed when rice was grown in Zn deficient soil. This deficiency not only decreased the Zn content but also diminished the bioavailable Zn, thereby exacerbating Zn malnutrition in humans (Zaman et al., 2018; Zubair et al., 2024). Zn insufficiency is the sixth leading cause of mortality and human disease. Worldwide, rice is second

only to wheat in harvested area (158 million hectares) and production (exceeding 755 million tonnes of milled rice in 2019) (Rahman and Zhang, 2023). Approximately 90% of global rice cultivation occurs in South Asia (58 million hectares), Southeast Asia (43 million hectares), and East Asia (31.5 million hectares). It serves as the primary staple meal for around half to two-thirds of the global population. Globally, around 3.5 billion individuals rely on rice for almost 20% of their daily caloric consumption. Moreover, more than 2 billion individuals in Asia obtain 80% of their energy requirements from rice. India possesses the greatest area devoted to paddy cultivation globally (44 million hectares) and ranks as the second-largest rice producer (89 million tonnes per annum); nonetheless, its productivity remains significantly lower (2.05 tonnes per hectare) than the world average (2.62 tonnes per hectare) (Marschner, 1995; Meenakshi et al., 2010; Singh and Prasad, 2014).

In India, rice constitutes approximately 24% of the total planted area. It accounts for 43% of total food grain output, 46% of total cereal production in the country, and exclusively provides 30% of the entire caloric intake in the Indian diet. Additionally, children below the age of 6 consume 118 g of rice each day (Meenakshi et al., 2010; Singh and Prasad, 2014). Considering population growth trends and per capita availability, the future need for rice production is estimated to be approximately 215 to 230 g per day, necessitating 109 to 117 million tonnes of rice production by 2025. The typical Zn concentration in rice tissue ranges from 25 to 100 ppm, with deficient symptoms manifesting when levels drop below 20 ppm (Marschner, 1995; Singh and Prasad, 2014). Considering the bioavailability of Zn from rice, rice bran is the predominant reservoir of Zn, containing over three times the amount found in the endosperm, while the polishing process diminishes the Zn level (Nakandalage et al., 2016).

Landraces represent a significant reservoir of genetic variation, superior nutritional and sensory quality products, and resilience consistent in challenging conditions. They were helpful in securing food security, until the emergence of formal plant breeding, which resulted in the creation of high-yielding varieties that

progressively replaced landraces. Currently, landraces are receiving renewed attention due to the escalation of genetic erosion (due to monoculture of cultivars) and the rising consumer demand for diverse and locally sourced food products (Lazaridi et al., 2024). They are indigenous variants of a cultivated plant species that have been adapted to their specific natural and cultural environments. India hosts numerous landraces, which exhibit significant morphological and genetic diversity, making them prime subjects for thorough investigation (Das et al., 2013). Rich in vital amino acids, vitamins, anthocyanins, and flavonoids, landraces can also help treat metabolic and noncommunicable diseases (Panda et al., 2024). A study conducted in rice of Kerala, revealed that the amount of essential elements, amylose content and antioxidants were high in landraces, compared to elite varieties (Veena et al., 2023). Similarly, a recent study on common beans revealed that landraces contained higher levels of key mineral components, vital fatty acids such as linolenic acid, and antioxidants such as anthocyanin, flavonoids, and ascorbate compared to superior varieties (Gorbe et al., 2025). Given the advantageous nutritional impacts of these landraces, biofortification initiatives must also concentrate on them. Until now, there are only a limited number of studies on landraces. The majority of research concentrate on a limited array of genotypes, neglecting traditional or landrace variants.

The presence of Zn in soil presents several constraints for its uptake by plants and subsequent accumulation in rice grains. Soil Zn is an essential mineral derived from natural processes and anthropogenic activities. There are five different types of it: chelated to primary weathering minerals, adsorbed or chelated organic, exchangeable, non-exchangeable, and water-soluble (Dhaliwal et al., 2019). The soil Zn concentration changes from 10 to 300 ppm, with a mean concentration of approximately 50 ppm. Numerous factors influence the phytoavailability of Zn, including pH, redox potential, organic matter and clay content, humus levels, cation exchange capacity, microbial activity, soil structure, moisture levels, and interactions with other soil components. Acidic soil contains the divalent cation Zn^{2+}, and basic soil contains the monovalent cation $ZnOH^{+}$. The increase of pH in alkaline soil results in decreased Zn availability due to calcareous conditions.

Organic matter can either solubilise or immobilise Zn, with the solubilised form being more accessible to plants (Baran et al., 2018). Soil microorganisms, including bacteria and arbuscular mycorrhizae, facilitate the solubilisation of Zn in the rhizosphere via acidification. The physical structure of soil and its ability to retain moisture play a crucial role in maintaining phytoavailable Zn. Well-aerated, porous soil with sufficient moisture can enhance Zn availability to plants (Benedet et al., 2020). However, phosphorus can negatively affect Zn uptake, as its application has been shown to reduce Zn levels in wheat and other staple crops (He et al., 2021). In contrast, nitrogen supplementation can enhance Zn availability in the soil and increase Zn accumulation in grains (Zhao et al., 2022).

Seed priming and the foliar application of Zn compounds can effectively mitigate these limitations. Seed priming is a cost-effective method that improves plant stress tolerance by enhancing seed quality. This technique initiates the metabolic processes associated with seed germination, stimulates photosynthesis, enhances antioxidant systems, and promotes growth and productivity in plants (Jarrar et al., 2024). Zn sources utilized in seed priming include inorganic, organic, chelated forms, and Zn nanoparticles (Zn-NPs). Common inorganic sources consist of zinc sulphate ($ZnSO_4$), zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$), zinc chloride ($ZnCl_2$), zinc nitrate ($Zn(NO_3)_2$), zinc carbonate ($ZnCO_3$), and zinc oxide (ZnO). Among these, $ZnSO_4$ and $ZnSO_4 \cdot 7H_2O$ are widely preferred due to their high solubility, minimal interaction with soil components, and cost-effectiveness (He et al., 2025). The most commonly used synthetic chelated form is Zn-EDTA, where Zn is bound to a chelating agent. However, synthetic chelators pose challenges such as poor biodegradability, rapid photodegradation, and potential toxicity when applied excessively, and also it is costly (Tayyari et al., 2024). Organic Zn chelators, such as Zn amino acid complexes, are gaining recognition for their efficiency in seed priming. These include Zn complexes with histidine (Zn-His), methionine (Zn-Met), glutamine (Zn-Gln), glycine (Zn-Gly), and arginine (Zn-Arg), which are frequently used in priming applications (Saini et al., 2024a).

Priming is an agricultural technique that enhances crop growth and nutritional quality. Nutripriming involves soaking seeds in a nutrient-rich solution for a specific period to improve nutrient uptake. This method not only provides essential nutrients but also facilitates biochemical processes during seed priming. It supports crops by fulfilling micronutrient needs, enhancing germination, seedling emergence, stand establishment, yield, and grain micronutrient accumulation. Additionally, it strengthens plants against both abiotic and biotic stresses (Kumari et al., 2025). Traditional fertilizers are primarily sourced from mining, a non-renewable process that contributes to environmental pollution. Moreover, when nutrients are applied to soil, their availability to plants is often limited by soil physicochemical properties, creating challenges in nutrient absorption (Irfan et al., 2024).

Foliar application of Zn fertilisers in agronomic biofortification demonstrates greater efficiency compared to soil application and can increase grain Zn content across diverse countries, environments, and agricultural management practices. The process demonstrates increased efficiency and cost-effectiveness, dependent upon the timing of application. Zn applied to the leaves is absorbed by the epidermis, then translocated to the phloem, ultimately reaching the grain. The cuticle and trichomes helps in the uptake of Zn applied to the leaves. Low doses of Zn are commonly employed to mitigate leaf scorching (Han et al., 2024). Foliar application of Zn compounds effectively addresses the physiological Zn requirements of plants, enhancing photosynthesis, improving antioxidant mechanism, and reducing lipid peroxidation (Sattar et al., 2022). Foliar application at advanced developmental stages increased Zn accumulation in both whole grain and endosperm. The concentration of Zn in the grain was elevated after foliar application during the booting, anthesis and the milking stages (Akça et al., 2022). The timing of foliar application has a significant impact on Zn distribution in the grain and it requires an evaluation for optimal results (Ram et al., 2024). The Zn content alone is insufficient to determine the bioavailable Zn in rice, as various compounds, including phytate, chelate the metals. To comprehend bioavailability, it is essential to explicitly

estimate the inhibitors that can complex with Zn, thereby reducing their absorptive capacity or bioavailability (Manzoor et al., 2024).

Biofortification is a strategy aimed at increasing the micronutrient content of crops; however, uptake and distribution of these nutrients within plant cells and organelles is complex, as it is regulated by various physiological and molecular processes. These processes involve the coordinated uptake, transport, storage, and redistribution of minerals, facilitated by membrane transporters that aid in the movement of nutrients into the seed endosperm (Roorkiwal et al., 2021). Developing biofortified crops with enhanced micronutrient levels holds promise for addressing global malnutrition. Plants absorb mineral nutrients from the soil through their root systems, which are then transported to aerial parts and distributed throughout the plant. These nutrients are essential for cellular structures and membranes within plant organelles. Their movement occurs through membrane carrier proteins, ion channels driven by electrochemical gradients, or active transport mechanisms facilitated by electrogenic pumps. Membrane transporters play a key role in cellular nutrient uptake, intercellular transport, distribution, and storage, particularly in the edible portions of crops (Che et al., 2023).

Several transporter families contribute to the uptake and movement of micronutrients within plants. These include zinc-regulated and iron-regulated transporter-like proteins (ZIP), Phosphorylation type ATPases (P-type ATPase), cation diffusion facilitators (CDF), natural resistance-associated macrophage proteins (NRAMP), cation exchangers (CAX), plant cadmium resistance (PCR) proteins, vacuolar iron transporters (VIT), and yellow stripe-like (YSL) transporters (Hu and Jiang, 2024; Mishra et al., 2024). These transporters regulate the movement of essential elements such as zinc (Zn), iron (Fe), cobalt (Co), cadmium (Cd), copper (Cu), manganese (Mn), and nickel (Ni). While many membrane transporter families have been identified and characterized in plants, limited information is available regarding their role in enhancing mineral accumulation in the edible parts of crops. Research using transgenic approaches suggests that modifying membrane

transporters can significantly improve the micronutrient content of food crops, offering a potential solution to micronutrient deficiencies (Krishna et al., 2023a).

The Zn-regulated, Iron-regulated transporter-like Protein (ZIP) was initially characterised in *Arabidopsis thaliana*, deriving its name from the Zn-regulated transporter (ZRT1 and ZRT2) genes of *Saccharomyces cerevisiae* and the iron-regulated transporter (IRT1) gene of *A. thaliana*. The transmembrane protein ZIP is anticipated to have eight transmembrane (TM) domains, with the variable region between the TM3 and TM4 domains contributing to the variable length of the protein. The TM4 region, which has an amphipathic helix and histidine-rich residues, is the most conserved part of the protein (Zhang et al., 2017). The crystal structure and transport mechanism of ZIP proteins in plants remain undetermined. The BbZIP protein from *Bordetella bronchiseptica*, a gram-negative bacterium has been extensively researched and documented. The structure features a unique 3+2+3TM arrangement, consisting of closely associated 8 TM helix domains. The entrance cavity is situated in the outer membrane orientated towards the cell exterior, whereas the exit cavity is positioned in the inner membrane, directed towards the cytoplasmic side. The BbZIP transport mode is characterised as an elevator-type transport mechanism. The structure of the BbZIP TM protein exhibits significant differences between its metal-unbound state and metal-bound state (Pang et al., 2023).

P1B-ATPases are transition metal transporters classified within the P-type ATPase superfamily, which is further divided into Cu/Ag and Zn/Cd/Co/Pb transporters. These proteins possess 6 to 8 transmembrane helices and are categorised into two groups: Cu/Ag transporters and Zn/Cd/Co/Pb transporters (Zhiguo et al., 2018). The N-terminal and C-terminal domains are essential for metal binding and the regulation of metal transport. The actuator (A) domain, nucleotide binding domain/ATP binding domain (N), phosphorylation domain (P), and transmembrane metal binding site/domain (TM MBD) are significant domains in these proteins (Mayerhofer et al., 2016). The A domain conveys alterations in the N domain to the TM regions and is responsible for dephosphorylation reactions. The C

terminal domain, characterised by a high concentration of Cys and His residues, exhibits a strong affinity for binding Zn and Cd, capable of chelating up to 10 Zn ions per C terminal. The transmembrane metal binding domain (TM MBD) in P1B-ATPases facilitates the recognition and transport of transition metals across the lipid bilayer via the transporter protein channel (Guo et al., 2024).

In Zn-transporting HMAs, conserved amino acid residues play a role in Zn binding and transport. The P domain exhibits a high degree of conservation among P type ATPases, featuring the transient phosphorylating Arg within the conserved sequence Asp-Lys-Thr-Gly (DKTG). The N/nucleotide binding domain exhibits the highest variability, capable of binding ATP and phosphorylating the conserved Arg in the P domain. The A domain is a globular phosphate linked to TM2 and TM3 via two extended linker sequences. The catalytic mechanism of Ca^{2+}-ATPase has been extensively studied (Tian et al., 2023). A significant conformational change in the protein facilitates the translocation of the ion across the membrane, involving multiple catalytic intermediates (Li et al., 2024). The E1/E2 state of the protein in the catalytic cycle, or the Post–Albers cycle, is widely recognised for its role in distinguishing the protein's differential affinity for nucleotides and ions. Rice contains nine members of heavy metal ATPases (OsHMAs). OsHMA2 is located in the node region and facilitates the loading of Zn into developing tissues and panicles. OsHMA2 serves as a primary Zn transporter, facilitating the translocation of Zn from roots to shoots and participating in the xylem loading of Zn (Williams and Mills, 2005; Argüello et al., 2007; Rosenzweig and Argüello, 2012; Satoh-Nagasawa et al., 2012; Smith et al., 2014; Batool et al., 2023; Tian et al., 2023).

In rice, 16 *OsZIP* genes (*OsZIP1–OsZIP16*) have been identified and studied under both Zn deficient and Zn sufficient conditions (Krishna et al., 2022). Among these, OsZIP1 and OsZIP9 are involved in Zn uptake, while OsZIP4, OsZIP5, and OsZIP8 contribute to Zn translocation from the roots to the shoots. Research suggests that OsZIP4 and OsZIP8 play a significant role in grain filling. ZIP transporters have also been characterized in other cereal crops, highlighting their importance in Zn transport (Huang et al., 2022). ZIP transporters facilitate the

movement of Zn and other essential micronutrients from external sources or intracellular stores into the cytoplasm (Amini et al., 2022). Additionally, members of the HMA (Heavy Metal ATPase) family, particularly HMA2 and HMA4, are responsible for transferring Zn from parenchymatous cells into the xylem, ensuring its distribution to aerial plant parts (Tian et al., 2023). While the functions of ZIP and HMA genes under Zn deficient conditions are well-documented in rice, their specific roles in seed biofortification remain largely unexplored. Further research on the involvement of ZIP and HMA transporters in nutrient enrichment could provide valuable insights into improving Zn availability in rice and other staple crops.

Seed priming is acknowledged for its prompt advantages in enhancing seed germination, seedling vitality, and total plant efficacy. Recent research indicate that priming may exert transgenerational effects, impacting growth, stress resilience, and nutrient accumulation in later generations (Liu et al., 2022). This mechanism, also known as stress memory or transgenerational priming, unfolds when primed plants convey physiological and molecular alterations to their progeny, resulting in improved adaptability and performance under analogous environmental situations (Louis et al., 2023). Primed parent plants typically yield seeds characterised by increased metabolic activity, superior nutrient reserves, and heightened resistance to abiotic stressors, including drought, salinity, and oxidative stress (Lukić et al., 2023). The observed effects are primarily ascribed to epigenetic modifications, changes in gene expression patterns, and enhanced activation of antioxidant and stress-responsive pathways (Yang et al., 2022; Ganie et al., 2024). Repriming, or exposing the subsequent generation to an additional round of seed priming, may further augment these advantageous effects. Repriming has been shown to enhance stress tolerance mechanisms, increase enzyme activity, and fortify the antioxidant defence system in plants (Sen et al., 2022). Furthermore, there is a lack of knowledge regarding the impact of priming and re-priming on the succeeding generation for augmenting Zn content in plants. Also, limited information is available on the effect of priming and re-priming in the subsequent generation.

Despite significant advancements in understanding biofortification strategies for rice, several critical gaps remain in the context of Zn biofortification. Most

studies focus on a limited number of genotypes, with little attention to traditional or landrace varieties. There is insufficient mechanistic understanding of how Zn is absorbed, translocated, and stored during priming and foliar spray treatments of Zn compounds and the role played by ZIP and HMA families of transporters under Zn deficient and sufficient conditions of Zn upon these treatments. The current research focuses on the effect of seed and seedling priming on enhancing Zn content in rice plants. It also investigates the physiological and biochemical responses of rice to priming by analysing key metabolic pathways, including photosynthetic efficiency, metabolite accumulation, antioxidant defence mechanisms, and stress marker regulation. Additionally, the study examines how Zn transporter activity is affected. Furthermore, it evaluates the supplementary effect of foliar spray at key reproductive stages in enhancing grain Zn content. The research also explores how priming influences the subsequent generation and assesses the impact of repriming on Zn enrichment and plant performance. The detailed objectives of this study are as follows.

OBJECTIVES

1. To screen the landraces and elite varieties of rice for their grain Zn content.
2. To analyse the dosage and duration of priming agents $ZnSO_4$ and $ZnNO_3$ that can specifically nutriprime different varieties of *Oryza sativa* L.
3. To study the effect of Zn nutripriming on rice plants by analyzing
    a. Growth and yield
    b. Antioxidation mechanism
    c. Photosynthetic performance
    d. Oxidative stress
    e. Metabolite accumulation
    f. Grain Zn concentration
4. To study the effect of foliar spraying of Zn compound at reproductive stages on the bioavailability of Zn in rice plants.

5. To select a suitable rice variety that can accumulate optimum Zn within grain without compromising yield.

6. To study the molecular mechanism behind the priming effect by analysing the expression of selected genes (*OsZIP1* to *10* and *OsHMA2*) involved in absorption and transport of Zn from soil to grains.

7. To analyse the effect of Zn nutripriming getting carried over into the subsequent generation.

2.1 Rice

The genus *Oryza* consists of 27 species. Based on cytogenetic analysis through molecular markers it was revealed that there are 11 distinct genotypes. In this six are diploids (2n = 24; AA, BB, CC, EE, FF, and GG) and the rest are allotetraploids (2n = 48; BBCC, CCDD, HHJJ, HHKK, and KKLL). It is believed that *Oryza* originated during middle Miocene era (13–15 million years ago). The two major cultivated species, are *O. sativa* L. and *O. glaberrima,* where *O. sativa* L. is distributed globally, and cultivated widely in Asia, while *O. glaberrima* is grown in Africa. The two major subspecies of the cultivated Asian rice, *O. sativa* L. sp. are *O. sativa* ssp. *japonica* and *O. sativa* ssp. *indica*. Asian cultivated rice is domesticated from ancestors of the wild rice species *O. rufipogonI* (perennial). African rice is closely related to *O. barthii,* thus it is considered as its wild relative. The Asian and African rice were independently domesticated from Asian and African wild rice species. The African rice is now getting replaced by the Asian rice due to its undesirable features, such as the easy shattering of seeds, brittle grain, and lower yields (Chen et al., 2019a). The practice of growing rice in the region of Kerala can be traced back to approximately 3000 BC. Despite being the primary dietary staple for the local population, Kerala exhibits a disparity between rice production and consumer demand, with the former falling short of the latter. From 1960 to 1969, there was an enormous rice shortage, accounting to 40.12%. This shortage further escalated to 83.45% from 2009 to 2010. The cultivation of rice underwent a significant reduction during the 1980s, with the total area decreasing from 8,500,000 hectares in 1980–81 to 1,980,000 hectares in 2017 (Blakeney et al., 2020).

The rice landraces found in the Kerala State of India are designated with names corresponding to several factors, including the cropping season, growing conditions, crop duration, morphological characteristics such as height, milled rice colour of husk, and grain shape. These landraces are also named based on

unique attributes like medicinal capabilities and other distinctive characteristics, such as scent. The presence of various ecological characteristics, scattered village communities, and a rich mix of ethnic and cultural backgrounds in Kerala have resulted in variations in the production of rice throughout the region. Several contributing factors, including an extensive agricultural history, diverse ecological conditions, and the necessity for distinct variations, have led to the emergence of numerous localised variants. In high-altitude areas, the cultivation techniques of slope and terrace farming need the utilisation of upland varieties, whilst irrigated conditions necessitate the use of distinct types. The cultivation of Pokkali in coastal regions necessitates the use of appropriate kinds, while the cultivation in inland regions with neutral soil necessitates freshwater wetland types. In the coastal regions of the Thrissur district, there is a requirement for submergence-tolerant varieties. The agroecological circumstances present in humid tropical environments necessitate the cultivation of specific cultivars that are well-suited to the unique characteristics of each of these conditions. The challenges related to rice production in Kerala could not be effectively addressed by a single variety, hence resulting in many landraces. The paddy fields in Kerala, once used for cultivating traditional rice landraces, have got transformed and resulted in the conversion of these fields into construction sites or for the cultivation of upland crops. Consequently, this process has depleted a significant number of primitive cultivars and indigenous rice landraces (Latha et al., 2013).

2.2 Zinc malnutrition and hidden hunger

Zn deficiency is the fifth leading risk factor for morbidity and mortality in developing countries and ranks eleventh globally (WHO, 2002). It represents a significant global issue, adversely affecting crop quality (Zulfiqar et al., 2024), in addition to causing yield loss and diminishing Zn content in grains (Tabesh et al., 2020). The United States in 1972 identified deficiency of Zn as the predominant deficiency of micronutrient in crop plants (Lindsay, 1972). The Green Revolution enhanced the productivity of essential food crops to accommodate the expanding population. However, an unforeseen consequence of this achievement was

micronutrient malnutrition (Graham et al., 2012). Nonetheless, it replaced the conventional high-nutrient varieties, leading to a decline in nutritional quality and contributing to soil degradation due to the heightened application of fertilisers and pesticides. The agricultural system has not adequately fulfilled the dietary needs for micronutrients, particularly among disadvantaged populations in developing countries. A transition to a sustainable agricultural system that prioritises nutritional considerations is necessary to address the issues arising from inadequate agricultural practices (Garg et al., 2018). Individuals in developing and economically disadvantaged nations rely on cereals as a primary source of sustenance. Their dietary diversity is somewhat restricted. They cannot afford a range of food items, including animal-based foods that are high in Zn. Zn deficiency in soil often correlates with Zn undernourishment in specific regions globally. Zn malnutrition affects nearly 50% of cereal farming areas, and cultivating these cereals in Zn-deficient soil diminishes the bioavailable Zn in these crops. Approximately half of the paddy fields exhibit Zn deficiency, resulting in low yields and poor nutritional quality of rice cultivated in these soils (Krithika and Balachandar, 2016).

Globally, more than 2 billion individuals experience micronutrient malnutrition, commonly referred to as hidden hunger (FAO, 2018); it adversely affects the health and socioeconomic status of a nation. Micronutrient malnutrition in India accounts for approximately 0.5% of GDP (Stein and Qaim, 2007). Healthy and productive human resources significantly enhance national development. The primary cause of Zn malnutrition is the consumption of Zn poor foods or diet, particularly those high in phytic acid, hence with less bioavailability of Zn (Clemens, 2014). The deficiency of Zn results from heightened demands, malabsorption and impaired utilisation. These issues may arise from physiological or pathological conditions that require elevated amount of Zn. Specific medications also adversely affect the availability of Zn (Ritchie et al., 2018). Certain individuals exhibit reluctance to consume animal-based foods due to their religious convictions. Vegans and vegetarians encounter similar challenges. Antinutrients like phytic acid, polyphenols and, calcium, as well as Zn concentrations in soil, influence the amount of Zn available in a plant-based diet (Choudhary et al., 2022).

Cereal grains, including rice and wheat, are typically consumed following milling and polishing processes. The concentration of Zn is higher in the outer layers and germ of the grain, whereas the inner starchy portion contains lower amounts (Senguttuvel et al., 2023). The Zn-rich regions are removed during the processes of dehulling and milling. Consequently, polished rice consumed by individuals contains minimal bioavailable Zn (Lu et al., 2013; Nakandalage et al., 2016; Bodeerath et al., 2024). Rice-wheat cropping represents a critical agricultural system in South Asia. In these areas, over cropping, improper application of fertilisers like potassium, phosphorus, and nitrogen, and poor handling of on-farm residue are the main causes of Zn shortage. Soil pH and Zn solubility are two physicochemical factors that worsen Zn deficiency (Farooq et al., 2019). Acidic soil containing high levels of calcium, organic matter, clay, or phosphorus limits the availability of Zn to plants (Noulas et al., 2018). Zn binds to soil particles more easily when it is associated with humus in the soil. Applying charcoal and compost to agricultural soils can raise fulvic and humic acid levels, which lowers the amount of Zn that is bioavailable (Li et al., 2019). By changing the pH of the soil, Zn can form compounds that are not soluble; hence, the careless application of organic manure might reduce the bioavailability of Zn. In Brazil, pig manure was applied extensively, which reduced the amount of Zn in soil (Benedet et al., 2020).

2.3 Zinc in soil

Both natural processes and human activity are the sources of Zn in the soil. The main natural source is rock weathering, whereas the anthropogenic sources are industrial and agricultural processes such as smelters and fertilisers high in Zn. The Zn concentration of soil is greatly influenced by the parent rock's properties, soil type, soil order, pH, climate, moisture content, and both microbial and human activity (Tsonev and Lidon, 2012). Water-soluble, exchangeable, non-exchangeable, adsorbed or chelated organic, and chelated to primary weathering minerals are the five different types of Zn found in soil. The availability of Zn in soil is influenced by the processes of absorption and desorption between soluble and adsorbed forms. In normal soil, the total Zn content varies between 10 and 300 μg/kg. About 50 μg/kg

of Zn are present in the soil on average. Plants cannot easily access all of the Zn in the soil and only very little Zn can be absorbed by plants from the soil (Stanton et al., 2022).

The amount of phytoavailable Zn in a given soil is indicated by the amount of Zn that can be extracted using DTPA (diethylenetriaminepentaacetic acid) (Sharma et al., 2013). When the amount of DTPA extractable Zn in soil is less than 0.8 µg/kg, the soil is considered Zn deficient (Wissuwa et al., 2008). Soil Zn phytoavailability is influenced by several factors. These include microbial activity, cation exchange capacity, pH, redox reactions, organic matter, humus, clay, soil structure, water content, and interactions with other elements. These elements, either alone or in different combinations, prevent plants from getting Zn. The solubility of zinc in soil is greatly affected by pH. In acidic conditions (below 7.7), Zn is mainly found as Zn^{2+}, a form that plants can easily absorb, which is a form readily accessible to plants. At basic pH levels (above 7.7), Zn exists primarily as the monovalent cation $ZnOH^{+}$. Plants can absorb this form to a certain extent. When the pH exceeds 9, Zn is present as $Zn(OH)_2$, and this cannot be absorbed by plants easily (Duffner et al., 2013; Laurent et al., 2024).

The significant reduction of Zn phytoavailability is observed under alkalinity. In the presence of $CaCO_3$ in the soil, Zn is adsorbed in a fixed calcium zincate form. Furthermore, an increase in soil pH leads to a reduction in the Zn phytoavailability due to calcareousness (Chahal et al., 2023). Soil organic matter can either enhance the solubility of Zn or cause its immobilisation. The Zn in solubilised form is readily accessible to plants, whereas the fixed form is unavailable to them (Dhaliwal et al., 2019). Baran et al. (2018) conducted a principal component analysis that demonstrated organic carbon as a critical factor influencing the soil Zn phytoavailability. In soil, organic matter is categorised into humin, fulvic acid and humic acid based on differential solubility. The Zn associated with them is typically in a locked form, rendering it least accessible to plants. The Zn in fulvic acids exhibits greater mobility and availability, attributed to its enhanced hydrophilicity. The soil, cation exchange capacity (CEC) is affected by organic matter. It refers to

the capacity of soil to retain and exchange cations. A high CEC in soil increases the exchangeable Zn for plant uptake (Chen et al., 2019b). Karimi et al. (2019) demonstrated that the application of biochar in saline soil enhanced the Zn phytoavailbility due to improved organic matter content and CEC.

Microorganisms such as arbuscular mycorrhizal fungi and zinc-solubilizing and mobilizing bacteria play a role in dissolving Zn in the rhizosphere. This process occurs through the acidification of the region via the secretion of phytosiderophores or phenolics. The use of Zn solubilising bacteria in conjunction with Zn supply enhanced the productivity and nutritional content of chickpeas (Ullah et al., 2020a). Soil structure and its ability to retain water are essential for maintaining plant-available Zn. Well-aerated, porous soil with sufficient moisture can improve Zn availability to some extent. Clay presence diminishes Zn availability (Natasha et al., 2022; Behera et al., 2024). Flooding can induce anaerobic conditions in the soil, thereby affecting the availability of Zn. Although it initially enhances the Zn availability, but eventually insoluble compounds are generated, thus reducing its solubility (Yang et al., 2021; Nath et al., 2024). Arid condition also reduces the phytoavailability of Zn. Phosphorus could hinder the Zn phytoavailability as they are antagonistic. The addition of phosphorus in both acidic and alkaline soils resulted in a decrease in DTPA-extractable Zn, as well as a reduction in Zn content in wheat (Chen et al., 2019b). The treatment of phosphorus reduced Zn, with reductions of 17% in wheat and 20% in maize (Zhang et al., 2024a). However, nitrogen enhances the availability of Zn in soil and increases the Zn content in grains (Liu et al., 2024a).

2.4 Role of zinc in plants

Zn is a vital nutrient for plants. The significance of Zn in plants was first established in maize in 1915 (Mazé, 1915). Zn deficiency in crop plants was initially recognised in India, as khaira disease of paddy cultivated on calcareous soils (Nene, 1966). An optimal Zn concentration is essential for proper physiological functions in plant cells. Zn plays a crucial role in maintaining the structural and functional integrity of biological membranes, facilitating transcription, translation, regulation,

and providing defence against various diseases (Sadeghzadeh, 2013; Yadav et al., 2024). It serves as a structural, catalytic, and signalling component both intracellularly and intercellularly. Zn is the sole metal necessary for all six enzyme classes. Zn is crucial for the function of metallo-enzymes that participate in metabolism. Plants necessitate an optimal concentration of Zn to fulfil their life cycle. The Zn in various plants typically scales from 30 to 100 ppm; levels exceeding this range are considered toxic, although certain hyperaccumulators can exceed these concentrations. The Zn below 15-20 ppm of dried leaves indicates deficient condition (Mitra, 2015).

Zn plays a crucial role in metabolic pathways, including tryptophan biosynthesis, and other phytohormones (Ghosh and Roychoudhury, 2024). Chlorophyll biosynthesis, pollen formation protection against photooxidative damage, and PSII repair are other critical processes in which it plays a role (Lilay et al., 2024). In 1940, Skoog reported that tomato plants exhibited severe symptoms of Zn deficiency, characterised by reduced stem elongation (Skoog, 1940). Severe Zn deficiency results in root apex necrosis, whereas mild Zn deficiency is associated with uneven chlorosis between veins, shortened internodes, epinasty, and reduced leaf size. Common symptoms in rice include increased mortality rates, stunted growth, leaf discolouration, and delayed flowering (Waqeel and Khan, 2022). The deficiency of Zn in plants causes stunted growth, chlorosis, smaller leaves, sterile spikelets (Das et al., 2018), decrease in fruit size, inhibition in shoot elongation, reduced Cu/Zn superoxide dismutase (SOD) activity, and enhanced photooxidation. It also cause oxidative damage of membranes, proteins, phospholipids, chlorophyll molecules, nucleic acids, SH group containing enzymes, IAA inhibition (Zaman et al., 2018), synthesis of impaired protein, increase in ROS, decrease in detoxification mechanism (Cakmak, 2000). A decrease in chlorophyll content, lower chl *a: b* ratio, reduction in PS II per leaf area, impaired quantum efficiency of PS II, and an overall decline in photosynthetic performance occurs in Zn-deficient plants (Chen et al., 2007).

The plants growing in similar conditions differ significantly in absorption, translocation, accumulation and storage of Zn. The rich sources of Zn within the plants include leafy vegetables, and the least available source includes cereals. It may be due to the immobile nature of Zn within the plant tissue, primarily through the phloem. Usually, the seeds, fruits, and tubers are phloem fed tissues; thus, the amount of Zn in these edible portions will be low. When compared to the cereals, the legumes contain more Zn. Amaranthaceae, Brassicaceae and Salicaceae accumulate more Zn content, whereas the Poaceae, Solanaceae and Linaceae acquire least Zn content (Gregory et al., 2017; Akhtar et al., 2019). Even though fruits are poor sources of Zn, pomegranate is rich in Zn. It contains Zn to the level of 9.97–17.29 mg kg^{-1} fresh weight (Tozzi et al., 2020). A study conducted in 8 citrus fruit cultivars found that the Zn concentration within them is in the range of 0.10-0.24 mg/100 g fresh weight (Czech et al., 2020). The Zn in edible portions of plants is dependent on the genetic makeup and environment of the plant.

2.5 Role of zinc in human

Zn, a transition metal ranks as the second most abundant divalent cation in humans. Cell biology of Zn encompasses critical processes including cell division, differentiation, growth, cellular transport, wound healing, insulin production and release, as well as the regulation of blood pressure and the endocrine system. The testes, muscle, liver, bones, and brain exhibit the highest concentrations of Zn. It is abundant in synaptic vesicles and plays essential roles in learning and memory processes (Stiles et al., 2024). The recommended daily intake of Zn varies by age, gender, and diet, ranging from 3 to 16 mg per day. The recommended dietary allowance of Zn is 11 mg/day for men and 8 mg/day for women, with increased daily requirements for newborns, infants, pregnant, and lactating women. The upper limit of consumption is 40 mg per day for adults. Since Zn is not stored in the body and lacks a long-term retention system, a daily intake is necessary to maintain optimal levels and support its functions (Poudel et al., 2024).

Zn is obtained by humans via a wide range of foods. Animal-based foods are more nutritious and bioavailable than plant-based foods. Zn is found in abundance in

oysters, beef, hog, lamb, chicken and their products are high in Zn content than other meats. Milk and milk products can also provide Zn to humans. Zn is also found in leafy vegetables, beans, tubers and cereals. Plant products, on the other hand, are less bioavailable due to the presence of antinutrients such as phytate (McClung, 2019; Chasapis et al., 2020). The physiological requirement of Zn is high during pregnancy, infancy, lactation and adolescence. Zn plays important role in immune system, sensory system, reproduction, oxidative stress, apoptosis, neurobehavioural development and also acts as a neurotransmitter (Prasad et al., 2014; Parveen et al., 2017; Gammoh and Rink, 2019).

In infants and children, Zn deficiency results in compromised neuro-behavioural function. Acrodermatitis enteropathica is a genetic disorder that is contributed by poor Zn absorption in humans (Ogawa et al., 2018). In humans, Zn deficiency leads to learning disability, decrease in physical growth, delay in sexual maturity, vulnerability to diarrhoea and pneumonia, malfunctioned immune system and increase in infections and mortality. Among elderly, Zn deficiency results in chronic non-healing ulcers, impaired taste sensitivity and compromised immune system. Imbalances in Zn levels are significant factors in the progression of cardiovascular diseases, cancer, diabetes, obesity, skin disorders, metal-related diseases, neurodegenerative diseases, and ageing (Chasapis et al., 2020). Excessive depletion of Zn may result in gastrointestinal or urinary tract disorders and bowel diseases. The deficiency compromises the immune system, making individuals more susceptible to pathogens and infections from microorganisms such as bacteria and viruses (Jin et al., 2024).

2.6 Biofortitifcation

Impoverished communities are unable to adopt alternative strategies to fulfil dietary Zn requirements, including enhancing food with Zn, diversifying dietary choices, and using Zn supplements. The primary approach to address Zn malnutrition in these areas is through the biofortification of staple food crops, such as rice. Biofortification represents the most practical, long-lasting, and economical approach for reaching marginalized populations. It refers to the process of enhancing

edible parts with readily available micronutrients and vitamins. The approach includes schemes such as conventional plant breeding, recombinant DNA technology, and agronomic biofortification. Traditional low-yielding cereal varieties possess a high nutrient density, including essential vitamins and minerals. Conventional plant breeding involves the identification of specific varieties, rich in nutrients as well as yield (Cakmak and Kutman, 2018). Physiological limitations and genetic barriers to the bioaccumulation of micronutrients in edible tissues can be overcome through the manipulation of the target taxon's genome using rDNA technology. Conventional breeding and genetic engineering methods have numerous limitations. Both processes are resource-intensive and expensive, with public unacceptability and ethical concerns regarding GMOs being particularly significant (Wei et al., 2012).

Agronomic biofortification serves as an effective method for delivering fertilizers to plants at the optimal stage and in the appropriate quantities and represents, the most widely accepted approach to biofortification (Bandara and Dissanayaka, 2024). The target concentration for Zn biofortification is 28 micrograms per kilogram of rice grain. Zn can be applied through several methods, including soil application, foliar application, root dipping, and micronutrient seed treatment or invigoration (Mamun et al., 2018). The efficient application method can significantly enhance grain Zn concentration. The root dipping of seedlings in Zn solution during transplantation resulted in a significantly enhanced accumulation of Zn in the grain compared to other treatments (Zaman et al., 2018).

2.6.1 Seed priming

The embryo contained in a seed represents a prospective plant. In it, the resources can enhance the potential of plant for success within its established niche. Seed priming is a practical, feasible, simple method, comprising of hydrating seeds along with mild treatments of various physical or chemical factors in a controlled way to initiate many metabolic pathways, and further these hydrated seeds are dried back to their original weight before sowing (Jisha et al., 2013; Farooq et al., 2019). An agronomic approach to tackle the nutrient deficiency of plants as well as in

humans is to treat the seeds with the deficient nutrient. Thus, seeds can be treated with macro- and micronutrients in order to reduce the nutrient deficiencies (Akhtar et al., 2019). In addition to this, seed priming with micronutrient improves the other nutritional components, such as protein content (Seddigh et al., 2016), and reduces antinutritional factors like Cd and phytate (Slamet-Loedin et al., 2015).

Nutripriming of seeds offers several advantages in comparison to soil application. The predominant sources of fertilisers are mined, which are finite. Additionally, mining raises environmental concerns due to pollution. Soil fertilization must navigate various barriers imposed by the physicochemical properties of soil to reach the plant. Nutripriming of seeds is cost-effective and environmentally advantageous due to its lower expense and reduced nutrient usage. This is application of minimal nutrient content to seeds, which can be done single handedly by a farmer for a small-scale cultivation. It can facilitate uniform seedling emergence and establishment, enhance stress tolerance, and improve productivity and grain quality. The dosage and duration of nutripriming are critical factors that affect its efficacy, which varies among different crops based on their genetic composition. Higher concentrations of priming over extended durations led to reduced growth metrics in the majority of crops, likely attributable to toxic side effects (Bandara and Dissanayaka, 2024). Initial studies are essential to determine the ideal nutrient levels and application timing for improving crop growth, yield, and quality.

2.6.2 Various sources of zinc for nutripriming

The various Zn sources used in seed priming include inorganic Zn sources, organic Zn sources, chelated Zn sources and Zn nanoparticles (Zn-NP). The inorganic source includes zinc sulphate ($ZnSO_4$), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), zinc chloride ($ZnCl_2$), zinc nitrate ($ZnNO_3$), zinc carbonate ($ZnCO_3$) and zinc oxide (ZnO). $ZnSO_4$ and $ZnSO_4.7H_2O$ are the most commonly used priming agents due to its solubility, less reactivity soil components and cheaper rate. The most common synthetic chelated form of Zn is Zn-EDTA, wherein the metal ion is coordinated with the chelating agent. The quantity of soluble part of Zn

present in all the above compounds varies (Naik and Das, 2007; Ostad et al., 2017). The synthetic Zn chelators have many ill effects like low biodegradability, quick photodegradability and release of toxic agents on application in high amounts and it is also costly. Therefore, organic Zn chelators like Zn amino acids are acquiring prominence as a priming agent. Zn amino acid complexes such as $Zn(His)_2$, $Zn(Met)_2$, $Zn(Gln)_2$, $Zn(Gly)_2$, $Zn(Arg)_2$ are commonly used for priming (Mirbolook et al., 2021). Nanoparticles are also attaining attraction nowadays as they have many superior characteristics when compared to the traditional sources of nutrients. Some of such traits are their high surface area-to-volume ratio, high stability, high adsorption, increased surface reactivity, etc. Thus, a low dosage of application can have a prominent impact on increased growth, yield and biofortification of crops (Rastogi et al., 2017; Mittal et al., 2020). The Zn nanoparticle (Zn NP) is one of the widely used NP in agricultural sector. It can be synthesised chemically by using chemical reagents (Neto et al., 2020) or biologically with the aid of microorganisms (Sabir et al., 2020) or plant extracts (Sabir et al., 2014). When used for priming and biofortification, the cost-effectiveness as well as the overall activity and effectiveness are usually considered before selecting a Zn source as a priming agent.

2.6.3 Foliar spray

Foliar application of Zn fertilizers in agronomic biofortification is more efficient than soil application and can enhance grain Zn content across various environments and agricultural management practices. The process is more efficient and economical, influenced by the timing of application. Zn applied to the leaves is absorbed by the epidermis, subsequently translocating to the phloem and ultimately reaching the grain. The cuticle and trichomes contribute to the absorption of Zn applied to the leaves. Low doses of Zn are typically utilized to prevent leaf scorching (Ram et al., 2016; Pal et al., 2021; Lian et al., 2023; Sánchez-Palacios et al., 2023). The enhancement of Zn concentration in brown and white rice is influenced by the type of fertilizer applied. The foliar Zn supplementation significantly enhances both grain Zn content and yield. Nutripriming is economically advantageous, as it necessitates a lower quantity of fertilizer compared

to alternative methods (Majda et al., 2019). Foliar application during advanced developmental stages enhanced the accumulation of Zn in both whole grain and endosperm. The amount of Zn in the grain increased following foliar application during the anthesis or boot stage and the milking stage (Sánchez-Palacios et al., 2023). The timing of foliar application significantly influences Zn distribution within the grain (Das et al., 2023). The optimal timing for foliar application to enhance grain Zn content should be evaluated, through systematic experiments and trials.

2.7. Possible roles of priming in influencing the increase of zinc level in plants

To fulfill any biofortification approach, a thorough knowledge of the transporter system is very essential. Transporter proteins are necessary for the translocation of metal ions into and out of the cell, microcompartmentation and for sequestering these ions in vacuoles to act as a reservoir to reduce toxicity. Mainly three transporter systems are elucidated for Zn transport. They are the ZIP (zinc–iron permease or ZRT, IRT-like proteins) family of transporters seen on plasma membrane. The CDFs (cation diffusion facilitator) like MTPs (metal tolerance protein), seen on tonoplasts and HMAs (heavy metal ATPase) a P-type ATPase, localised on plasma membrane, tonoplast and endomembrane system (Caroli et al., 2020), which are detailed in section 2.12.

On seed invigoration (seed priming or seed coating) with Zn, an increase in yield factors was noticed, which may be due to increased expression of Zn transporters like HMAs. There is evidence for the expression of HMAs (HMA9) transporter proteins in anther of rice and *Arabidopsis* on application of Zn. The increased Zn in anther has many positive aspects such as increased pollen fertilisation and seed set as discussed earlier. Thus, it could be concluded that these transporters translocate Zn into androecium, leading to effective fertilization and seed set, bringing about an increase in yield parameters (Lee et al., 2007). This can be confirmed by the male sterility of nas4x-2 mutant line of *Arabidopsis* during Zn deficiency. In these mutants, the nicotinamine (NA) was completely absent,

affecting NA-mediated transportation of Zn to anthers via the transporters (Schuler et al., 2012).

Brutus (BTS) a Zn-containing protein is able to regulate the expression of genes needed for Zn translocation and accumulation. BTS is an E3 ubiquitin ligase having a Zn finger protein domain. This ubiquitin ligase can add ubiquitin and regulate Fer-like Deficiency-Induced Transcription Factor (FIT) transcription factor, which aids in the expression of genes needed for Fe uptake and the genes for the transporters like IRT. Along with Fe, non-specific uptake of Zn also takes place through this transporter (Long et al., 2010). FIT can also regulate the expression of *Nicotinamine Synthase (NAS)* genes (Rodríguez-Celma et al., 2019). The *NAS* synthesises low molecular weight chelators like nicotinamine (NA) and deoxymugineic acid (DMA) which can chelate Zn. The Zn-NA complex thus formed aid in long distance transport of Zn via xylem and phloem into the seed (Lee et al., 2011; Clemens et al., 2013). The plant cadmium resistance 1 (PCR1) is a transmembrane protein aiding in the translocation of Zn. In rice, it is present in roots at seedling stage, while at reproductive stage it is seen on I and II internodes and on spikelet. Hence it can be proposed that this transporter may also have a role in Zn accumulation in grains (Song et al., 2015). Therefore, during seed priming this metal can certainly regulate the key genes discussed above reflecting in biofortification.

2.8 Effect of zinc enrichment in plants

Seed priming influences plants positively and helps the plant to tide over various difficult situations that it encounters during its life cycle. Zn nutripriming of seeds has beneficial impact on germination parameters, seedling establishment, various yield attributes and Zn content in edible tissues (Farooq et al., 2012), and also many metabolic processes get stimulated (Gupta et al., 2016). The use of nutriprimed seeds in Zn-deficient soils are more efficient, as seeds with high Zn content perform better in Zn-deficient soil than seeds with a smaller Zn content (Cakmak and Kutman, 2018). There is a trend of increase in Zn content in edible portions on increasing the seed treatment concentrations of Zn. However, at higher

concentrations, germination and growth are inhibited due to phytotoxicity (Reis et al., 2018; Carvalho et al., 2019).

2.8.1 Zinc in germination and seedling establishment

Seed priming increased the germination rate, reduced mean germination time and provided synchronised germination and early establishment of seedlings in crop plants. When mungbean seeds were nutriprimed for 6 h in 0.1 M $ZnSO_4$ solution, germination was increased by 53% when sown in a sandy loam soil with an alkaline pH of 8 (Haider et al., 2020). This may be due to the involvement of Zn in activating many enzymes needed for key metabolism like those involved in anaerobic respiration like alcohol dehydrogenase and is also crucial for the production of proteins (Cakmak and Kutman, 2018).

Zn induced auxin production may be the reason for cell elongation and cell division, thus helping in root and shoot elongation. The distribution of Zn in coleoptile and radicle gives further proof for this. On germination, Zn present in the seeds is mobilised to growing regions of coleoptile and radicle and gets concentrated more at the tips. This feature shows the requirement of Zn in enzymes, carbohydrate metabolism as well as in transcription and translation, thus aiding in the increase of various growth parameters (Ozturk et al., 2006; Broadley et al., 2012). During DNA replication for the formation of initiation complex and replication fork, Minichromosome Maintenance (MCM) proteins are needed. They possess various domains including a Zn finger domain for their activity. Along with this protein, several other proteins with Zn finger domain are also necessary for replication (Shultz et al., 2007). Also during cell division Zn is necessary for the polymerisation and stability of microtubules (Domart et al., 2020).

Transcription factors regulate gene expression and thus have role in development, signal transduction and metabolism. GATA Zn transcription factors are DNA binding proteins which regulate various developmental processes in plants including flower development (Zhu et al., 2020). The role of GATA Zn finger transcription factor, Blue Micropylar End 3 (BME3) in breaking seed dormancy, is well documented in *Arabidopsis* plant. It positively influences the transition from

dormancy to germination, by enabling the radicle to come out by overcoming the various mechanical constrains that block germination. This can be correlated with the observation that seeds with malfunctioned BME3 showing deeper dormancy when compared with the normal populations (Liu et al., 2005). On priming the seeds with Zn, this metal may possibly influence this transcription factor and thereby enhance the germination parameters.

During germination, ROS are produced continuously as a result of the metabolic activities occurring in mitochondria, peroxisomes, glyoxysomes and chloroplast. Although ROS accumulation can be harmful, low levels of the same can provide disease resistance, apoptosis of aleurone layer, deterioration of endosperm, cell signalling and maintenance of redox potential. The level of ROS should be maintained between a threshold level called "oxidative window" for breaking the seed dormancy. Below it, the seed will remain in dormant stage, and above this level, it results in production of anomalous seedlings. Thus, in order to break dormancy and increasing the seed vigour, the cell's antioxidant machineries should perform optimally. For this, Zn-containing SOD and catalase enzymes appear to help the seed to increase its vigour by controlling the ROS levels (Ma et al., 2017).

2.8.2 Zinc in yield, yield related traits

The efficacy of Zn in promoting the grain yield and yield components can be inferred by analysing the productive panicle number, grains per panicle and grain weight. Through seed treatments with Zn, all these attributes can be increased (Farooq et al., 2018). In Zn deficient soil, nutripriming of maize seeds with optimised time and concentration of $ZnSO_4$ solution enhanced the yield by 27% (Harris et al., 2007). In a field research done in Faisalabad and Sialkot, $ZnSO_4$ primed seeds enhanced the yield of rice plants by 31% and 40%, respectively (Farooq et al., 2018). The use of $ZnSO_4$ solution to prime the rice seeds enhanced the output by 23% (Zulfiqar et al., 2020). In the case of chickpea, priming with $ZnSO_4$ solution improved the yield by 24% and 15% in the desi and kabuli varieties, respectively (Ullah et al., 2020b). Zn seed priming was the most efficient strategy for increasing wheat grain production in rice–wheat cropping systems, increasing

output by 56% in conventional tillage and 60% in conservation tillage (Nadeem et al., 2020). The reason behind this can be deduced by the fact that Zn is necessary for the pollen tube growth and fertilisation by facilitating pollen–stigma interaction (Pandey et al., 2006). For effective fertilisation and seed setting, the pollen from the androecium has to reach the female gametophyte present in the gynoecium, and for this, the pollen tube grows and moves through the transmission tract of the style. The extracellular space needed for the pollen tube growth is produced by programmed cell death (PCD), the *No Transmitting Tract* gene encoding a C2H2/C2HC zinc finger transcription factor facilitates this (Cascallares et al., 2020). In addition to this, the movement is also facilitated by the expression of *Central Cell Guidance (CCG)* gene encoding a transcription factor with a conserved N terminal zinc β-ribbon domain in the central cell of the mature female gametophyte (Chen et al., 2007; Wang et al., 2010; Li and Yang, 2020). Thus, Zn has a prominent role in effecting the fertilisation and seed set.

The greater growth attributes reflected in plants as a result of Zn are due to the involvement of Zn in photosynthesis. Carbonic anhydrase, a Zn metalloenzyme, shows pivotal role in photosynthetic organisms as it is crucial for the activity of the enzyme Rubisco. Carbonic anhydrase catalyses the reversible conversion of bicarbonate to CO_2, the substrate needed for Rubisco (Qiao et al., 2014; Dimario et al., 2017). Thus, the role of Zn in the activity of carbonic anhydrase and the indirect influence in the rate of photosynthesis is evident by the decrease in carbonic acid concentration, carbonic anhydrase mRNA as well as photosynthetic rate during Zn deficient condition (Sasaki et al., 1998; Polishchuk, 2021). Aldolase, a Zn-dependent enzyme, is vital for growth and development of all organisms including plants. It plays an essential role in carbohydrate metabolisms by catalysing key reactions in reductive pentose phosphate cycle, metabolic processes such as Embden–Meyerhof–Parnas (EMP) pathway and gluconeogenesis (Lv et al., 2017). Hence, it can be inferred that this enzyme assists in enhancing photosynthetic capacity, growth rate and the biomass increase by hastening the regeneration of RuBP by converting fructose-1,6 bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Raines, 2022; Meloni et al., 2023).

2.8.3 Effect of zinc and priming on photosynthetic machinery and function

Zn is inevitable for the proper functioning of photosynthetic machinery. It is crucial for chlorophyll biosynthesis and PSII repair mechanisms. Seed priming with Zn enhanced the photosynthetic pigments as well as efficiency of photosynthesis in various plants under normal as well as stress conditions. In wheat seed priming with ZnO NPs enhanced the chlorophyll a, b and total chlorophyll content by 48 to 50%. The photo protective pigment carotenoid was also enhanced by 34% upon seed priming, when compared to control plants. Chl *a* fluorescence parameters give the efficiency of photosystem, especially the PSII and various parameters that have significance in photosynthetic mechanisms can be deducted from the fluorescence data. Polyphasic fluorescence transients (O–J–I–P) best describes the electron transport activity at various stages in the thylakoid reactions of photosynthesis. It was noticed that the efficiency of water splitting complex denoted by Fv/Fo was enhanced by more than 50% on seed priming with Zn. Likewise there was also an enhancement of more than 50% in number of active reaction centres per chlorophyll denoted by RC/ABS. The driving force for photosynthesis, the plant vitality indicator called performance index (PI) based on absorption, and cross-section were significantly enhanced under seed priming with Zn. Energy pipe leaf model also revealed significant enhancement in absorption, trapping of photons and electron transport upon seed priming with Zn. Therefore, seed priming with Zn can efficiently absorb the light energy, transfer it to the reaction centre, and can efficiently use for photochemistry, eventually causing upregulation of photosynthesis in primed plants (Rai-Kalal and Jajoo, 2021).

In spinach, salt stress reduced the photosynthetic pigments, photosynthetic rate, stomatal conductance, transpiration rate and water use efficiency. These decreases in photosynthetic features were reverted upon application of Zn via seed priming and foliar spray (Ahmad et al., 2024). Likewise, in wheat also the salinity stress reduced the chlorophyll a, b and total chlorophyll content. However, seed priming with ZnO NPs reduced the stress effect by enhancing the pigment content, thus photosynthesis in plants (Zafar et al., 2024). In maize under Co stress, there was

a reduction in chlorophyll content, photosynthetic efficiency (Fv/Fm), photosynthetic rate, stomatal conductance and transpiration rate. The ultrastructure of chloroplast and stomatal aperture were also seriously harmed by Co stress. However, seed priming with Zn restored the normal photosynthetic activities and alleviated the stress effects of Co (Salam et al., 2022). Moradi and Siosemardeh, (2023) demonstrated that seed priming and foliar application of Zn compounds can enhance the photosynthetic pigments in rice plants. In wheat, induction of drought stress negatively affected the photosynthetic rate, stomatal conductance, total chlorophyll content, carotenoid content, chl *a* fluorescence parameters such as Fv/Fm, quantum efficiency of photosystem II (ϕ_{PSII}), the photochemical fluorescence quenching (q_P), the capture and the electron transport rate (ETR). These effects were ameliorated and all these photosynthestic parameters were upregulated on application of seed priming and foliar spray with $ZnSO_4$ (Pavia et al., 2019).

2.8.4 Effect of zinc and priming on antioxidant system

Plant experiences oxidative stress, ROS are generated during normal metabolism as well as gets elevated during stress conditions. To maintain homeostasis and balancing between its production and detoxification, plants have an efficient antioxidant system within it. Antioxidants are molecules which can hamper the production of free radicals either by inhibiting the reaction or quenching it and prevent cell damage (Dumanović et al., 2021). The antioxidant system is comprised of enzymatic and non-enzymatic agents. The enzymatic agents include superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), glutathione peroxidase (GPX), Guiacol peroxidase (GPOX), glutathione reductase (GR), monodehydroascorbate reductase (MDHR), dehydroascorbate reductase (DHR). The non-enzymatic antioxidants include ascorbic acid (AA), glutathione (GSH), α-tocopherol, carotenoids, flavonoids, phenols, and anthocyanin (García-Caparrós et al., 2021).

The SOD act as the first line of defence against oxidative damage and is omnipresent in every cell. This enzyme catalyses the conversion of $O_2^{\bullet-}$ radicals to

H_2O_2 and molecular oxygen (O_2). Depending upon the metal group present on them, they are classified into three types in plants; copper and zinc (Cu/Zn-SODs) (seen in chloroplasts, cytosol and mitochondria), manganese (Mn-SODs) (seen in mitochondria) and iron (Fe-SODs) (seen in peroxisomes and mitochondria). The catalase enzymes convert hydrogen peroxide into water and oxygen and are mainly seen in the peroxisomes and mitochondria. They are of 3 groups; group 1 (seen in photosynthetic tissues), group 2 (seen in vascular tissues), group 3 (seen in seeds and reproductive tissues). APX detoxifies H_2O_2 to water using ascorbate as an electron donor and are seen in mitochondria, chloroplasts, and peroxisomes (García-Caparrós et al., 2021).

In Sorghum under salinity stress, seed priming with Zn enhanced the activity of enzymatic antioxidants such as catalase, APX, peroxidase and SOD (Hassan et al., 2024). In wheat under drought stress also the application of seed priming with Zn enhanced the activity of enzymatic antioxidants (Abbas et al., 2023; El-Shazoly et al., 2025). And also under salinity stress, the wheat plants primed with Zn showed enhancement in enzymatic antioxidant activities (Zafar et al., 2024). Similarly in ground nut treated with Zn compounds as seed priming an upregulated activity of various enzymatic antioxidants such as SOD, GPX, catalase was observed (Ashwini et al., 2024). In rapeseed also the treatment with ZnO NPs enhanced the activity of enzymes such as SOD, POD and CAT under salinity stress, alleviating the ill effects of salinity (El-Badri et al., 2021). In maize plants, under Co stress, the activity of various enzymatic antioxidants were high upon priming the plants with Zn through seed priming, to thrive in the adverse condition of metal stress (Salam et al., 2022). Seed priming wheat plants with ZnO NPs also, enhanced the enzymatic antioxidant system within them (Rai-Kalal and Jajoo, 2021). Seed priming with Zn in *Vigna mungo* (L.) enhanced the activity of enzymatic antioxidants under As stress (Banerjee et al., 2023).

The non-enzymatic antioxidants, such as ascorbic acid directly scavenges $O_2^{\bullet-}$, $^{\bullet}OH$ and $^1O_2$, and can reduce H_2O_2 to H_2O through the APX reaction. Glutathione is a thiol tripeptide (γ-glutamylcysteinyl-glycine) functioning as a

scavenger for H_2O_2, $^1O_2$, $^{\bullet}OH$ and $O_2^{\bullet-}$. It interacts with various biomolecules by forming adducts through glutathiolation with reactive electrophiles or by reducing them in the presence of ROS or organic free radicals, resulting in GSSG as a byproduct. The oxidized form can be reverted to GSH by 'de novo' synthesis or through the action of glutathione reductase (GR) there by maintaining a cellular GSH reserve (García-Caparrós et al., 2021). Plants have a plethora of bioactive compounds called secondary metabolites to ward off both abiotic and biotic stresses. The class of phenolic compounds plays a crucial role in mitigating the effects of stress. The shikimate or phenylpropanoid pathway synthesises more than 8000 molecules under this category. They play a critical role in the physiology of plants by scavenging ROS, photoprotection, enzyme activation, and cell signalling mechanisms. The flavonoids, a primary class of phenolics, include anthocyanin as a subgroup (Khanday et al., 2024). They have antioxidant activity, thereby neutralising the ROS formed in the plants. They also maintain the membrane integrity and osmoprotection. Some of them have the ability to chelate toxic metals. They can regulate antioxidant enzyme activity and stress-responsive gene expression (Saini et al., 2024b).

In wheat under drought stress, the plants raised from seed priming with Zn showed better tolerance by the enhanced amount of non-enzymatic antioxidants such as flavonoids and total phenolics present in them (El-Shazoly et al., 2025). Seed priming with Zn in *Vigna mungo* (L.) enhanced the amount of non-enzymatic antioxidants such as total phenolics and flavonoids under As stress (Banerjee et al., 2023). Under water deficit condition in maize, the application of Zn seed priming, reduced the stress effects in these plants by enhancing the non-enzymatic antioxidants such as ascorbate, flavonoids, anthocyanin and total phenolics content (Saeed et al., 2023). The ascorbate and glutathione content was enhanced upon seed priming with Zn and enhanced survival in rice plants grown under As stress (Choudhury et al., 2022).

2.8.5 Effect of zinc priming on oxidative stress

A complex physiological, biochemical, and molecular phenomenon occurring at normal or elevated levels under stressful conditions constitutes oxidative stress. This form of stress occurs when the homeostasis between production and detoxification of ROS becomes disrupted. ROS are substances containing one or more activated oxygen molecules and free radicals derived from molecular oxygen. They cause oxidative stress by oxidizing cell macromolecules and constituents. In biological systems, the ROS that impart oxidative stress includes singlet O_2 ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$) (Demidchik, 2015). They cause disruption in nucleotide structure, lipid peroxidation, membrane damage and protein oxidation. The major site of production of ROS in a cell includes the apoplast, chloroplast, mitochondria and peroxisome (García-Caparrós et al., 2021).

The initial response to oxidative stress is considered, the formation of a superoxide radical ($O_2\bullet$), which is generated by electron transport reactions taking place in chloroplasts and mitochondria. Even though, they are short lived, they can generate other ROS. They produce more stable and reactive ROS like hydroperoxyl radicals ($HO^{\bullet-}$), these ROS reacts to produce another ROS called H_2O_2. ($O_2\bullet$-) can also reduce Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, respectively, which interact with H_2O_2 to produce •OH (Demidchik, 2015; García-Caparrós et al., 2021). H_2O_2 is more stable, moderately active ROS and is membrane permeable, possibly through aquaporin. They are generated in various organelles, such as mitochondria, chloroplast, nucleus, peroxisome and endoplastic reticulum (Demidchik, 2015; García-Caparrós et al., 2021). Hydroxyl radicals ($^{\bullet}OH$) is the more reactive ROS that can damage nucleic acid, protein and lipids. The transition metals like Fe^{2+} react with H_2O_2 to produce Fe^{3+} +$^{\bullet}OH$ + OH^{-}(Demidchik, 2015; García-Caparrós et al., 2021). Singlet O_2 ($^1O_2$) is a ROS generated at the PSII light harvesting complex during energy transfer between the chlorophyll molecule and molecular oxygen during photosynthesis. During stress conditions, the balance between their formation and scavenging mechanisms in chloroplast is lost creating deleterious effects to the photosystems (Dogra and Kim, 2020; Wang et al., 2024).

Maize plants grown under Co stress, showed enhanced oxidative stress as evidenced from the high H_2O_2, $O_2^{\bullet-}$, and MDA content (lipid peroxidation). However, seed priming these plants decreased these contents, helping the plant to overcome the stress effects (Salam et al., 2022). In wheat plants, seed primed with ZnO NPs is reported to have a decrease of 35% in lipid peroxidation (Rai-Kalal and Jajoo, 2021). In faba bean under drought stress, the seed priming treatment with Zn reduced the MDA content, thus helped the plant to tide over the stress condition (Farooq et al., 2021). The H_2O_2, $O_2^{\bullet-}$, and MDA content, the markers for oxidative stress were high in blackgram under As stress. However, seed priming with Zn compound have reduced the generation of these ROS molecules (Banerjee et al., 2023). In rice plants grown under Cd stress, the exogenous application of Zn decreased the $O_2^{\bullet-}$, and MDA content, thus the viability of cells were more in them (Faizan et al., 2021).

2.9 Zinc biofortification achievements through seed nutripriming and foliar spray

Seed priming with $ZnSO_4$ solution increased the yield and yield-related traits and grain Zn content in barley and wheat. However, an increase in concentration and time of application has negative effect on the growth and yield. This may be due to the cytotoxic effect of high concentration of Zn. Seed priming as well as seed coating of two varieties of wheat with $ZnSO_4.7H_2O$ increased the stand establishment, emergence, yield and yield-related traits, and also grain Zn content in continuous two seasons of experiments (Hassan et al., 2019). In three wheat varieties, different types of Zn amino acid chelate were used to study the effect on growth, yield and biofortification efficiency. The biofortification efficiency of each varied with the source of Zn and genetic makeup of the wheat variety. The different Zn amino acid chelates used were Zn-histidine [Zn $(His)_2$], Zn-glutamine [Zn $(Gln)_2$], Zn-arginine [Zn $(Arg)_2$] and Zn-glycine [Zn $(Gly)_2$]. When compared to other priming agents, and soil application of Zn fertilisers, Zn-histidine and Zn-arginine increased grain Zn content (Seddigh et al., 2016). Seed priming with $ZnSO_4$ and $ZnCl_2$ in sugar beet increased the Zn concentration, making it a potential

biofortification candidate. The Zn level increase achieved through Zn seed priming could support various functions of plants such as increase in photosynthetic rate, transpiration and stomatal conductance of the plant (Carmona et al., 2020). Seed invigoration by priming and coating of chickpea with $ZnSO_4$ increased the bioavailable grain Zn content along with the yield and yield-related traits (Ullah et al., 2020b).

Farooq and co-workers (2018) studied the yield and grain Zn content of aromatic rice grain based on various Zn application methods including seed priming and seed coating in direct seeded and transplanted rice production systems. Even though there were no marked differences in yield among the treatments, there were considerable differences in grain Zn content. When compared to seed coating, seed priming was more effective in increasing the grain Zn content (Farooq et al., 2018). On calcareous soils, seed priming of rice with 0.5% $ZnSO_4$ solution could increase grain Zn content, when compared to control plants without any Zn treatment (Imran et al., 2015).

Seed priming with Zn is a good option to increase growth, yield parameters and grain Zn content of mungbean (*Vigna radiata* (L.) Wilczek) (Haider et al., 2020). Field trials of wheat and chickpea, using $ZnSO_4$ as a priming agent, increased the grain Zn content (Harris et al., 2007). A comparative study of different modes of Zn treatments in maize was done to identify the potential of each method for biofortification. The results showed that seed coating method was more effective in increasing the grain Zn content. Seeds of two cultivars of common bean Sadri and Talash analysed for the influence of Zn amino acid synthetic chelators like Zn-methionine [$Zn(Met)_2$] and Zn-histidine [$Zn(His)_2$] over soil applied Zn fertilisers. The analysis revealed that $Zn(Met)_2$ was more efficient in increasing the seed Zn content in Sadri cultivar while the $Zn(His)_2$ was more efficient in Talash cultivar. Seed priming using [$Zn(His)_2$] and [$Zn(Met)_2$] increased the grain Zn concentration by 8.5 to 33.8% and 16.5 to 34.3%, respectively (Tabesh et al., 2020b). Yet another success was the use of NPs. The combined action of ZnO-NP and FeO-NP increased Zn content in ragi (Mahmoud et al., 2019).

2.10 Zinc uptake from soil and translocation

The mobility of Zn from the rhizosphere to the root system of a plant is determined by the plant type, root characteristics, physicochemical properties of soil and various plant microbe interactions. The roots absorb Zn as a divalent Zn ion form. Like all the nutrients, the need for Zn by plants during various phases of the life cycle is different. Thus, according to the genetic makeup and physiological need for Zn, plants can increase the solubility and absorption of Zn from the soil by acidifying the rhizosphere. In plants, the root can exudate proton, organic acids like citric acid, malic acid and oxalic acid. Plants can actively absorb the Zn by hyperpolarising the root cell plasma membrane with the aid of ATPase. In cereal crops, there is an additional mechanism and it is by releasing phytosiderophores into the soil, which forms complexes with Zn, aiding in the absorption of Zn (Gupta et al., 2016). Arbuscular mycorrhizae (AM) can alter the physicochemical properties of soil, and through phosphatase, dehydrogenase activities and releasing glomalin glycoprotein, they facilitate the uptake of Zn from the soil. A field study in maize for increasing grain Zn content by the activity of zinc solubilising bacteria and AM mycorrhizae has found promising results (Suganya et al., 2020b).

The charged Zn^{2+} ion does not freely diffuse across lipid bilayer membranes (Eide, 2006). In soil, Zn initially penetrates the root cell wall free space through a diffusion mechanism (Gupta et al., 2016). The transport of Zn^{2+} into the cortex occurs through either the symplastic or apoplastic pathway (Kumar et al., 2016). Zn transporter proteins are essential for the uptake of Zn^{2+} into cells and the export from intracellular compartments (Hacisalihoglu et al., 2001; Hacisalihoglu and Kochian, 2003; Kumar et al., 2016). The ZIP and other Zn transporters facilitate the transport of Zn^{2+} ions across cellular membranes into the cytoplasm. Subsequently, Zn^{2+} ions traverse the casparian band, endodermis, and xylem parenchyma cells, which then facilitate the loading of Zn into the xylem (Krishna et al., 2023a).

Within the plant, transporter proteins are necessary for translocation and storage of Zn in edible portions. These proteins play an essential role in the translocation of metal ions into and out of the cell, microcompartments, and

sequestering these ions in vacuoles to act as a reservoir or reduce toxicity. Mainly three transporter systems are elucidated for Zn transport. They are the ZIP (zinc–iron permease or ZRT, IRT-like proteins) family of transporters seen on the plasma membrane. The CDFs (cation diffusion facilitator) like MTPs (metal tolerance protein), seen on tonoplasts and HMAs (Heavy Metal ATPase) a P-type ATPase, localised on the plasma membrane, tonoplast and endomembrane system (Caroli et al., 2020). Usually, Zn is transported within the plant as Zn^{2+} or complexed with protein, amino acids and organic acids. In the xylem, translocation of Zn occurs mainly as Zn^{2+} or complexed with histidine or nicotianamine (NA) while in the phloem, Zn is mostly seen complexed with small proteins and NA. Zn can reach the storage tissues via xylem or phloem (White and Broadley, 2009).

2.11 Storage and redistribution of zinc in plants

The optimal concentration of Zn in the cytoplasm is necessary for the proper functioning of cells. Zn accumulation and movement within cells play a key role in biofortification (Thomine and Vert, 2013). Membrane transporters are essential for the movement, retention, and allocation of Zn within intracellular organelles. The vacuole serves as a crucial cell organelle for the storage of excess Zn in the cytoplasm. Multiple transporters facilitate the storage of Zn in the vacuole and its redistribution to other needy parts. In *Arabidopsis* AtMPT1 and AtMPT3 are situated in the vacuolar membrane and play a role in the sequestration of Zn within the vacuole (Arrivault et al., 2006). AtHMA3 has been demonstrated to participate in vacuolar Zn sequestration (Morel et al., 2009). In rice, OsMTP1, OZT1 belonging to the CDF family and OsHMA3 mainly serves as a transporter located in the tonoplast that sequesters Zn into vacuoles (Lan et al., 2013; Cai et al., 2019; Ning et al., 2023). Zn transporters are also seen on cellular organelles, including mitochondria, chloroplasts, the endoplasmic reticulum, and the golgi apparatus. Each of them serves a distinct function in the intracellular transport of Zn within plant cells. Transporters located on cellular and intercellular organelles play a critical role in Zn homeostasis. Limited information exists regarding the functional role of transporters within inter-cellular organelles.

2.12 Zinc transporters involved in bifortification of zinc in seeds.

Enhancing biofortification requires knowledge of the movement of Zn from soil to the edible part of the plant. In plant cells, the membrane transporters have a specific function; they aid in maintaining homeostasis and fortifying seeds with Zn and Fe. Major crops have been found to express and localize different membrane transporters in tissue-specific ways. Certain membrane transporters actively participate in Zn grain filling and are expressed during the crop's seed development stage. The expression of *AtZIP4*, *AtZIP6*, *AtZIP9*, and *AtIRT3*, in seed formation of *Arabidopsis* plays role in regulation of Zn balance at this stage, thus they might help in Zn biofortification (Lee et al., 2021). The flag leaves of maize (*ZmZIP1* to *11*) (Mondal et al., 2014), and wheat (*ZIP1*, *3*, *7*, *9* and *15*) (Deshpande et al., 2018) express a large number of ZIP transporters, during seed development. In seed biofortification, membrane transporters expressed in flag leaves might be crucial. *ZmZIPs* are up regulated at different phases of maize embryo and endosperm development, according to gene expression studies. Late in the embryo's development, the *ZmZIP6* and *ZmIRT1* genes are expressed. *ZmZIP5* is expressed in the midway of seed development, whereas *ZmZIP4* is significantly expressed at the early stages (Li et al., 2013). The membrane transporters that cause Zn buildup in seeds of other plants are subjected to very less research. It appears that overexpressing the Zn transporter can improve the enhancement of Zn in the edible parts. For instance, the tapioca plant has higher Zn concentration in its edible sections when *AtZIP1* and *AtMTP1* are overexpressed (Gaitán-Solís et al., 2015).

2.12.1 Zinc-regulated, Iron-regulated transporter-like Protein (ZIP)

In plants, Zn-regulated, Iron-regulated transporter-like Protein (ZIP) was first characterised in *Arabidopsis thaliana*. It was named after the Zn regulated transporter (ZRT1 and ZRT2) genes of *Saccharomyces cerevisiae* and the iron-regulated transporter (IRT1) gene of *A. thaliana* due to its similarity with them (Grotz et al., 1998; Guerinot, 2000). The IRTI gene is an iron transporter gene (Eide et al., 1996) while ZRT1 and ZRT2 genes codes for high-affinity and low affinity Zn transporter respectively in yeast cells (Zhao and Eide, 1996a, 1996b; Grotz et al.,

1998; Guerinot, 2000). The transmembrane protein, ZIP is predicted to possess eight transmembrane (TM) domain and both the terminals is seen on the outer membrane facing extracellularly. The variable region between the TM3 and TM4 domain accounts for the variable length of the protein, and these regions are abundant in histidine residue and predicted to be the metal binding site. The TM4 with amphipathic helix containing histidine rich residues is the highly conserved region of the protein (Guerinot, 2000; Ishimaru et al., 2005; Eide, 2006; Chen et al., 2008).

Understanding the structure of a protein is crucial for investigating its function and regulation inside a cell. However, the crystal structure of ZIP proteins in plants is yet to be determined. The crystal structure and transport mechanism of the ZIP protein from the bacteria *Bordetella bronchiseptica* (BbZIP) have been thoroughly studied and documented (Zhang et al., 2017; Pang et al., 2023). Comparing to other membrane transporters, an unique structure, a rare 3+2+3TM structural design is found for BbZIP. The TM2, TM4, TM5, and TM7 becomes the inner bundle, whereas the TM4 and TM5 are the inner most, while the rest of the TMs becomes the outer bundle. The entrance cavity is observed within the outer membrane facing the cell exterior, while the exit cavity is located on the inner membrane, facing the cytoplasmic side. Significantly, negative charges exist in both cavities. The BbZIP crystallized in the presence of $CdCl_2$ was identified with 4 metal binding sites (M1 to M4) and Zn^{2+}-substituted BbZIP revealed 7 metal binding sites (M1 to M7) (Zhang et al., 2017, 2023).

The transport mechanism in BbZIP was explained as an elevator-type transport mechanism. There is a dramatic difference in the structure of the BbZIP TM protein in its metal unbound apostate and metal-bound state (Cd). The metal chelating residues in the M1 and M2 sites can be categorized into three distinct classes. The residues that underwent no significant structural changes (N178 of TM4, D208 of TM5, and E240 of TM6), the residues that change in the orientation of their side chains, moving away from the M1 site (E181 of TM4, Q207, and E211 of TM5), the residues in which the side chains and Cαs moved away from the M1 site (H177 of TM4 and M99 of TM2). Thus, the M1 site was dissembled in the

apostate. The analysis of the metal-bound and unbound forms of the protein indicated the presence of two distinct functional domains. Domain I is composed of TM1, TM4, TM5, and TM6, while domain II consists of TM2, TM3, TM7, and TM8. The change in the Cαs of H177 and M99 makes structural change in the domain I and domain II relative to one another making a substantial structural change in the whole transport protein. Comparing domain I and II in the apostate and metal bound state revealed a sliding movement of domain I at a hinge site in the extracellular region TM6 making a 9° rigid movement of domain I relative to domain II. Hence, a change in the conformation of the protein between outward-facing conformation (OFC) during apostate and inward-facing conformation (IFC) on metal binding occur during the transport of metals (Zhang et al., 2023).

The computational analysis revealed the structure and functions of rice ZIPs (OsZIP) (Krishna et al., 2022; Mohammed et al., 2023). There were variations in the amino acid residues found in rice, specifically for metal binding and chelation functions. Compared to other OsZIPs, the OSZIP 13 and 16 exhibited more conservation and similarity to BbZIP. His177 is crucial in releasing metal and is present in all the OsZIPs except OsZIP16. Glu211 is crucial for metal binding in all OsZIPs, except in OsZIP1 (Ala243) and OsZIP2 (Ala248). The Zn atom is bound to four residues: Glu181, Gln207, Glu211, and a water molecule. The Glu181 and Glu211 residues remained constant in all OsZIPs. However, Glu173 and Glu240 substituted the Gln207 residue and a water molecule in OsZIP16 and Glu253 and Asn30 in OsZIP13 (Krishna et al., 2022).

2.12.2 The P_{1B}-ATPases: HMA (Heavy Metal ATPase)

The P_{1B}-ATPases are transition metal transporters, belonging to the P-type ATPase superfamily, and is divided into two groups, Cu/Ag and Zn/Cd/Co/Pb transporters (Williams and Mills, 2005). It is modelled to have 6 to 8 TM helices and both the N terminal and C terminal domains with metal binding domain are seen on the intracellular part. The N terminal and C terminal of these proteins have important role in metal binding as well as in the regulation of metal transport (Eren et al., 2007). Majority of the P_{1B}-ATPases have 8 TM helices. The other important

domains present in them are the actuator (A) domain, nucleotide binding domain/ATP binding domain (N), the phosphorylation domain (P) with a conserved asp residue for phosphorylation and transmembrane metal binding site/domain (TM MBD). The domains A, P and N have catalytic roles. The sequence conserved in TM 6, 7 and 8 are predicted to have role in metal binding (Argüello et al., 2007). The A domain is thought to transmit the changes in the N domain to the TM regions and accounts for dephosphorylating reactions (Smith et al., 2014). They use energy from ATP hydrolysis for translocating the metals across the membrane (Rosenzweig and Argüello, 2012). A crystalline structure for the HMA protein is still lacking, and its functional characterisation is not studied well.

The C and N terminal domains are rich in Cys and His residues, having role in the overall functioning of the transporter. In plants, the N terminal domain of Zn^{2+} HMA /ATPases is nearly 70 amino acids long, possessing the conserved C^{17}-C-X-X-E^{21} sequence (X- S, T, P, and A), where X is an amino acid sequence that can coordinate the metal with thiol or carboxyl group. Studies in the AtHMA2 and AtHMA4 had enriched the structure as well as the function of both N and C terminal metal binding domains. The C17, C18, and E21 of the N terminal metal binding domain participate in Zn^{2+} and Cd^{2+} coordination. Lack of N terminal metal binding domain as well as mutations in any of the above mentioned amino acids, reduced ATPase activity without compromising metal binding to transmembrane metal binding domain (Eren et al., 2007). The C terminal domain of 11 His residues and 26 Cys residues, which can bind Zn and Cd with high affinity and can chelate up to 10 Zn per C terminal. The removal of His and/or Cys from this terminal had reduced the content of these metals. Thus, the C terminal have role in chelating/sensing the metal as well as regulation in the transport of metals through the transporter (Baekgaard et al., 2010).

The transmembrane metal binding domain (TM MBD) in the P_{1B}-ATPases is responsible for recognition and transport of transition metals across the lipid bilayer through the transporter protein channel. In Zn transporting HMAs, the conserved amino acid residues Cys, Pro, Cys from TM6, Lys from TM7 and Asp and Gly from

TM8, contribute to Zn binding and transportation (Argüello et al., 2007). The P domain, is extremely conserved among the P type ATPase, the transient phosphorylating Arg is present in the conserved sequence Asp-Lys-Thr-Gly (DKTG). It also contain Thr-Gly-Asp-Asn (TGDN) and Gly-Asp-Gly-x-Asn-Asp (GDGXND) domain essential for Mg^{2+} binding. The most variable domain is the N/nucleotide binding domain. It can binds with the ATP and can phosphorylate the conserved Arg in the P domain. The A domain is a globular phosphate connected to the TM2 and TM3, by 2 long linker sequence, they are flexible and have conserved sequence Thr-Gly-Glu (TGE) (Palmgren and Nissen, 2011; Lekeux et al., 2019).

The catalytic mechanism or transport mechanism of the protein is well studied in Ca^{2+}-ATPase. A large conformational change occurs in the protein for translocating the ion across the membrane with several catalytic intermediates. E1/E2 state of the protein in the catalytic cycle or the Post–Albers cycle is universally accepted to identify the differential affinity of the protein towards nucleotide and ions. In the E1 state, the ion binding site, present in the centre of metal binding TM domain, show high affinity towards the ions, hence it gets bind to the binding site, causing a conformational change in the P domain and N domain. In P domain Mg^{2+} ion get binds, to a site near to the conserved Arg, thus, the Arg in P domain is accessible for phosphorylation, and the protien becomes E1-P state. This creates a rotation in A domain, thus the ions gets diffused from the binding site and exit from the protein. Now the protein becomes in E2-P conformation. A further change in the conformation of A loop bring its TGE loop in proximity with the phosphorylated Arg, enabling a nucleophilic attack on the phosphate bond, dephosphorylating the protein and the protein is now in E2 conformation. This causes again the rotation of A domain to its initial position and the transporter protein converts back into the E1 state (Kühlbrandt, 2004). Thus rather than pushing the ions through the membrane the ions are fixed in the site and the site gets opened to either side of the membrane, enabling the translocation of ion from one side of the membrane to the other side (Palmgren and Nissen, 2011).

Rice possesses nine members of HMAs (OsHMAs) in which OsHMA1–3 are members of the Zn/Co/Cd/Pb subfamily, while OsHMA4–9 are members of the Cu/Ag subfamily. The OsHMA9 can also translocate Zn (Takahashi et al., 2012a, 2012b). OsHMA1, OsHMA7, and OsHMA8 are found in the chloroplast membrane, OsHMA3 is found in the tonoplast, and OsHMA2, OsHMA4, OsHMA5, OsHMA6, and OsHMA9 are found in the plasma membrane (Tian et al., 2023). OsHMA2, a plasma membrane located transporter, is a major Zn transporter for translocation of Zn from roots to shoots. It is seen in the pericycle of root, and involve in xylem loading of Zn (Stanton et al., 2022). OsHMA2 is also seen in node region and help in the loading of Zn to the developing tissues and panicles (Yamaji et al., 2013). It can also translocate Cd. The C terminal region is very critical for the transportation of Cd, but for Zn it is not necessary, but the N terminal domain is very essential for the translocation of Zn (Satoh-Nagasawa et al., 2012). Thus in biofortification efforts the C terminal can be truncated or modified in such a way that can hinder the Cd uptake without affecting the Zn uptake.

2.12.3 Other transporter proteins

2.12.3.1 Yellow Stripe-Like (YSL) Family

They are metal transporters, belonging to the oligopeptide transporters (OPT) family, that transport small peptides and aminoacid derivatives (Curie et al., 2009). They are proton-coupled symporter and are well known for transporting Fe, Mn and Cu in plants (Didonato et al., 2004). They are found to be important for long distance translocation of metals and translocate metals as metal- chelated complexes such as metal-nicotianamine (NA), metal-DMA and metal–phytosiderophore (PS) complexes (Chen et al., 2023). The YS1 protein was first discovered in Fe deficient maize and named after the phenotype. They showed yellowish stripe on the leaves, owing to Fe deficiency induced chlorosis due to inefficient chlorophyll synthesis. Later similar proteins were discovered in other plants and named as yellow stripe like protein (YSL) (Curie et al., 2001; Andresen et al., 2018). The YSLs is predicted to have 12 to 14 TM domains, in which the both the N terminal and C terminal is seen extracellular (Wang et al., 2022a). The transport mechanism of this protein is

identified to be a proton-coupled elevator-like in Barley (*Hordeum vulgare* L.) (Yamagata et al., 2022). HvYS1 protomer has two domains YS core domain and scaffold domain.

There are 18 YSL transporters present in the rice, most of them functions to translocate Fe^{3+}-DMA, Fe^{2+}-NA to nearby cells, long distance, developing tissues and seeds (Song et al., 2024). In Zn deficient plant, higher amount of Zn-DMA is observed. Thus, chelated Zn have importance in long distance translocation of Zn within the plant (Suzuki et al., 2008). The Zn- chelated complexes have identical structure with Fe- chelated complexes. At the same time in theory, the causes for Zn deficiency in soil is almost same for Fe deficiency. Thus for plants practically it is more efficient in terms of energy, to translocate both of these metals with identical transporters (Wirén et al., 1996). In rice Zn-chelated with DMA or NA is essential for the absorption as well as intracellular transport as well as long distance transport (Weiss et al., 2021). The OsYSL may be responsible for this translocation, further studies has to be carried out in this direction, to find a candidate OsYSL transporter for translocating Zn-DMA within the rice plant. The role of YSL in maize for the absorption and translocation of Zn is characterised. There is the need of 19 ZmYSLs, ZmYSL2, 5, 7, 9, 12 to for the translocation of Zn along with Fe. All of these have definite role during grain filling in maize (Song et al., 2024).

2.12.3.2 Metal tolerance protein (MTPs)

The Cation diffusion facilitator (CDF) family of transporters was initially documented in the late 1990s, and its first identified member, CzcD, was found to be involved in the resistance to heavy metals in metal-resistant bacterium *Cupriavidus metallidurans*. Plant CDFs are frequently referred to as MTPs (Metal Tolerance Proteins) (Kolaj-Robin et al., 2015). The initial plant CDF protein discovered is ZAT (Zn transporter of *Arabidopsis thaliana*). ZAT was renamed as AtMTP1 (Metal Tolerance Protein 1) because of its role in *Arabidopsis* heavy metal tolerance (Yuan et al., 2012). Metal tolerance proteins (MTP) are antiporters that facilitate the transit of divalent cations (Zn^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}, Ni^{2+}, and Mn^{2+}) in plants by exchanging Me^{2+} ions with H^+ or K^+ ions contributing significantly to plant metal

tolerance and homeostasis. They are categorised into three types: Mn-CDF, Zn-CDF, or Fe/Zn-CDF, depending on their specificities for these metals. The plant MTPs can be further classified into seven distinct categories based on their evolutionary relationships. Groups 1, 12, and 5 belong to the Zn-CDFs; groups 6 and 7 belong to the Fe/Zn-CDF; and 8 and 9 belong to the Mn-CDF (Montanini et al., 2007; Gustin et al., 2011).They are present in several membranes such as golgi apparatus, and vacuolar membranes. They are efflux proteins and facilitate the movement of metals out of the cytoplasm, either into the extracellular environment or into organelles (Ricachenevsky et al., 2013; Gao et al., 2020; Wang et al., 2022b). There are 10 MTPs in rice (OsMTPs). Out of this, 7 OsMTPs (OsMTP1, OsMTP5, OsMTP6, OsMTP7, OsMTP8, OsMTP9, and OsMTP12) were categorised into their respective number groups (group no. 1, 5, 6, 7, 8, 9, and 12). The OsMTP8.1 was placed in group 8, and MTP11 and MTP11.1 were placed in group 9 (Chen et al., 2013; Ueno et al., 2015; Ram et al., 2019).

2.12.3.3 Natural Resistance-Associated Macrophage Protein (NRAMP) Family

The Nramp (natural resistance-associated macrophage proteins), are a group of proton/metal transporter proteins conserved throughout evolution in all organisms ranging from prokaryotes to higher plants and humans. The substrates include Fe^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+} and Ni^{2+}. The Nramp family plays a crucial role in metal absorption, transport, storage, and intra- and intercellular trafficking in plants. The protein is predicted to contain 10 to 12 TMDs, with both C and N terminal seen at the cytoplasm (Ullah et al., 2018; Ishida and Corcino, 2022)**.** The crystal structure of the Nramp protein homolog in *Staphylococcus capitis* DMT (ScaDMT) and in the radiation resistant bacterium, *Deinococcus radiodurans* (DraNramp) revealed the mechanism of metal transport in these proteins (Ehrnstorfer et al., 2014; Bozzi et al., 2016). There are 7 NRAMPs in rice, namely OsNRAMP 1-7 (Mani and Sankaranarayanan, 2018).

2.12.3.4 Zinc induced facilitator like family (ZIFL)

ZIFL, transporter of mugineic acid (TOM) and vacuolar mugineic acid transporter (VMT) all are homologous and belong to the major facilitator

superfamily (MSF) transporter family. The Major Facilitator Superfamily (MFS) is the biggest group of secondary transport carriers in living organisms, consisting of at least 29 families (Saier et al., 1999; Ricachenevsky et al., 2013). Haydon and Cobbett, (2007) described Zn-induced facilitator (ZIF1) family of transporters in *Arabidopsis thaliana*. The AtZIF1 transporter plays a significant role in maintaining the balance of Zn in the plant body and the transcription of AtZIF1 is enhanced in response to an excess supply of Zn. AtZIF1 proteins are specifically seen on the tonoplast. They are likely play a role in the transportation of Zn to the vacuole. Nicotianamine, phytochelatin, and His are known to serve as metal-binding ligands in plants. However, it seems improbable that these ligands have significance in binding Zn in vacuoles. Organic acids, such as citrate and malate, have been identified as ligands in plant vacuoles and are thus suitable candidates for the ZIF1 substrate in regulating Zn homeostasis. Several tonoplast transporters for citrate or malate have been discovered; however, none belong to the major facilitator superfamily (MFS). Considering that ZIF1 exhibits the highest resemblance to MFS proteins, which facilitate transportation through substrate-H^+ antiport, and possesses protein sequence motifs commonly conserved across these MFS members, it is plausible to suggest that ZIF1 may provide this function in *A. thaliana*. ZIF1 is crucial in *A. thaliana's* ability to tolerate Zn by actively engaging in the process of sequestering Zn in vacuoles (Haydon and Cobbett, 2007).

Rice *ZIFL* genes were identified through computational analysis and named, ZIFL1 to 13. Their expression in various tissue during vegetative as well as reproductive stages were analysed. It was found that *OsZIFL5* and *10* were expressed during the vegetative stages. *OsZIFL2, 3,5,7,10,12* were expressed in flag leaves. *OsZIFL5, 9, 7, 10, 12* were expressed in panicles during various stages of grain filling (Ricachenevsky et al., 2011). Thus manipulating any of them, may have role in increasing the grain Zn content. It was interesting to see the result of a knock out for a vacuolar phytosiderophore transporter OsVMT/OsZIFL12. They functions as a vacuolar mugineic acid transporter and translocate the DMA to the vacuole. *OsVMT* exhibits an increased expression in the tonoplast of parenchyma cell bridges of node I, where there is a significant accumulation of Fe and Zn. OsVMT plays a

role in storing DMA in the vacuoles. Disrupting this gene enhances Fe and Zn in polished rice grains due to solubilisation of stored metals in the node. The rice homologs of OsVMT i.e. OsTOM1 and OsTOM2 facilitate the transportation of DMA and are found in the plasma membrane. However, the subcellular location of OsTOM3, again a rice homolog has yet to be studied (Che et al., 2019).

The Transporter of MAs (TOM1) acts as a DMA efflux transporter in rice. Zn-induced facilitator 1 (ZIF1), equivalent to TOM1 in *Arabidopsis*, has been recognised as an efflux transporter of NA. During seed development, nutrients are conveyed from leaves to the embryo and endosperm through vascular bundles. During germination, the nutrients that have accumulated in the endosperm are delivered to the embryo through the epithelium. Expression of TOM2 was detected in the scutellum, dorsal vascular bundles and epithelium of the developing seeds, while TOM3 was seen in the aleurone and anther. These data indicate that the secretion and transportation of DMA by the TOM family have significance in plant nutrition (Nozoye et al., 2011, 2015).

2.12.3.5 Pleiotropic drug resistance (PDR) protein

Bacterial ATP binding cassette (ABC) transporters facilitate the entry of many substrates. *Paracoccus denitrificans* possesses two Zn ABC transporter systems, ZnuABC and AztABCD, which exhibit overlapping functions in Zn-deficient environments. Both systems have homologs in the human pathogenic bacteria *Citrobacter koseri* and certain strains *of Klebsiella pneumoniae*. The plant pathogen *Agrobacterium tumefaciens*, also have homologs of these proteins for Zn import (Meléndez et al., 2020). No homologs in plants is discovered until now for these transporters. Recently a pleiotropic drug resistance protein (OsPDR20) belonging to ABCG subfamily of ABC transporters was identified to transport Zn in rice. It is localised on plasma membrane, predicted to have 7 TM helices and is crucial for the growth and seed formation in rice (Chao et al., 2023). The importance of ABC transporters in metal homeostasis is attaining importance nowadays. More work in this direction has to be done to get a complete understanding about metal transport in plants.

2.12.3.6 Other proteins

Besides these transporting proteins discussed above, additional categories of metal-binding proteins play a role in controlling the storage, distribution, and balance of metals. Metallochaperones are a diverse group of proteins that have the ability to bind metal ions and facilitate their movement to specific areas within cells by utilizing membrane-bound metal transporters. Metallochaperone proteins usually possess a heavy metal-binding domain (HMA) with a well-preserved Cys-X-X-Cys (X: any amino acid) motif. This motif adopts a Beta-Alfa-Beta-Alfa fold structure, which enables the protein to bind heavy metals like Cd, Cu, or Zn ions. Metallochaperones can be categorized into two subfamilies based on their structure. The first subfamily, heavy metal-associated plant proteins (HPPs), comprises metallochaperones with only 1-2 HMA domains. The second subfamily, heavy metal-associated isoprenylated plant proteins (HIPPs), includes metallochaperones with an extra C-terminal isoprenylation motif (Rono et al., 2019). Rice possess 59 HIPPs genes. OsHIPP33 is a constitutively expressed protein found in the plasma membrane and nucleus. The expression of OsHIPP33 is enhanced in response to Zn and Fe stress. The mutation of OsHIPP33 had a substantial negative impact on rice, causing decrease in the various yield parameters. Additionally, there was a decrease observed in Zn and Fe in straw, husk, and brown rice (Cao et al., 2022).

Another class of such proteins are Metallothioneins (MTs). They are small proteins with low molecular weight and high cysteine content. They are central players in maintaining metal balance, detoxifying harmful substances, and removing reactive oxygen species (ROS). Plant metallothioneins (MTs) are categorized into four distinct categories (MT1–4) according to the configuration of cysteine residues. They have distinct expression patterns in various plant organs. Roots generally express type 1 MTs; leaves express type 2 MTs; fruits express type 3 MTs; and seeds express type 4 MTs. Through transcriptome analysis, it was found that *OsMT2b* and *OsMT2c*, exhibited predominant and constant expression in the phloem of expanded and dispersed vascular bundles in the nodes and in anther. Disruption of

either *OsMT2b* or *OsMT2c* resulted in elevated Zn levels in the nodes while reducing the distribution of Zn to the panicle. This indicates that both OsMT2b and OsMT2c have a significant function primarily in allocating Zn to grain using chelation and subsequent transportation of Zn in the phloem of rice (Lei et al., 2021).

Phytic acid (PA), can chelate Zn and make it less bioavailable, hence considered as antinutrients in Zn biofortifcation perspective. PA is the primary storage form of phosphate (P) stored in the seeds of plants. A multidrug resistance-associated proteins (MRPs), belonging to ABCC type vacuolar transporters called PA-MRP transporters, are involved in phytate transport. Manipulating this transporter can reduce the PA content in seed and can increase the bioavailability of Zn (Colombo et al., 2020; Cominelli et al., 2020). The PHO1 gene family transporters located in plasma membrane (OsPHO1; 1 and OsPHO1; 2) and SULTR-like phosphorus distribution transporter (SPDT) controls the supply and allocation of phosphorus in rice grains. The Node I of rice had the highest level of SPDT expression and mutating these transporter proteins may substantially improve Zn bioavailability (Kumar et al., 2021).

2.13 Role of ZIP and HMA involved in zinc uptake, long-distance transport, remobilization, and grain filling in rice plant

In rice, the uptake of Zn by the root cells is facilitated by OsZIP1, OsZIP3 and OsZIP9 of the ZIP family transporters. The Zn can be sequestered in vacuoles by the activity of Heavy Metal ATPase proteins like OsHMA2 and OsHMA3. In the root cells, it is complexed with nicotianamine (NA), and through the epidermis, cortex and endodermis it reaches the xylem either by apoplast or symplast (Huang et al., 2020, 2022). Transporter proteins like OsHMA2, OsZIP4, OsZIP5 and OsZIP8 participate to load Zn to the root xylem and further root to shoot loading of Zn occurs (Chen et al., 2008; Liu et al., 2019; Yang et al., 2020). In xylem, Zn is usually present as soluble ions or complexed with small molecules like amino acids. The distribution of Zn to various plant parts is dependent upon the physiological growth stage and nutritional status of the plant. During vegetative growth, it is

allocated to developing leaves and during grain filling stage to the grain. However, to achieve maximum growth potential critical minimum concentration of Zn ranging 20 to 25 mg/g should be maintained in dry flag leaves. In xylem unloading and phloem loading, OsZIP4 have a functional role. The OsZIP3 and OsHMA2 present in the nodes also contribute to xylem unloading (Yamaji et al., 2013; Sasaki et al., 2015; Mu et al., 2021).

Zn accumulation in grain can be by the continuous absorption of Zn from the soil during the grain filling stage or by remobilization from the shoot system. The rice nodes act as a hub to distribute Zn from the xylem to the developing tissues like panicles. The node contains two major vascular bundles, enlarged vascular bundle (EVB) and diffuse vascular bundle (DVB). The EVB connects lower node with leaves while the DVB that surrounds EVB connects to upper nodes and panicle. To distribute Zn to panicle, inter-vascular transport with the aid of transporter proteins is needed (Yamaji et al., 2015). So, from the xylem of EVB, Zn is unloaded to the apoplast by OsZIP3 located on the xylem transfer cells. Through symplastic loading, Zn reaches to the intervening parenchyma cells and gets unloaded to the apoplast of DVB by an unidentified translocator. From there Zn is transported to the phloem of DVB via OsHMA2. The route and translocators for loading of Zn to the grain by relocation from leaves is unclear. It is assumed that Zn from chloroplast and vacuole of mesophyll cells are loaded to apoplast and from there it is loaded to the phloem. In phloem tissues, Zn is usually seen complexed with nicotianamine (NA) (Kawakami and Bhullar, 2018).

However, within the grain, there are no plasmodesmatal connections between the maternal tissue and filial tissues like endosperm and embryo. The transport beyond the nucellar tissue may be apoplastic, and the transporters like OsZIP4 and OsZIP8 may contribute to grain Zn loading (Yamaji and Ma, 2014, 2017). In early grain filling stages, Zn is distributed to the endosperm through the aleurone layer. But later when starch is laid down, this distribution slows or ceases (Nakandalage et al., 2016; Mitani-Ueno et al., 2018; Huang et al., 2022). The possible reason for this in the perspective of the plant, it is efficient and profitable to

store these nutrients needed for germination to carry out enzymatic reactions, photosynthesis, antioxidant potential in the embryo than in endosperm, when the cost of transporting nutrients is considered. If it is stored in endosperm, again the same expenditure of energy is needed to transport it back to the embryo (Lu et al., 2013).

2.14 Regulation of membrane transporter genes in rice plant

The uptake, distribution, translocation, storage and maintenance of Zn homeostasis depends on the membrane transport system and ligands, which can bind with Zn. These systems has to be tightly regulated to cope up with both shortage and surplus Zn conditions (Liao et al., 2022).

2.14.1 Transcriptional regulations

Two transcription factors (TFs) bZIP19 and bZIP23, belonging to the basic-region leucine-zipper (*bZIP*) transcription factor gene family was identified in *Arabidopsis*, which are transcriptional regulators under Zn shortage (Assunção et al., 2010). They all belong to the F group of *Arabidopsis* basic-leucine zipper proteins (F-bZIPs), distinguished by a cysteine and histidine-rich motif at the N-terminus. They interact with a 10 bp Zn Deficiency Response Element (ZDRE; RTGTCGACAY) located in the promoter region of their target gene. The target genes encompass the transporters within the ZIP family (Castro et al., 2017) and can act as a Zn sensor. In active conformation, it is a dimer and are dissociated into monomers during inactivation. They increase the transcription of Zn transporter genes by binding with ZDRE domain during deficiency of Zn. During Zn surplus condition, the excess Zn binds to the Cys/His-rich Zn Sensor Motif (ZSM). This cause a conformational change in the TFs, and hampers the dimerization of protein, thus it cannot bind with the DNA, so that transcription of Zn transporter genes are halted (Lilay et al., 2021; Assunção, 2022). In rice, the Zn deficiency response is controlled by OsbZIP48 acting as an equivalent of AtbZIP19 and AtbZIP23 (Lilay et al., 2020). OsbZIP48 regulate the expressions of *OsZIP4* and *OsZIP8* (Hu et al., 2024). To activate OsbZIP48, phosphorylation by protein kinase RLCK160 is required (Liu et al., 2024b).

Another family of transcription factors (TFs) peculiar to plants represented by the NAC (NAM, ATAF, and CUC) TFs also control Zn homeostasis. 151 NAC transcription factors have been discovered in rice. A comparative investigation of the transcriptome of several rice types cultivated under sufficient and insufficient Zn levels revealed that 27 NAC transcription factors responded under Zn shortage. Of the 27 NAC genes, ONAC32 (ONAC056), ONAC120, ONAC017 (ONAC030), and OsNAC300 were strongly activated in response to a lack of Zn. In this OsNAC15 is an important transcription factor which functions in Zn deficiency tolerance. OsNAC15 is localised in the nucleus, and its expression is downregulated under conditions of Zn shortage. OsNAC15 directly interacts with the promoters of *OsZIP7* and *OsZIP10*, leading to the suppression of their transcription (Zhan et al., 2022).

The TITANIA (TTA) in rice (OsTTA) is a constitutively expressed transcription factor located in the nucleus, characterised by a plant homeodomain-finger (PHD-finger) domain. It enhances the expression of metal transporters such as OsZIP and OSNramp needed for Zn uptake and translocation, thus promoting the uptake of metals necessary for optimal plant growth through transcriptional regulation of these metal transporters (Tanaka et al., 2018). miRNA can post translationally regulate TFs and transporter proteins. In maize the miRNA166d regulate bZIP TFs (Gao et al., 2019). As mentioned above the bZIP TF can regulate the expression of transporter proteins. MicroRNAs (miRNAs) are small non-coding, 22 nucleotides long, regulatory RNA molecules that can regulate activity of gene posttranscriptional. Zeng et al. (2019) conducted a transcriptome analysis in rice. The study identified 68 miRNAs, including 38 unique miRNAs and 5 that are sensitive to copper, which exhibited distinct expression patterns. The differentially expressed genes identified may serve as potential candidates for future biofortification efforts (Zeng et al., 2019).

2.14.2 Epigenetic regulation

Epigenetics is the heritable changes in chromosomes by DNA methylation, histone modification and chromatin remodelling without altering the gene sequence.

It controls many cellular events (Brito et al., 2020). Zn functions as a modulator by serving as a cofactor binding to active or allosteric sites for epigenetic enzymes such as DNA methyltransferase (DNA MTase), histone acetyltransferase (HAT), histone deacetylase (HDAC), histone demethylase, histone E3-ubiquitin ligases (EUBLs), and histone deubiquitinating module (DUBm) complexes. HATs utilise Zn ions to attach to DNA through their Zn finger motif, while HDACs possess Zn in their active sites, which is essential for the hydrolase process. In addition, DNMTs are recognised to have several Zn binding sites (Brito et al., 2020; Yusuf et al., 2021).

Zn deficiency can also be linked to DNA hypomethylation via the methionine synthetase (MTR) and a decrease in the activity of DNA methyltransferases (DNMTs). Zn also plays a crucial function in preserving the structural integrity of Zn finger domains (ZnDs), which are involved in epigenetic processes. Recently, it was reported that these motifs may exert a novel genomic influence by functioning as methyl-CpG binding proteins (MBPs). These proteins have the ability to identify and attach to methylated DNA, which then triggers subsequent changes in gene expression (Noronha et al., 2022). Fujisawa and colleagues have demonstrated that Zn shortage results in the loss of enzymatic activity of the histone acetyltransferase KAT7, which in turn leads to reduced acetylation of histone H3 at Lys14 (H3K14ac) in Human Embryonic Kidney (HEK), HEK293A cells. The reduction in H3K14ac results in the increased expression of ZIP10. This allows the importation of Zn from sources outside the cell to maintain the balance of Zn within the cell. These findings indicate that cells react to a lack of Zn by transforming it into an epigenetic signal to initiate cellular reactions (Fujisawa et al., 2023).

In maize, upon induction of Zn stress, when compared to the control, the expression of ZIP transporters such as *ZIP1, ZIP2, ZIP3,ZIP4, ZIP5, ZIP6, ZIP7* and *ZIP8* dropped. The expression levels of *HDA102, HD2b, HD2c,* and *HDA106* were reduced, while the expression of *HD2a* was elevated. Upon exposure to Zn, the expression levels of *DNMT1, DNMT2a, DNMT2b*, were reduced compared to the control group. Conversely, the expression levels of *MET3a* and *MET3c* were

elevated. This shows the epigenetic regulators may play a crucial role in Zn acquisition in maize (Shafiq et al., 2020). Ou and co-workers experimentally proved the transgenerational inheritance of epigenetic modification in rice subjected to various heavy metal stress (Ou et al., 2012; Cong et al., 2019). A recent study in rice explores the epigenetic mechanism behind resistance to mercury (Hg) and uncovers a conflicting link between Hg and Zn (Zn) (Cong et al., 2024). Hence, to modulate epigenetic enzymes and epigenetic mechanisms, the various Zn transporters and Zn binding proteins have to be tightly regulated. Further studies has to be done in this area of research to get a full picture on how the Zn homeostasis in rice plant is maintained by regulating the various proteins involved in absorption, sequestration, translocation.

2.15 Priming memory and effect on subsequent generation

Priming can result in the retention of previously experienced stress memories after the event has concluded, a process referred to as priming memory. When a second attack of this stress occur, then the plant can fight back more swifty and strongly. This memory can be either retained only in the current generation (somatic memory), or sometimes, it can pass on to the subsequent generations (transgenerational memory). Thus, this is an effective method for evading stress in plants (Leuendorf et al., 2020; Yang et al., 2022; Ganie et al., 2024). In a nutshell, priming memory can be summarized as follows. The first event in this is stress perception and signalling. These stress stimuli may be either environmental stresses (biotic or abiotic) or by applying priming treatments (chemical, physical). These creates stress memory through transcriptional (gene expression changes, transcription factor, chromatin modulation) and post transcriptional as well as translational regulation (chromatin modulation, DNA methylation variation, non-coding RNAs). All of these separately or cumulatively cause ROS creation and scavenging, hormone biosynthesis and signalling, protein metabolism and enzymes activities as well as metabolite production. All the changes in these pathways eventually creates stress memory by biochemical changes, epigenetic imprinting, hormonal changes and signalling changes, leading to phenotypic adaptation. Thus

creates three types of priming memory, somatic (short and long term), intergenerational (direct from parent to offspring) and transgenerational (multiple generations). In the absence of primary stress effect, by utilising the techniques of seed priming (GABA, JA, SA), one can induce cross stress tolerance through priming memory. This could be established via epigenomics (sRNA mediated, DNA methylation, chromatin changes), transcriptomics (TFs, antioxidant genes, transporter genes, hormonal genes), proteomics (ROS scavenging proteins, chaperones, key metabolic proteins) and metabolomics (amino acids, sugars, antioxidants, secondary metabolites) (Turgut-Kara et al., 2020; Liu et al., 2022).

3.1. Plant material and growth conditions

A total of thirty rice varieties were selected for the study. In 2018, a survey was done by the Kerala Agricultural University in collaboration with the Kerala Department of Agricultural Development and Farmers Welfare. They had created a directory of the growers, locations, and landraces (KAU, 2018). With the help of this, fifteen landraces were collected from Padma Shri Cheruvayal Raman, Wayanad, Kerala, India, who had a good repository of landraces, which are very unique and poorly studied (Table 1). Fifteen widely cultivated hybrid rice varieties were collected from regional rice research station (RARS) Pattambi, Palakkad, Kerala, India (Table 1). The rice grains were dried, dehusked manually and the brown rice was ground using a mortar and pestle, followed by sieving through a 0.5 mm mesh. The resulting powder was then sealed in an airtight container and stored for further analysis. All the experiments were carried out in triplicate.

The seeds from eight varieties selected (Adukkan, Annapoorna, Gandhakashala, Jyothi, Kumkumashali, Mullankayama, Ponmani and Uma) based on the initial set of experiment were germinated in paper cups (240mL). The experiment was conducted in the polyhouse of the Department of Botany, University of Calicut, Kerala, India. Farm soil from the university botanical garden was used for the experiment and the various properties of the soil are detailed in Table 2. The seeds were first surface sterilised with 0.1% sodium hypochlorite for 1min and after that, it was washed well with double distilled water to remove any trace of the sodium hypochlorite. For priming the seeds, the seeds were incubated in $ZnSO_4$ (0.25M, 0.5M, 0.75M and 1M) and $ZnNO_3$ solution (0.25M, 0.5M, 0.75M and 1M) for 6, 12, 18 and 24h, then it was washed with double distilled water, and dried back into the original dry weight. For seedling priming, the rice seedlings of 1 leaf old (5d after sowing) were foliar sprayed with the $ZnSO_4$ or $ZnNO_3$ nutrient solutions (0.25, 0.5, 0.75%). The spray was done in the evening with utmost care taken to prevent the Zn compounds from infiltrating the soil. Seeds without any treatments were

taken as the control. Good quality irrigation water was supplied to meet the crop's requirements. On 14 d after sowing, the total chlorophyll, carotenoid and MDA content were analysed in leaf samples (Fig. 1A).

The optimum concentration and duration were fixed for both the priming agents based on the above experiments. Thus the treatments include, control plants (without treatment), seed priming with $ZnSO_4$ (0.5M, 18h), seed priming with $ZnNO_3$ (0.5M, 18h), seedling priming with $ZnSO_4$ (0.5%), seedling priming with $ZnNO_3$ (0.5%), combined treatments of seed priming and seedling priming with $ZnSO_4$ and combined treatments of seed priming and seedling priming with $ZnNO_3$. The eight rice varieties (Adukkan, Annapoorna, Gandhakashala, Jyothi, Kumkumashali, Mullankayama, Ponmani and Uma) were germinated in paper cups and on 10 d after sowing it was transplanted to plastic pots (16cm height and 17cm diameter). The experiments were triplicated with a completely randomised block design. At physiological maturity, the yield parameters and grain Zn content were analysed. Based on the result, two rice varieties (Annapoorna and Kumkumashali) were selected along with $ZnNO_3$ as the most successful priming agent. Further study was carried out only on these two rice varieties and $ZnNO_3$ as priming agent. Farm soil from the university botanical garden was used for the experiment and the various properties of the soil are detailed in Table 2. The treatments include control plants (no treatment), seed priming with $ZnNO_3$ (0.5M, 18h), seedling priming with $ZnNO_3$ (0.5%) and combined treatments of seed priming and seedling priming with $ZnNO_3$. Photosynthetic, antioxidant, oxidative stress and metabolic parameters were analysed on the 45d after sowing. Yield parameters and bioavailable Zn were analysed at maturity. To assess the bioavailable Zn, supplementary foliar applications ($ZnNO_3$ (0.5%)) were conducted at different reproductive phases (booting, flowering, and milky stages) (Fig. 1B).

Based on the above study, rice genotype Kumkumashali was selected to study the impact of seed priming and foliar spray at reproductive stages on growth, biomass, Zn and other nutrients levels and yield, as well as expression of Zn transporter genes under Zn deficient and sufficient conditions. Seed priming ($ZnNO_3$

Table 1. List of rice varieties used in this study

Sl. No.	Rice Variety	Type	Bran Colour
1	Adukkan	landrace	Red
2	Cheattuveliyan	landrace	Red
3	Kannichennellu	landrace	Red
4	Kumkumashali	landrace	Red
5	Kuruva	landrace	Red
6	Marathondi	landrace	Red
7	Onnamottan	landrace	Red
8	Paalthondimatta	landrace	Red
9	Rakthashali	landrace	Red
10	Veliyan	landrace	Red
11	Gandhakashala	landrace	White
12	Jeerakashala	landrace	White
13	Mullankayama	landrace	White
14	Paalthondivella	landrace	White
15	Urunikayama	landrace	White
16	Aishwarya	hybrid	Red
17	Annapoorna	hybrid	Red
18	Jyothi	hybrid	Red
19	Kanchana	hybrid	Red
20	Manuvarna	hybrid	Red
21	Samyukhtha	hybrid	Red
22	Uma	hybrid	Red
23	Vaishakh	hybrid	Red
24	Varsha	hybrid	Red
25	Vytila 6	hybrid	Red
26	Akshaya	hybrid	White
27	Neeraja	hybrid	White
28	Ponmani	hybrid	White
29	Supriya	hybrid	White
30	Swetha	hybrid	White

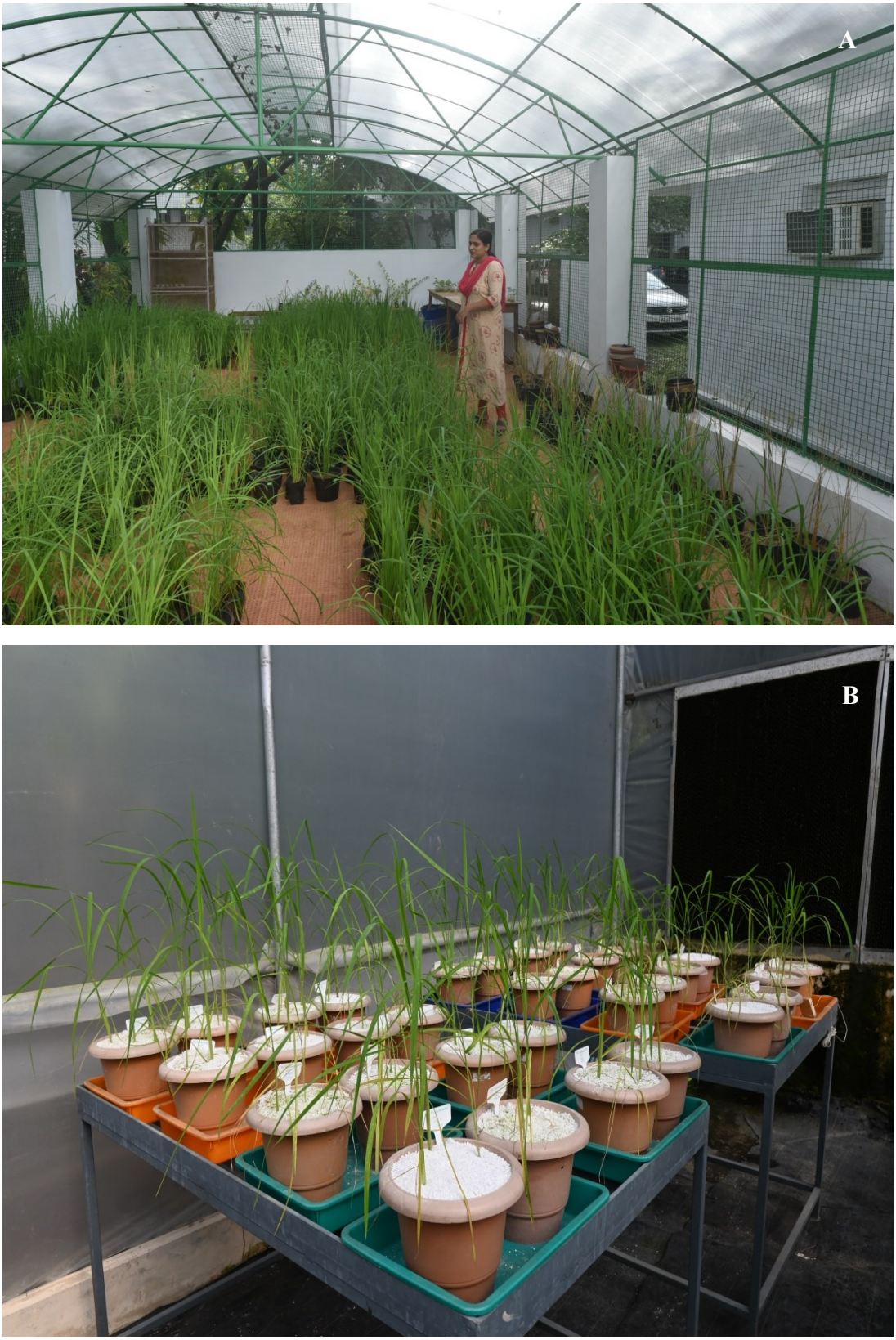


Figure 1. Rice plants grown in poly house of Department of Botany, University of Calicut (A), Department of Bio Sciences; (B) Rajagiri College of Social Sciences.

Table 2. Properties of the soil used for growing the rice seeds after seed priming treatment

Soil properties	
pH	5.95
EC	0.503 (mS/cm)
TDS	265 (mg/L)
OC	1.86 %
Organic matter	4.65 %
Available Nitrogen	369.60 (kg/ha)
P	7.62 (kg/ha)
K	1034.88 (kg/ha)
DTPA extractable zinc	6.05 (mg/kg)
Fe	135.25 %
Sand	72.5 %
Clay	16.25 %
Silt	11.25 %
Soil texture	Sandy loam

(0.5 M, 18h)) and FS ($ZnNO_3$ (0.5 %)) concentrations were selected for this experiment based on the results of previous experiments. The experiment was conducted in the green house at the Department of Biosciences, Rajagiri College of Social Sciences, Kochi, Kerala, India, from January, 2023 to April, 2023. Seeds were grown on horticultural grade perlite (Astra Chemical, Chennai, Tamil Nadu, India) in a plastic pot (25 × 33.5 cm), and subjected to various treatments including; 1) Zn deficient control (negative control), 2) Zn sufficient control (positive control), 3) Zn deficient condition with seed priming, 4) Zn sufficient condition with seed priming, 5) Zn deficient condition with foliar spray, 6) Zn sufficient condition with foliar spray, 7) Zn deficient condition with combined treatments of seed priming and foliar spray and 8) Zn sufficient condition with combined treatments of seed priming and foliar spray. The basal nutrient solution was prepared based on the previously reported protocol (Ceasar et al., 2014) containing modified levels of Zn [Zn deficient condition (0.05 µM) and Zn sufficient condition (1.5 µM)] (Krishna et al., 2023b) as noted in table 3. The nutrient solution was given on every three days. A total of 10 to 15 seeds were initially sown. After 15d of growth, the plants were thinned to three plants per plot. For the FS experiment, 0.5% $ZnNO_3$ were sprayed on to the plant at booting, flowering, and milky stages of rice using a sprayer. A total of three replicates were maintained for each experiment.

To study the second-generation effects of seed priming on plants raised from seeds acquired in the previous experiment. Seeds were selected from controls (Zn deficient and Zn sufficient condition) and combined seed priming and foliar application (Zn deficient (seed priming+ foliar spray) and Zn sufficient (seed priming+ foliar spray)). The seeds were either non-primed or primed in the second stage. The seeds were germinated in paper cups and on 10 d after sowing it was transplanted to plastic pots (16cm height and 17cm diameter). Farm soil from the university botanical garden was used for the experiment and the various properties of the soil are detailed in Table 2. The experiments were triplicated with a completely randomised block design. At physiological maturity, the yield parameters and grain Zn content were analysed.

3.2. Mineral analysis

Extraction: A 0.2 g dried sample of the rice tissues (grain powder, root, node I, flag leaves, and panicle) were transferred to a Kjeldahl flask and 20 mL of concentrated HNO_3 and 1 mL of H_2O_2 were added and heated in a heating mantle until the solution became colourless. After complete digestion, the solution was cooled, filtered and made up to 50 mL with distilled water.

Estimation: Zinc (Zn) and other essential elements were analysed using the inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7800, Germany). The ICP-MS instrument was calibrated with an internal multi-element standards (Merck, Germany). The operating specification during the measurement was as follows: RF Power – 1550 W, gas flow rate: plasma gas – 15.0 $L \cdot min^{-1}$, auxiliary gas – 0.9 $L \cdot min^{-1}$, nebuliser gas – 1.01 $L \cdot min^{-1}$; scan mode – peak hooping.

3.3. Estimation of amylose

Extraction: A mixture containing 0.1 g of the powdered rice grain, 1 mL ethanol (95%) and 9 mL sodium hydroxide (1N) was heated in a boiling water bath for 15 min. The resulting solution was cooled and made up to 100 mL with distilled water in a 100 mL volumetric flask.

Estimation: The amylose-iodine blue complex method was used to estimate the amylose content in the rice grains. A 5 mL aliquot of the solution was collected in a test tube, and then 1 mL of acetic acid (1N) and 2 mL of 0.2% iodine solution were added. The sample was incubated in the dark for 20 min. After incubation, absorbance was measured at 620 nm in a spectrophotometer (Multiskan, Thermo Fisher Scientific, Vantaa, Finland) against a reagent blank and the amylose content was calculated using a calibration curve made from standard potato amylose (Singh and Juliano, 1977).

Table 3. Composition of nutrient solution

Name of the Nutrient	Working Concentration	MW	Amt/Litre	0.5 M Stock (1 L)	1000x stock (1 L)	0.2 M stock (1L)	0.1 M Stock	Volume required for 10 L
$Ca(NO_3)_2$	2.0 mM	164.0		82 gm				40 ml
$MgSO_4$	0.5 mM	120.3				24.06 gm		25 ml
KCl	0.1 mM	74.5				14.9 gm		5 ml
H_3BO_3	10 µM	61.8	618 µg	Micro salt stock	618 mg	Micro salt stock		10 ml
$MnCl_2$.$4H_2O$	0.5 µM	197.9	98.95 µg		98.9 mg			
***$ZnCl_2$**		136.2			68.1 µg			
$CuCl_2$. $2H_2O$	0.2 µM	170.4	34.08 µg		34.08 mg			
Na_2MoO_4	0.1 µM	241.95	24.1 µg		24.1 mg			
K_2SO_4	0.3 mM	174.2	174.2			34.84 gm		varies
KH_2PO_4	0.7 mM	136.08	136.08				13.6 gm	varies
FeEDTA	0.1 mM	367.0				73.4 gm		5 ml
pH = 6.0 (using 0.1 M H_2SO_4 or 0.1 M NaOH)								

***Varied for different concentration of Zn (Deficient: 0.05 µM Zn and Sufficient 1.5 µM Zn)

3.4. Estimation of photosynthetic parameters

3.4.1. Pigment estimation

Extraction: For the estimation of pigments, 500 mg of leaf tissue was homogenised in 5 mL of 80% acetone and centrifuged at 5000 rpm for 6 min at 4°C.

Estimation: The photosynthetic pigments were estimated spectrophotometrically (Multiskan, Thermo Fisher Scientific, Vantaa, Finland). The absorbance of the extract was read at 470, 646, 663 and 750nm against a blank (80% acetone). The calculations for chlorophyll and carotenoid were made according to Arnon (1949) and Lichtenthaler and Wellburn, (1983) respectively.

$$\text{Total chlorophyll} = \frac{20.12(A_{646} - A_{750}) + 8.02(A_{663} - A_{750})}{\text{Fresh weight of the sample}} \times \text{volume}$$

$$\text{Carotenoids} = \frac{1000(A_{470}) + 3.27(\text{Chl a} - \text{Chl b})}{\text{Fresh weight of the sample} \times 229} \times \text{Volume}$$

3.4.2. Chlorophyll stability index

Extraction: 200 mg of leaf tissue was immersed in 10 mL distilled water and incubated in two different conditions. Half of the sets were incubated at 50^0C for 1h, while the other half at room temperature. Later the water was drained out and 5mL dimethyl sulphoxide (DMSO) was added and incubated at 70^0C, for 2h until the leaves became pale.

Estimation: The chlorophyll stability index (CSI%) was estimated as per Mishra et al. (2016). Then the chlorophyll content was estimated according to Arnon (1949).

CSI= [1-(C1/C2)] * 100

C1- chlorophyll content of temperature treated sample

C2- chlorophyll content of untreated sample

3.4.3. Chlorophyll *a* fluorescence measurements

The chlorophyll *a* (Chl *a*) fluorescence analysis was performed using Plant Efficiency Analyzer (Handy PEA; Hansatech Ltd., King's Lynn, Norfolk, UK). After 20 min of dark adaptation, the upper surface of the leaves was illuminated with 650 nm light of 3000 $\mu molm^{-2}s^{-1}$ intensity. Various chl *a* fluorescence parameters (Table 4), were analysed, OJIP curve and radar plots were prepared using the Biolyzer HP3 software (Bioenergetics Laboratory, University of Geneva, Switzerland).

3.4.4. Photosystem (PS) I and II activities

Isolation of thylakoid membranes: Using a pre-chilled mortar and pestle, 500 mg of fresh tissue was homogenised in 5 mL of ice-cold isolation buffer (pH 7.8) that contained 400 mM sucrose, 10 mM NaCl, and 20 mM tricine. The homogenate was centrifuged at 4°C for 6 min at 5000 rpm after being filtered through four layers of cheesecloth. The pellet was dissolved in 500 M suspension buffer (pH 7.5) after centrifugation, which contained 100 mM sucrose, 10 mM NaCl, 20 mM HEPES [N(2-hydroxyethyl) piperazine-N (2-ethanesulphonic acid)], and 2 mM $MgCl_2$ and was kept at 4°C.

Estimation of chlorophyll content of thylakoid membranes: Using Arnon's method, the chlorophyll content was assessed (1949). To test tube containing 3 mL of 80% acetone (v/v), 40 µL of the thylakoid solution was introduced. Using a vortex mixer, the contents of the tubes were thoroughly combined. The homogenate was then centrifuged for 5 min at 5000 rpm to separate off the supernatant. The absorbance was measured at 645, 663, and 750 nm in comparison to a solvent blank (80% acetone). The following equation was used to determine the overall chlorophyll content in the thylakoids.

Total chl = 20.12 (A646-A750) + 8.02 (A663-A750) × Dilution factor

Estimation of thylakoid electron transport activities:

Estimation of PSI activity: Stock solutions of 500 mM of DCMU (3-(3,4-

Table 4. Detailing of the different fluorescence parameters studied

Parameters	Description
Phases in induction curve	
O=F_O	Minimal fluorescence/ first step of chl *a* fluorescence transient
I=F_I	Intermediate step in the chl *a* fluorescence transient at 2 ms
J=F_J	Intermediate step in the chl *a* fluorescence transient at 30ms
P=F_P=F_M	Maximal fluorescence level/ final step of chl *a* fluorescence transient
K	Intermediate step in the chl *a* fluorescence transient at 0.3 ms
OJ-phase	It represents the reduction of the acceptor side of PSII
JI-phase	It represents the reduction of the PQ (Plastoquinone) pool
IP-phase	It represents the reduction of the acceptor side PS I
Other JIP parameters	
F_V	Maximal variable fluorescencce (F_M-F_O)
F_V/F_M	It represent maximum quantum yield of PSII
F_V/F_O	It represents the maximum efficiency of water splitting complex
SFI $_{ABS}$	An indicator of PSII structure and functioning
V_J	Relative variable fluorescence at phase J of the fluorescence induction curve
V_I	Relative variable fluorescence at phase I of the fluorescence induction curve
PI $_{ABS}$	Performance index of PSII on absorption basis
PI $_{TOTAL}$	Performance index of electron flux to the final PSI electron acceptors
Yield parameters	
φPo	Maximum quantum yield of primary PSII photochemistry (at t = 0)
φ (Do)	Quantum yield of energy dissipation
φ(Eo)	Quantum yield (at t = 0) for electron transport from QA^- to plastoquinone

Phenomenological energy flux	
ABS/CSm	Absorption of energy per excited cross-section (CS) approximated by F_M
TRo/CSm	Excitation energy flux trapped by PSII of a photosynthesizing sample cross-section (CS) approximated by F_M
ETo/CSm	Electron flux transported by PSII of a photosynthesizing sample cross-section (CS) approximated by F_M
DIo/CSm	Heat dissipation of excitation energy by PSII of a photosynthesizing sample cross-section (CS) approximated by F_M

dichlorophenyl)-1,1-dimethylurea), 10 mM of DCPIP (2,6-dichlorophenolindophenol), 500 mM of ascorbate, 5 mM of MV (methyl viologen), and 1 M of NaN_3 were prepared for the determination of PSI activity. In suspension buffer, a 2 mL reaction mixture including 20 µL of each DCMU, DCPIP, ascorbate, and MV, 10 µL of NaN_3, and 40 µL of thylakoid extract was mixed together (pH 7.5). DCPIP was used as a synthetic electron donor, while MV was used as an artificial electron acceptor, to evaluate PSI activity in terms of oxygen consumption.

Artificial electron acceptors and donors were used to block certain steps of the light-dependent electron transport in the thylakoid membrane in order to estimate the PSI and PSII activities based on the O_2 uptake/evolution from/into the media. Initially, PSII activity was inhibited by adding DCMU to the medium in order to measure PSI activity. The artificial electron donors ascorbate and DCPIP in the medium, where ascorbate served as a reductant by donating electrons to DCPIP, and further, the electrons given by DCPIP to plastocyanin, were transferred to PSI, sustained the electron transport to PSI. Electrons from PSI are bypassed to an artificial electron acceptor, MV in the reaction mixture. MV reacts with oxygen molecules in the medium and produce H_2O_2. Further the dissociation of H_2O_2 to form oxygen and H_2O by the action of catalase is arrested by NaN_3 added in the reaction mixture. Thus the oxygen consumption by activity of PSI alone is measured by oxygen electrode system.

Estimation of PSII activity: In suspension buffer, a 2 mL reaction mixture containing 20 µL pBQ (50 mM) and 40 µL thylakoid extract was prepared (pH 7.5). PSII activity was measured in terms of oxygen evolution by using PBQ as an artificial electron acceptor.

To measure the PSII activity, artificial electron acceptor pBQ was added in the medium and it will scavenge the electrons from plastoquinone. The transfer of electron from plastoquinone to cytochrome is terminated and so the activity of PSII alone was measured. Splitting of water for transferring of electrons to PSII result in evolution of oxygen molecules in the medium and it was measured by oxygen electrode system.

Thylakoids were extracted from leaves according to Puthur (2000), using a Clark-type oxygen electrode (DW1/AD, Hansatech, Norflok, UK) linked to a digital control box (OXYG1, Hansatech). A 100W halogen lamp was used to irradiate the sample with white light at a saturating intensity of 1800 mol photons $m^{-2}s^{-1}$ in order to assess the light-dependent O_2 uptake/evolution (LS2, Hansatech). The amount of O_2 consumed (by PSI) or evolved (by PSII) per minute per milligrams of chlorophyll was used to express the PSI and PSII activity. The temperature of thylakoid extraction procedure and assay was maintained at 4°C. All the experiments were preferably done in dark conditions.

Preparation of pBQ: To prepare p-benzoquinone, 0.5 g of hydroquinone was dissolved in 5 mL of distilled water in a beaker and stirred well to dissolve it completely. In a separate beaker, 1 g of potassium dichromate was dissolved in 10 mL of distilled water, and the solution was placed in an ice bath to maintain a low temperature. Then, 1 mL of concentrated sulphuric acid was slowly added to the potassium dichromate solution with continuous stirring. The hydroquinone solution was then added dropwise to the cold oxidizing mixture while continuously shaking or stirring to ensure uniform mixing. As the reaction progresses, a yellow precipitate of pBQ was formed. The reaction mixture was allowed to stand for a few minutes to complete precipitation, and the yellow crystals are then collected by filtration using filter paper. The filtered crystals are transferred onto a watch glass and dried in an oven at approximately 50°C until completely dry. The final product was stored in a dry, airtight container away from light and moisture.

3.4.5. Photosynthetic gas exchange measurements

Leaf photosynthetic gas exchange parameters were analysed in the morning (09:00 a.m. to 12:00 p.m.) by using a LI-6400 portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). Leaf surfaces were cleaned and dried with tissue paper prior to being placed in the leaf chamber for gas exchange measurements. Measurements were recorded on fully expanded leaves, with readings taken between 9:00 and 10:00 AM under room temperature and ambient CO_2 conditions. The internal light source in the LI-6400 was adjusted to an intensity of 1500 μmol m^{-2} s^{-1}

to maintain consistent and uniform lighting throughout all measurements. Leaf gas exchange parameters, including net photosynthetic rate (Pn, μmol m^{-2} s^{-1}), stomatal conductance (gs, μmol m^{-2} s^{-1}), and transpiration rate (E, mmol m^{-2} s^{-1}), were measured in rice leaves. Pn, gs, and E were computed utilising the equations established by (Caemmerer and Farquhar, 1981).

3.5. Estimation of antioxidant system

3.5.1 Enzymatic antioxidant system assay

3.5.1.1 Superoxide dismutase (SOD, EC 1.15.1.1)

Extraction: In a pre-chilled mortar, 500 mg of tissue was ground into a fine powder along with liquid nitrogen. The tissue was then homogenised with 100 mM phosphate buffer (pH 7.8) and centrifuged the homogenate for 15 min at 4°C and 14,000 rpm. The enzyme assay was performed using the supernatant.

Enzyme Assay: The modified protocol of Giannopolitis and Ries (1977) was employed to estimate the SOD activity in the fresh sample. The capacity of SOD to impede the photochemical reduction of nitroblue tetrazolium (NBT) was evaluated by monitoring its activity. The reaction mixture composed of 0.1 mL of 1.5 M sodium carbonate, 0.3 mL of 0.13 M methionine, 0.3 mL of 10 μM EDTA, 0.3 mL of 13 μM riboflavin, and 0.3 mL of 0.63 mM nitrobluetetrazolium, as well as 0.1 mL of enzyme extract. Phosphate buffer (50 mM, pH 7.8) was employed to prepare the reaction mixture to a maximum volume of 3 mL. Various assay systems were established, including dark-control, light-control, and test samples. Controls contained only the assay mixture, without the enzyme extract. The light-controls were illuminated under a fluorescent lamp for 30 min, while the dark-controls were maintained in darkness. Additionally, one set of test samples (tubes containing assay mixtures with enzyme extract) was illuminated, while the other set was maintained in darkness. The illuminated control functioned as an absolute light control, while the dark control served as a blank. The absorbance of the developed blue colour at 560 nm was recorded against the blank to quantify the formazan accumulation in various tubes using a spectrophotometer (Multiskan, Thermo Fisher Scientific,

Vantaa, Finland). The results were reported in units of SOD mg^{-1} protein. The enzyme activity that inhibited the photo reduction of NBT to blue formazan by 50% was defined as one unit of SOD.

3.5.1.2 Guaiacol peroxidase (GPOX, EC 1.11.1.7)

Extraction: 500 mg of tissue was ground into a fine powder in a pre-chilled mortar with liquid nitrogen. The tissue was subsequently homogenised with 100 mM phosphate buffer (pH 7.8) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was used to conduct the enzyme assay, which was transferred to a test tube and stored in a cold bath.

Enzyme Assay: The method of Gaspar et al. (1975) was employed to measure the GPOX activity in the fresh samples. The hydrogen peroxide (H_2O_2) dependent oxidation of guaiacol was followed by the measurement of GPOX activity at 420 nm (extinction coefficient 26.6 mM^{-1} cm^{-1}). The 3 mL assay mixture composed of 100 mM phosphate buffer (pH 7.8), with 30 µL of 1% guaiacol, and 20 µL of enzyme extract. The blank was generated without the enzyme extract in the reaction mixture. The components were thoroughly combined. In order to activate the enzyme, 12 µL of H_2O_2 were introduced. The UV-VIS spectrophotometer was employed to measure the increase in absorbance at 420 nm caused by the oxidation of guaiacol immediately following the addition of hydrogen peroxide. The measurements were taken at 30 s intervals over a 3 min period. The amount of enzyme that resulted in the formation of 1 µM of tetraguaiacol per minute was defined as one unit of GPOX activity.

3.5.1.3 Ascorbate peroxidase (APX, EC 1.11.1.11)

Extraction: In a pre-chilled mortar, 5 mL of extraction medium was used to homogenise 500 mg of fresh tissue. The extraction buffer composed of 100 mM potassium phosphate buffer (pH 7.0), which contained 0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 10 mM NaCl, 0.5 mM KH_2PO_4, and 1 mM ascorbate. The homogenate was centrifuged at 4°C for 4 min at 15000 rpm and filtered through two layers of cheese cloth. The resulting supernatant was utilised for the enzyme assay.

Enzyme Assay: The method of Nakano and Asada (1981) was employed to evaluate the APX activity of the fresh samples. The cytosolic APX activity was evaluated by monitoring the decrease in absorbance at 290 nm because of AsA oxidation. The 3 mL assay system was composed of 100 mM potassium phosphate buffer (pH 7.0) and 0.5 mM AsA and 0.1 mM EDTA. The enzyme reaction was initiated by adding 10 µL of 100 mM H_2O_2 to the buffer, which resulted in a final reaction mixture containing 0.1 mM H_2O_2. To this 20 µL of cytosolic enzyme extract was added. Monitoring the decrease in absorbance at 290 nm was conducted to follow the H_2O_2 dependent oxidation of AsA (ε=2.8 $mM^{-1}cm^{-1}$). One unit was defined as the quantity of enzyme that oxidised 1 µmol of AsA per min at room temperature under the aforementioned conditions.

3.5.1.4 Catalase (CAT, EC 1.11.1.6)

Extraction: In a medium that contained 100 mM phosphate buffer (pH 7.0), 500 mg of plant tissue was homogenised using a pre-chilled glass mortar and pestle. The homogenate was filtered through two layered muslin cloth. The filtrate was subsequently centrifuged at 16,000 rpm for 15 min at 4°C in a refrigerated centrifuge (Thermo Scientific- Legend X1R, Kalkberg, Germany). The enzyme assay was conducted using the supernatant.

Enzyme assay: The method of Kar and Mishra (1976) was employed to ascertain the activity of CAT in the fresh samples. The activity of CAT was assessed by observing a decrease in absorbance at 240 nm for a period of 1 min after the decomposition of H_2O_2. One unit of the enzyme was defined as µmoles of H_2O_2 decomposed per min per mg of protein. The assay system composed of 2.4 mL of 100 mM phosphate buffer (pH 7.0), 0.3 mL of enzyme extract, and 0.3 mL of 30 mM H_2O_2. The enzyme extract and phosphate buffer were combined thoroughly in a test tube using a pipette. To initiate enzyme activity, H_2O_2 was introduced to this. Enzyme activity was measured at 240 nm for 90 s at 15 s intervals immediately following the addition of H_2O_2. The CAT activity was quantified in µmol H_2O_2 oxidised per min per g of fresh weight.

3.5.2 Non-enzymatic antioxidants system assay

3.5.2.1 Ascorbate (AsA) content

Extraction: The leaf tissue was weighed (500 mg) and homogenised with 5 mL of 5% (w/v) TCA. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C after being filtered through a muslin cloth. the supernatant was collected and employed to estimate the AsA content.

Estimation: The method of Chen and Wang (2002) was employed to estimate the AsA content. A well-mixed aliquot of 0.3 mL of the supernatant was combined with 0.3 mL of 200 mM NaH_2PO_4. This mixture was successfully supplemented with 0.5 mL of 10% (v/v) TCA, 0.4 mL of 42% (v/v) H_3PO_4, 0.4 mL of 4% (w/v) bipyridyl (dissolved in 70% alcohol), and 0.2 mL of 3% $FeCl_3$ (w/v). The mixture was incubated at 42°C for 15 min. The absorbance was measured at 524 nm immediately following incubation, and the AsA content was determined using a standard curve that was constructed using varying concentrations of AsA.

3.5.2.2 Glutathione (GSH) content

Extraction: The leaf tissue (500 mg) was homogenised with 6 mL of 5% (w/v) TCA. After filtered through a filter paper, the homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected to estimate the reduced glutathione content.

Estimation: The estimation of GSH content was conducted following the methodology of Chen and Wang (2002). An aliquot of 0.5 mL of the supernatant was combined with 2.6 mL of 150 mM NaH_2PO_4 buffer (pH 6.8) and 0.18 mL of 3 mM 5 dithio-bis-2-nitrobenzoic acid (DTNB), which was previously dissolved in 100 mM phosphate buffer at pH 6.8. After 5 min, absorbance was measured at 412 nm, and GSH content was determined using a standard curve based on varying concentrations of reduced glutathione.

3.5.2.3. Estimation of total phenolics

Extraction: An amount of 0.1 g of tissue was homogenised in 5 mL of 80% ethyl alcohol to extract total phenols, and a supernatant was obtained by centrifugation at 10,000 rpm for 20 min at 4°C.

Estimation: A suitable aliquot of the supernatant was made up to 3 mL with distilled water, supplemented with 1N Folin-Ciocâlteu reagent (FCR) and shaken thoroughly. After 3 min incubation, 20% Na_2CO_3 was added and the mixture was heated in a boiling water bath for 1 min. The absorbance was measured against a blank sample at 650 nm by using a UV-VIS spectrophotometer. Catechol was used as a standard to determine the total phenolics content (Folin and Denis, 1915).

3.5.2.4. Estimation of flavonoids

Extraction: A 0.1 g tissue sample was homogenised with 3 mL of acidified methanol (MeOH:HCl:H_2O in a ratio 79:1:20) and incubated for 24 h at room temperature.

Estimation: The spectrophotometric determination of flavonoids, at a wavelength of 315 nm and a extinction coefficient of 33 $mM^{-1}cm^{-1}$ was carried out according to Mirecki and Teramura (1984).

3.5.2.5. Estimation of anthocyanins

Extraction: 0.1 g of powdered rice sample was mixed with acidified methanol (MeOH:HCl, 99:1) and incubated at 4 ºC for 24 h.

Estimation: Anthocyanin content was determined by measuring the absorbance at 530 and 657 nm in a UV-VIS spectrophotometer against reagent blank. The anthocyanin content was calculated based on the molar extinction coefficient of 29600 L mol^{-1} cm^{-1} for cyanidin-3-glucoside equivalents as described by Mancinelli et al. (1975).

3.6. Oxidative stress

3.6.1. Superoxide ($O_2^{\cdot-}$) content

Extraction: Two hundred mg of seedling were cut into 1×1mm pieces and immersed in 10 mM potassium phosphate buffer (pH 7.8) that contained 0.05% NBT and 10 mM sodium nitrate ($NaNO_2$).

Estimation: The superoxide concentration was assessed according to the methodology outlined by Doke, (1983). The mixture was maintained in a water bath at 85°C for 15 min. Subsequent to incubation, the mixture was promptly transferred to an ice bath to reduce the temperature. Following cooling, the absorbance of the combination was assessed at 580 nm. Sodium nitrate ($NaNO_2$) served as the reference standard.

3.6.2. Hydrogen peroxide content

Extraction: 200 mg of tissue was weighed and homogenised in 5 mL of 0.1% ice cold trichloroacetic acid. The homogenate underwent centrifugation at 12,000 rpm for 15 min. The supernatant obtained was utilised for the quantification of hydrogen peroxide.

Estimation: The hydrogen peroxide concentration was assessed according to the methodology outlined by Junglee et al. (2014). 1 mL of the supernatant was combined with 1 mL of potassium phosphate buffer (pH 7), followed by the addition of 1 mL of 1M potassium iodide. The absorbance of the combination was quantified at 390 nm. Hydrogen peroxide served as the standard.

3.6.3. Lipid peroxidation estimation

Extraction: MDA was extracted from 300 mg of leaf tissue by homogenising in 5 mL of 5% trichloroacetic acid (TCA) and centrifuging at 12,000 rpm for 5 min.

Estimation: The lipid peroxidation was estimated in terms of malondialdehyde (MDA) content (Heath and Packer, 1968). 2 mL of supernatant was taken in a test tube and an equal amount of 0.5% thiobarbituric acid (TBA)

prepared in 20% TCA was added to it and heated for 30 min at 95°C. Optical density was measured against a reaction blank at 532 and 600 nm in a UV-VIS spectrophotometer and MDA was calculated using its molar extinction coefficient of 155 $mM^{-1}cm^{-1}$.

3.6.4. Electrolyte leakage (EL%)

The estimation of electrolyte leakage (EL%) was conducted as per the methodology outlined by Lutts et al. (1996), with modifications. 500 mg of fresh tissue were sectioned into 10 mm^2 segments and deposited in tubes containing 50 mL of distilled water. The samples were maintained at 4°C for 24 h, thereafter brought to room temperature, and electrical conductivity was measured (EC1). The tissue was subsequently autoclaved at 120°C for 15 min, after which the electrical conductivity (EC2) was re-evaluated using a conductivity meter (Eutech, Cyberscan 600). The EL% was computed as follows:

$$EL\% = (EC_1/EC_2) \times 100$$

3.6.5. Membrane stability index (MSI)

The membrane stability index (MSI) was assessed according to the methodology outlined by Sairam et al. (1997). 500 mg of fresh tissue was sectioned into segments measuring 10 mm^2 and deposited in tubes containing 20 mL of distilled water in two separate sets. One sample was maintained at 40°C for 1 h, and electrical conductivity (C1) was assessed using a conductivity meter (Eutech, Cyberscan 600, Vernon Hills, USA). A separate set was maintained in a boiling water bath (100°C) for 1 h, and its electrical conductivity (C2) was then measured. The MSI was computed as follows:

$$MSI = [1 - (C_1/C_2)] \times 100$$

3.7. Metabolites

3.7.1. Total soluble sugar

Extraction: 500 mg leaf tissue was homogenized in 80% ethyl alcohol (v/v) using a clean glass mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was collected.

Estimation: The total soluble sugar was estimated using the method proposed by Dubois et al. (1956). To 100 µL of aliquot 300 µL of distilled water was added and made up to 1 mL. To this 200 µL of 5% phenol was added and shaken well. Further, 1000 µL of concentrated sulphuric acid was added through the sides of the tubes and mixed well. Absorbance of the resultant solution is taken in a UV-VIS spectrophotometer at 490 nm against a reaction blank. D-glucose was used as the standard.

3.7.2. Total free amino acids

Extraction: 500 mg of fresh samples was homogenized with 80% (v/v) ethanol. The extract was centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was saved.

Estimation: Total free amino acids were determined according to the method of Moore and Stein, (1948). 100 µL of the sample was made up to 1 mL with distilled water and 1 mL of ninhydrin reagent was added into it. Tubes were kept in boiling water bath for 15 min. On removal from water bath 5 mL of diluent (equal volume of water and n-propanol) was added to it and shaken well. This mixture was incubated at room temperature for 15 min and absorbance was read at 570 nm using a UV-VIS Spectrophotometer against a reagent blank. Standard curve was plotted by using leucine as standard.

Preparation of reagent: Reagent solution was prepared by dissolving 2 g of ninhydrin and 300 mg of hydrindantin in 75 mL of methyl cellosolve. The solution was stirred carefully to avoid air bubbles in the solution. To this solution 25 mL of sodium acetate buffer (pH 5.5) was added and the resulting reddish reagent solution was immediately transferred to an amber coloured bottle. The reagent was freshly prepared.

3.7.3. Total protein

Extraction: 200 mg of leaf tissue was homogenized in 2 mL of phosphate buffer (pH 7) using mortar and pestle and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was saved.

Estimation: The total protein content was estimated according to Bradford (1976). A 100 µL aliquot was taken and madeup to 1mL with phosphate buffer (pH 7) and 1 mL Bradford reagent was added. Absorbance was recorded against a reaction blank at 595 nm. Bovine serum albumin (BSA) was used as the standard.

3.8. Estimation of agro-morphological, biomass and yield traits

The traits including plant height, shoot and root dry weight, root length, crown root number, tiller number/plant, reproductive tiller/plant, panicle length, grains per panicle, harvest index (%), and 100-grain weight were recorded in all plants subjected to different treatments. All the traits were analysed after the maturation of the rice. Plant height, root length and panicle length were measured manually using a graduated scale. Crown root number, tiller number/plant, reproductive tillers/plant, and grains per panicle were counted manually. For analysis of shoot dry weight and root dry weight, 100-grain weight, shoots, root and grains were separated and placed at 60˚C for 48 hours in a hot air oven until the weights turned to be stable, and dry weights were analysed using a weighing balance. Harvest index was calculated according to the following formula: HI (%) = grain weight/straw weight + grain weight × 100.

3.9 Estimation of phytic acid

Extraction: Phytate was extracted from 0.1 g of powdered brown rice with 2.5% HCl in a shaker for 2 h and then centrifuged at 5000rpm for 15min.

Estimation: 900 µL of the supernatant was mixed with 300 µL of Wade reagent [$FeCl_2$ (0.03%) in sulfosalicylic acid (0.3%)], and absorbance was taken at 500 nm in a UV-VIS spectrophotometer. Sodium phytate was used as the standard for the assay (Latta and Eskin, 1980).

3.10. Bioavailable zinc

The bioavailability of Zn in control and various Zn-treated plants (seed priming, seedling priming and foliar spray) was analysed using two methods. Phytate: Zinc (PA: Zn) molar ratio (Morris and Ellis, 1989) and tri-variate model (Miller et al., 2007).

PA: Zn molar ratio was calculated as per the following equation

$$PA:Zn\ molar\ ratio\ = \frac{PA\ in\ mg\ kg^{-1}\ /\ 660}{Zn\ in\ mg\ kg^{-1}\ /\ 65}$$

Where 660 is the molar mass of PA, and 65 is the molar mass of Zn.

The bioavailability of Zn as per the tri-variate model was calculated as per the following equation

$$TAZ = 0.5\ [A_{max} + TDZ + K_R\ (1+TDP/K_p)^2 - [Amax + TDZ + K_R\ (1+TDP/K_p)^2 - 4\ A_{max} + TDZ]^{1/2}]$$

Where TAZ is total daily absorbed Zn, TDZ is daily dietary Zn, TDP is daily dietary PA, A_{max} is maximum Zn absorption (0.091), K_R is equilibrium dissociation constant of Zn-receptor binding reaction (0.033), K_P is equilibrium dissociation constant of Zn-PA binding reaction (0.680).

3.11. Analysis of gene expression patterns in rice plant

Expressions of *OsZIP* and *OsHMA* genes were analyzed in root, node I, flag leaf and panicle of rice grown under deficient and sufficient conditions of Zn upon various Zn treatments. The detailed protocols for designing of gene-specific primer, screening and validation of gene-specific primer, total RNA extraction, cDNA synthesis, and RT-PCR and qRT-PCR analysis are given below.

3.11.1. RNA isolation and cDNA synthesis

Total RNA was isolated from root, node I, flag leaf and panicle tissues using NucleoSpin® RNA Plant kit (MACHERYNAGEL GmbH & Co. KG, Germany)

according to the manufacturer's instructions during milky stage of rice plant under all the treatments. The quantity and purity of isolated RNA were measured using a NanoDrop-spectrophotometer (ND-2000, Thermo Scientific, Wilmington, DE, USA). The cDNA was synthesized from 500 ng each of total RNA using iScript Reverse transcription kit (Biorad) according to the manufacturer's instructions. This kit specifically contained genomic DNA wipe-out to eliminate any gDNA contamination from isolated RNA during cDNA synthesis.

3.11.2. qRT-PCR analysis

Ten ZIP (*OsZIP1-OsZIP10*) and 1 HMA (*OsHMA2*) genes were included for expression analysis by qRT-PCR analysis in the present study. The primers (*OsZIP1-OsZIP10*) and (*OsHMA2*) were used from an all ready published article (Ning et al., 2023). Details of primer is given in the Table 5. For qRT-PCR, 10µL reactions were employed containing 5µL of 2x SsoFast EvaGreen Supermix (Bio-Rad Laboratories, USA), 4µL cDNA (1:50 dilution) and 1µL primer mix (500 nM each forward and reverse primer). The cycling conditions for qPCR were enzyme activation at 95°C for 30s, denaturation at 95°C for 5s, annealing, and extension at 60°C for 5s (35 cycles). Finally, melting curve analysis was performed by increasing the temperature from 65 to 95°C with 0.5°C increments followed by signal capture. The cycle threshold (Ct) values of rice were analyzed using the $2^{-\Delta\Delta Ct}$ method. We used the Osubiquitin (Osuq) constitutive gene for normalization of CT value of expression values. The constitutive gene was selected for this study based on already available reports (Ning et al., 2023). Three biological replicates, each consisting of three technical replicates, were used for qRT-PCR analysis.

3.12. Statistical analysis

The data were analysed using MS Excel, SPSS software and R studio (R version 4.2.0). The graphs were plotted using MS Excel. One-way ANOVA followed by Duncan post- hoc test at 5% probability level was analysed using the SPSS software (Version 16.0, SPSS Inc., Chicago, USA). Results were expressed as mean values ± SE. All the assays were carried out in three replicates. Different letters indicate significant differences among the treatments. The correlation and

principal component analysis (PCA) were generated in R studio. Only numeric variables were selected for correlation and PCA analysis to ensure accurate computation. The analysis was conducted using the ggplot2, corrplot, FactoMineR, and factoextra packages in R. For correlation analysis, a Pearson correlation matrix was computed using the `cor()` function, and the correlation plot was generated using the `corrplot()` function. PCA was performed to reduce dimensionality and identify key variables contributing to the variation among samples. The dataset was scaled and centered, and PCA was conducted using the `PCA()` function from the FactoMineR package. A PCA biplot was constructed using `fviz_pca_biplot()` to represent both variables and individual observations in the principal component space. Additionally, box plots were generated to compare variations in Zn content and other traits across different treatments and rice varieties. The ggplot2 package was used to create box plots, with treatment groups or varieties on the x-axis and trait values on the y-axis. The geom_boxplot() function was applied to display the median, quartiles, and outliers, and custom colours were assigned to differentiate between groups.

3.13. Chemicals

Analytical reagent (AR) or guaranteed reagent (GR) chemicals were purchased from Himedia, SRL and Merck companies. Bovine serum albumin (BSA), riboflavin, methyl viologen, 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea (DCMU) were purchased from Sigma-Aldrich Co., USA.

Table 5. Details of primers used for gene expression analysis

Gene name	Forward primer	Reverse Primer	Product size with intron (bp)	Product size without intron (bp)	Tm (°C)
OsZIP1	TTTCGGACGTTTGTTGGTTC	TCCTGAAACTTTGGTTGGAGT		161	61.5
OsZIP2	GCATTGTTCAGGCTAATTTTAAGG	GGCAGTTGAGCTATGCACATTG		102	59.5
OsZIP3	TCACTGAGGCCGTCGTCAATCAGG	ACGACAAGTGCGGTCGAGCTGT		131	59.5
OsZIP4	CATGAAGACCAAGGTGCAGAGAAGG	TCACGCCCAGATGGCGATCA	1055	221	65.7
OsZIP5	TCTACATGGCACTTGTCGATCTC	GACATGGATGCAGATCCAAGCA		110	62.4
OsZIP6	ACCAGGTTCTACGAGGGCAAGCA	AGCGGCGCCTTGTTGTCGTT		121	59.5
OsZIP7	GGTGCAGAGCAAAGGCAAGCT	AATTTCCTCTACATTAGTCCCTGA		146	62.4
OsZIP8	ATCTTCTTCTCGCTAACCACAC	GCAGCCGCTGCGTCGAGAAT		126	59.5
OsZIP9	GCTCAGTTAAAGAACTTCTCTGC	CGACATCGAGTCCAGAATTCC		119	59.5
OsZIP10	TTTCGGACGTTTGTTGGTTC	TCCTGAAACTTTGGTTGGAGT		153	59.5
OsHMA2	CATAGTGAAGCTGCCTGAGATC	GATCAAACGCATAGCAGCATCG		135	59.5
OsUiquitin	GCTCCGTGGCGGTATCAT	CGGCAGTTGACAGCCCTAG	1625	117	59.5

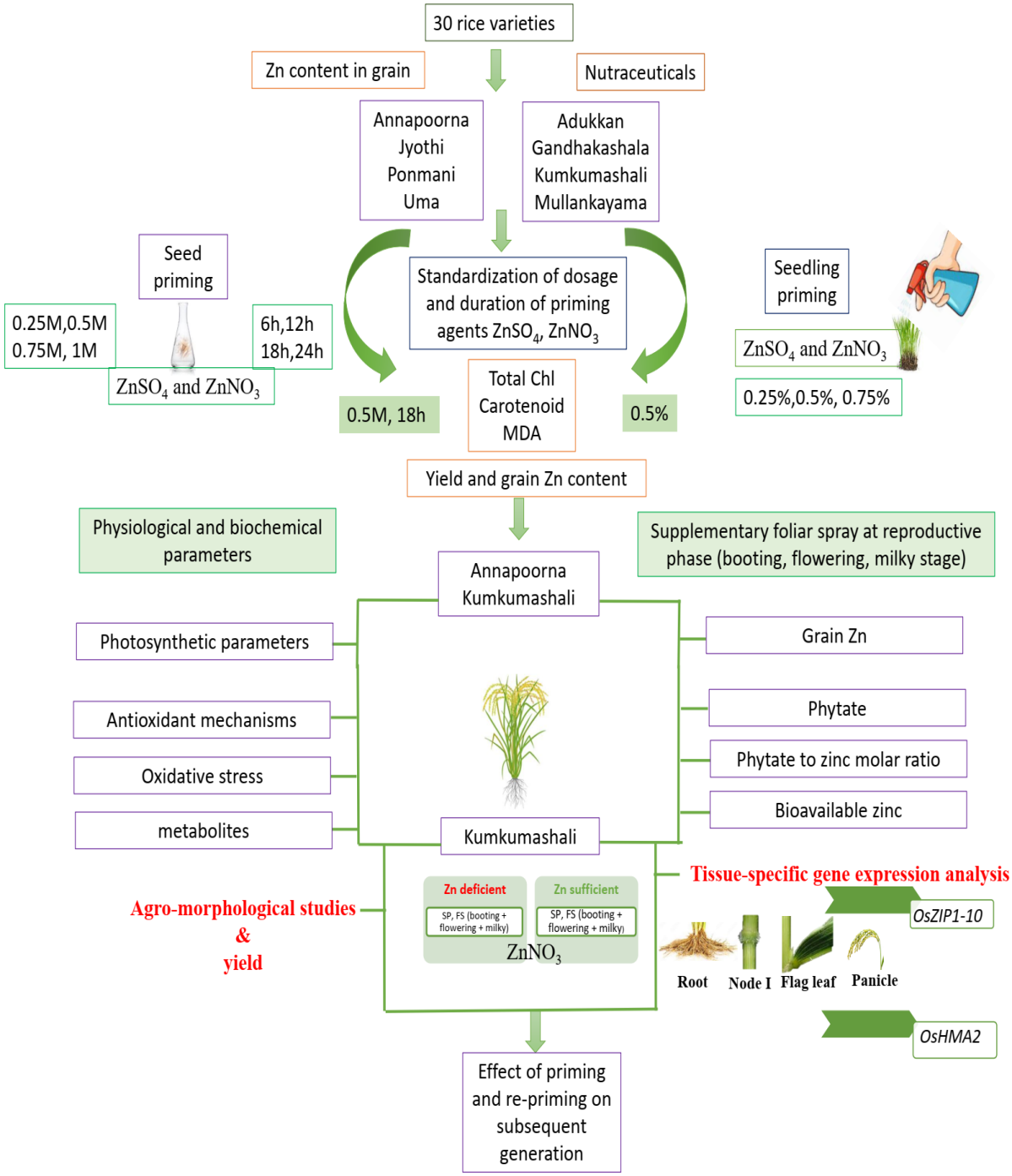


Figure 2. Schematic representation of the work

4.1 Screening of rice varieties for biofortification with Zn

Initially thirty rice varieties were screened, including fifteen landraces and fifteen hybrid varieties, for quantifying the content of Zn in brown (unpolished) rice grain. The landrace Kumkumashali had the highest concentration of Zn (31.84 mg/kg DW), whereas the hybrid rice variety Uma had the lowest (18.93 mg/kg DW). Adukkan, which is a landrace and locally known for its resistance towards water flood, also had a higher Zn content (31.09 mg/kg DW). Two aromatic landraces, Gandhakashala (28.18 mg/kg DW), a GI-tagged rice, and Mullankayama (31.32 mg/kg DW), were also high in Zn content. On average, 29.5 mg/kg of Zn was present in all the studied samples. The amount of other micro- and macro-elements present in the study samples were also analysed. The range of iron (Fe) in the studied samples was between 1.28 and 4.29 mg/kg DW, with a mean content of 2.96 mg/kg DW. The study samples had a boron (B) range of 0.44 to 1.27 mg/kg DW. A mean concentration of 3.28 mg/kg DW of copper (Cu) was also observed in the brown rice grain. The mean concentration of manganese (Mn) was 25.76 mg/kg DW, ranging from 13.80 to 38.06 mg/kg DW. Molybdenum (Mo) was also present in the studied rice samples, with an average concentration of 0.68 mg/kg DW. The maximum content of microelement nickel (Ni) was 0.22 mg/kg DW and minimum was 0.12 mg/kg DW. The macroelement calcium (Ca) was in the range of 78.10 to 154.41 mg/kg DW, while potassium (K) had a mean concentration of 1.8 g/kg DW. The highest concentration of Mg was 1.82 g/kg DW, and the lowest was 1.07 g/kg DW (Table 6). The landraces had an enriched content of essential elements compared to the hybrid elite varieties.

The presence of antioxidants and antihyperglycemic compounds in rice grains, such as total phenolics, flavonoids, and anthocyanin content were also investigated. The results revealed that these compounds were higher in pigmented rice varieties when compared to the non-pigmented ones. The total phenolics ranged from 0.20 to 0.85 mg/g DW, while flavonoids had a maximum concentration of 0.98

mmol/g DW and a minimum of 0.07 mmol/g DW. In the case of anthocyanin, the landrace Kumkumashali had the highest concentration (0.55g/kg DW), while Urunikayama had the lowest (0.01 g/kg DW), a non-pigmented landrace. The determined amylose contents during the experiment varied from 17% to 36%. Compared to all other rice varieties used in the study, the landrace Kumkumashali had the highest amylose content (36%), and this value corresponds to a high amylose-containing rice variety (Fig. 3). Principal component analysis (PCA) was conducted with respect to both elemental and nutraceutical factors. This method simplifies large data sets into a new variable while maintaining the maximum variability of the data without compromising on information and variance. Moreover, the correlation between all the variables under study can be inferred from the PCA plot. The first two axes PC1 and PC2 represent 37.3% of the original variability. From the plots, it is determined that, there is a positive correlation between the total phenolics, flavonoids, and anthocyanins. Zn exhibited a positive correlation with B, Ca, Cu, K, Mg, Mn, Mo, and Ni (Fig. 4).

Based on these observations, four hybrid elite varieties, viz. Annapoorna, Jyothi, Ponmani, and Uma, and four landraces such as Adukkan, Gandhakashala, Kumkumashali, and Mullankayama were selected for further biofortification studies. Of these, Adukkan and Kumkumashali are pigmented rice, while Gandhakashala and Mullankayama are aromatic.

4.2 Standardization of dosage and duration of treatments with seed priming agents

4.2.1 Seed priming

The seeds of selected eight rice varieties of rice (Adukkan, Annapoorna, Gandhakashala, Jyothi, Kumkumashali, Mullankayama, Ponmani and Uma) were treated with various concentrations of priming agents $ZnSO_4$ (0, 0.25, 0.5, 0.75 and 1M) and $ZnNO_3$ (0, 0.25, 0.5, 0.75 and 1M) for 6, 12, 18 and 24h duration. The optimum concentration and duration of priming agents were selected by analysing the total chlorophyll content (Table 7 & 10), carotenoid content (Table 8 &11) and MDA content (Table 9 & 12) of 14 d old plants raised from primed seeds, compared

Table 6. Concentration of different essential elements in the grain of studied rice varieties

Rice Variety	B (mg/kg DW)	Cu (mg/kg DW)	Fe(mg/kg DW)	Mn (mg/kg DW)	Mo (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)	Ca (mg/kg DW)	K (g/kg DW)	Mg (g/kg DW)
Adukkan	$0.58\pm0.70^{i-k}$	3.27 ± 0.04^{fg}	3.63 ± 0.13^{c}	28.24 ± 0.24^{j}	0.93 ± 0.03^{bc}	$0.17\pm0.07^{f-h}$	31.09 ± 0.05^{g}	110.79 ± 0.84^{i}	2.34 ± 0.13^{b}	1.63 ± 0.24^{f}
Cheattuveliyan	$0.54\pm0.41^{k-n}$	2.68 ± 0.39^{jk}	1.84 ± 0.15^{j}	25.35 ± 0.25^{l}	0.63 ± 0.10^{e}	$0.20\pm0.01^{a-c}$	28.33 ± 0.26^{i}	111.20 ± 0.90^{hi}	1.90 ± 0.16^{m}	1.54 ± 0.14^{k}
Kannichennellu	0.91 ± 0.32^{c}	$2.71\pm0.26^{i-k}$	4.16 ± 0.06^{ab}	27.02 ± 0.02^{k}	0.62 ± 0.08^{ef}	$0.13\pm0.08^{k-n}$	$25.30\pm0.32^{j-m}$	86.12 ± 1.25^{n}	1.99 ± 0.81^{i}	1.55 ± 0.37^{j}
Kumkumashali	$0.63\pm0.34^{h-j}$	2.44 ± 0.17^{kl}	1.44 ± 0.00^{k}	32.23 ± 0.11^{g}	0.84 ± 0.06^{cd}	$0.15\pm0.08^{h-j}$	39.84 ± 0.15^{a}	114.14 ± 1.06^{ef}	2.15 ± 0.09^{c}	1.82 ± 0.14^{a}
Kuruva	0.45 ± 0.08^{o}	5.00 ± 0.03^{b}	1.28 ± 0.02^{k}	21.20 ± 0.13^{q}	0.37 ± 0.02^{g}	$0.15\pm0.09^{g-j}$	$29.69\pm0.07^{g-i}$	116.27 ± 0.86^{de}	1.33 ± 0.46^{z}	1.27 ± 0.81^{v}
Marathondi	$0.64\pm0.30^{f-i}$	4.61 ± 0.07^{c}	2.66 ± 0.04^{h}	21.75 ± 0.10^{p}	0.50 ± 0.04^{f}	$0.16\pm0.10^{f-i}$	$25.37\pm0.39^{j-l}$	78.10 ± 0.06^{o}	1.90 ± 1.65^{n}	1.41 ± 1.05^{r}
Onnamottan	$0.54\pm0.29^{k-m}$	$3.04\pm0.01^{g-i}$	3.06 ± 0.03^{f}	31.28 ± 0.16^{h}	$0.88\pm0.01^{b-d}$	$0.14\pm0.06^{i-l}$	35.16 ± 0.15^{de}	90.22 ± 0.22^{m}	2.03 ± 0.89^{g}	1.71 ± 1.05^{d}
Paalthondimatta	$0.72\pm0.27^{e-f}$	4.60 ± 0.04^{c}	3.06 ± 0.06^{f}	29.45 ± 0.06^{i}	0.59 ± 0.05^{ef}	$0.18\pm0.00^{d-f}$	31.24 ± 1.17^{g}	85.30 ± 0.40^{n}	1.97 ± 0.36^{l}	1.42 ± 0.17^{q}
Rakthashali	$0.76\pm0.15^{d-e}$	$2.92\pm0.10^{h-k}$	2.56 ± 0.05^{h}	25.37 ± 0.11^{l}	0.53 ± 0.02^{ef}	$0.14\pm0.07^{i-m}$	25.93 ± 1.12^{jk}	$112.69\pm1.19^{f-i}$	2.15 ± 0.08^{c}	1.73 ± 0.98^{c}
Veliyan	$0.50\pm0.03^{l-o}$	3.88 ± 0.08^{d}	2.58 ± 0.01^{h}	18.44 ± 0.01^{t}	0.30 ± 0.01^{g}	$0.15\pm0.10^{h-j}$	23.13 ± 0.49^{n}	86.11 ± 0.81^{n}	1.49 ± 0.13^{v}	1.22 ± 1.01^{w}
Gandhakashala	0.44 ± 0.22^{o}	$1.87\pm0.11^{m-o}$	4.13 ± 0.11^{ab}	35.57 ± 0.08^{d}	0.93 ± 0.05^{bc}	$0.15\pm0.08^{i-k}$	28.18 ± 0.56^{i}	116.40 ± 0.77^{d}	2.05 ± 0.53^{f}	1.51 ± 0.17^{l}
Jeerakashala	$0.68\pm0.39^{e-h}$	5.35 ± 0.08^{a}	4.29 ± 0.04^{a}	37.21 ± 0.07^{c}	0.55 ± 0.02^{ef}	$0.17\pm0.12^{e-g}$	$24.08\pm0.88^{l-n}$	93.53 ± 0.90^{l}	1.58 ± 0.21^{u}	1.38 ± 0.18^{s}
Mullankayama	$0.65\pm0.42^{f-i}$	5.02 ± 0.02^{b}	3.07 ± 0.06^{f}	30.90 ± 0.42^{h}	0.58 ± 0.03^{ef}	$0.15\pm0.08^{h-j}$	31.32 ± 0.84^{g}	86.27 ± 0.06^{n}	2.10 ± 0.20^{d}	1.59 ± 0.21^{i}
Paalthondivella	$0.83\pm0.40^{c-d}$	5.32 ± 0.05^{ab}	4.02 ± 0.02^{b}	23.57 ± 0.08^{o}	0.79 ± 0.07^{d}	$0.13\pm0.11^{j-m}$	37.24 ± 0.34^{c}	85.55 ± 0.93^{n}	2.03 ± 0.08^{h}	1.66 ± 0.13^{e}
Urunikayama	$0.62\pm0.10^{h-j}$	3.79 ± 0.02^{de}	2.53 ± 0.03^{h}	38.06 ± 0.06^{a}	0.64 ± 0.04^{e}	$0.19\pm0.09^{c-e}$	29.85 ± 1.29^{gh}	110.73 ± 0.45^{i}	2.08 ± 0.64^{e}	1.60 ± 0.07^{h}
Aishwarya	0.47 ± 0.27^{no}	2.20 ± 0.08^{lm}	2.87 ± 0.02^{g}	18.64 ± 0.04^{t}	0.59 ± 0.02^{ef}	$0.14\pm0.03^{i-m}$	23.56 ± 0.51^{mn}	106.12 ± 1.06^{j}	1.75 ± 0.20^{p}	1.45 ± 0.13^{o}
Annapoorna	$0.56\pm0.02^{j-m}$	4.62 ± 0.05^{c}	3.63 ± 0.06^{c}	37.69 ± 0.08^{b}	0.60 ± 0.01^{ef}	$0.14\pm0.07^{i-m}$	39.36 ± 0.57^{ab}	126.83 ± 0.63^{b}	1.74 ± 0.28^{q}	1.81 ± 1.33^{b}
Jyothi	$0.60\pm0.05^{h-k}$	3.46 ± 0.09^{ef}	2.30 ± 0.05^{i}	16.45 ± 0.01^{u}	0.63 ± 0.05^{e}	$0.12\pm0.03^{m-n}$	$25.51\pm0.39^{j-l}$	$113.0\pm0.40^{f-i}$	1.86 ± 0.32^{o}	1.44 ± 0.12^{p}
Kanchana	1.01 ± 0.17^{b}	2.04 ± 0.03^{mn}	$3.51\pm0.02^{c-e}$	14.66 ± 0.14^{v}	0.77 ± 0.02^{d}	0.12 ± 0.06^{n}	$24.33\pm0.51^{k-n}$	100.20 ± 0.36^{k}	1.34 ± 0.28^{y}	1.07 ± 0.31^{z}

Manuvarna	0.49±0.13^{m-o}	3.21±0.02^{f-h}	3.41±0.08^{de}	28.33±0.01^{j}	1.67±0.05^{a}	0.14±0.05^{j-n}	24.31±0.11^{k-n}	118.24±0.87^{d}	1.40±0.23^{x}	1.13±0.17^{y}
Samyukhtha	0.58±0.20^{i-k}	1.97±0.03^{m-o}	2.67±0.01^{h}	19.91±0.02^{s}	0.99±0.03^{b}	0.12±0.03^{mn}	31.00±0.34^{g}	111.40±0.69^{hi}	1.74±0.28^{q}	1.44±0.13^{p}
Uma	0.63±0.09^{g-j}	1.64±0.07^{o}	2.95±0.04^{fg}	13.80±0.03^{w}	0.76±0.01^{d}	0.13±0.07^{k-n}	18.93±0.26^{o}	116.62±0.57^{d}	1.72±0.21^{r}	1.37±0.21^{s}
Vaishakh	0.72±0.20^{ef}	5.16±0.05^{ab}	3.11±0.04^{f}	33.58±0.07^{f}	0.38±0.01^{g}	0.21±0.07^{a-c}	25.69±0.29^{j-l}	108.13±0.18^{j}	1.45±0.11^{w}	1.07±0.08^{z}
Varsha	0.72±0.09^{e-g}	2.57±0.06^{k}	3.33±0.01^{e}	24.10±0.09^{n}	0.60±0.01^{ef}	0.22±0.03^{a}	33.21±0.68^{f}	121.52±0.13^{c}	2.05±0.18^{f}	1.64±0.15^{f}
Vytila 6	0.65±0.02^{f-i}	1.79±0.00^{no}	3.02±0.01^{fg}	16.41±0.08^{u}	0.53±0.03^{ef}	0.19±0.01^{b-d}	26.33±0.48^{j}	108.17±0.36^{j}	1.72±0.18^{s}	1.50±0.07^{m}
Akshaya	1.27±0.14^{a}	2.68±0.00^{jk}	2.16±0.00^{i}	34.86±0.04^{e}	0.94±0.02^{bc}	0.21±0.02^{ab}	37.81±0.30^{bc}	154.41±0.26^{a}	2.40±0.14^{a}	1.62±0.18^{g}
Neeraja	0.58±0.01^{i-l}	2.76±0.01^{i-k}	3.12±0.02^{f}	20.54±0.03^{r}	0.56±0.01^{ef}	0.17±0.00^{e-g}	33.97±0.13^{ef}	111.63±0.02^{g-i}	2.03±0.13^{h}	1.46±0.03^{n}
Ponmani	0.59±0.02^{i-k}	4.12±0.00^{d}	2.98±0.00^{fg}	24.91±0.01^{m}	0.59±0.01^{ef}	0.16±0.00^{f-i}	36.36±0.23^{cd}	118.18±0.04^{d}	1.99±0.00^{j}	1.35±0.03^{t}
Supriya	0.62±0.06^{h-k}	2.13±0.00^{l-n}	2.98±0.01^{fg}	24.90±0.03^{m}	0.51±0.01^{ef}	0.15±0.00^{h-j}	29.02±0.21^{hi}	121.27±0.01^{c}	1.64±0.00^{t}	1.15±0.01^{x}
Swetha	0.62±0.04^{h-k}	1.68±0.01^{o}	2.52±0.01^{h}	18.38±0.01^{t}	0.62±0.01^{ef}	0.12±0.00^{i-n}	30.12±0.11^{gh}	113.60±0.01^{fg}	1.98±0.02^{k}	1.29±0.03^{u}

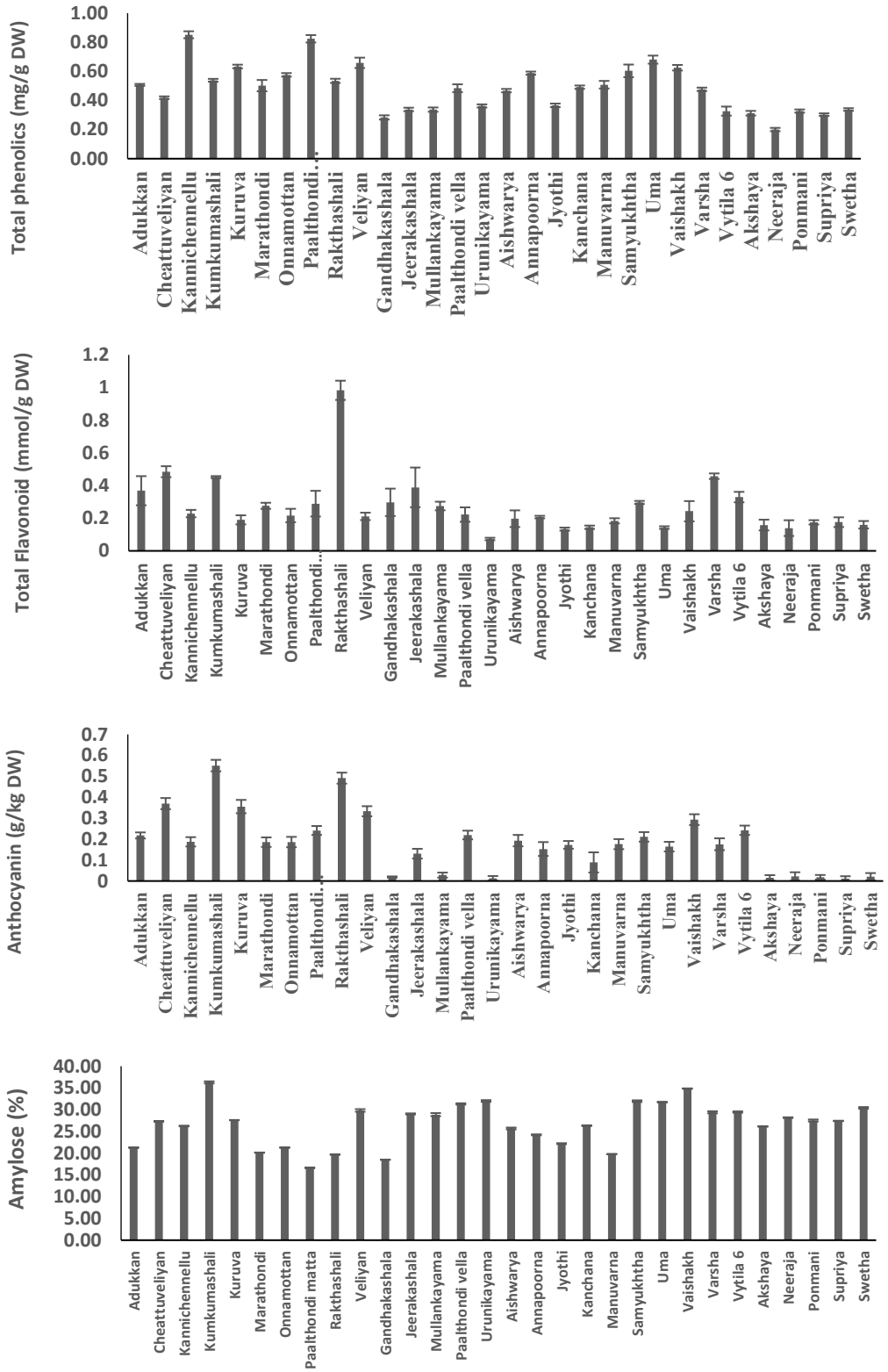


Figure 3. Total phenolics (A), Flavonoids (B), Anthocyanin (C), and Amylose (D) content of the studied rice samples. Values shown are mean ± SE

Table 7. Total chlorophyll content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnSO_4$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	Total chlorophyll (mg/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	1.33±0.03^{d}	1.5±0.03^{d}	1.35±0.01^{c}	1.68±0.19^{c}	1.03±0.01^{d}	0.94±0.02^{d}	1.86±0.06^{d}	1.37±0.1^{e}
0.25	6	1.55±0.01^{c}	2.18±0.11^{b}	1.36±0.01^{c}	2.13±0.1^{b}	1.26±0.01^{c}	0.99±0.01^{c}	2.22±0.02^{a}	1.8±0.08^{b}
0.5	6	1.86±0.02^{a}	2.59±0.03^{a}	1.56±0.02^{a}	2.65±0.06^{a}	1.62±0.01^{a}	1.14±0.01^{a}	2.26±0.04^{a}	1.98±0.04^{a}
0.75	6	1.76±0.02^{b}	1.59±0.27^{d}	1.47±0.02^{b}	2.72±0.05^{a}	1.57±0.01^{b}	1.08±0.03^{b}	2.38±0.04^{b}	1.57±0.01^{c}
1	6	1.56±0.01^{c}	1.92±0.01^{c}	1.41±0.01^{b}	2.69±0.13^{a}	1.55±0.18^{b}	1.04±0.01^{b}	2.19±0.02^{c}	1.22±0.01^{d}
0	12	1.36±0.02^{e}	1.53±0.03^{c}	1.35±0.03^{c}	1.69±0.06^{d}	1.04±0.02^{e}	0.95±0.03^{d}	1.89±0.06^{d}	1.38±0.01^{e}
0.25	12	1.74±0.01^{c}	2.52±0.01^{b}	1.65±0.02^{b}	2.29±0.15^{c}	1.43±0.16^{d}	1.05±0.01^{c}	2.37±0.02^{b}	1.93±0.03^{b}
0.5	12	1.92±0.01^{a}	2.62±0.02^{a}	1.85±0.02^{a}	2.67±0.01^{a}	1.95±0.01^{a}	1.23±0.01^{a}	2.59±0.07^{a}	2.08±0.08^{a}
0.75	12	1.84±0.01^{b}	2.63±0.02^{a}	1.66±0.02^{b}	2.54±0.01^{b}	1.76±0.01^{b}	1.14±0.01^{b}	2.16±0.07^{c}	1.8±0.08^{c}
1	12	1.66±0.02^{d}	2.55±0.02^{b}	1.65±0.02^{b}	2.53±0.02^{b}	1.63±0.03^{c}	1.09±0.01^{c}	1.63±0.03^{e}	1.59±0.03^{d}
0	18	1.32±0.02^{d}	1.5±0.02^{c}	1.34±0.02^{d}	1.68±0.02^{e}	1.02±0.01^{e}	0.93±0.02^{c}	1.85±0.04^{d}	1.36±0.03^{d}
0.25	18	1.81±0.01^{b}	2.22±0.02^{b}	1.97±0.02^{b}	2.73±0.09^{b}	1.57±0.01^{d}	1.16±0.04^{b}	2.59±0.08^{b}	2.57±0.01^{b}
0.5	18	1.93±0.04^{a}	2.9±0.01^{a}	2.26±0.06^{a}	3.23±0.06^{a}	2.35±0.01^{a}	1.37±0.02^{a}	2.89±0.01^{a}	2.6±0.31^{a}
0.75	18	1.85±0.01^{b}	2.49±0.13^{b}	1.94±0.01^{b}	2.82±0.02^{c}	2.02±0.01^{b}	1.14±0.01^{b}	2.4±0.05^{b}	2.55±0.02^{b}
1	18	1.74±0.01^{c}	1.09±0.8^{d}	1.86±0.01^{c}	2.5±0.01^{d}	1.94±0.01^{c}	1.13±0.01^{b}	2.18±0.04^{c}	2.21±0.16^{c}
0	24	1.36±0.03^{e}	1.54±0.03^{d}	1.36±0.01^{d}	1.69±0.03^{d}	1.04±0.01^{e}	0.96±0.02^{c}	1.87±0.01^{e}	1.38±0.02^{d}
0.25	24	1.82±0.06^{b}	2.13±0.3^{c}	1.95±0.01^{b}	2.79±0.05^{a}	1.69±0.01^{c}	1.1±0.01^{a}	1.78±0.04^{d}	1.8±0.08^{b}
0.5	24	1.9±0.01^{a}	2.74±0.2^{a}	2.1±0.03^{a}	2.44±0.05^{b}	2.23±0.02^{a}	1.14±0.01^{a}	2.38±0.02^{a}	1.93±0.01^{a}
0.75	24	1.67±0.02^{c}	2.49±0.05^{b}	1.76±0.03^{c}	2.76±0.11^{a}	1.85±0.01^{b}	1.03±0.01^{b}	2.16±0.07^{b}	1.61±0.04^{c}
1	24	1.5±0.03^{d}	2.07±0.07^{c}	1.38±0.01^{d}	2.36±0.07^{c}	1.14±0.01^{d}	0.98±0.01^{c}	1.89±0.03^{c}	1.03±0.01^{e}

Table 8. Carotenoid content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnSO_4$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	Carotenoid (mg/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	0.29±0.1^{c}	0.33±0.01^{e}	0.25±0.01^{c}	0.35±0.02^{c}	0.33±0.01^{b}	0.14±0.01^{a}	0.39±0.02^{d}	0.32±0.02^{d}
0.25	6	0.31±0.01^{bc}	0.50±0.01^{c}	0.29±0.01^{b}	0.41±0.02^{b}	0.35±0.01^{ab}	0.14±0.01^{a}	0.46±0.01^{c}	0.42±0.01^{b}
0.5	6	0.35±0.01^{a}	0.58±0.01^{a}	0.33±0.01^{a}	0.54±0.01^{a}	0.37±0.01^{a}	0.16±0.02^{a}	0.5±0.01^{a}	0.46±0.01^{a}
0.75	6	0.32±0.01^{ab}	0.54±0.01^{b}	0.31±0.01^{b}	0.56±0.00^{a}	0.35±0.01^{ab}	0.15±0.01^{a}	0.51±0.01^{b}	0.38±0.01^{c}
1	6	0.31±0.02^{bc}	0.43±0.01^{d}	0.3±0.01^{b}	0.55±0.02^{a}	0.33±0.01^{b}	0.14±0.01^{a}	0.46±0.01^{c}	0.33±0.01^{d}
0	12	0.28±0.1^{c}	0.34±0.01^{d}	0.26±0.01^{c}	0.36±0.02^{e}	0.33±0.03^{c}	0.15±0.01^{b}	0.39±0.01^{d}	0.33±0.01^{e}
0.25	12	0.32±0.01^{c}	0.53±0.01^{b}	0.31±0.01^{b}	0.48±0.02^{d}	0.35±0.01^{b}	0.16±0.01^{b}	0.5±0.01^{b}	0.47±0.01^{b}
0.5	12	0.38±0.01^{a}	0.59±0.01^{a}	0.35±0.02^{a}	0.61±0.01^{a}	0.41±0.02^{a}	0.21±0.01^{a}	0.54±0.01^{a}	0.52±0.01^{a}
0.75	12	0.35±0.01^{b}	0.58±0.01^{a}	0.33±0.02^{b}	0.51±0.00^{c}	0.35±0.02^{b}	0.16±0.01^{b}	0.46±0.01^{c}	0.42±0.01^{c}
1	12	0.33±0.01^{c}	0.50±0.02^{c}	0.31±0.01^{b}	0.55±0.01^{b}	0.25±0.01^{d}	0.16±0.01^{b}	0.36±0.01^{d}	0.38±0.01^{d}
0	18	0.29±0.01	0.34±0.02^{e}	0.25±0.03^{d}	0.34±0.02^{e}	0.32±0.01^{d}	0.14±0.05^{d}	0.38±0.02^{e}	0.31±0.01^{e}
0.25	18	0.33±0.1^{b}	0.63±0.01^{b}	0.33±0.01^{c}	0.62±0.01^{b}	0.38±0.01^{b}	0.17±0.01^{c}	0.55±0.03^{b}	0.6±0.01^{b}
0.5	18	0.41±0.01^{a}	0.70±0.02^{a}	0.4±0.02^{a}	0.69±0.02^{a}	0.44±0.02^{a}	0.29±0.02^{a}	0.64±0.02^{a}	0.66±0.02^{a}
0.75	18	0.35±0.01^{b}	0.59±0.01^{c}	0.36±0.02^{b}	0.59±0.01^{c}	0.39±0.02^{b}	0.2±0.01^{b}	0.5±0.01^{c}	0.56±0.01^{c}
1	18	0.31±0.02^{b}	0.55±0.01^{d}	0.33±0.01^{c}	0.52±0.01^{d}	0.36±0.01^{c}	0.18±0.01^{bc}	0.47±0.01^{d}	0.51±0.01^{d}
0	24	0.28±0.3^{c}	0.35±0.01^{d}	0.27±0.01^{d}	0.35±0.02^{e}	0.34±0.01^{b}	0.15±0.01^{c}	0.39±0.03^{d}	0.34±0.02^{d}
0.25	24	0.32±0.01^{b}	0.53±0.01^{b}	0.29±0.01^{c}	0.60±0.01^{a}	0.36±0.01^{b}	0.2±0.01^{b}	0.4±0.01^{cd}	0.42±0.01^{b}
0.5	24	0.4±0.01^{a}	0.64±0.01^{a}	0.38±0.01^{a}	0.51±0.02^{c}	0.41±0.01^{a}	0.23±0.02^{a}	0.52±0.02^{a}	0.47±0.01^{a}
0.75	24	0.3±0.01^{b}	0.51±0.01^{b}	0.34±0.01^{b}	0.58±0.01^{b}	0.34±0.01^{b}	0.19±0.01^{b}	0.46±0.01^{b}	0.39±0.01^{c}
1	24	0.26±0.01^{c}	0.43±0.01^{c}	0.32±0.01^{b}	0.47±0.02^{d}	0.33±0.01^{b}	0.18±0.01^{b}	0.42±0.01^{c}	0.3±0.01^{e}

Table 9. MDA content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnSO_4$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	MDA (µmol/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	3.74±0.02^{a}	0.41±0.01^{a}	3.35±0.02^{a}	0.46±0.01^{a}	3.65±0.02^{a}	3.41±0.03^{a}	0.40±0.01^{a}	0.42±0.04^{a}
0.25	6	3.57±0.02^{c}	0.39±0.01^{a}	3.26±0.02^{b}	0.46±0.01^{a}	3.55±0.01^{b}	3.06±0.01^{c}	0.39±0.01^{a}	0.38±0.01^{b}
0.5	6	3.44±0.01^{d}	0.39±0.04^{a}	3.04±0.01^{c}	0.43±0.04^{b}	3.44±0.01^{c}	2.73±0.01^{e}	0.39±0.00^{a}	0.36±0.06^{b}
0.75	6	3.57±0.02^{c}	0.38±0.02^{a}	3.23±0.02^{c}	0.44±0.02^{ab}	3.54±0.01^{b}	2.94±0.02^{d}	0.38±0.01^{a}	0.32±0.03^{c}
1	6	3.66±0.02^{b}	0.40±0.01^{a}	3.25±0.01^{c}	0.46±0.01^{a}	3.57±0.01^{b}	3.32±0.01^{b}	0.37±0.01^{a}	0.36±0.02^{a}
0	12	3.75±0.02^{a}	0.41±0.01^{a}	3.34±0.02^{a}	0.45±0.01^{a}	3.64±0.02^{a}	3.40±0.03^{a}	0.39±0.01^{a}	0.41±0.02^{a}
0.25	12	3.44±0.01^{c}	0.40±0.02^{a}	3.18±0.01^{b}	0.39±0.02^{b}	3.44±0.01^{b}	2.94±0.01^{d}	0.36±0.01^{a}	0.33±0.01^{b}
0.5	12	3.28±0.01^{e}	0.35±0.01^{b}	3.02±0.01^{d}	0.39±0.01^{b}	3.36±0.02^{c}	2.03±0.01^{e}	0.35±0.01^{b}	0.33±0.04^{b}
0.75	12	3.34±0.01^{d}	0.37±0.05^{b}	3.19±0.01^{b}	0.43±0.05^{a}	3.45±0.02^{b}	2.84±0.01^{c}	0.35±0.01^{b}	0.32±0.02^{b}
1	12	3.55±0.02^{b}	0.40±0.01^{a}	3.16±0.03^{c}	0.45±0.01^{a}	3.44±0.02^{b}	3.17±0.02^{b}	0.36±0.01^{a}	0.33±0.03^{b}
0	18	3.77±0.01^{a}	0.42±0.01^{a}	3.31±0.01^{a}	0.47±0.01^{a}	3.66±0.01^{a}	3.42±0.02^{a}	0.40±0.03^{a}	0.43±0.04^{a}
0.25	18	3.35±0.02^{d}	0.35±0.00^{c}	3.19±0.01^{b}	0.45±0.01^{a}	3.43±0.01^{b}	2.85±0.02^{c}	0.32±0.00^{c}	0.31±0.00^{bc}
0.5	18	2.94±0.01^{e}	0.31±0.00^{d}	2.94±0.01^{d}	0.37±0.01^{c}	2.96±0.02^{d}	2.16±0.02^{e}	0.30±0.00^{c}	0.29±0.08^{c}
0.75	18	3.36±0.01^{c}	0.39±0.00^{b}	3.03±0.01^{c}	0.38±0.01^{c}	3.32±0.01^{c}	2.65±0.02^{d}	0.32±0.00^{c}	0.31±0.04^{bc}
1	18	3.43±0.01^{b}	0.41±0.01^{a}	3.03±0.01^{c}	0.41±0.01^{b}	3.44±0.01^{b}	3.03±0.02^{b}	0.35±0.00^{b}	0.33±0.05^{b}
0	24	3.72±0.01^{a}	0.40±0.01^{a}	3.31±0.01^{b}	0.45±0.01^{a}	3.55±0.02^{a}	3.41±0.01^{a}	0.39±0.01^{a}	0.39±0.02^{a}
0.25	24	3.43±0.01^{d}	0.39±0.02^{a}	3.28±0.01^{b}	0.43±0.02^{b}	3.52±0.01^{a}	3.02±0.01^{b}	0.34±0.00^{b}	0.36±0.01^{a}
0.5	24	3.02±0.01^{e}	0.37±0.01^{b}	3.26±0.01^{c}	0.44±0.01^{ab}	3.15±0.01^{c}	2.27±0.01^{d}	0.35±0.01^{b}	0.33±0.01^{b}
0.75	24	3.55±0.01^{b}	0.39±0.00^{a}	3.28±0.01^{b}	0.42±0.01^{b}	3.44±0.02^{b}	2.85±0.01^{c}	0.36±0.03^{a}	0.35±0.01^{b}
1	24	3.47±0.17^{c}	0.41±0.03^{a}	3.36±0.02^{a}	0.47±0.03^{a}	3.52±0.01^{a}	3.26±0.01^{d}	0.38±0.02^{a}	0.39±0.01^{a}

Table 10. Total chlorophyll content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	Total chlorophyll (mg/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	1.33±0.03^{c}	1.5±0.03^{e}	1.35±0.01^{e}	1.35±0.01^{e}	1.03±0.01^{e}	0.96±0.02^{d}	1.62±0.03^{d}	1.37±0.1^{d}
0.25	6	1.41±0.1^{b}	1.93±0.08^{d}	1.44±0.1^{d}	1.86±0.02^{c}	1.35±0.1^{d}	1.1±0.1^{c}	2.11±0.02^{b}	2.5±0.04^{a}
0.5	6	1.55±0.01^{a}	2.55±0.04^{a}	1.85±0.01^{a}	2.05±0.19^{b}	1.85±0.01^{a}	1.35±0.01^{a}	2.24±0.44^{a}	2.44±0.06^{b}
0.75	6	1.53±0.01^{a}	2.26±0.08^{b}	1.55±0.01^{c}	2.24±0.01^{a}	1.57±0.01^{b}	1.25±0.01^{b}	2.11±0.03^{b}	2.15±0.08^{c}
1	6	1.41±0.01^{b}	2.05±0.01^{c}	1.65±0.03^{b}	1.66±0.12^{d}	1.42±0.03^{c}	1.04±0.02^{c}	2.06±0.03^{c}	2.49±0.08^{b}
0	12	1.34±0.03^{e}	1.54±0.02^{e}	1.36±0.01^{d}	1.36±0.02^{e}	1.06±0.01^{e}	0.97±0.02^{e}	1.61±0.09^{d}	1.35±0.01^{d}
0.25	12	1.64±0.01^{c}	2.22±0.08^{d}	2.17±0.02^{b}	2.59±0.01^{b}	1.63±0.01^{c}	1.43±0.04^{c}	2.33±0.03^{c}	2.48±0.01^{c}
0.5	12	1.87±0.02^{a}	3.01±0.01^{a}	2.24±0.04^{a}	2.73±0.12^{a}	2.24±0.01^{a}	1.65±0.02^{a}	2.58±0.44^{a}	2.63±0.01^{a}
0.75	12	1.75±0.02^{b}	2.57±0.07^{b}	2.18±0.04^{b}	2.19±0.17^{d}	1.74±0.01^{b}	1.5±0.02^{b}	1.75±0.44^{b}	2.5±0.01^{b}
1	12	1.55±0.01^{d}	2.39±0.05^{c}	2.04±0.03^{c}	2.23±0.01^{c}	1.66±0.02^{d}	1.35±0.03^{d}	1.76±0.11^{b}	2.54±0.04^{b}
0	18	1.33±0.02^{e}	1.51±0.04^{e}	1.35±0.01^{c}	1.33±0.04^{e}	1.04±0.01^{e}	0.95±0.02^{d}	1.61±0.01^{d}	1.36±0.03^{e}
0.25	18	1.98±0.01^{b}	2.55±0.03^{c}	2.34±0.01^{a}	2.53±0.05^{b}	1.94±0.01^{c}	1.35±0.01^{c}	2.58±0.03^{b}	2.82±0.23^{b}
0.5	18	2.05±0.01^{a}	3.35±0.06^{a}	2.35±0.09^{a}	3.06±0.09^{a}	2.43±0.01^{a}	1.84±0.01^{a}	3.22±0.08^{a}	3.25±0.03^{a}
0.75	18	1.87±0.01^{c}	3.18±0.11^{b}	2.33±0.01^{a}	2.47±0.01^{c}	2.07±0.04^{b}	1.57±0.01^{b}	2.24±0.35^{c}	2.51±0.05^{c}
1	18	1.64±0.02^{d}	1.7±0.18^{d}	2.13±0.01^{b}	2.22±0.02^{d}	1.85±0.01^{d}	1.5±0.02^{b}	1.63±0.39^{d}	2.48±0.12^{d}
0	24	1.35±0.03^{d}	1.53±0.03^{e}	1.37±0.01^{d}	1.34±0.01^{d}	1.07±0.01^{e}	0.99±0.02^{c}	1.62±0.04^{d}	1.38±0.02^{e}
0.25	24	1.87±0.01^{b}	2.29±0.04^{b}	2.24±0.01^{b}	1.85±0.01^{b}	2.2±0.03^{b}	1.54±0.01^{a}	1.93±0.15^{b}	2.62±0.01^{b}
0.5	24	1.96±0.01^{a}	2.7±0.02^{a}	2.47±0.01^{a}	2.92±0.01^{a}	2.21±0.01^{a}	1.57±0.01^{a}	2.48±0.05^{a}	2.82±0.23^{a}
0.75	24	1.74±0.01^{c}	2.02±0.03^{c}	2.05±0.01^{c}	1.85±0.01^{b}	2.13±0.02^{c}	1.19±0.03^{b}	1.85±0.11^{c}	2.31±0.14^{d}
1	24	1.35±0.01^{d}	1.59±0.06^{d}	1.35±0.01^{d}	1.55±0.01^{c}	1.26±0.01^{d}	1.04±0.01^{c}	1.89±0.06^{c}	2.46±0.03^{c}

Table 11. Carotenoids content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	Carotenoids (mg/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	0.29±0.01^{d}	0.33±0.01^{d}	0.25±0.01^{d}	0.34±0.01^{c}	0.32±0.01^{c}	0.15±0.01^{b}	0.32±0.01^{d}	0.32±0.02^{b}
0.25	6	0.31±0.01^{c}	0.44±0.01^{c}	0.28±0.01^{c}	0.41±0.01^{b}	0.33±0.01^{b}	0.17±0.01^{b}	0.42±0.01^{c}	0.49±0.01^{a}
0.5	6	0.37±0.01^{a}	0.54±0.01^{a}	0.35±0.01^{a}	0.52±0.01^{a}	0.39±0.01^{a}	0.21±0.01^{a}	0.54±0.01^{a}	0.51±0.01^{a}
0.75	6	0.33±0.01^{b}	0.5±0.01^{b}	0.33±0.01^{ab}	0.49±0.01^{a}	0.38±0.01^{a}	0.18±0.01^{b}	0.41±0.01^{c}	0.51±0.01^{a}
1	6	0.31±0.01^{c}	0.45±0.01^{c}	0.31±0.02^{bc}	0.43±0.01^{b}	0.36±0.01^{a}	0.17±0.01^{b}	0.45±0.01^{b}	0.50±0.02^{a}
0	12	0.29±0.02^{c}	0.34±0.02^{d}	0.29±0.01^{c}	0.35±0.02^{e}	0.32±0.01^{c}	0.16±0.01^{d}	0.34±0.01^{d}	0.34±0.02^{c}
0.25	12	0.32±0.01^{b}	0.52±0.01^{c}	0.26±0.01^{c}	0.56±0.01^{b}	0.34±0.01^{c}	0.22±0.01^{c}	0.53±0.01^{ab}	0.52±0.00^{ab}
0.5	12	0.39±0.02^{a}	0.65±0.01^{a}	0.4±0.01^{a}	0.65±0.01^{a}	0.44±0.01^{a}	0.29±0.03^{a}	0.57±0.01^{a}	0.56±0.01^{a}
0.75	12	0.33±0.02^{b}	0.57±0.01^{b}	0.35±0.01^{b}	0.51±0.01^{c}	0.38±0.01^{b}	0.28±0.01^{b}	0.54±0.02^{a}	0.54±0.01^{a}
1	12	0.33±0.01^{b}	0.51±0.01^{c}	0.32±0.01^{b}	0.49±0.01^{d}	0.37±0.01^{b}	0.20±0.02^{c}	0.46±0.01^{c}	0.55±0.01^{a}
0	18	0.28±0.01^{e}	0.33±0.01^{e}	0.25±0.01^{d}	0.34±0.02^{d}	0.33±0.01^{d}	0.14±0.01^{d}	0.33±0.01^{d}	0.32±0.02^{d}
0.25	18	0.34±0.01^{c}	0.55±0.01^{c}	0.31±0.01^{c}	0.57±0.01^{b}	0.37±0.01^{c}	0.25±0.01^{c}	0.57±0.01^{b}	0.59±0.01^{b}
0.5	18	0.43±0.01^{a}	0.79±0.02^{a}	0.42±0.01^{a}	0.79±0.02^{a}	0.47±0.02^{a}	0.33±0.01^{a}	0.71±0.02^{a}	0.73±0.01^{a}
0.75	18	0.38±0.01^{b}	0.69±0.02^{b}	0.37±0.01^{b}	0.58±0.02^{b}	0.41±0.01^{b}	0.28±0.03^{b}	0.57±0.01^{b}	0.54±0.01^{c}
1	18	0.32±0.01^{d}	0.42±0.01^{d}	0.33±0.02^{c}	0.48±0.01^{c}	0.41±0.01^{b}	0.26±0.01^{c}	0.46±0.01^{c}	0.55±0.01^{c}
0	24	0.3±0.01^{b}	0.35±0.03^{d}	0.27±0.01^{c}	0.36±0.01^{d}	0.35±0.01^{b}	0.16±0.01^{d}	0.35±0.01^{c}	0.36±0.02^{d}
0.25	24	0.3±0.03^{b}	0.49±0.01^{b}	0.3±0.01^{b}	0.51±0.01^{b}	0.36±0.01^{b}	0.30±0.01^{b}	0.45±0.01^{b}	0.56±0.00^{a}
0.5	24	0.4±0.01^{a}	0.59±0.01^{a}	0.41±0.01^{a}	0.6±0.01^{a}	0.42±0.01^{a}	0.31±0.02^{a}	0.55±0.01^{a}	0.59±0.01^{a}
0.75	24	0.32±0.01^{b}	0.44±0.01^{c}	0.29±0.01^{b}	0.51±0.01^{b}	0.40±0.01^{a}	0.27±0.01^{b}	0.45±0.01^{b}	0.51±0.01^{b}
1	24	0.3±0.01^{b}	0.37±0.01^{d}	0.26±0.01^{c}	0.43±0.01^{c}	0.35±0.01^{b}	0.25±0.01^{c}	0.42±0.01^{b}	0.47±0.00^{c}

Table 12. MDA content in eight different rice varieties subjected to various seed priming concentrations and duration of treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Concentration (M)	Duration (h)	MDA (µmol/g FW)							
		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
0	6	3.75±0.02^{a}	1.35±0.02^{a}	3.34±0.02^{a}	0.48±0.01^{a}	3.64±0.02^{a}	3.40±0.03^{a}	0.51±0.03^{a}	0.43±0.04^{a}
0.25	6	3.53±0.01^{c}	1.23±0.00^{b}	3.24±0.02^{c}	0.47±0.01^{ab}	3.34±0.02^{d}	3.25±0.02^{b}	0.49±0.02^{b}	0.37±0.06^{b}
0.5	6	3.35±0.01^{d}	1.17±0.02^{c}	3.15±0.01^{d}	0.46±0.03^{abc}	3.23±0.01^{e}	3.02±0.00^{d}	0.46±0.01^{bc}	0.35±0.04^{b}
0.75	6	3.63±0.01^{b}	1.05±0.05^{d}	3.28±0.01^{b}	0.45±0.01^{bc}	3.44±0.01^{c}	3.17±0.02^{c}	0.44±0.04^{c}	0.31±0.07^{c}
1	6	3.63±0.01^{b}	1.09±0.04^{d}	3.28±0.01^{b}	0.44±0.01^{d}	3.55±0.01^{b}	3.22±0.00^{b}	0.43±0.01^{c}	0.35±0.01^{b}
0	12	3.76±0.02^{a}	1.34±0.02^{a}	3.33±0.01^{a}	0.45±0.01^{a}	3.63±0.02^{a}	3.39±0.03^{a}	0.51±0.02^{a}	0.42±0.04^{a}
0.25	12	3.43±0.01^{d}	0.98±0.03^{b}	3.16±0.02^{c}	0.43±0.01^{ab}	3.35±0.02^{c}	3.16±0.01^{b}	0.42±0.01^{b}	0.32±0.01^{b}
0.5	12	3.14±0.01^{e}	0.95±0.02^{b}	3.08±0.01^{d}	0.41±0.02^{bc}	2.96±0.02^{d}	2.94±0.00^{d}	0.41±0.02^{bc}	0.32±0.04^{b}
0.75	12	3.59±0.01^{b}	0.96±0.14^{b}	3.16±0.00^{c}	0.40±0.01^{bc}	3.35±0.01^{c}	3.05±0.02^{c}	0.45±0.01^{b}	0.31±0.02^{b}
1	12	3.53±0.01^{c}	1.00±0.02^{b}	3.28±0.01^{b}	0.41±0.01^{bc}	3.43±0.01^{b}	3.18±0.01^{b}	0.43±0.02^{b}	0.32±0.04^{b}
0	18	3.74±0.02^{a}	1.36±0.02^{a}	3.35±0.02^{a}	0.47±0.01^{a}	3.65±0.02^{a}	3.41±0.03^{a}	0.52±0.03^{a}	0.43±0.02^{a}
0.25	18	3.35±0.01^{d}	0.84±0.13^{d}	3.36±0.31^{a}	0.40±0.01^{b}	3.15±0.01^{d}	2.88±0.01^{c}	0.46±0.00^{b}	0.26±0.01^{c}
0.5	18	2.85±0.01^{e}	0.71±0.01^{e}	2.86±0.01^{c}	0.36±0.02^{cd}	2.73±0.01^{e}	2.02±0.00^{d}	0.37±0.02^{c}	0.26±0.05^{c}
0.75	18	3.45±0.02^{c}	0.92±0.07^{c}	3.18±0.01^{b}	0.39±0.03^{bc}	3.26±0.03^{c}	2.95±0.00^{b}	0.44±0.01^{b}	0.29±0.04^{c}
1	18	3.55±0.02^{b}	0.99±0.06^{b}	3.18±0.02^{b}	0.39±0.02^{bc}	3.32±0.01^{b}	2.95±0.00^{b}	0.47±0.01^{b}	0.32±0.02^{b}
0	24	3.73±0.02^{a}	1.36±0.02^{a}	3.32±0.01^{a}	0.46±0.01^{a}	3.62±0.02^{a}	3.38±0.03^{a}	0.49±0.02^{a}	0.41±0.02^{a}
0.25	24	3.43±0.01^{d}	0.99±0.01^{c}	3.14±0.00^{b}	0.42±0.01^{bc}	3.35±0.02^{d}	3.04±0.00^{c}	0.44±0.02^{b}	0.35±0.02^{bc}
0.5	24	3.02±0.01^{e}	0.94±0.29^{d}	2.97±0.01^{d}	0.44±0.01^{b}	3.07±0.01^{e}	2.57±0.02^{d}	0.42±0.03^{b}	0.32±0.01^{c}
0.75	24	3.56±0.02^{c}	0.98±0.04^{c}	3.27±0.03^{c}	0.45±0.02^{b}	3.43±0.01^{c}	3.04±0.00^{c}	0.49±0.03^{a}	0.34±0.04^{c}
1	24	3.66±0.02^{b}	1.13±0.16^{b}	3.34±0.00^{a}	0.47±0.09^{a}	3.53±0.01^{b}	3.16±0.02^{b}	0.50±0.05^{a}	0.38±0.16^{b}

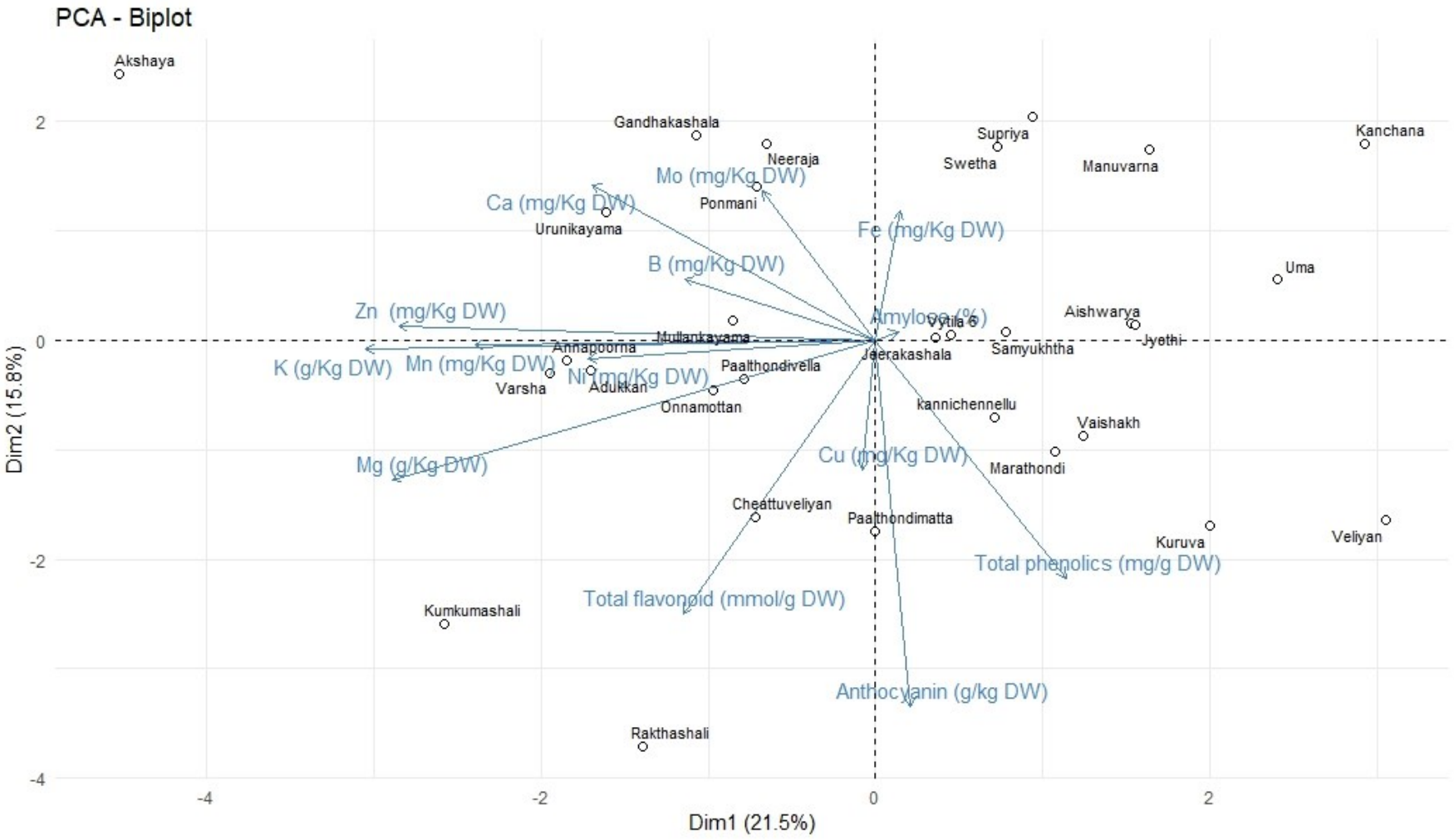


Figure 4. Principal component analysis of elemental and nutraceutical factors in the studied rice varieties

to the control plants (without any treatment). The maximum increase in total chlorophyll and carotenoid content and decrease in MDA (lipid peroxidation) content were observed in plants, raised from seeds primed with 0.5M $ZnSO_4$ and $ZnNO_3$ for 18h duration. Thus for seed priming, with $ZnSO_4$ and $ZnNO_3$ the concentration and duration were fixed as 0.5M and 18h.

4.2.2 Seedling priming

The priming of seedlings was done at one leaf stage (5 d after sowing) as foliar spray. The selected rice varieties were foliar sprayed with various concentration of priming agents $ZnSO_4$ (0, 0.25, 0.5 and 0.75%) and $ZnNO_3$ (0, 0.25, 0.5 and 0.75%) at one leaf stage. On 14 d after sowing, the total chlorophyll content, carotenoid content and MDA content were analysed (Table 13-15). The maximum increase in leaf pigment and decrease in MDA content was seen in plants sprayed with 0.5% $ZnSO_4$ and $ZnNO_3$. Therefore, the dosage of seedling priming was fixed as 0.5% $ZnSO_4$ and $ZnNO_3$.

4.3 Agronomic, yield characteristics and grain Zn content in selected rice varieties

4.3.1 Agronomic and yield characteristics

The application of Zn as seed priming and seedling priming enhanced the yield traits in the studied rice samples. The seed priming with $ZnSO_4$, enhanced the plant height, the highest increase was in Annapoorna and the lowest was in Adukkan. The increased seed setting percentage by priming with $ZnSO_4$ (7 to 17%) was in the order Kumkumashali > Gandhakashala > Annapoorna > Adukkan > Jyothi > Uma > Mullankayama > Ponmani. Under seed priming with $ZnSO_4$, the HI was highest in Kumkumashali (14%) and lowest in Adukkan. For Annapoorna, Gandhakshala and Mullankayama, there was more than 10% increase in HI, under seed priming with $ZnSO_4$, when compared to the control plants. When compared with other studied rice varieties, there was an increase of 11% in 100-grain weight in Kumkumashali raised from seeds primed with $ZnSO_4$, when compared to control plants, while in Ponmani, the enhancement of this parameter was the least. Seed priming with $ZnSO_4$ enhanced the tiller number/plant (9 to 14%), reproductive

tillers/plant (11 to 18%), and grains per panicle (10 to 22%) in all the varieties under study (Table 16-23).

The seed priming with $ZnNO_3$ enhanced the plant height in the range of 3 to 13% in Gandhakashala and Kumkumashali. The highest enhancement in seed setting was observed in Kumkumashali, followed by Gandhakashala, while Mullankayama showed the lowest increment. There was an increase in HI with $ZnNO_3$ priming and the highest increment was in Kumkumashali (16%), followed by Annapoorna (15%), Gandhakashala (14%) and Ponmani (11%). The highest increase in 100-grain weight was observed in Kumkumashali followed by Annapoorna when compared to untreated control plants. Seed priming with $ZnNO_3$ increased tiller number/plant also by 15 to 28%, reproductive tillers/plant by 17 to 28% and grains per panicle by 14 to 25% in all the rice varieties under investigation (Table 16-23).

The seedling priming with $ZnSO_4$ enhanced the plant height, seed setting, HI and 100- grain weight in all the rice varieties under study. The Annapoorna and Kumkumashali exhibited highest increase in plant height (>10%). Seedling priming also enhanced the seed setting in Kumkumashali (12%), Annapoorna (11%) and Gandhakashala (10%). The highest enhancement in HI was observed in Gandhakashala (11%). In general, the seedling priming enhanced the HI in the studied rice samples. 100-grain weight was increased by 8% in Annapoorna and 17% in Kumkumashali, when compared to the control samples. For other varieties, the increase was lesser when compared to the above mentioned rice varieties. Seedling priming with $ZnSO_4$ showed the least increment in tiller number/plant, reproductive tillers/plant and grains per panicle in all the rice varieties under study (Table 16-23).

Seedling priming with $ZnNO_3$ also showed an enhancement in plant height, seed setting percentage, HI and 100-grain weight, when compared to untreated control plants of the studied 8 rice varieties. The highest increase in plant height was observed in Kumkumashali (26%) and the lowest was in Gandhakashala (3%), for others it was above 5%. The increase in seed setting was above 10% in Kumkumashali, Annapoorna and Gandhakashala. The highest enhancement in HI

Table 13. Total chlorophyll content in eight different rice varieties subjected to various seedling priming concentrations of $ZnSO_4$ and $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Agent	Concentration (%)	Total chlorophyll (mg/g FW)							
$ZnSO_4$		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
	0	2.14±0.04^{c}	2.18±0.04^{c}	2.32±0.11^{c}	2.50±0.11^{c}	2.85±0.04^{c}	2.53±0.08^{c}	3.18±0.04^{c}	2.89±0.08^{c}
	0.25	2.39±0.01^{b}	2.49±0.01^{b}	3.19±0.13^{b}	2.91±0.04^{b}	3.13±0.00^{b}	3.24±0.02^{b}	3.63±0.04^{b}	3.64±0.02^{b}
	0.5	3.28±0.02^{a}	3.38±0.02^{a}	4.01±0.04^{a}	4.13±0.03^{a}	3.89±0.05^{a}	3.73±0.03^{a}	4.26±0.01^{a}	4.13±0.03^{a}
	0.75	2.05±0.03^{d}	2.15±0.03^{c}	2.25±0.04^{d}	2.37±0.02^{d}	2.75±0.03^{d}	2.49±0.02^{d}	3.09±0.01^{d}	2.69±0.02^{d}
$ZnNO_3$	0	2.16±0.04^{c}	2.26±0.04^{c}	2.02±0.11^{c}	2.52±0.11^{c}	2.88±0.04^{c}	2.59±0.08^{c}	3.08±0.04^{c}	2.99±0.08^{c}
	0.25	2.42±0.02^{b}	2.52±0.02^{b}	2.71±0.04^{b}	4.19±0.13^{b}	3.33±0.04^{b}	3.14±0.02^{b}	3.66±0.00^{b}	3.54±0.02^{b}
	0.5	3.56±0.02^{a}	3.86±0.02^{a}	3.93±0.03^{a}	4.65±0.04^{a}	3.96±0.01^{a}	3.88±0.07^{a}	4.29±0.05^{a}	4.28±0.07^{a}
	0.75	1.83±0.08^{d}	1.93±0.08^{d}	1.99±0.02^{c}	2.50±0.04^{c}	2.79±0.01^{d}	2.41±0.05^{d}	2.96±0.03^{d}	2.81±0.05^{d}

Table 14. Carotenoid content in eight different rice varieties subjected to various seedling priming concentrations of $ZnSO_4$ and $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Agent	Concentration (%)	Carotenoid (mg/g FW)							
$ZnSO_4$		Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
	0	0.52±0.02^{d}	0.53±0.02^{d}	0.63±0.01^{d}	0.54±0.01^{d}	0.80±0.02^{d}	0.76±0.02^{d}	0.81±0.02^{c}	0.76±0.02^{d}
	0.25	0.62±0.01^{b}	0.61±0.01^{b}	0.77±0.04^{b}	0.68±0.04^{b}	0.93±0.01^{b}	0.98±0.01^{b}	0.93±0.01^{b}	0.98±0.01^{b}
	0.5	0.82±0.01^{a}	0.81±0.02^{a}	0.94±0.01^{a}	0.85±0.01^{a}	1.15±0.02^{a}	1.07±0.01^{a}	1.20±0.02^{a}	1.07±0.01^{a}
	0.75	0.58±0.01^{c}	0.57±0.01^{c}	0.69±0.02^{c}	0.60±0.01^{c}	0.85±0.01^{c}	0.80±0.01^{c}	0.80±0.02^{c}	0.88±0.01^{c}
$ZnNO_3$	0	0.53±0.02^{d}	0.51±0.02^{c}	0.64±0.02^{d}	0.53±0.02^{d}	0.81±0.02^{d}	0.78±0.02^{c}	0.90±0.01^{d}	0.78±0.02^{c}
	0.25	0.61±0.01^{c}	0.62±0.01^{b}	0.74±0.01^{b}	0.65±0.01^{b}	0.93±0.01^{b}	1.06±0.02^{b}	0.95±0.01^{c}	1.06±0.02^{b}
	0.5	0.89±0.02^{a}	0.88±0.00^{a}	0.97±0.01^{a}	0.88±0.01^{a}	1.21±0.02^{a}	1.12±0.02^{a}	1.15±0.02^{a}	1.12±0.02^{a}
	0.75	0.67±0.01^{b}	0.48±0.01^{c}	0.68±0.01^{c}	0.59±0.01^{c}	0.90±0.01^{c}	0.80±0.01^{c}	1.05±0.01^{b}	0.80±0.01^{c}

Table 15. MDA content in eight different rice varieties subjected to various seedling priming concentrations of $ZnSO_4$ and $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

MDA (µmol/g FW)									
Agent	Concentration (%)	Adukkan	Annapoorna	Gandhakashala	Jyothi	Kumkumashali	Mullankayama	Ponmani	Uma
$ZnSO_4$	0	0.92±0.01^{b}	0.91±0.01^{b}	0.92±0.01^{a}	0.92±0.01^{a}	0.75±0.01^{a}	0.98±0.02^{b}	0.84±0.01^{a}	1.0±0.02^{a}
	0.25	0.76±0.03^{b}	0.76±0.03^{c}	0.80±0.01^{b}	0.87±0.01^{b}	0.71±0.01^{b}	0.94±0.01^{c}	0.75±0.01^{b}	0.94±0.01^{c}
	0.5	0.72±0.00^{c}	0.74±0.01^{c}	0.57±0.01^{c}	0.80±0.01^{c}	0.54±0.01^{c}	0.75±0.01^{d}	0.66±0.02^{c}	0.75±0.01^{d}
	0.75	0.98±0.04^{a}	0.98±0.04^{a}	0.93±0.01^{a}	0.90±0.01^{a}	0.77±0.01^{a}	1.13±0.09^{a}	0.72±0.08^{b}	0.99±0.09^{b}
$ZnNO_3$	0	0.93±0.01^{a}	0.90±0.01^{a}	0.92±0.01^{b}	0.90±0.01^{a}	0.75±0.01^{a}	1.01±0.02^{b}	0.74±0.01^{a}	1.01±0.02^{a}
	0.25	0.79±0.00^{b}	0.79±0.01^{b}	0.87±0.01^{c}	0.89±0.01^{a}	0.64±0.01^{b}	0.84±0.01^{c}	0.71±0.01^{a}	0.84±0.01^{b}
	0.5	0.72±0.01^{c}	0.72±0.01^{c}	0.79±0.01^{d}	0.66±0.01^{b}	0.51±0.02^{c}	0.62±0.05^{d}	0.64±0.01^{b}	0.62±0.05^{c}
	0.75	0.94±0.04^{a}	0.92±0.04^{a}	0.98±0.01^{a}	0.91±0.01^{a}	0.77±0.08^{a}	1.12±0.09^{a}	0.75±0.01^{a}	0.99±0.09^{a}

Table 16. Agronomic and yield parameters and grain Zn content in Adukkan subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	160.30 ± 0.36^{e}
	Tiller no	3.67 ± 0.33^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	65.80 ± 0.58^{f}
	Grains per panicle	41.00 ± 0.58^{e}
	100 grain weight	2.85 ± 0.01^{f}
	HI %	16.37 ± 0.09^{d}
	Grain Zn (mg/kg)	23.30 ± 0.05^{c}
T1 	Plant height (cm)	167.90 ± 0.86^{c}
	Tiller no	4.00 ± 0.58^{a}
	Reproductive tiller no	2.67 ± 0.88^{a}
	Seed setting %	70.83 ± 0.88^{cd}
	Grains per panicle	46.67 ± 0.88^{c}
	100 grain weight	3.06 ± 0.03^{bcd}
	HI %	16.94 ± 0.06^{cd}
	Grain Zn (mg/kg)	25.15 ± 0.04^{b}
T2 	Plant height (cm)	170.00 ± 0.58^{c}
	Tiller no	4.33 ± 0.33^{a}
	Reproductive tiller no	3.00 ± 0.58^{a}
	Seed setting %	72.83 ± 0.88^{bc}
	Grains per panicle	47.33 ± 0.88^{c}
	100 grain weight	3.09 ± 0.01^{abc}
	HI %	17.47 ± 0.09^{bc}
	Grain Zn (mg/kg)	26.85 ± 0.55^{a}

T3 	Plant height (cm)	164.17 ± 0.93^{d}
	Tiller no	3.67 ± 0.88^{a}
	Reproductive tiller no	2.67 ± 0.88^{a}
	Seed setting %	68.07 ± 0.88^{e}
	Grains per panicle	43.33 ± 0.88^{de}
	100 grain weight	3.02 ± 0.04^{cd}
	HI %	16.76 ± 0.15^{ef}
	Grain Zn (mg/kg)	24.41 ± 0.05^{bc}
T4 	Plant height (cm)	165.17 ± 1.48^{d}
	Tiller no	4.00 ± 0.58^{a}
	Reproductive tiller no	3.00 ± 0.58^{a}
	Seed setting %	69.40 ± 0.58^{de}
	Grains per panicle	45.00 ± 0.58^{cd}
	100 grain weight	2.98 ± 0.04^{d}
	HI %	16.87 ± 0.03^{de}
	Grain Zn (mg/kg)	24.90 ± 0.12^{bc}
T5 	Plant height (cm)	183.83 ± 0.92^{b}
	Tiller no	4.67 ± 0.33^{a}
	Reproductive tiller no	3.33 ± 0.88^{a}
	Seed setting %	73.37 ± 0.88^{ab}
	Grains per panicle	50.33 ± 1.20^{b}
	100 grain weight	3.13 ± 0.03^{ab}
	HI %	17.89 ± 0.52^{ab}
	Grain Zn (mg/kg)	26.85 ± 1.20^{a}
T6 	Plant height (cm)	190.07 ± 0.32^{a}
	Tiller no	5.00 ± 0.58^{a}
	Reproductive tiller no	3.67 ± 0.33^{a}
	Seed setting %	75.37 ± 0.33^{a}
	Grains per panicle	53.67 ± 0.88^{a}
	100 grain weight	3.16 ± 0.03^{a}
	HI %	18.47 ± 0.07^{a}
	Grain Zn (mg/kg)	27.90 ± 0.29^{a}

Table 17. Agronomic and yield parameters and grain Zn content in Annapoorna subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	92 ± 0.36^{d}
	Tiller no	5.33 ± 0.33^{c}
	Reproductive tiller no	4.33 ± 0.33^{c}
	Seed setting %	76 ± 0.4^{f}
	Grains per panicle	69.33 ± 4.33^{c}
	100 grain weight	2.17 ± 0.01^{e}
	HI %	16.54 ± 0.06^{e}
	Grain Zn (mg/kg)	22.38 ± 0.2^{f}
T1 	Plant height (cm)	107 ± 0.85^{b}
	Tiller no	6.00 ± 0.58^{abc}
	Reproductive tiller no	5.00 ± 0.58^{abc}
	Seed setting %	86 ± 0.41^{d}
	Grains per panicle	83.67 ± 0.33^{b}
	100 grain weight	2.34 ± 0.02^{c}
	HI %	18.54 ± 0.09^{bcd}
	Grain Zn (mg/kg)	24.85 ± 0.04^{d}
T2 	Plant height (cm)	109 ± 0.81^{b}
	Tiller no	6.33 ± 0.33^{abc}
	Reproductive tiller no	5.33 ± 0.33^{abc}
	Seed setting %	89 ± 0.4^{c}
	Grains per panicle	85.67 ± 1.45^{b}
	100 grain weight	2.38 ± 0.01^{b}
	HI %	19.07 ± 0.1^{bc}
	Grain Zn (mg/kg)	26.55 ± 0.55^{c}

T3 	Plant height (cm)	103±0.97^{c}
	Tiller no	5.33 ± 0.33^{c}
	Reproductive tiller no	4.33 ± 0.33^{c}
	Seed setting %	84±0.42^{e}
	Grains per panicle	80.33 ± 0.88^{b}
	100 grain weight	2.27±0.02^{d}
	HI %	17.82±0.06^{d}
	Grain Zn (mg/kg)	23.24±0.1^{e}
T4 	Plant height (cm)	104±0.98^{c}
	Tiller no	5.67 ± 0.33^{c}
	Reproductive tiller no	4.67 ± 0.33^{bc}
	Seed setting %	86±0.4^{d}
	Grains per panicle	81.67 ± 0.33^{b}
	100 grain weight	2.33±0.01^{c}
	HI %	18.14±0.52^{cd}
	Grain Zn (mg/kg)	23.88±0.06^{e}
T5 	Plant height (cm)	113±1.02^{a}
	Tiller no	7.00 ± 0.58^{ab}
	Reproductive tiller no	6.00 ± 0.58^{ab}
	Seed setting %	91±0.41^{b}
	Grains per panicle	92.33 ± 0.88^{a}
	100 grain weight	2.43±0.02^{b}
	HI %	19.49±0.58^{b}
	Grain Zn (mg/kg)	28.55±0.23^{b}
T6 	Plant height (cm)	118±3.4^{a}
	Tiller no	7.33 ± 0.67^{ab}
	Reproductive tiller no	6.33 ± 0.67^{a}
	Seed setting %	94±0.4^{a}
	Grains per panicle	95.67 ± 0.88^{a}
	100 grain weight	2.44±0.03^{a}
	HI %	21.15±0.61^{a}
	Grain Zn (mg/kg)	30.1±0.08^{a}

Table 18. Agronomic and yield parameters and grain Zn content in Gandhakashala subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	113±0.92^{c}
	Tiller no	3.33 ± 0.67^{a}
	Reproductive tiller no	3.33 ± 0.67^{a}
	Seed setting %	64±0.58^{e}
	Grains per panicle	107.33 ± 1.20^{f}
	100 grain weight	1.19±0.01^{d}
	HI %	19.88±0.06^{c}
	Grain Zn (mg/kg)	22.1±0.04^{c}
T1 	Plant height (cm)	138±0.7^{b}
	Tiller no	3.33 ± 0.67^{a}
	Reproductive tiller no	3.33 ± 0.67^{a}
	Seed setting %	75±0.58^{bc}
	Grains per panicle	127.00 ± 0.58^{c}
	100 grain weight	1.39±0.02^{ab}
	HI %	22.54±0.09^{b}
	Grain Zn (mg/kg)	24.85±0.04^{b}
T2 	Plant height (cm)	142±0.69^{b}
	Tiller no	3.67 ± 0.33^{a}
	Reproductive tiller no	3.67 ± 0.33^{a}
	Seed setting %	77±0.88^{b}
	Grains per panicle	129.67 ± 0.88^{b}
	100 grain weight	1.4±0.01^{a}
	HI %	23.07±0.1^{b}
	Grain Zn (mg/kg)	26.05±0.1^{a}
T3 	Plant height (cm)	112±0.94^{b}
	Tiller no	3.33 ± 0.67^{a}
	Reproductive tiller no	3.33 ± 0.33^{a}
	Seed setting %	71±0.88^{d}
	Grains per panicle	120.00 ± 0.58^{e}
	100 grain weight	1.31±0.02^{c}
	HI %	21.82±0.06^{b}
	Grain Zn (mg/kg)	23.61±0.05^{bc}

T4 	Plant height (cm)	127±1.01[b]
	Tiller no	3.33 ± 0.33[a]
	Reproductive tiller no	3.33 ± 0.33[a]
	Seed setting %	73±1.15[cd]
	Grains per panicle	123.67 ± 0.88[d]
	100 grain weight	1.33±0.01[bc]
	HI %	22.14±0.52[b]
	Grain Zn (mg/kg)	24.1±0.06[b]
T5 	Plant height (cm)	151±0.21[a]
	Tiller no	4.00 ± 0.58[a]
	Reproductive tiller no	4.00 ± 0.58[a]
	Seed setting %	80±0.58[b]
	Grains per panicle	141.33 ± 0.88[a]
	100 grain weight	1.43±0.02[ab]
	HI %	23.49±0.58[a]
	Grain Zn (mg/kg)	26.05±0.07[a]
T6 	Plant height (cm)	157±2.33[a]
	Tiller no	4.33 ± 0.33[a]
	Reproductive tiller no	4.33 ± 0.33[a]
	Seed setting %	83±0.58[a]
	Grains per panicle	143.67 ± 0.67[a]
	100 grain weight	1.47±0.01[a]
	HI %	25.15±0.06[a]
	Grain Zn (mg/kg)	27.1±0.09[a]

Table 19. Agronomic and yield parameters and grain Zn content in Jyothi subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	84.50 ± 2.08^{e}
	Tiller no	8.67 ± 0.33^{c}
	Reproductive tiller no	7.67 ± 0.33^{b}
	Seed setting %	76.03 ± 0.88^{e}
	Grains per panicle	92.67 ± 1.45^{e}
	100 grain weight	2.35 ± 0.02^{e}
	HI %	17.17 ± 0.09^{e}
	Grain Zn (mg/kg)	25.20 ± 0.05^{d}
T1 	Plant height (cm)	91.47 ± 0.30^{cd}
	Tiller no	9.67 ± 0.33^{bc}
	Reproductive tiller no	8.67 ± 0.33^{ab}
	Seed setting %	81.50 ± 0.58^{c}
	Grains per panicle	103.00 ± 1.15^{bc}
	100 grain weight	2.46 ± 0.03^{d}
	HI %	18.14 ± 0.06^{cd}
	Grain Zn (mg/kg)	26.85 ± 0.04^{cd}
T2 	Plant height (cm)	94.73 ± 0.97^{bc}
	Tiller no	10.00 ± 0.58^{bc}
	Reproductive tiller no	9.00 ± 0.58^{ab}
	Seed setting %	84.50 ± 0.58^{b}
	Grains per panicle	106.00 ± 2.00^{b}
	100 grain weight	2.53 ± 0.01^{c}
	HI %	18.67 ± 0.09^{bc}
	Grain Zn (mg/kg)	27.75 ± 0.55^{bc}

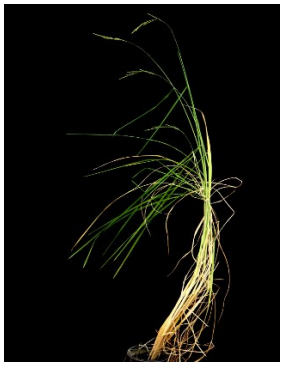
T3 	Plant height (cm)	87.60 ± 0.65^{de}
	Tiller no	9.00 ± 0.58^{c}
	Reproductive tiller no	8.00 ± 0.58^{b}
	Seed setting %	78.70 ± 0.58^{d}
	Grains per panicle	98.00 ± 0.58^{d}
	100 grain weight	2.51 ± 0.04^{cd}
	HI %	17.42 ± 0.10^{e}
	Grain Zn (mg/kg)	26.11 ± 0.05^{cd}
T4 	Plant height (cm)	92.27 ± 1.19^{cd}
	Tiller no	9.33 ± 0.33^{bc}
	Reproductive tiller no	8.33 ± 0.33^{ab}
	Seed setting %	80.37 ± 0.88^{cd}
	Grains per panicle	101.33 ± 0.88^{cd}
	100 grain weight	2.50 ± 0.02^{cd}
	HI %	17.74 ± 0.06^{de}
	Grain Zn (mg/kg)	26.60 ± 0.12^{cd}
T5 	Plant height (cm)	99.23 ± 3.76^{b}
	Tiller no	10.67 ± 0.33^{ab}
	Reproductive tiller no	9.00 ± 0.58^{ab}
	Seed setting %	85.20 ± 0.58^{ab}
	Grains per panicle	115.00 ± 0.58^{a}
	100 grain weight	2.69 ± 0.01^{b}
	HI %	19.09 ± 0.52^{ab}
	Grain Zn (mg/kg)	28.55 ± 1.20^{ab}
T6 	Plant height (cm)	106.27 ± 1.97^{a}
	Tiller no	11.67 ± 0.33^{a}
	Reproductive tiller no	9.67 ± 0.33^{a}
	Seed setting %	87.20 ± 0.58^{a}
	Grains per panicle	118.00 ± 0.58^{a}
	100 grain weight	2.80 ± 0.01^{a}
	HI %	19.67 ± 0.07^{a}
	Grain Zn (mg/kg)	29.60 ± 0.29^{a}

Table 20. Agronomic and yield parameters and grain Zn content in Kumkumashali subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	186 ± 1.44^{g}
	Tiller no	7.00 ± 0.58^{c}
	Reproductive tiller no	5.33 ± 0.33^{d}
	Seed setting %	61 ± 0.88^{f}
	Grains per panicle	84.67 ± 0.88^{e}
	100 grain weight	1.32 ± 0.03^{d}
	HI %	18.38 ± 0.15^{e}
	Grain Zn (mg/kg)	25.65 ± 0.14^{f}
T1 	Plant height (cm)	192 ± 0.29^{d}
	Tiller no	8.00 ± 0.58^{bc}
	Reproductive tiller no	6.33 ± 0.33^{cd}
	Seed setting %	70 ± 0.88^{cd}
	Grains per panicle	103.67 ± 1.45^{bc}
	100 grain weight	1.39 ± 0.02^{b}
	HI %	20.75 ± 0.09^{bcd}
	Grain Zn (mg/kg)	28.65 ± 0.04^{d}
T2 	Plant height (cm)	191 ± 0.65^{c}
	Tiller no	8.33 ± 0.33^{bc}
	Reproductive tiller no	6.67 ± 0.33^{bc}
	Seed setting %	72 ± 0.88^{c}
	Grains per panicle	106.33 ± 0.88^{b}
	100 grain weight	1.4 ± 0.01^{b}
	HI %	21 ± 0.1^{bc}
	Grain Zn (mg/kg)	30.35 ± 0.05^{c}

T3 	Plant height (cm)	190±0.44^{f}
	Tiller no	7.33 ± 0.33^{c}
	Reproductive tiller no	6.00 ± 0.00^{cd}
	Seed setting %	67±0.58^{e}
	Grains per panicle	100.33 ± 0.88^{cd}
	100 grain weight	1.36±0.01^{c}
	HI %	20.32±0.06^{d}
	Grain Zn (mg/kg)	27.04±0.1^{e}
T4 	Plant height (cm)	191±0.43^{e}
	Tiller no	7.67 ± 0.67^{c}
	Reproductive tiller no	6.33 ± 0.33^{cd}
	Seed setting %	69±0.88^{de}
	Grains per panicle	98.33 ± 1.20^{d}
	100 grain weight	1.38±0.01^{c}
	HI %	20.64±0.052^{cd}
	Grain Zn (mg/kg)	27.68±0.04^{e}
T5 	Plant height (cm)	205±2.02^{b}
	Tiller no	9.33 ± 0.33^{ab}
	Reproductive tiller no	7.67 ± 0.33^{ab}
	Seed setting %	72±0.58^{b}
	Grains per panicle	119.00 ± 2.89^{a}
	100 grain weight	1.39±0.02^{ab}
	HI %	21.99±0.17^{b}
	Grain Zn (mg/kg)	32.67±0.05^{b}
T6 	Plant height (cm)	206±1.45^{a}
	Tiller no	10.00 ± 0.58^{a}
	Reproductive tiller no	8.00 ± 0.58^{a}
	Seed setting %	76±0.58^{a}
	Grains per panicle	121.00 ± 0.58^{a}
	100 grain weight	1.41±0.01^{a}
	HI %	22.48±0.06^{a}
	Grain Zn (mg/kg)	34.49±0.06^{a}

Table 21. Agronomic and yield parameters and grain Zn content in Mullankayama subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	164.00 ± 2.31^{f}
	Tiller no	2.33 ± 0.88^{a}
	Reproductive tiller no	2.33 ± 0.88^{a}
	Seed setting %	66.80 ± 0.58^{f}
	Grains per panicle	51.67 ± 1.20^{f}
	100 grain weight	2.34 ± 0.06^{c}
	HI %	16.07 ± 0.09^{f}
	Grain Zn (mg/kg)	24.40 ± 0.05^{d}
T1 	Plant height (cm)	183.93 ± 1.10^{cd}
	Tiller no	2.33 ± 0.33^{a}
	Reproductive tiller no	2.33 ± 0.33^{a}
	Seed setting %	71.33 ± 0.88^{cd}
	Grains per panicle	57.00 ± 0.58^{cd}
	100 grain weight	2.41 ± 0.01^{bc}
	HI %	17.24 ± 0.06^{cd}
	Grain Zn (mg/kg)	25.75 ± 0.04^{cd}
T2 	Plant height (cm)	188.13 ± 0.63^{bc}
	Tiller no	2.67 ± 0.88^{a}
	Reproductive tiller no	2.67 ± 0.88^{a}
	Seed setting %	72.67 ± 0.33^{bc}
	Grains per panicle	59.00 ± 0.58^{c}
	100 grain weight	2.49 ± 0.01^{ab}
	HI %	17.77 ± 0.09^{bc}
	Grain Zn (mg/k g)	26.65 ± 0.55^{bc}

T3 	Plant height (cm)	175.33 ± 1.45^{e}
	Tiller no	2.33 ± 0.33^{a}
	Reproductive tiller no	2.33 ± 0.33^{a}
	Seed setting %	68.37 ± 0.88^{ef}
	Grains per panicle	54.00 ± 0.58^{ef}
	100 grain weight	2.43 ± 0.03^{bc}
	HI %	16.52 ± 0.10^{ef}
	Grain Zn (mg/kg)	25.01 ± 0.05^{cd}
T4 	Plant height (cm)	182.00 ± 1.53^{d}
	Tiller no	2.33 ± 0.33^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	69.70 ± 0.58^{de}
	Grains per panicle	56.33 ± 0.88^{de}
	100 grain weight	2.44 ± 0.02^{bc}
	HI %	16.84 ± 0.06^{de}
	Grain Zn (mg/kg)	25.50 ± 0.12^{cd}
T5 	Plant height (cm)	192.33 ± 1.20^{b}
	Tiller no	2.67 ± 0.33^{a}
	Reproductive tiller no	2.33 ± 0.33^{a}
	Seed setting %	74.17 ± 0.88^{ab}
	Grains per panicle	62.67 ± 0.88^{b}
	100 grain weight	2.47 ± 0.01^{ab}
	HI %	18.19 ± 0.52^{ab}
	Grain Zn (mg/kg)	27.45 ± 1.20^{ab}
T6 	Plant height (cm)	198.33 ± 0.88^{a}
	Tiller no	3.00 ± 0.58^{a}
	Reproductive tiller no	3.00 ± 0.58^{a}
	Seed setting %	76.17 ± 0.33^{a}
	Grains per panicle	65.67 ± 0.88^{a}
	100 grain weight	2.57 ± 0.04^{a}
	HI %	18.77 ± 0.07^{a}
	Grain Zn (mg/kg)	28.50 ± 0.29^{a}

Table 22. Agronomic and yield parameters and grain Zn content in Ponmani subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	118±0.23^{d}
	Tiller no	10.00 ± 0.58^{c}
	Reproductive tiller no	8.67 ± 0.33^{b}
	Seed setting %	77±0.58^{e}
	Grains per panicle	117.33 ± 3.93^{d}
	100 grain weight	2.14±0.02^{a}
	HI %	14.72±0.06^{f}
	Grain Zn (mg/kg)	20.38±0.2^{b}
T1 	Plant height (cm)	124±0.44^{c}
	Tiller no	11.00 ± 0.58^{bc}
	Reproductive tiller no	10.00 ± 0.58^{ab}
	Seed setting %	82±0.58^{c}
	Grains per panicle	134.67 ± 0.88^{c}
	100 grain weight	2.24±0.05^{a}
	HI %	15.74±0.09^{cd}
	Grain Zn (mg/kg)	21.85±0.04^{b}
T2 	Plant height (cm)	126±0.61^{b}
	Tiller no	11.33 ± 0.33^{bc}
	Reproductive tiller no	10.33 ± 0.33^{ab}
	Seed setting %	85±0.58^{b}
	Grains per panicle	138.33 ± 0.88^{bc}
	100 grain weight	2.2±0.02^{a}
	HI %	16.27±0.1^{bc}
	Grain Zn (mg/kg)	23.55±0.02^{a}

T3 	Plant height (cm)	122±0.58^{c}
	Tiller no	10.00 ± 0.58^{c}
	Reproductive tiller no	9.00 ± 0.58^{b}
	Seed setting %	79±0.88^{de}
	Grains per panicle	129.33 ± 3.18^{c}
	100 grain weight	2.19±0.02^{a}
	HI %	15.02±0.06^{e}
	Grain Zn (mg/kg)	21.11±0.05^{b}
T4 	Plant height (cm)	123±1.52^{c}
	Tiller no	10.67 ± 0.33^{c}
	Reproductive tiller no	9.67 ± 0.33^{b}
	Seed setting %	81±0.58^{cd}
	Grains per panicle	131.67 ± 2.73^{c}
	100 grain weight	2.25±0.02^{a}
	HI %	15.34±0.52^{de}
	Grain Zn (mg/kg)	21.6±0.03^{b}
T5 	Plant height (cm)	127±0.15^{b}
	Tiller no	12.67 ± 0.88^{ab}
	Reproductive tiller no	10.33 ± 0.33^{ab}
	Seed setting %	86±0.88^{b}
	Grains per panicle	147.00 ± 3.21^{ab}
	100 grain weight	2.23±0.03^{a}
	HI %	16.69±0.07^{ab}
	Grain Zn (mg/kg)	23.55±0.01^{a}
T6 	Plant height (cm)	130±1.01^{a}
	Tiller no	13.33 ± 0.33^{a}
	Reproductive tiller no	11.67 ± 0.88^{a}
	Seed setting %	88±0.58^{a}
	Grains per panicle	154.00 ± 7.00^{a}
	100 grain weight	2.22±0.01^{a}
	HI %	17.27±0.18^{a}
	Grain Zn (mg/kg)	24.6±0.01^{a}

Table 23. Agronomic and yield parameters and grain Zn content in Uma subjected to seed and seedling priming with $ZnSO_4$ and $ZnNO_3$. C (control), T1 (seed priming with $ZnSO_4$), T2 (seed priming with $ZnNO_3$), T3 (seedling priming with $ZnSO_4$), T4 (seedling priming with $ZnNO_3$), T5 (combined treatment of seed and seedling priming with $ZnSO_4$), T6 (combined treatment of seed and seedling priming with $ZnNO_3$). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Control 	Plant height (cm)	95.07 ± 1.27^{d}
	Tiller no	3.67 ± 0.33^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	75.73 ± 0.88^{e}
	Grains per panicle	108.33 ± 0.88^{f}
	100 grain weight	2.38 ± 0.01^{c}
	HI %	17.77 ± 0.09^{d}
	Grain Zn (mg/kg)	25.00 ± 0.05^{d}
T1 	Plant height (cm)	101.70 ± 0.99^{c}
	Tiller no	4.00 ± 0.58^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	80.90 ± 0.58^{c}
	Grains per panicle	121.00 ± 0.58^{c}
	100 grain weight	2.42 ± 0.01^{b}
	HI %	18.52 ± 0.10^{c}
	Grain Zn (mg/kg)	26.65 ± 0.04^{cd}
T2 	Plant height (cm)	104.23 ± 0.13^{b}
	Tiller no	4.33 ± 0.88^{a}
	Reproductive tiller no	3.00 ± 0.58^{a}
	Seed setting %	84.00 ± 0.58^{b}
	Grains per panicle	126.00 ± 0.58^{b}
	100 grain weight	2.42 ± 0.01^{b}
	HI %	19.52 ± 0.10^{ab}
	Grain Zn (mg/kg)	27.55 ± 0.55^{bc}

T3 	Plant height (cm)	101.37 ± 0.09^{c}
	Tiller no	3.67 ± 0.33^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	78.30 ± 0.58^{d}
	Grains per panicle	114.00 ± 1.15^{e}
	100 grain weight	2.43 ± 0.01^{b}
	HI %	18.44 ± 0.06^{c}
	Grain Zn (mg/kg)	25.91 ± 0.05^{cd}
T4 	Plant height (cm)	102.80 ± 0.51^{c}
	Tiller no	4.00 ± 0.58^{a}
	Reproductive tiller no	2.67 ± 0.33^{a}
	Seed setting %	80.07 ± 0.88^{cd}
	Grains per panicle	118.00 ± 0.58^{d}
	100 grain weight	2.44 ± 0.02^{b}
	HI %	18.97 ± 0.09^{bc}
	Grain Zn (mg/kg)	26.40 ± 0.12^{cd}
T5 	Plant height (cm)	107.63 ± 0.82^{b}
	Tiller no	4.33 ± 0.88^{a}
	Reproductive tiller no	3.00 ± 0.58^{a}
	Seed setting %	85.07 ± 0.37^{ab}
	Grains per panicle	128.00 ± 0.58^{ab}
	100 grain weight	2.56 ± 0.01^{b}
	HI %	19.39 ± 0.52^{ab}
	Grain Zn (mg/kg)	28.35 ± 1.20^{ab}
T6 	Plant height (cm)	110.03 ± 0.69^{a}
	Tiller no	4.67 ± 0.33^{a}
	Reproductive tiller no	3.33 ± 0.88^{a}
	Seed setting %	86.80 ± 0.58^{a}
	Grains per panicle	130.00 ± 0.58^{a}
	100 grain weight	2.58 ± 0.01^{a}
	HI %	19.97 ± 0.07^{a}
	Grain Zn (mg/kg)	29.40 ± 0.29^{a}

was observed in Gandhakashala (12%), while the lowest enhancement was found in Adukkan. Likewise, the highest increment in 100-grain was seen in Kumkumashali, while Uma showed the lowest increment. The tiller number/plant (7 to 14%), reproductive tillers/plant (7 to 18%) and grains per panicle (8 to 18%) was enhanced upon seedling priming with $ZnNO_3$ in all the rice varieties when compared to untreated control plants (Table 16-23).

The combined priming treatments at seed and seedling stages enhanced the height, seed setting, HI and 100 grain weight more efficiently in all the studied rice varieties, when compared to the control plants. The highest enhancement in plant height by combined priming treatments of $ZnSO_4$ was in Kumkumashali (34%), followed by Annapoorna (22%). In all the studied rice varieties, the increment in seed setting was above 10%, out of which the highest enhancement was in Kumkumashali (25%) and Annapoorna (20%). The highest increase in HI was seen in Gandhakashala (20%) and the lowest was in Adukkan (9%). The 100-grain weight was increased in all the rice varieties, and the response of Kumkumashali was higher compared to others. The tiller number/plant (23 to 33%), reproductive tillers/plant (25 to 43%), and grains per panicle (18 to 40%) was enhanced in all the varieties under study upon combined priming treatments at seed and seedling stages with $ZnSO_4$ (Table 16-23).

The combination of priming treatments at both seed and seedling with $ZnNO_3$ enhanced the plant height from 11 to 34% in various rice varieties taken for the study. In all the rice varieties, the seed setting percentage was significantly enhanced when compared to control plants and Kumkumashali, Annapoorna and Gandhakashala showed the highest increment as compared to others. The highest increase in HI was observed in Annapoorna (28%), while the lowest increase was in Uma (12%). The 100-grain weight was also enhanced in all the rice varieties under study, when compared to the control plants. The plants emerged from seeds primed with $ZnNO_3$ and seedlings primed with the same compound, enhanced the tiller number/plant by 34 to 44%, reproductive tillers/plant by 37 to 50% and grains per panicle by 20 to 42% (Table 16 to 23).

4.3.2 Grain Zn content

Seed priming with $ZnSO_4$ increased the grain Zn content in all the studied rice varieties, and the highest increase was observed in Kumkumashali followed by Annapoorna. The order of enhancement in grain Zn content was Annapoorna > Kumkumashali > Gandhakashala > Ponmani > Adukkan > Uma > Jyothi > Mullankayama, when compared to untreated control plants upon seed priming with $ZnNO_3$. The seedling priming with both Zn compounds enhanced the grain Zn content in all the studied rice varieties, but the seed priming was more effective than seedling priming. When compared to the individual application of $ZnSO_4$ and $ZnNO_3$ as seed and seedling priming, the higher enhancement in grain Zn content was observed in the combined priming treatments. The combined priming treatments with $ZnSO_4$ showed an enhancement between 13 to 27% in which Kumkumashali and Annapoorna showed the highest increase, while, Mullankayama and Jyothi showed the lowest increase. More than 15% of increase in grain Zn content was observed in all the rice varieties upon combined priming treatments with $ZnSO_4$ as well as $ZnNO_3$. Kumkumashali and Annapoorna showed an increase above 30% (Table 16 to 23).

4.3.3 Principal component and correlation analysis

The first principal component represents 41% variation, while the second component represents 27.2%, and together they represent 68.2% of variability. It visualizes the relationships between rice varieties, Zn biofortification treatments, and key agronomic traits, including Zn content, plant height (PH), tiller number (TN), reproductive tillers (RT), seed setting (SS), grain yield components (grains per panicle, GPP; 100-grain weight, 100 GW), and harvest index (HI). The rice varieties are well separated into distinct groups, indicating clear differences in their agronomic traits. Zn shows a strong positive correlation with harvest index (HI), highlighting that Zn biofortification strategies contribute to both nutrient enrichment and yield efficiency. Zn has a moderate positive correlation with seed setting (SS), showing that Zn biofortification strategies can enhance nutritional quality without reducing grain yield. Thus, it is clear that rice varieties that cluster near the Zn and

HI are ideal for Zn biofortification program, as it enhance grain Zn content without compromising yield. Accordingly, varieties positioned closer to Zn, HI, and SS in the PCA plot are the best performers for biofortification and yield improvement. Kumkumashali and Annapoorna are clustered near Zn, HI, SS and RT, indicating these varieties have higher Zn accumulation and good yield efficiency. Thus, Kumkumashali and Annapoorna can be selected for further analysis (Fig. 5).

Out of the two priming agents, the best performing one can be selected from the boxplot. Combined treatments of seed and seedling priming with $ZnNO_3$ (ZN SP + SGP) has the highest median Zn content. The combined treatments of seed and seedling priming with $ZnSO_4$ (ZS SP + SGP) also shows high Zn content, but lower than combined treatment with $ZnNO_3$. Seed priming with $ZnNO_3$ (ZN SP) has a higher Zn content than seedling priming with $ZnNO_3$ (ZN SGP), indicating seed priming alone is better than seedling priming alone. Therefore, the best priming agent was selected for further analysis was $ZnNO_3$. Based on the above observations, two rice varieties, Annapoorna, and Kumkumashali were selected for further analysis as they responded more to the various priming treatments. On comparing $ZnSO_4$ and $ZnNO_3$, $ZnNO_3$ gave better results, thus this priming agent was selected for further treatments.

4.4 The effect of seed priming and seedling priming in photosynthetic features, antioxidant system, oxidative damage, membrane stability and metabolites.

4.4.1 The effect of seed priming and seedling priming on photosynthetic parameters

Several photosynthetic traits in rice plants were improved by priming with Zn compounds. A notable enhancement in photosynthetic pigments was seen in various treatments relative to the control plants.

4.4.1.1 Photosynthetic pigments

The seed priming with $ZnNO_3$ enhanced the total chlorophyll content in Annapoorna and Kumkumashali by 15 and 12% respectively. The seedling priming with $ZnNO_3$ also enhanced total chlorophyll content in Annapoorna (12%) and

Kumkumashali (11%). When compared to individual application of seed and seedling priming, the combined treatments of seed priming and seedling priming caused the highest enhancement in total chlorophyll content. The combined treatments of seed and seedling priming with $ZnNO_3$ enhanced total chlorophyll content in Annapoorna (39%) and Kumkumashali (43%), when compared to control plants. The seed priming and seedling priming with $ZnNO_3$, enhanced carotenoid content in Annapoorna (47 and 27%) and Kumkumashali (27 and 16%). However, the combined treatments of seed and seedling priming with $ZnNO_3$, enhanced carotenoid content by 54% in Annapoorna, and 51% in Kumkumashali, on comparison with control plants (Fig. 6).

4.4.1.2 Chlorophyll stability index (CSI)

The Chlorophyll Stability Index (CSI) values for each treatment indicate the relative stability of chlorophyll in response to the various treatments. In Annapoorna, CSI showed an enhancement under seed priming (16%), when compared to control plants. Whereas seedling priming enhanced the CSI to a minimal level (9%). The combined application of seed priming and seedling priming brought about a significant enhancement in CSI (28%), in primed plants. Similar trend in CSI was also observed in Kumkumashali. The seed priming, seedling priming and combined treatment of primings increased CSI by 20, 13 and 30% respectively, when compared to control plants. These results suggest that the combined priming treatments of $ZnNO_3$ are more effective in enhancing chlorophyll stability in plants (Fig. 6).

4.4.1.3 Photosystem (PS) I and II activity

Application of Zn had an impact on the photosystem activities of the photosynthesis. The activities of photosystem I and II were increased under priming treatments. The seed priming with $ZnNO_3$ enhanced PSI activity in Annapoorna (23%) and Kumkumashali (31%). Compared to seed priming, seedling priming was less efficient and it enhanced PSI activity by 14% in Annapoorna and 17% in Kumkumashali. The combined treatments of seed and seedling priming further enhanced the PSI activity, when compared to individual treatments and the increase

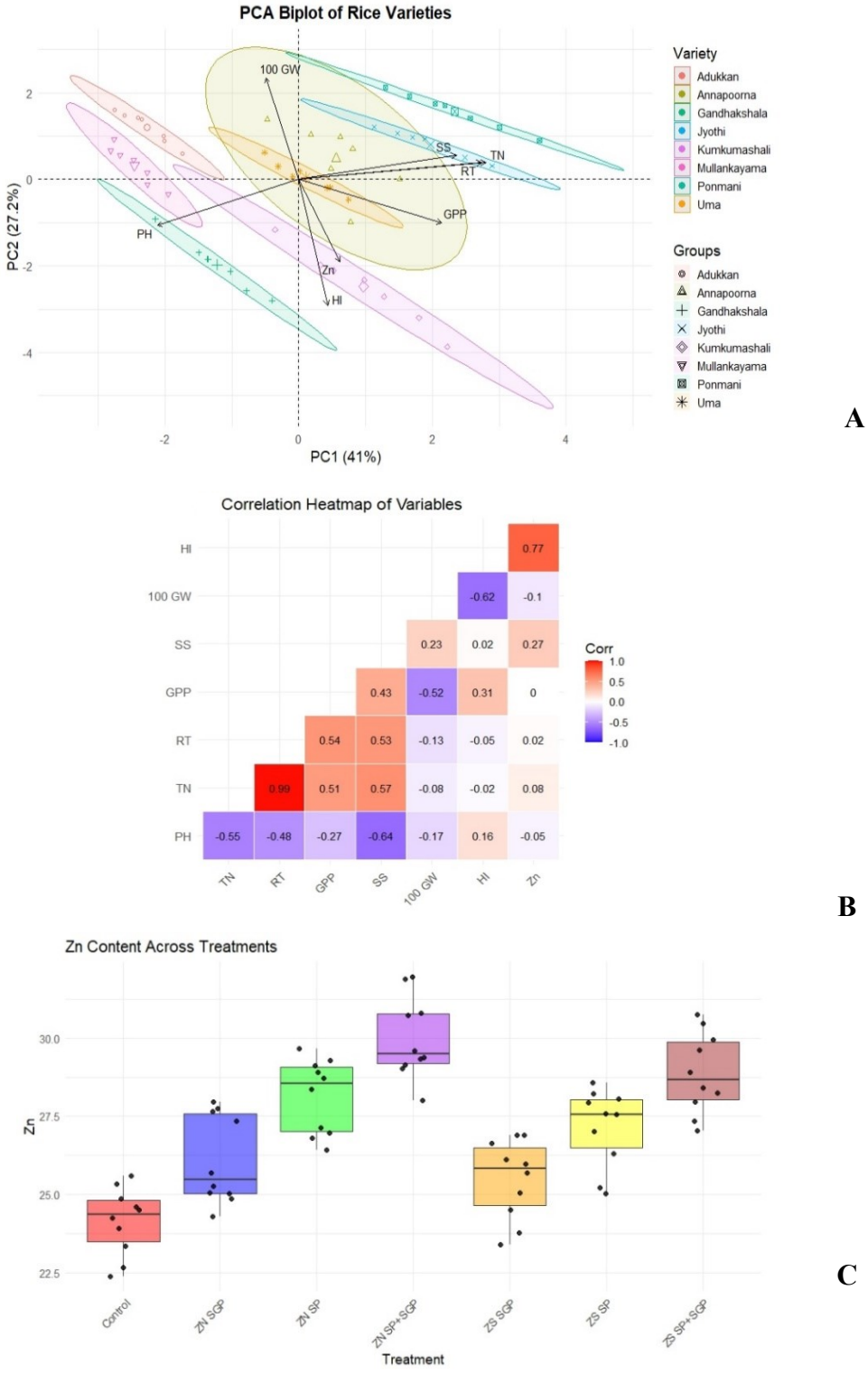


Figure 5. Principal component analysis (A), Correlation plot (B) for agronomic and yield parameters and grain Zn content in the studied rice varieties subjected to various priming treatments, Boxplot (C) showing variation in grain Zn content across various priming treatments. ZN SGP ($ZnNO_3$ seedling priming), ZN SP ($ZnNO_3$ seed priming), ZN SP + SGP ($ZnNO_3$ seed priming + seedling priming), ZS SGP ($ZnSO_4$ seedling priming), ZS SP ($ZnSO_4$ seed priming), ZS SP + SGP ($ZnSO_4$ seed priming + seedling priming)

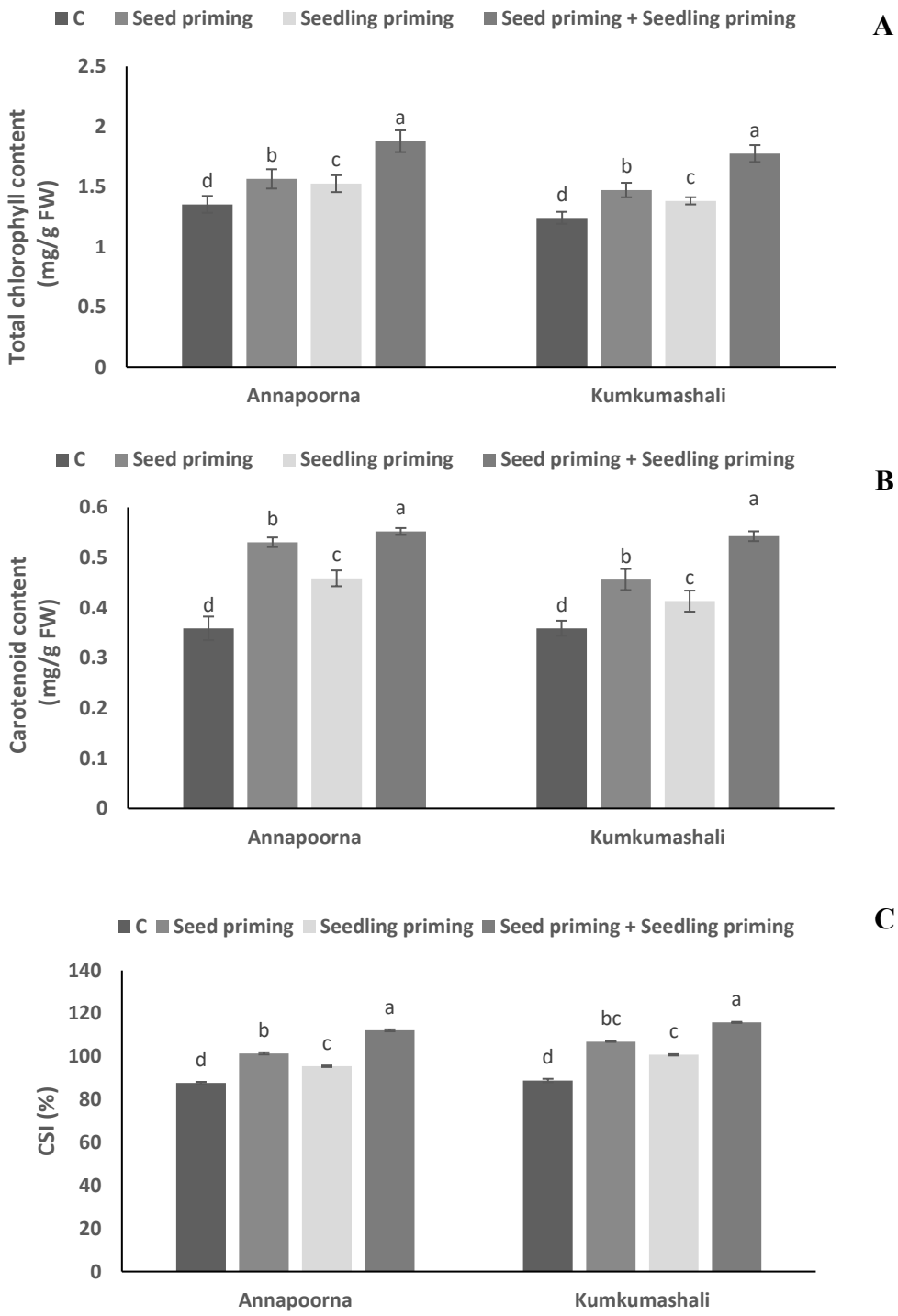


Figure 6. Total chlorophyll content (A), Carotenoid content (B), Chlorophyll stability index (CSI %) (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

was 52% in Kumkumashali and 43% in Annapoorna. The seed priming enhanced PSII activity in Annapoorna (17%) and Kumkumashali (18%). Similarly, seedling priming enhanced the PSII activity in both the studied rice varieties (8 to 10%) but the enhancement was higher during seed priming. The combined treatments of seed and seedling priming showed highest enhancement of PSII activity in Annapoorna by 38%, and Kumkumashali by 39% (Fig. 7).

4.4.1.4 **Chlorophyll *a* fluorescence**

The primary photochemistry was analysed using Chl *a* fluorescence parameters to evaluate any significant changes occurring because of various Zn treatments. The OJIP curve is a transient chlorophyll *a* fluorescence induction curve that reflects the sequential reduction of electron transport components in photosystem II (PSII) when a dark-adapted leaf is exposed to light. It consists of four distinct phases: O (origin), J (2–3 ms), I (30–50 ms), and P (peak), providing insights into photosynthetic efficiency, energy transfer, and stress responses in plants. Both the rice varieties under study showed a general trend on various treatments of Zn. There was an increase in F_0, and a substantial increase in fluorescence at O-J, J-I and I-P phases in all the treatments. This increase was highest in the combined application of seed priming and foliar spray of $ZnNO_3$. Priming with $ZnNO_3$ reduced initial fluorescence significantly compared to the control, indicating improved light-harvesting efficiency at the start. Priming enhanced light-harvesting efficiency (O phase), improved electron transport (J phase) and better energy use (I phase) (Fig. 8 & 9).

The radar plot illustrates the impact of several $ZnNO_3$ treatments on the fundamental and biophysical parameters of the JIP test. The performance indices ($PI_{(abs)}$ and $PI_{(csm)}$) are crucial metrics reflecting the vitality of the plant. The structural and functional stability of PSII can be ascribed to these characteristics, and in the treatments of seed and seedling priming with $ZnNO_3$, both indices were significantly elevated. The seed priming enhanced $PI_{(abs)}$ in Annapoorna and Kumkumashali by 211 and 138% respectively. In Annapoorna, the seedling priming and combined treatments of seed and seedling priming enhanced the $PI_{(abs)}$ by 74

and 203% respectively, while in Kumkumashali the enhancement was by 131 and 142% respectively. The $PI_{(csm)}$ was also enhanced by various treatments of $ZnNO_3$, in Annapoorna and it was enhanced by 280, 100 and 300% upon seed priming, seedling priming and combined treatment of seed and seedling priming. There was also an also an enhancement of $PI_{(csm)}$ in Kumkumashali upon application of $ZnNO_3$ via seed priming (210%), seedling priming (184%) and combined treatments of seed priming and seedling priming (233%). The maximal quantum yield for primary photochemistry (φPo), indicating the overall efficiency of PSII, was enhanced by all the priming treatments with $ZnNO_3$, whereas the quantum yield of energy dissipation (φDo) was diminished. All priming treatments enhanced the quantum yield for electron transport of the active PSII reaction center (φEo).

The seed priming, seedling priming and combined treatments of seed and seedling priming in Annapoorna enhanced φPo by 24, 15 and 25% respectively, whereas, φEo by 37, 15 and 38% respectively. However, φDo was decreased by 35, 23 and 37% upon seed priming, seedling priming and combined treatments of seed and seedling priming in Annapoorna plants. The primed plants of Kumkumashali also showed significant enhancement in φPo and φEo and reduction in φDo parameters, when compared to control plants. The seed priming enhanced φPo and φEo by 15 and 30% respectively, while decreased φDo by 30%. The seedling priming also showed an enhancement of 14 and 34%, for φPo and φEo respectively, whereas a 29% decrease was observed for φDo. The combined treatment of seed and seedling priming also showed increase in φPo and φEo by 15 and 35% respectively. There was a 30% decrease for φDo upon combined treatments of seed and seedling priming. Maximal fluorescence (Fm), was increased upon seed priming in Annapoorna and Kumkumashali by 22 and 30% respectively, whereas seedling priming showed a 15 and 23% increase respectively, which was lower than seed priming. The combined treatments of seed and seedling priming showed a significant enhancement in Fm by 32 % in Annapoorna and 37% in Kumkumashali (Fig. 10).

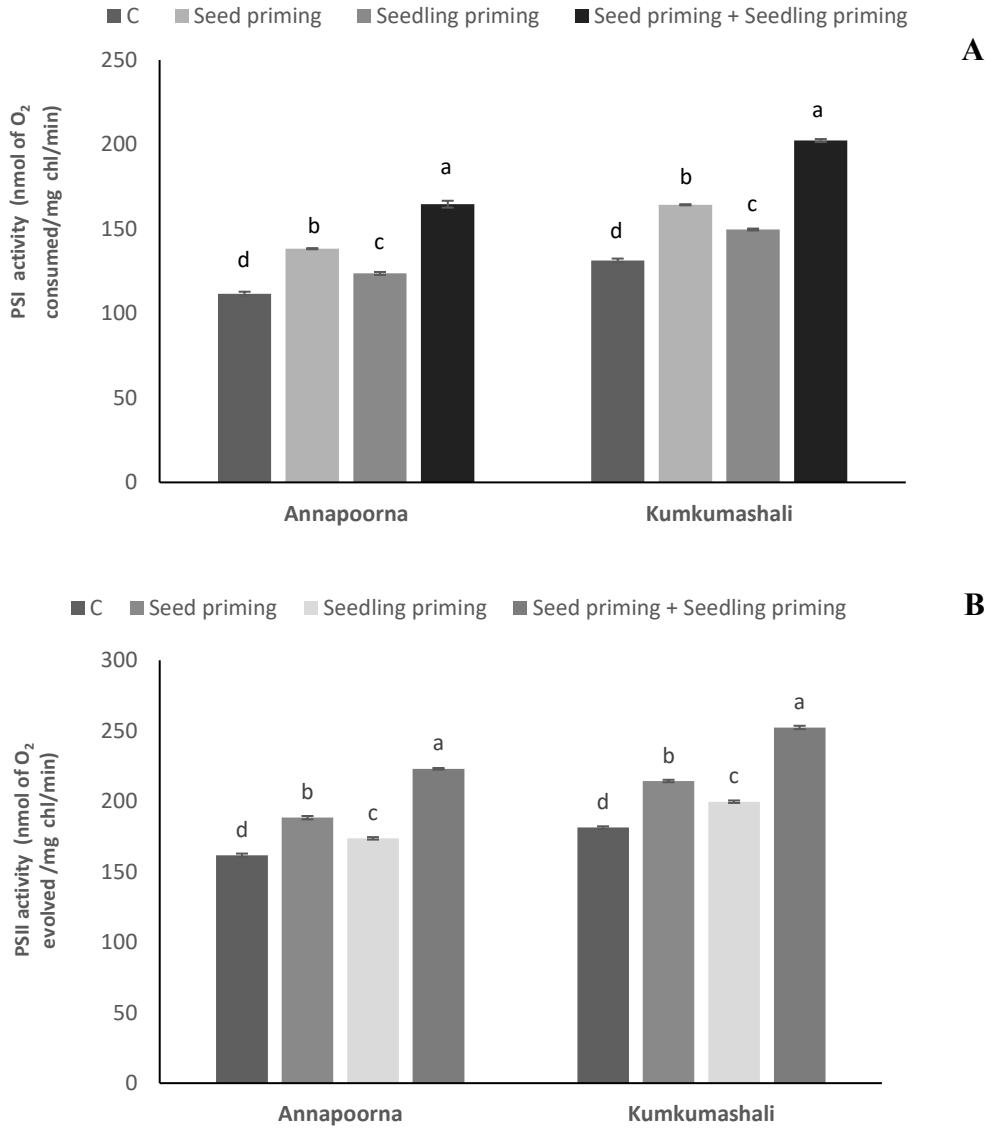


Figure 7. Photosystem I activity (A), Photosystem II activity (B) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

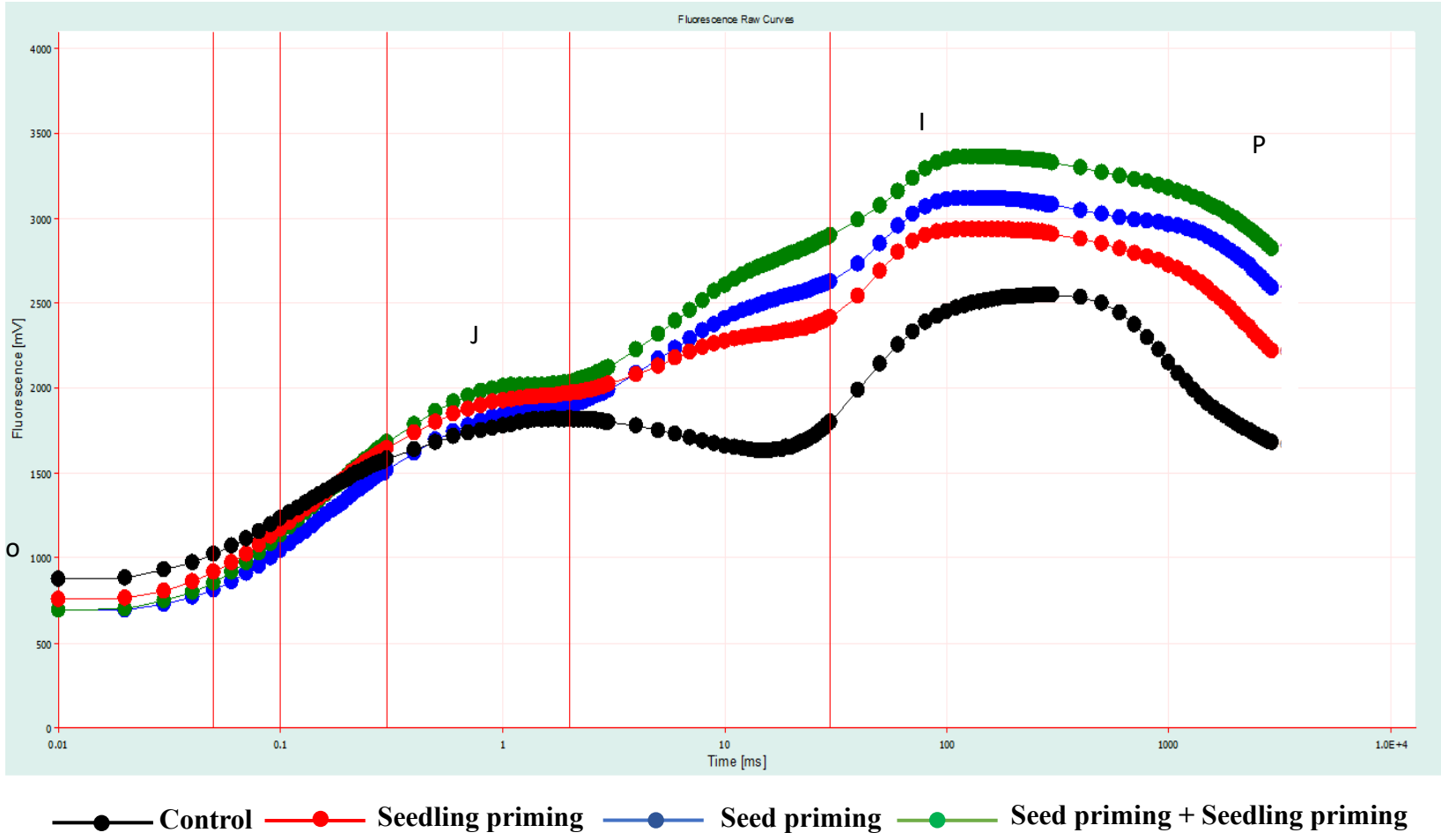


Figure 8. Chlorophyll *a* fluorescence transient curves measured in the leaf of Annapoorna subjected to various priming treatments with $ZnNO_3$

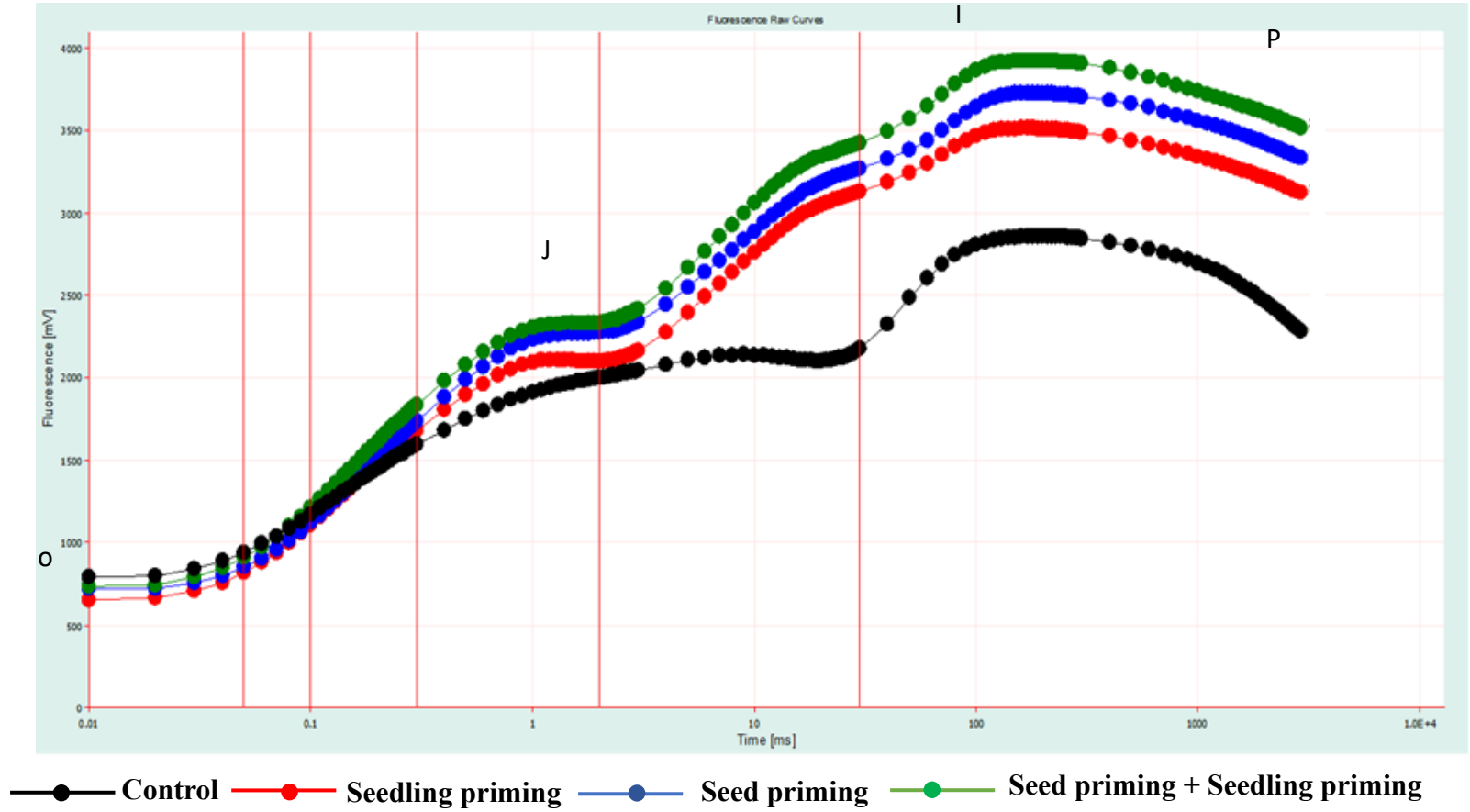


Figure 9. Chlorophyll *a* fluorescence transient curves measured in the leaf of Kumkumashali subjected to various priming treatments with $ZnNO_3$

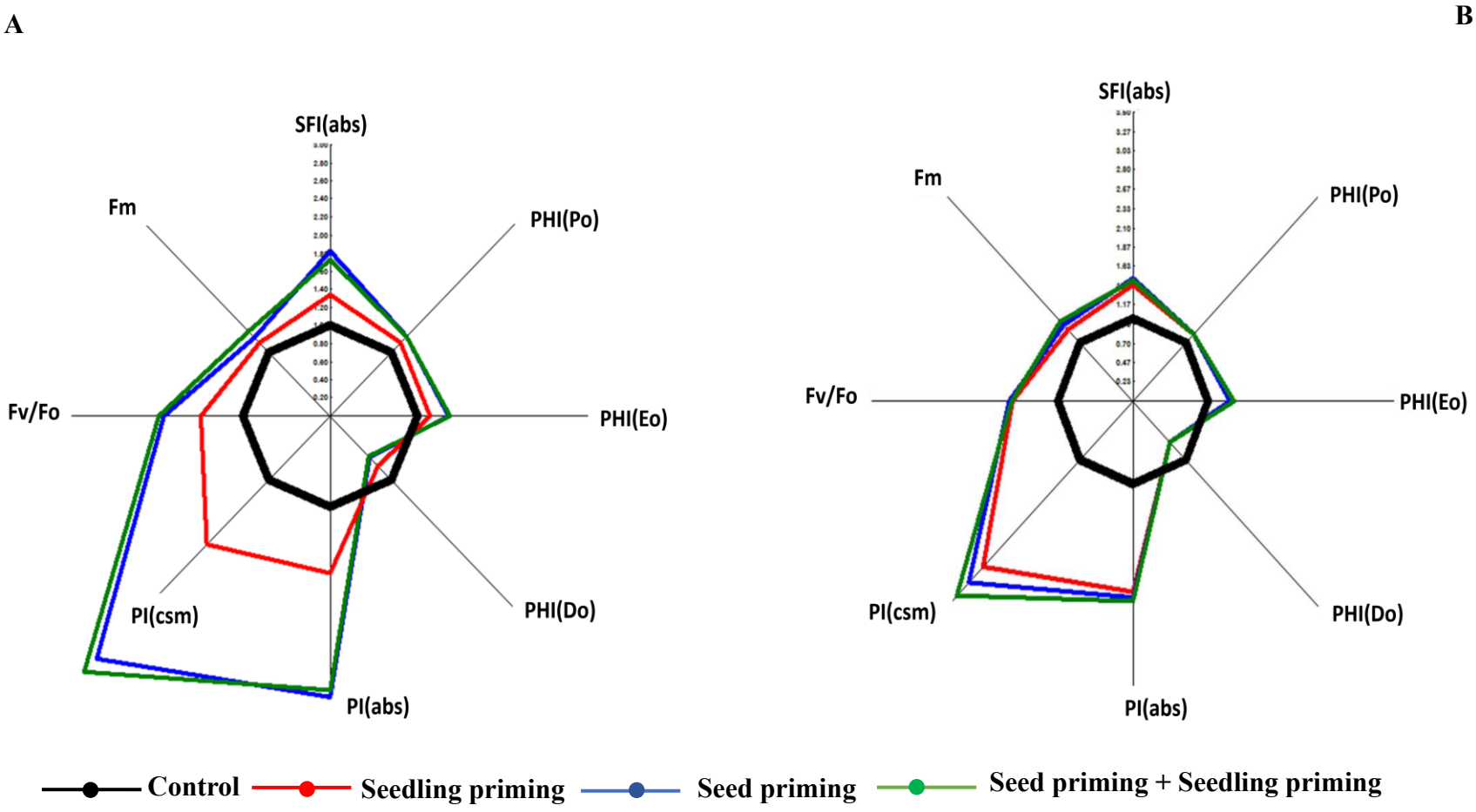


Figure 10. Radar plot of Annapoorna (A) and Kumkumashali (B) subjected to various priming treatments with $ZnNO_3$

The efficiency of the water-splitting complex (Fv/Fo) significantly increased in all plants exposed to $ZnNO_3$ priming treatments. In Annapoorna the Fv/Fo was increased by seed priming (91%), seedling priming (49%) and combined treatments of seed and seedling priming (97%), when compared to control plants. Likewise, in Kumkumashali, various treatments of $ZnNO_3$, enhanced Fv/Fo by 66%. The SFI, PSII structure function index also showed significant enhancement on various treatments of $ZnNO_3$. Annapoorna showed a significant enhancement in SFI by 82, 34 and 72% upon seed priming, seedling priming and combined treatments of seed and seedling priming respectively. An enhancement of 48, 41 and 46% was recorded in Kumkumashali following seed priming, seedling priming and combined treatments of seed and seedling priming. When compared to individual priming treatments, the highest enhancement in all these studied parameters were seen in combined priming treatments at seed and seedling stages (Fig. 10).

The energy pipeline leaf model of the photosynthetic apparatus was analysed to visualize the phenomenological energy fluxes per cross section of PSII upon various Zn priming methods in all the varieties under study. The results revealed that the various fluorescence parameters (ABS/CSm, TRo/CSm, ETo/CSm, DIo/CSm) had significant variation among various priming treatments of $ZnNO_3$ in primed plants, when compared to control plants. Among the various priming treatments, seedling priming showed the least, but significant enhancement in ABS/CSm, TRo/CSm and ETo/CSm in both the rice varieties under study. In Annapoorna, the enhancement was by 15, 33 and 33% respectively; while in Kumkumashali it was by 23, 41 and 65% respectively for ABS/CSm, TRo/CSm and ETo/CSm. The seed priming showed an enhancement in ABS/CSm by 22 and 30%, TRo/CSm by 51 and 50%, ETo/CSm by 67 and 69% respectively in Annapoorna and Kumkumashali. Across all the treatments, the combined treatment of seed and seedling priming showed the highest increment in ABS/CSm, TRo/CSm and ETo/CSm. In Annapoorna and Kumkumashali, the combined treatment enhanced ABS/CSm (32 and 37% respectively), TRo/CSm (65 and 57% respectively) and ETo/CSm (83 and 85% respectively), in primed plants, on comparison with control plants. Under various priming treatments with $ZnNO_3$, the DIo/CSm decreased, indicating

effective energy utilization for photochemistry and less wastage in the form of dissipated energy. The seed priming, seedling priming and combined treatments of seed and seedling priming, decreased DIo/CSm by 21, 11 and 16% respectively in Annapoorna, and by 10, 13 and 4% in Kumkumashali. The rice varieties taken for study, Annapoorna and Kumkumashali could very well absorb and trap the energy but Kumkumashali utilises the absorbed energy more efficiently in electron transport (Fig. 11 & 12).

4.4.1.5 Leaf gas exchange parameters

The various treatments of Zn had significant effect on the photosynthetic rate (Pn), transpiration (E) and stomatal conductance (gs) in the rice plants taken for study. In Annapoorna, seed priming with $ZnNO_3$ enhanced the Pn by 24%. Seedling priming showed a moderate increase, when compared to untreated control plants. The most effective treatment was combined treatments of seed and seedling priming and showed the highest increment (46%). In Kumkumashali the seed priming (28%) increased the Pn in primed plants when compared to control plants. The seedling priming also showed a moderate enhancement in Pn (11%) when compared to control plants. The combined treatments of seed and seedling priming had a considerable enhancement in Pn (51%). Out of the all treatments, the combined priming application of $ZnNO_3$ at seed and seedling stages showed a significant increase of Pn in both the rice varieties (Fig. 13)

Seed priming with $ZnNO_3$ enhanced Gs in Annapoorna (21%) and Kumkumashali (28%). The Gs enhancement was moderate upon seedling priming in Annapoorna (11%) and Kumkumashali (18%). In an overview, the highest increment of Gs was obtained in combined treatments of seed and seedling priming in both the rice varieties, and it was by 44% in Annapoorna, and 45% in Kumkumashali. The various treatments of Zn priming had an impact on the E of all the rice varieties under study. In Annapoorna, the seed priming, seedling priming and combined priming treatments with $ZnNO_3$ enhanced the E by 29, 15 and 49% respectively. While compared to Annapoorna, Kumkumashali was more efficient and the enhancement of E in primed plants was by 35% with seed priming, 16%

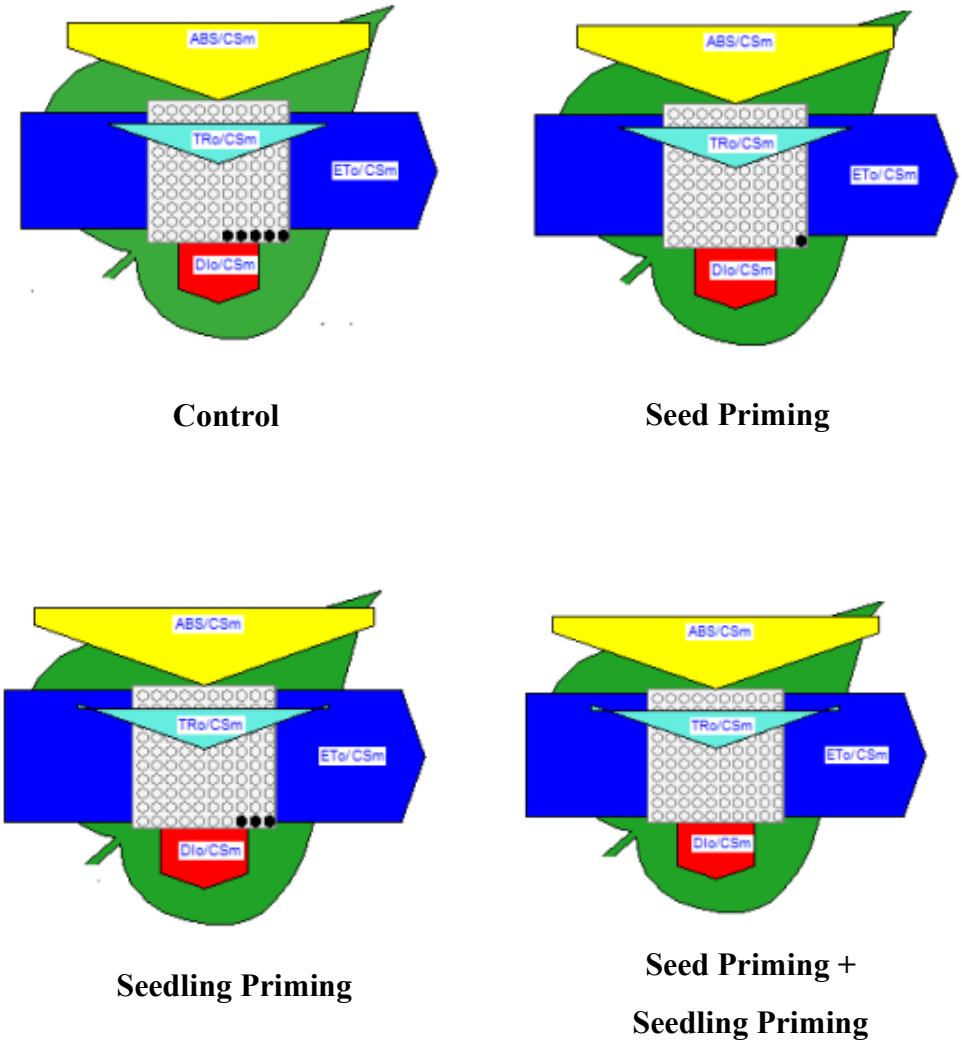


Figure 11. Leaf pipeline model showing the proportion of phenomenological energy flux parameters (calculated per cross section) in Annapoorna subjected to various priming treatments with $ZnNO_3$

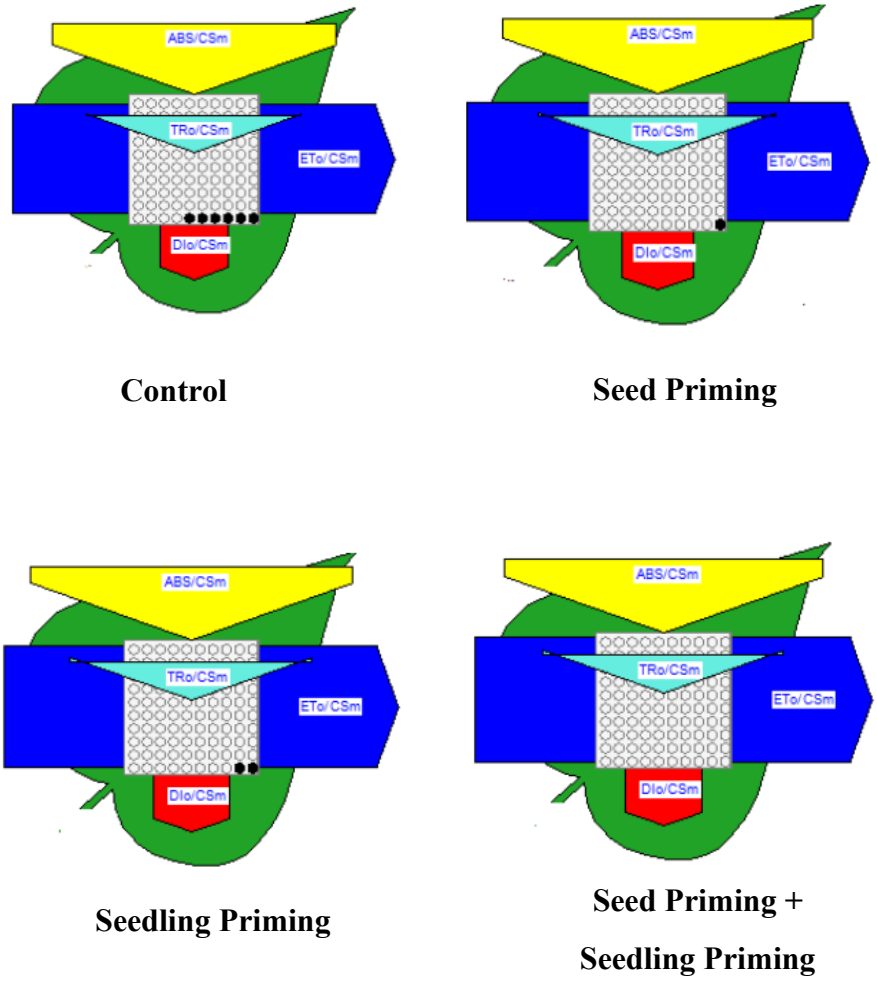


Figure 12. Leaf pipeline model showing the proportion of phenomenological energy flux parameters (calculated per cross section) in Kumkumashali subjected to various priming treatments with $ZnNO_3$

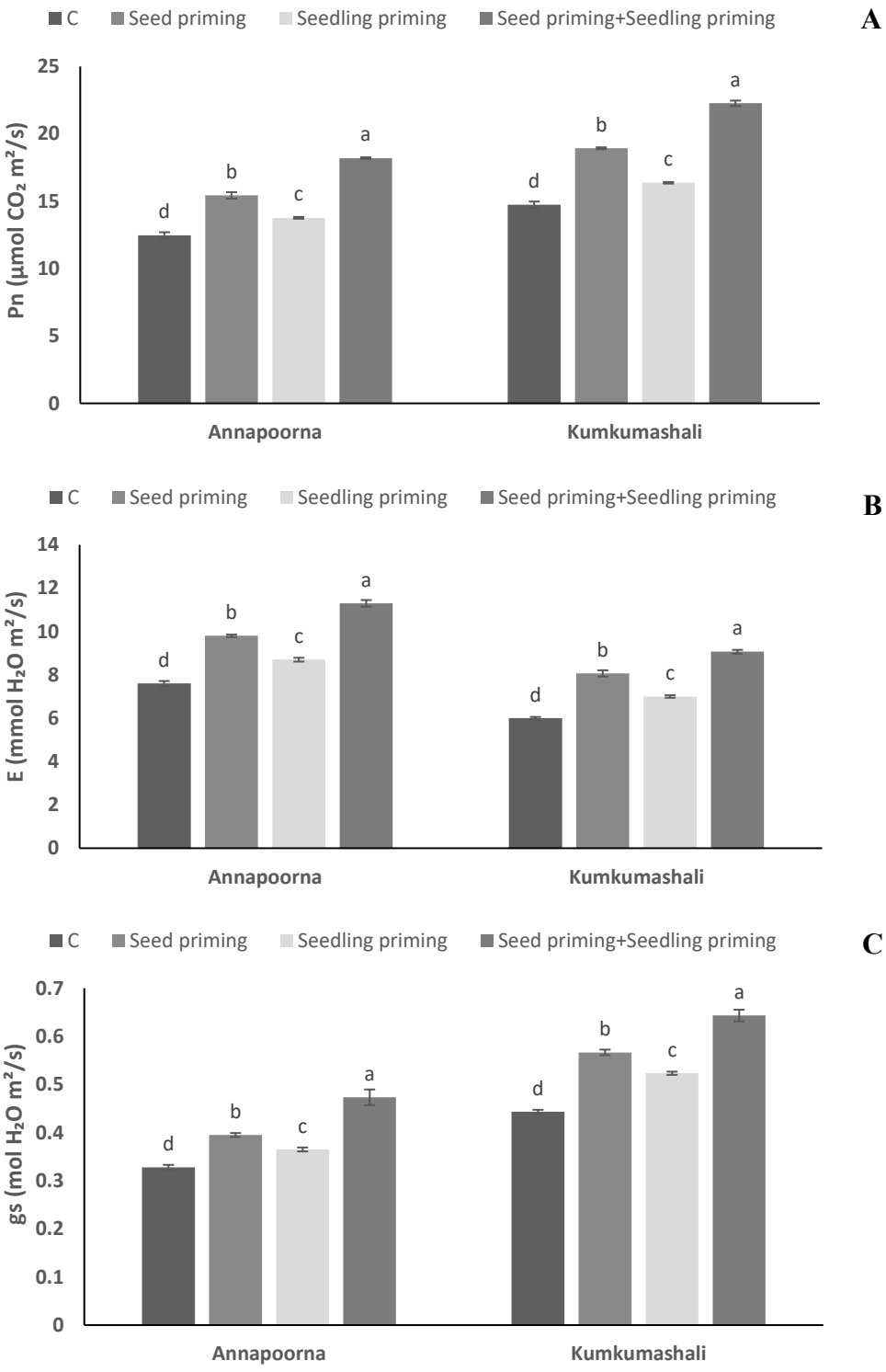


Figure 13. Photosynthetic rate (Pn) (A), Transpiration rate (E) (B) and stomatal conductance (gs) (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

with seedling priming and 51% with combined treatments of seed and seedling primings (Fig. 13).

4.4.2 The effect of seed priming and seedling priming on antioxidant mechanism

4.4.2.1 Enzymatic antioxidants

4.4.2.1.1 SOD activity

A consistent enhancement in SOD activity was seen in the rice varieties under study on various priming treatments with Zn, when compared to untreated plants. In Annapoorna, the seed priming, and combined treatments of seed and seedling priming increased SOD activity by 20 and 36% respectively. Kumkumashali responded most significantly to all the treatments. Seed priming and seedling priming enhanced the SOD activity, by 21% and 13% respectively in primed plants, when compared to control plants. However, the increase was much more (48%), when combined treatments of seed and seedling priming were carried out (Fig. 14).

4.4.2.1.2 GPOX activity

Kumkumashali demonstrated the most significant enhancement in GPOX activity, and the combined application of seed and seedling priming showed the highest enhancement (56%), followed by Annapoorna (52%). The seed priming increased GPOX activity in primed plants of Annapoorna (19%) and Kumkumashali (24%), when compared to control plants and it was lesser than the combined treatment. The seedling priming exhibited the lowest increment in GPOX activity (14%) when compared to control plants in both the rice varieties (Fig. 14).

4.4.2.1.3 APX activity

Seed priming resulted in an enhancement of APX activity in Annapoorna (17%) and Kumkumashali (19%) on comparison with control plants. When seedling was primed, the APX activity increased to a lesser extent (9%) in primed plants, when compared to untreated control plants. The highest enhancement in APX

activity was observed on combined application of seed and seedling priming, in both the rice varieties under study and the increase was 43% in Annapoorna and 48% in Kumkumashali (Fig. 14).

4.4.2.1.4 CAT activity

The most significant enhancement in CAT activity was shown by Kumkumashali (37%) and Annapoorna (28%) upon combined application of seed and seedling priming when compared to control plants. The seed priming also enhanced CAT activity in Annapoorna (16%) and Kumkumashali (16%) relative to control plants. The least increase in CAT activity was seen during seedling priming in both the rice varieties under study (10%) (Fig. 14).

4.4.3 Non-enzymatic antioxidants

4.4.3.1 Ascorbate content

Seed priming enhanced ascorbate content in Annapoorna by 13%. The seedling priming also enhanced ascorbate content, but was lower than seed priming and it was only by 6%. The highest enhancement in ascorbate content was seen under combined treatments of seed and seedling priming (23%). Kumkumashali was highly responsive to all the treatments, when compared to Annapoorna. Seed priming, seedling priming and combined treatments of seed and seedling priming increased the ascorbate content by 13, 8 and 30% respectively in primed plants when compared to untreated plants. Comparing all the treatments, the combined application of seed and seedling priming generated better enhancement in ascorbate content in both the rice plants (Fig. 15).

4.4.3.2 Glutathione content

The Glutathione content in primed plants was enhanced when compared to control plants. Across all the treatments, seedling priming showed the least enhancement in glutathione content, and it was 12% in Kumkumashali, followed by Annapoorna (10%). In Annapoorna and Kumkumashali, combined treatments of

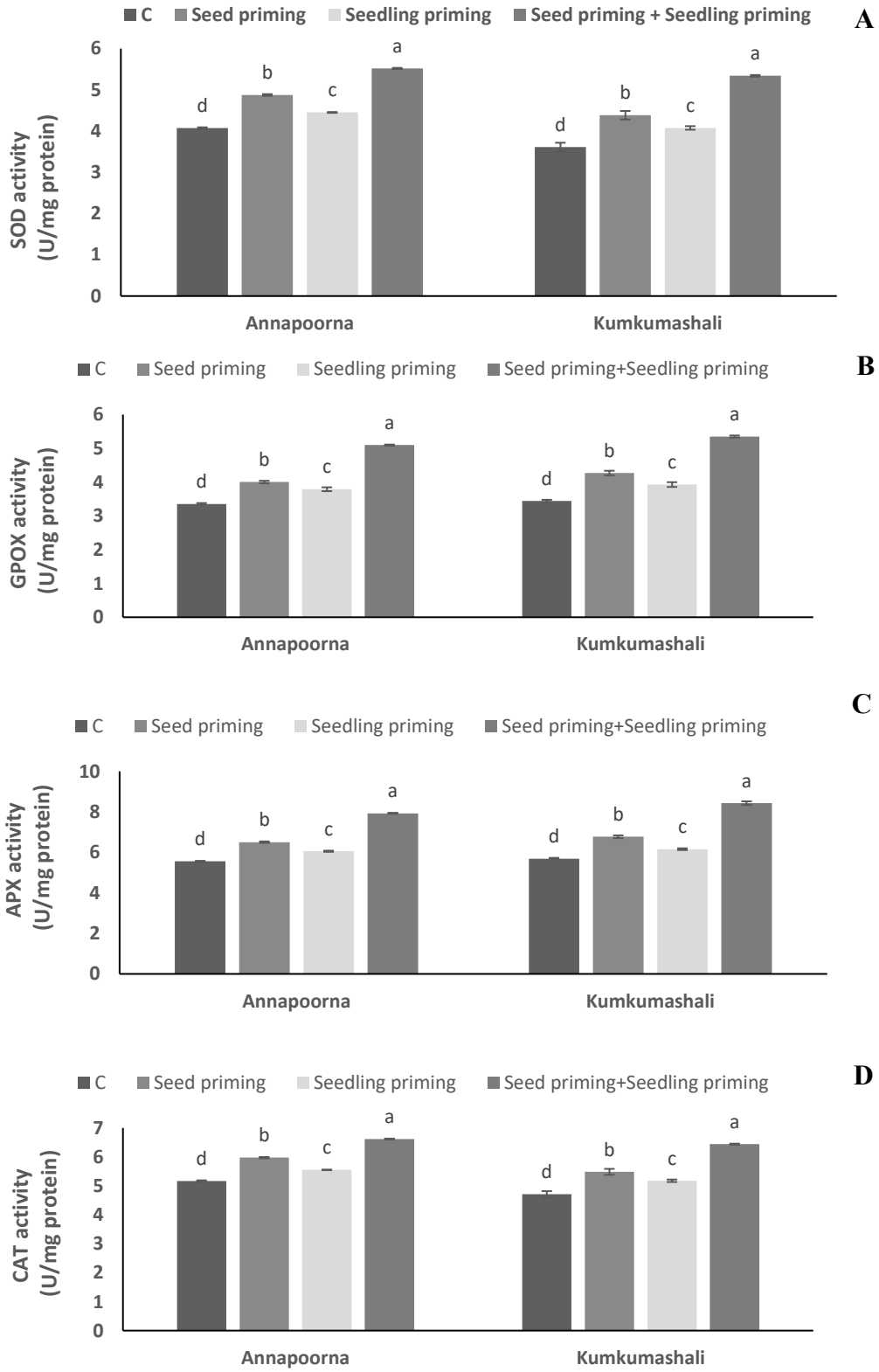


Figure 14. SOD (A), GPOX (B), APX (C) and CAT (D) activities in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

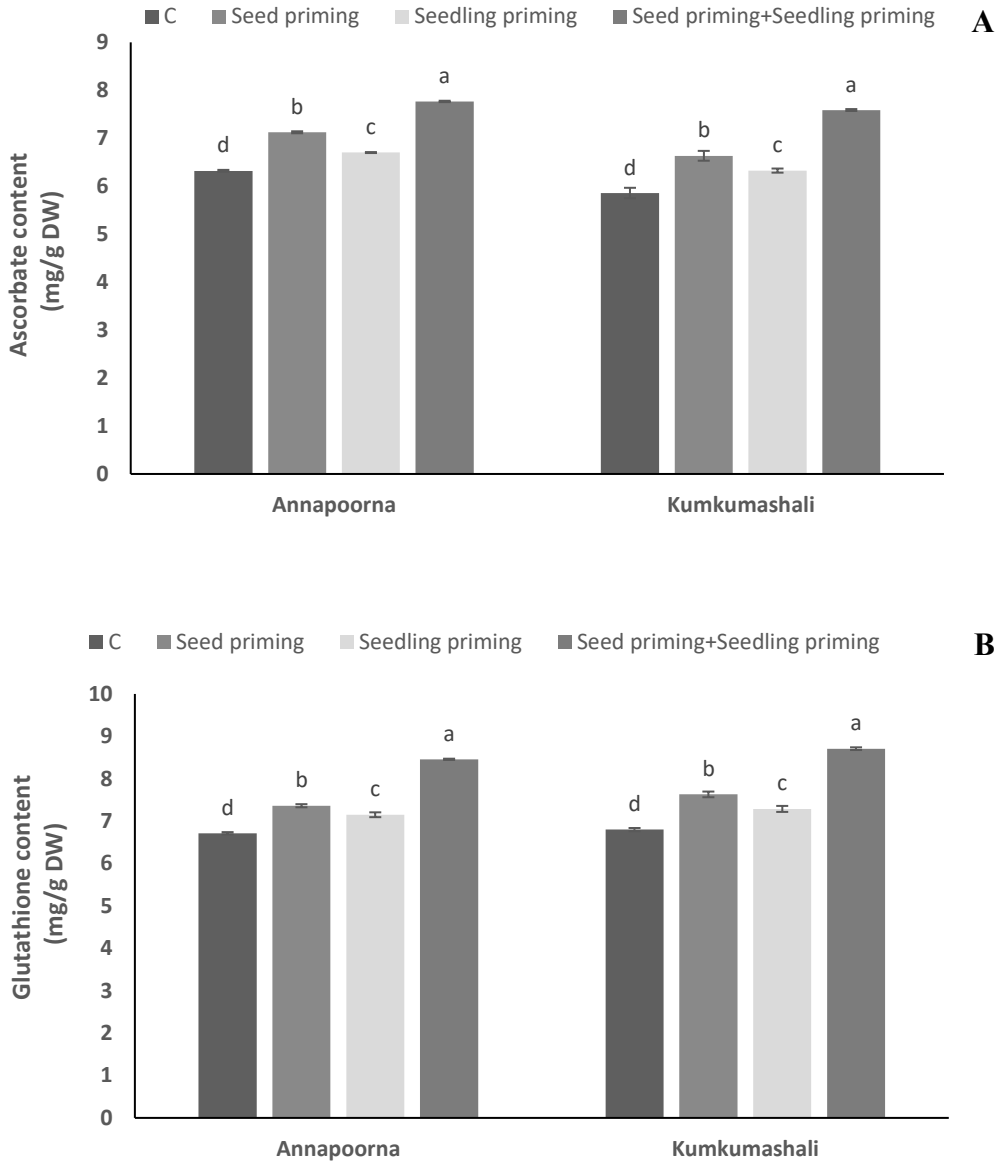


Figure 15. Ascorbate content (A), Glutathione content (B) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

seed and seedling priming enhanced the glutathione content to 26% and 28% respectively in comparison to control plants (Fig. 15).

4.4.3.3 Total phenolics content

Seed priming in Annapoorna and Kumkumashali enhanced total phenolics content to the extent of 13% and 14% respectively. Seedling priming enhanced total phenolics content in Annapoorna, and Kumkumashali to a lesser extent. The highest increment in total phenolics was observed in combined priming treatments at seed and seedling stages in Kumkumashali (34%) followed by Annapoorna (22%) (Fig. 16).

4.4.3.4 Flavonoids

The flavonoids content was enhanced by the Zn priming methods in the rice varieties with least increment recorded in plants subjected to seedling priming and highest increment in plants subjected to combined treatments of both seed and seedling priming. In Annapoorna, the seed priming (16%) and combined application of seed and seedling priming (38%) enhanced flavonoid content in primed plants, on comparing with control plants. Likewise, in Kumkumashali, application of Zn as seed priming (16%) and in combination with seed and seedling priming (47%) enhanced flavonoid accumulation. However, in both these varieties, the seedling priming with the $ZnNO_3$ also enhanced the flavonoid content, but to a lesser extent, than those observed in earlier cases (Fig. 16).

4.4.3.5 Anthocyanin content

Maximum enhancement in the anthocyanin content was recorded in the combined treatments of seed and seedling priming with $ZnNO_3$ when compared to control in the studied rice varieties. Kumkumashali showed the highest increment in anthocycanin content with a significant increase of 46%, followed by Annapoorna (38%). However, seed priming showed lesser increase of anthocyanin content in Annapoorna (21%) and Kumkumashali (25%), when compared to control plants. Eventhough seedling priming also enhanced the anthocyanin content in all the

studied rice varieties, but it was to a lesser extent, when compared to seed priming and combined treatments of both priming methods (Fig. 16).

4.4.4 The effect of seed priming and seedling priming on oxidative damage and membrane integrity

4.4.4.1 Superoxide content

Superoxide content decreased in all the treatments in both the rice varieties under study, when compared to control plants. In Annapoorna, the superoxide level decreased significantly in all the treatments, with the highest decrease in combined treatments of seed and seedling priming (40%). The seed priming and seedling priming (7 to 15%) also showed a decrease in superoxide content in primed plants on comparison with untreated plants. In Kumkumashali, across all the Zn treatments, combined treatments of seed and seedling priming (45%) showed substantial decrease in superoxide content. Seed priming with $ZnNO_3$ (18%) also showed decrease in superoxide content in primed plants, but was lower than combined treatment. While seedling priming showed the least reduction across all the priming treatments (Fig. 17).

4.4.4.2 Hydrogen peroxide content

The various treatments with $ZnNO_3$ reduced the hydrogen peroxide (H_2O_2) content in the rice varieties under study. Across the various treatments of Zn, the most substantial reduction in H_2O_2 was seen in combined treatments of seed and seedling priming. The seed priming and combined treatment of seed and seedling priming reduced H_2O_2 content in Annapoorna (13 and 35%) and Kumkumashali (16 and 41%), when compared to non-primed plants. In all the rice varieties, the seedling priming with $ZnNO_3$ showed least reduction in H_2O_2 content (Fig. 17).

4.4.4.3 Malondialdehyde content (MDA)

Both the rice varieties showed a decrease in malondialdehyde (MDA) content across all the $ZnNO_3$ treatments. Kumkumashali showed the highest decrease (44%) followed by Annapoorna (38%) upon combined treatments of seed

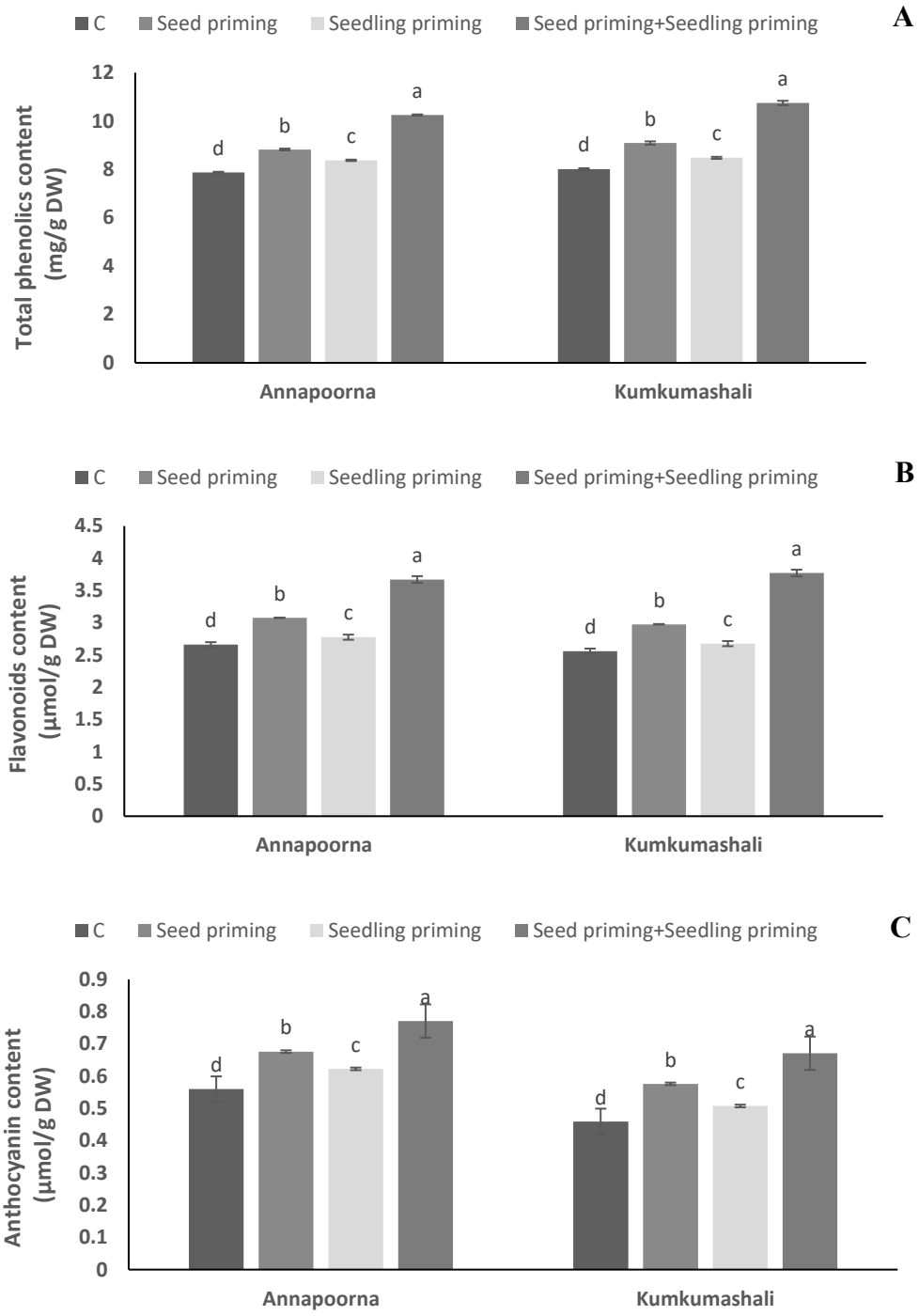


Figure 16. Total phenolics (A), Flavonoids (B) and Anthocyanin content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

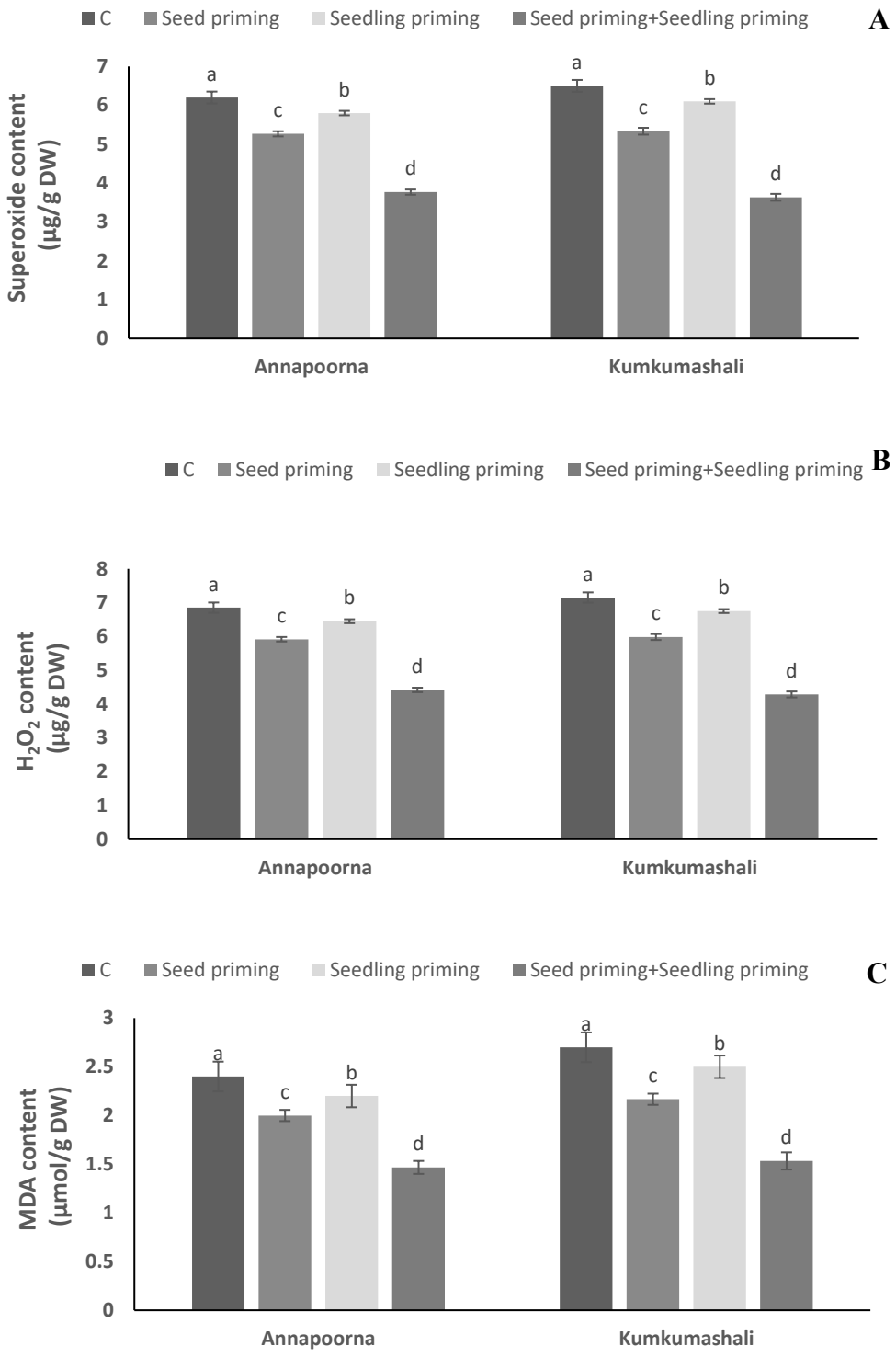


Figure 17. Superoxide content (A), H_2O_2 content (B) and MDA content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

and seedling priming. Seed priming also showed significant decrease in MDA content, but was lesser than combined priming and it was by 17% in Annapoorna and 20% Kumkumashali. When compared to other treatments, seedling priming had lesser impact (Fig. 17).

4.4.4.4 Electrolyte leakage

The electrolyte leakage was reduced in the rice varieties under study due to various treatments of $ZnNO_3$. Seed priming and combined treatments of seed and seedling priming gave the best results, while seedling priming alone had least effect. The combined application causes the highest reduction of electrolyte leakage in the rice varieties under study. This reduction was more profound in Kumkumashali (35%), followed by Annapoorna (31%). The seedling priming reduced electrolyte leakage in Annapoorna (8%) and Kumkumashali (10%) to only a lesser extent. However, the seed priming reduced the electrolyte leakage content in Annapoorna by 17% and Kumkumashali by 20%, when compared to untreated plants (Fig. 18).

4.4.4.4.5 Membrane stability index

All treatments with $ZnNO_3$ showed considerable increase in membrane stability index of the primed rice varieties under study on comparison with control plants. Kumkumashali consistently showed the highest increase in membrane stability index under all $ZnNO_3$ treatments, with the highest enhancement recorded in combined treatments of seed and seedling priming (34%) and Annapoorna closely follows Kumkumashali, with an increase of 32%. Seed priming also increased membrane stability index in Annapoorna (18%) and Kumkumashali (19%), when compared to control plants, but to a lesser extent. The seedling priming showed least increment in membrane stability, compared to other treatments (Fig. 18).

4.4.5 The effect of seed priming and seedling priming on metabolites

4.4.5.1 Total soluble sugars

Priming with $ZnNO_3$ enhanced the amount of total soluble sugars in primed plants when compared to control plants. Moreover, this enhancement was highest in combined treatments of seed and seedling priming, and it was by 33% in

Annapoorna and 35% in Kumkumashali, when compared to individual treatments. The seed priming also increased the soluble sugars content in Annapoorna (19%) and Kumkumashali (21%), while seedling priming showed least increment (Fig. 19).

4.4.5.2 Total free amino acids

The free amino acids content enhanced in the rice varieties upon different priming treatments of $ZnNO_3$, when compared to control plants. Out of the two rice varieties under study, Kumkumashali showed the highest increase in free amino acids content under combined treatments of seed and seedling priming (33%) followed by Annapoorna (29%). Among seed priming, seedling priming and combined treatments of seed and seedling priming, the least increment was shown in plants subjected to seedling priming. Seed priming improved free amino acids content in Annapoorna by 11% and Kumkumashali by 13% (Fig. 19).

4.4.5.3 Total protein

The total protein content was enhanced in rice varieties subjected to different priming treatments in both the rice varieties compared to control plants. The highest enhancement was recorded in combined treatments of seed and seedling priming. The seedling priming enhanced total protein content to a lesser extent (10 to 13%), while seed priming elevated total protein content by 16% and 19% in primed plants of Annapoorna and Kumkumashali respectively, when compared to control plants. In Annapoorna, the highest increase in total protein content was observed under combined treatments of seed and seedling priming (37%). Kumkumashali also showed a significant enhancement in protein content upon combined treatments of seed and seedling priming (44%), when compared to untreated control plants (Fig. 19).

4.4.6 Principal component and correlation analysis

The PCA plots represents the relationships between treatments, varieties and different physiological traits. The first component represents 78.5% variability, while the second component represents 15.2% variability and together, total variance is 93.7%. Traits located towards the right (positive Dim1) (e.g., total

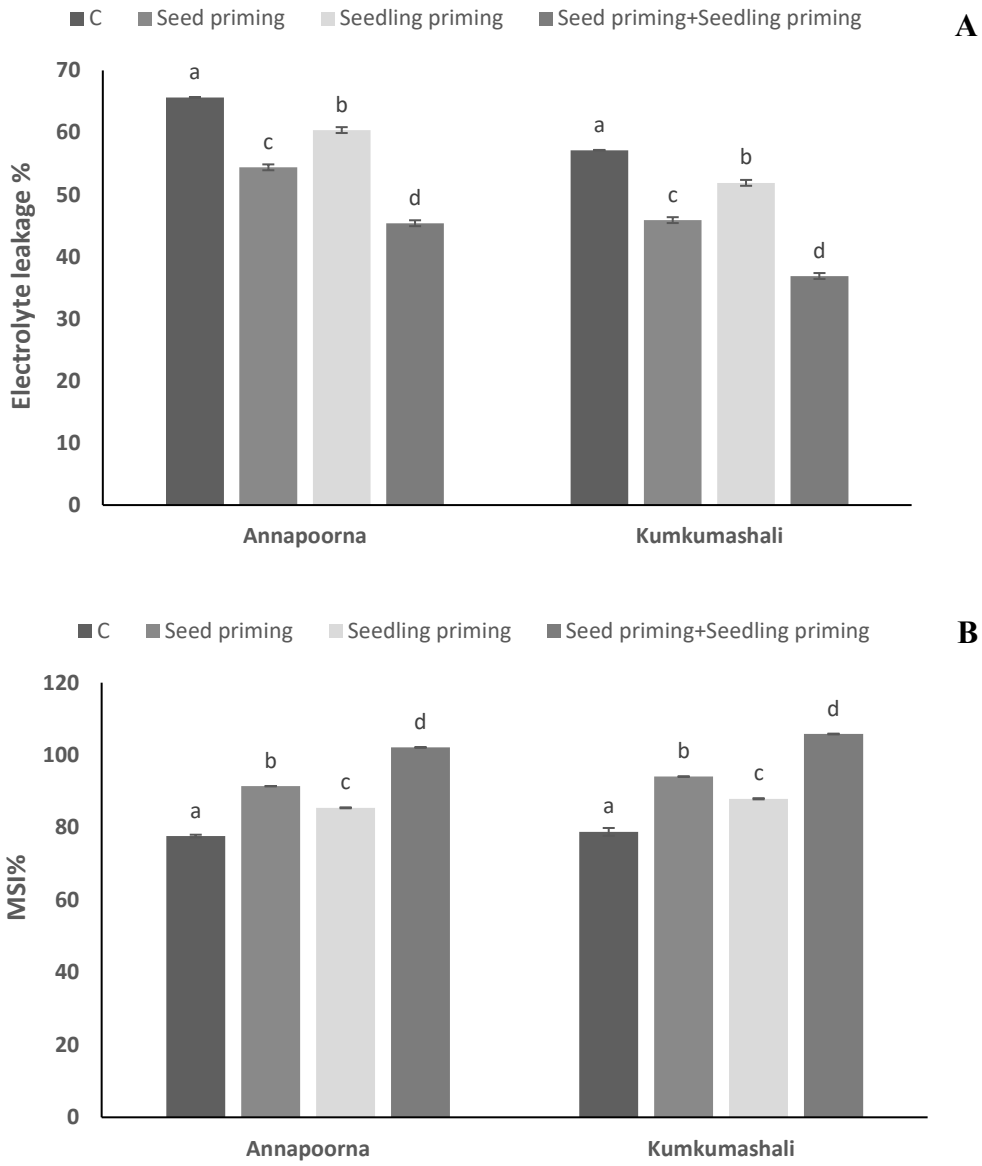


Figure 18. Electrolyte leakage (A) and Membrane stability index (B) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

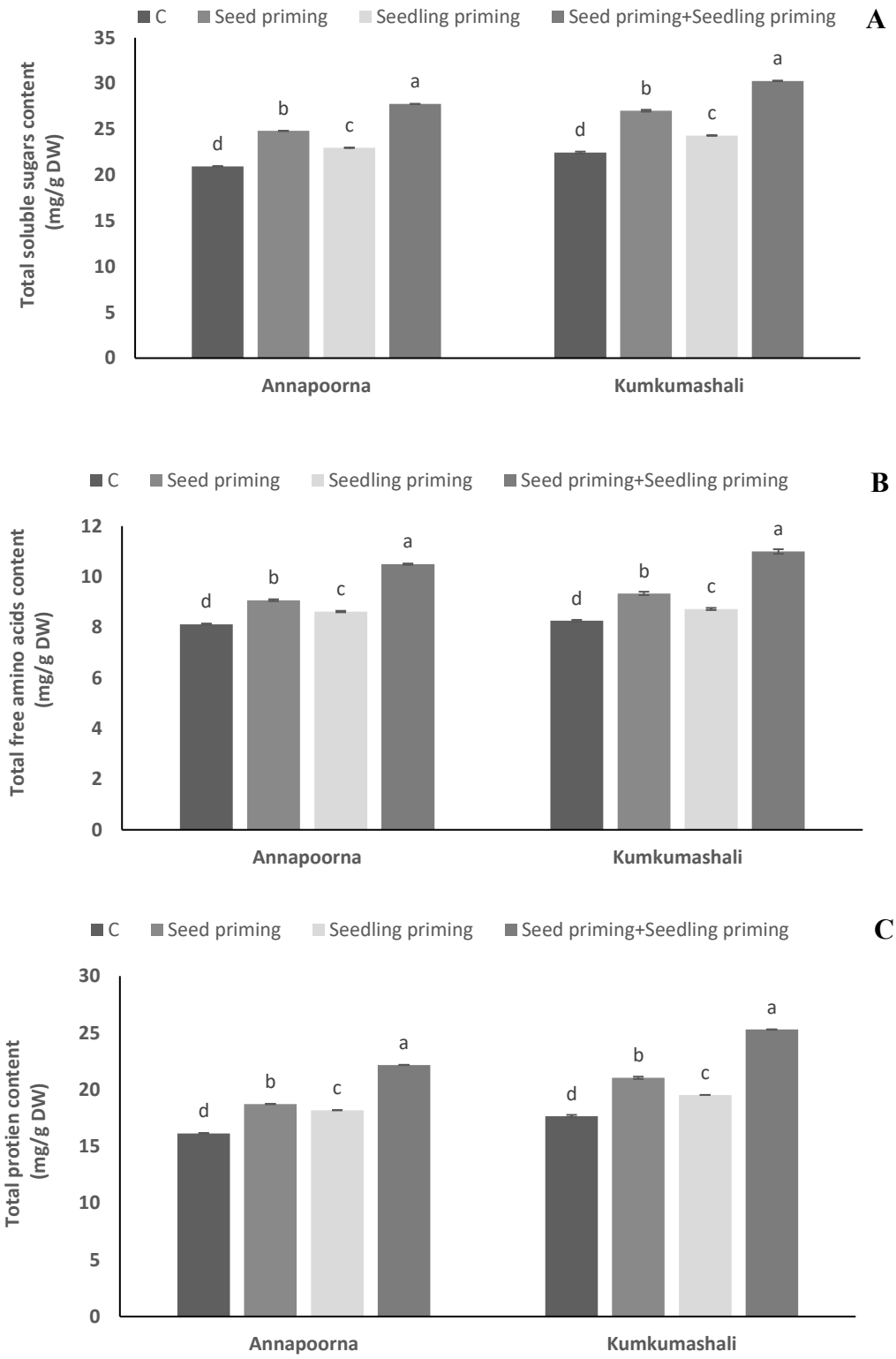


Figure 19. Total soluble sugars (A), Total Free amino acids (B) and Total protein content (C) in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

chlorophyll, anthocyanin, ascorbate, carotenoids, flavonoids, SOD, CAT, APX, phenolics, glutathione, PSII, PSI, sugar, MSI, etc.) are positively correlated with better physiological performance. Traits on the left side (e.g., MDA, H_2O_2, SO, EL, DIo/CSm, PHI(Do)) are related to detrimental effects. Kumkumashali is mainly clustered towards the right, where most beneficial traits (photosynthetic efficiency, antioxidant activity, sugar, protein, glutathione, etc.) are located. This suggests that Kumkumashali responds better to treatments, exhibiting better physiological performance. Annapoorna is more towards the left, where MDA, H_2O_2, SO, EL are located. This suggests that Annapoorna performs less efficiently compared to Kumkumashali. Treatments that shift data points to the right (e.g., seed priming (SP) and combined treatments of seed and seedling priming (SP+SGP) in Kumkumashali) appear to be the most effective in improving plant health and performance. Control and seedling priming (C, SGP) tend to be more on the left, suggesting lower efficiency (Fig. 20 & 21).

Red regions (high positive correlation) indicate strong associations between traits that improve plant performance. Photosynthetic efficiency (PSI, PSII, Pn, Fv/Fo) correlates well with antioxidants (APX, SOD, CAT, Glutathione) and sugar metabolism. These relationships suggest that better photosynthesis leads to enhanced metabolic efficiency. Blue regions (negative correlation) highlight oxidative damage (MDA, EL, H_2O_2, SO) that negatively affect growth traits. Kumkumashali outperforms Annapoorna, showing strong associations with photosynthesis, antioxidants, and sugar metabolism (Fig. 20 & 21).

4.5 The effect of seed priming, seedling priming and additional foliar spray at different reproductive stages (booting, flowering, milky stage) on zinc content, phytic acid, phytic acid to zinc molar ratio and bioavailable zinc

Foliar sprays of $ZnNO_3$ were applied to two rice varieties during the reproductive phases, specifically at the booting, flowering, and milky stages of rice growth. Here additional to seed priming, seedling priming and combined treatments of seed and seedling priming, additional foliar spray during each of the three reproductive stages (booting + flowering + milky stage) were administrated.

Subsequently, investigated the Zn content, phytic acid (PA) content, phytic acid to Zn molar ratio, and bioavailable Zn under each treatment to identify the most efficacious method for biofortification.

4.5.1 Zinc content

Priming and administration of foliar spray at reproductive stages had an impact on the Zn present in the grain. There was higher enhancement in Zn content in the grain, subjected to combined treatments of seed and seedling priming, while seedling priming alone gave the least increment in grain Zn content. In Annapoorna, seed priming along with additional foliar spray at reproductive stages displayed significant increment in grain Zn content by 34% when compared to control plants. While with seedling priming it was only 17%, and was much lesser than seed priming. Furthermore, the combined seed and seedling priming in conjunction with foliar spray at reproductive stages showed the highest increment in grain Zn content (47%) (Table 24).

In Kumkumashali, the foliar application of $ZnNO_3$ during all the stages of reproduction on plants raised from seed priming showed a 34% enhancement in grain Zn content, while the seedling primed plants showed the least enhancement (17%) and combined seed and seedling priming showed the highest enhancement (48%) in grain Zn content when compared to control plants (Table 24).

4.5.2 Phytic acid (PA) content

Priming with $ZnNO_3$ compounds during various stages of reproduction sufficiently reduced the phytic acid (PA) content in rice grains. In Annapoorna, seed priming along with foliar spray at booting + flowering + milky stages resulted in 20% lowering of grain PA content when compared to control plants. Similarly, the grain PA content was decreased upon seedling priming together with foliar spray, but this decrease was very low compared to other priming treatments and it was decreased only by 10%. Furthermore, the combined seed and seedling priming with $ZnNO_3$ along with foliar spray with $ZnNO_3$ at booting + flowering + milky stages showed the highest reduction in grain PA content by 25% (Table 24).

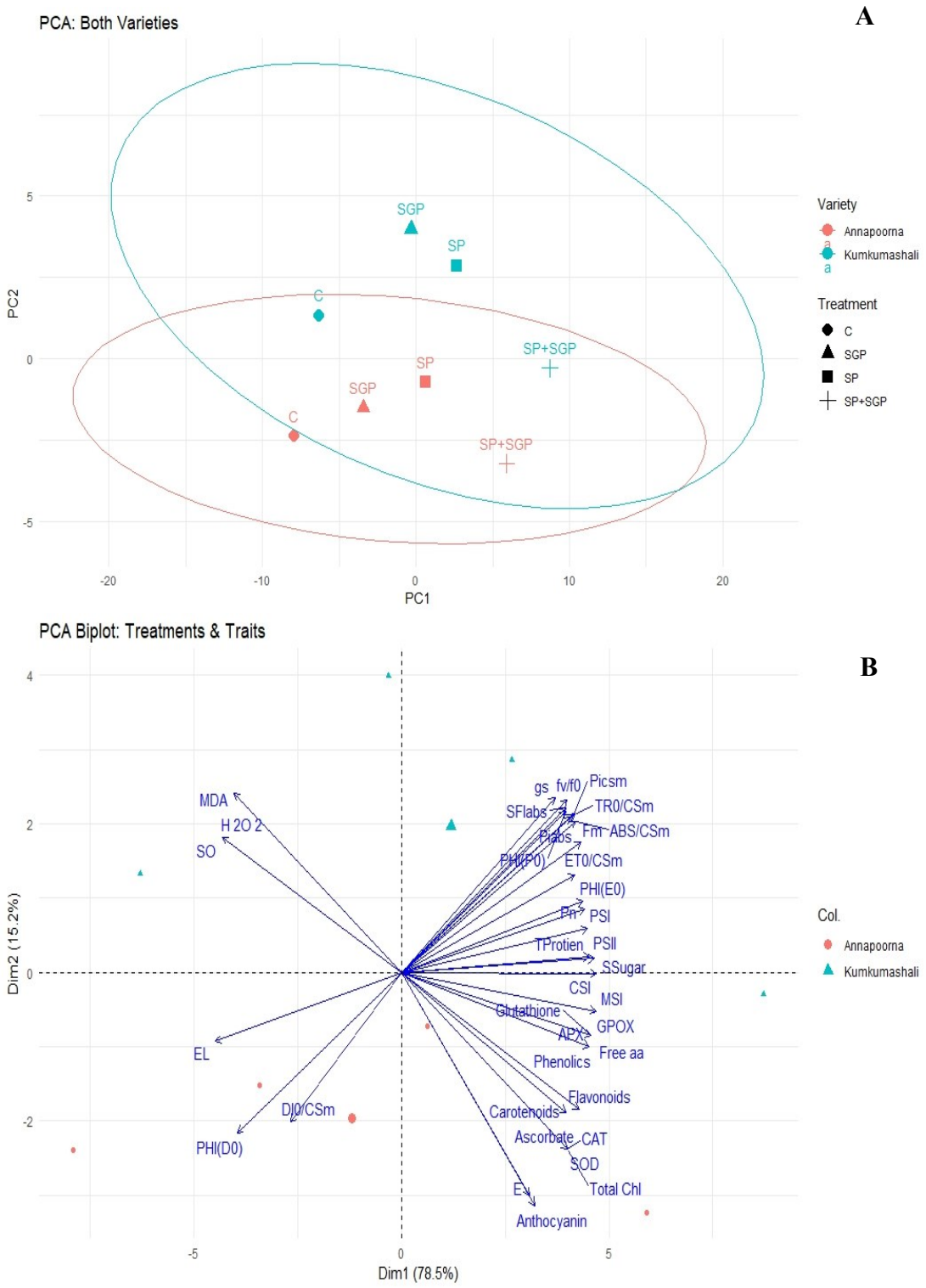


Figure 20. Principal component analysis (PCA) plot representing distribution of treatments (A) and PCA variable biplot (B) representing the relationship between physiological and biochemical traits and treatment effects in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. C (control), SP (seed priming), SGP (seedling priming), SP + SGP (seed priming + seedling priming)

A

Annapoorna Correlation Matrix

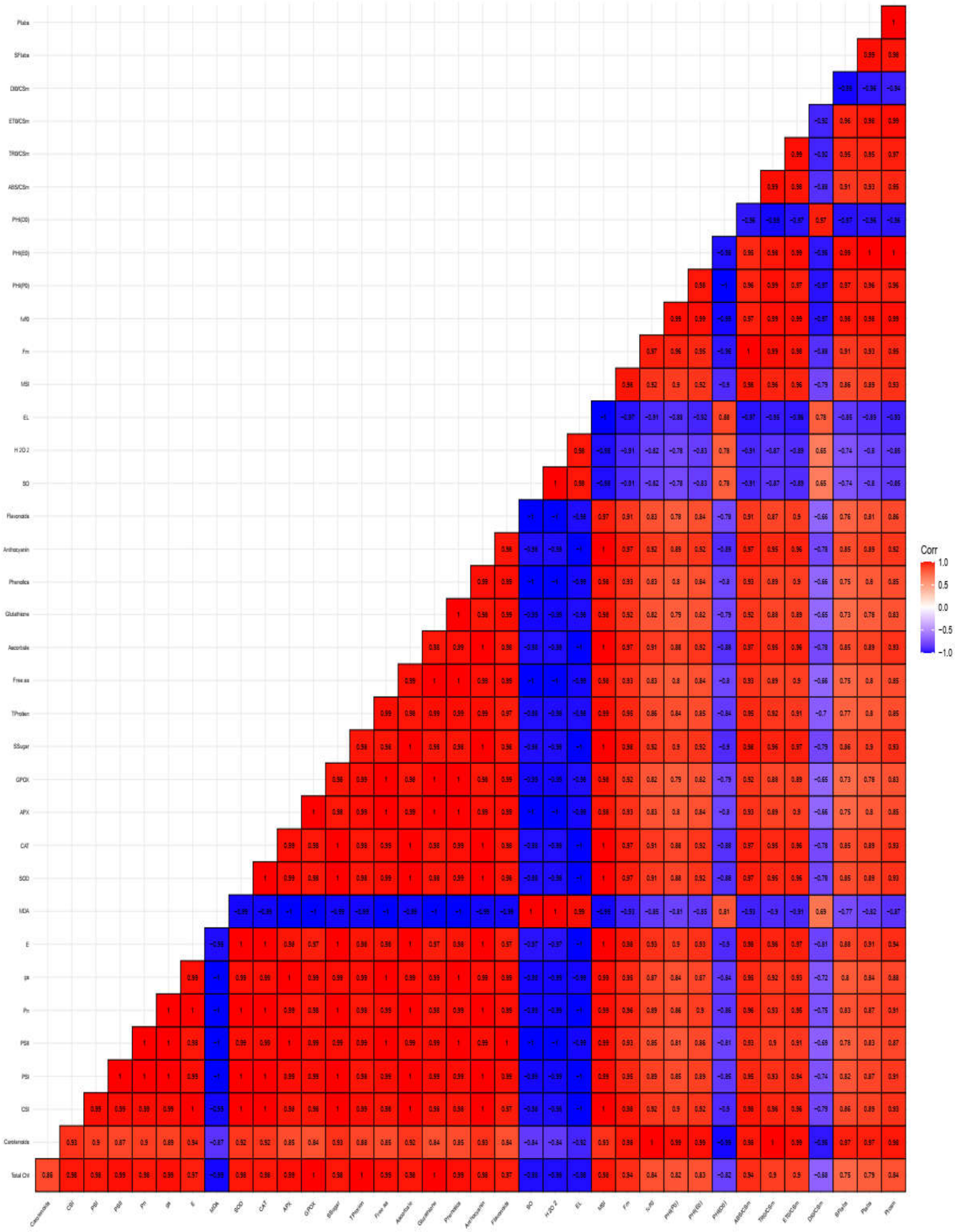


Figure 21. The correlation matrix heatmaps of Annapoorna subjected to various priming treatments with $ZnNO_3$ showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in red and negative in blue. It ranges from -1 to 1, where -1 means a perfect negative linear relationship between variables, 1 indicates a perfect positive linear relationship between variables and 0 indicates there are no relationship between studied variables

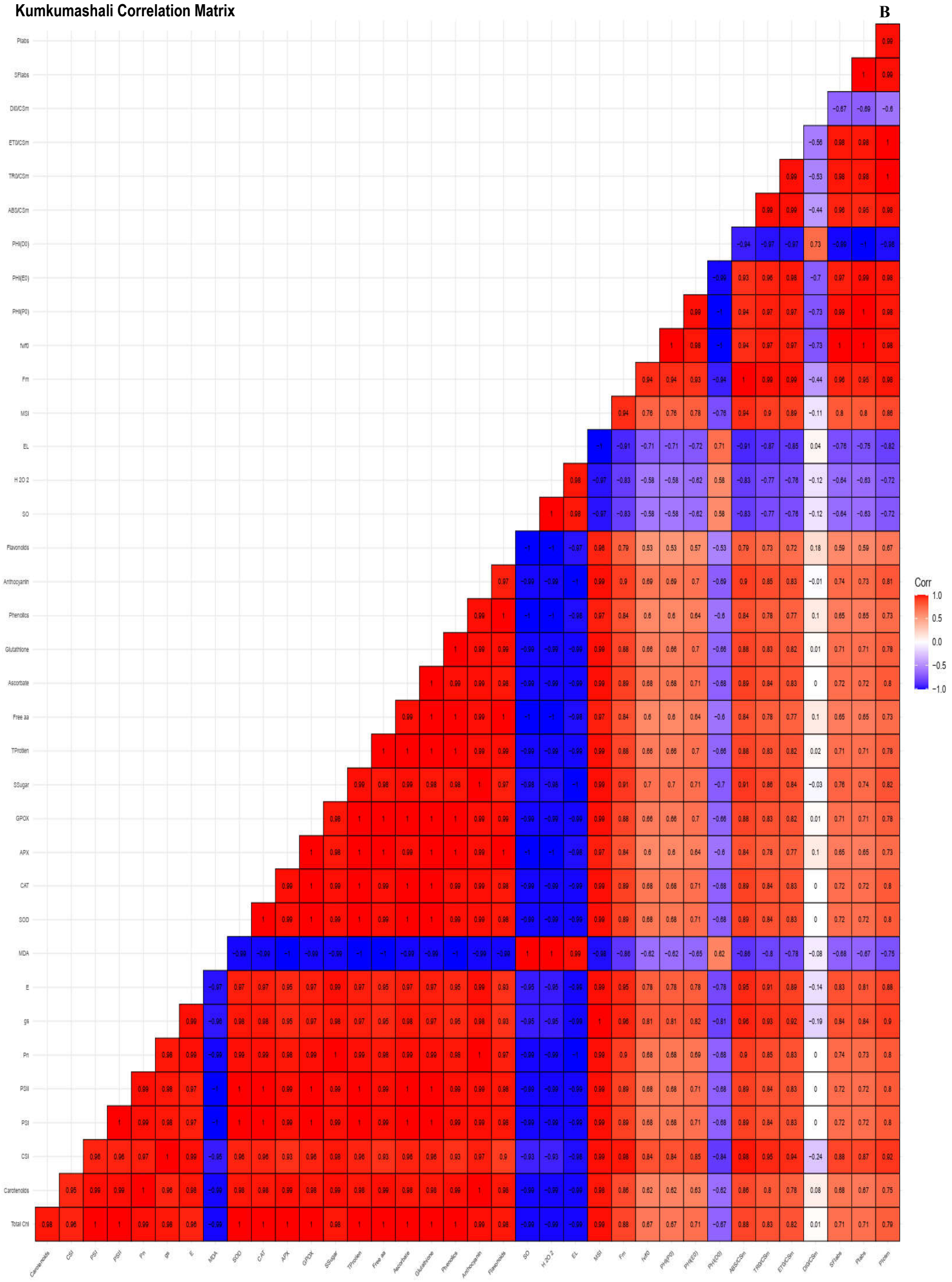


Figure 21. The correlation matrix heatmaps of Kumkumashali subjected to various priming treatments with $ZnNO_3$ showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in red and negative in blue . It ranges from -1 to 1, where -1 means a perfect negative linear relationship between variables, 1 indicates a perfect positive linear relationship between variables and 0 indicates there are no relationship between studied variables

Correlation Matrix for Zn Treatments (Annapoorna and Kumkumashali)

C

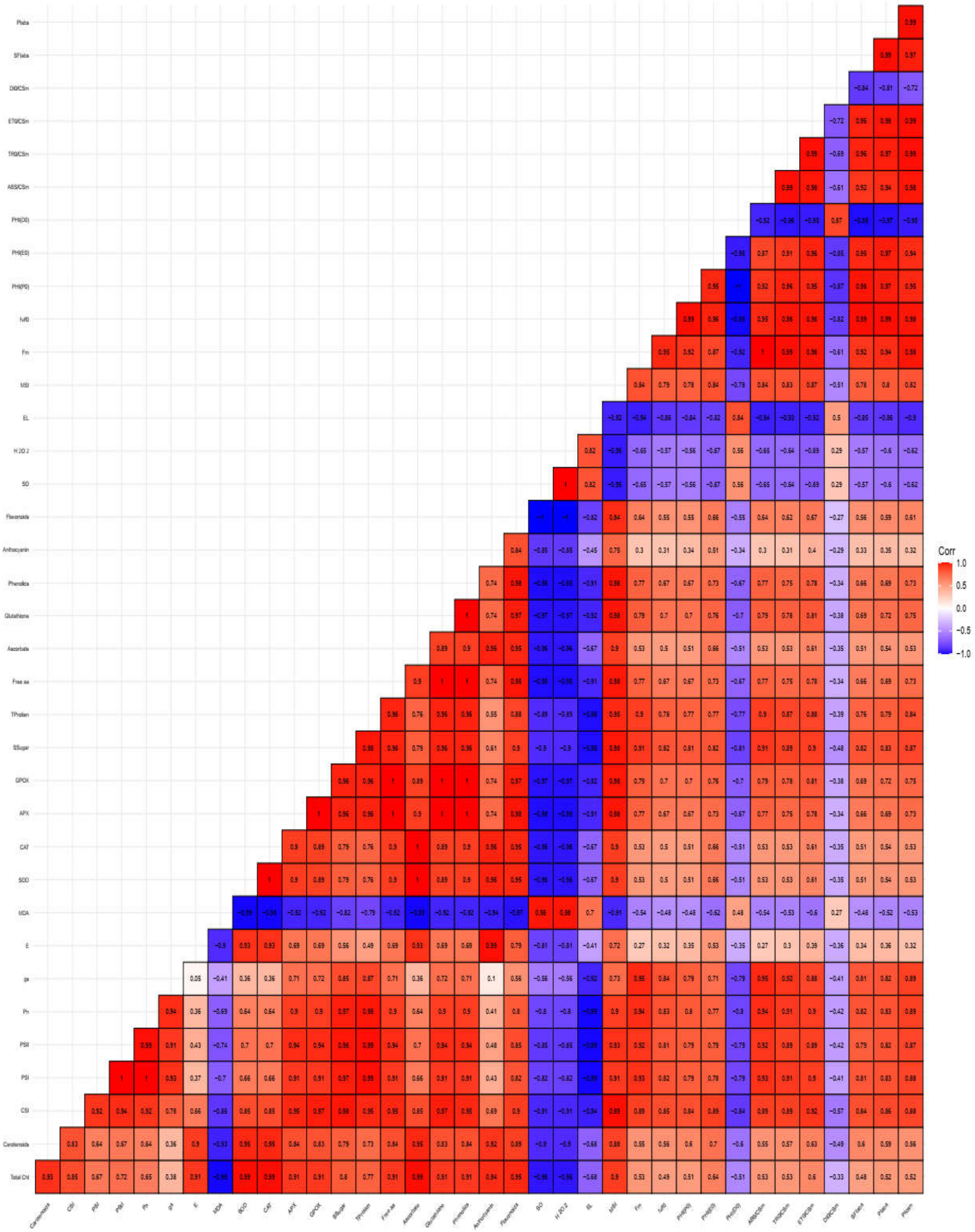


Figure 21. The correlation matrix heatmaps of Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$ showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in red and negative in blue. It ranges from -1 to 1, where -1 means a perfect negative linear relationship between variables, 1 indicates a perfect positive linear relationship between variables and 0 indicates there are no relationship between studied variables

Table 24. Zinc, phytic acid, phytic acid to Zn molar ratio and bioavailable Zn in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

	Zn		PA		PA:Zn		Bioavailable Zn	
	Annapoorna	Kumkumashali	Annapoorna	Kumkumashali	Annapoorna	Kumkumashali	Annapoorna	Kumkumashali
Control	22.38 ± 0.20^{e}	25.65 ± 0.21^{e}	4.94 ± 0.01^{a}	4.53 ± 0.03^{a}	21.70 ± 0.27^{a}	17.30 ± 0.21^{a}	2.12 ± 0.02^{e}	2.52 ± 0.03^{e}
Seed priming	26.55 ± 0.55^{c}	30.35 ± 0.55^{c}	4.45 ± 0.04^{c}	4.06 ± 0.03^{c}	16.75 ± 0.27^{c}	13.39 ± 0.33^{c}	2.63 ± 0.07^{c}	3.11 ± 0.07^{c}
Seedling priming	23.88 ± 0.06^{d}	27.68 ± 0.06^{d}	4.80 ± 0.01^{b}	4.27 ± 0.02^{b}	20.10 ± 0.03^{b}	15.42 ± 0.09^{b}	2.31 ± 0.01^{d}	2.77 ± 0.01^{d}
Seed priming + Seedling priming	30.10 ± 0.08^{b}	34.49 ± 0.24^{b}	4.31 ± 0.00^{d}	3.85 ± 0.02^{d}	14.33 ± 0.04^{d}	11.17 ± 0.07^{e}	3.08 ± 0.01^{b}	3.63 ± 0.03^{b}
Seed priming+ Foliar spray	29.98 ± 0.08^{b}	34.23 ± 0.13^{b}	3.97 ± 0.03^{e}	3.44 ± 0.01^{f}	13.24 ± 0.12^{e}	10.06 ± 0.05^{f}	3.06 ± 0.01^{b}	3.59 ± 0.02^{b}
Seedling priming + Foliar spray	26.29 ± 0.13^{c}	30.09 ± 0.13^{c}	4.44 ± 0.01^{c}	3.69 ± 0.03^{e}	16.88 ± 0.04^{c}	12.26 ± 0.06^{d}	2.60 ± 0.02^{c}	3.07 ± 0.02^{c}
Seed priming + Seedling priming+ Foliar spray	32.86 ± 0.40^{a}	38.06 ± 0.21^{a}	3.73 ± 0.03^{f}	3.23 ± 0.01^{g}	11.34 ± 0.14^{f}	8.48 ± 0.06^{g}	3.42 ± 0.05^{a}	4.08 ± 0.03^{a}

In Kumkumashali, seed priming and additional foliar spray at reproductive stage displayed lowering in grain PA content by 24%, when compared to control plants. Similarly, the grain PA content was decreased upon seedling priming along with foliar spray at booting + flowering + milky stage and it was only by 19%, which was the lowest reductions among the different treatments of $ZnNO_3$. However, the combined seed and seedling priming along with foliar spray at three different reproductive stages showed the highest reduction (29%) in grain PA content (Table 24).

4.5.3 Phytic acid to zinc molar ratio

PA to Zn (PA:Zn) molar ratio gives an idea about the phytoavailable Zn present in the given tissue. In the present study, the PA: Zn molar ratio was decreasing in plants subjected to various Zn treatments. In Annapoorna, seed priming (23%), seedling priming (7%), seed and seedling priming in combination (34%) decreased the PA: Zn molar ratio in grain. Seed priming along with foliar spray, displayed further lowering (39%) in grain PA: Zn molar ratio when compared to control plants. Similarly, the grain PA: Zn molar ratio was decreased upon seedling priming together with foliar spray (22%), but it was lower than seed priming along with foliar spray. Furthermore, the combined treatment of seed and seedling primings in conjunction with foliar spray showed the highest reduction (48%) in grain PA:Zn molar ratio (Table 24).

In Kumkumashali, seed priming, seedling priming, seed and seedling priming in combination decreased the PA: Zn molar ratio in grain by 23%, 11% and 35% respectively. The seed priming and foliar application during all the stages of reproduction (booting, flowering and milky stages) reduced PA: Zn molar ratio by 42%. However, the seedling priming along with foliar spray at reproductive stages showed the least reduction of 29%. The highest reduction in PA: Zn molar ratio was recorded under combined treatments of seed and seedling priming together with foliar spray at reproductive stages (51%) when compared to control plants (Table 24).

4.5.4 Bioavailable Zn

Bioavailable Zn was analysed based on trivariate method in order to find how the bioavailability of Zn was changed in the rice varieties upon various treatments of $ZnNO_3$. It was found that there were substantial difference in bioavailable Zn in both the rice varieties by different priming treatments and foliar spray at various stages of reproduction, when compared to untreated control plants. In Annapoorna, seed priming, seedling priming, combined treatments of seed and seedling priming enhanced the bioavailability of Zn in grain by 24%, 9% and 45% respectively. Seed priming along with foliar spray displayed an increment of 44% in bioavailable Zn, which was much higher than seedling priming along with foliar spray, when compared to control plants. The bioavailable Zn in grain was increased upon seedling priming together with foliar spray by 22%, which was lesser than other priming treatments. The combined treatments of seed and seedling priming in conjunction with foliar spray at booting + flowering + milky stages (61%) showed the highest increment in bioavailable Zn (Table 24).

In Kumkumashali, 23%, 10% and 44% enhancement in the bioavailable Zn in grain was recorded in rice subjected to seed priming, seedling priming and combined treatments of seed and seedling priming respectively. Combined application of seed priming and foliar spray showed a significant enhancement (42%) in bioavailable Zn when compared to control plants. Similarly, bioavailable Zn in grain was increased upon seedling priming together with foliar spray (22%), but to a lesser extent than other treatments. However, the maximum enhancement in bioavailable Zn (62%) was observed in plants subjected to combined treatments of seed and seedling priming in combination with foliar spray at various reproductive stages, when compared to control plants (Table 24).

4.5.5 Principal component and correlation analysis

PC1 (84.51%), represents the largest variation in the data. It primarily distinguishes treatments based on Zn content and bioavailable Zn. While, PC2 (12.84%), explains additional variation, mainly influenced by PA (phytic acid) and PA:Zn molar ratio. Kumkumashali and Annapoorna are clearly separated, showing

distinct responses to different treatments. Treatments such as combined treatments of seed and seedling priming, combined treatments of seed priming and foliar spray and combined treatments of seed and seedling priming and foliar spray (SP+SGP, SP+FS, SP+SGP+FS) are positioned towards the right in PC1, suggesting that these treatments significantly contributed to Zn enrichment. Control (C) and seedling priming (SGP) treatments are located towards the left, indicating relatively lower Zn levels. Zn and bioavailable Zn vectors point towards the right, indicating that treatments falling in this direction such as combined treatments of seed and seedling priming, combined treatments of seed priming and foliar spray and combined treatments of seed and seedling priming and foliar spray (SP+SGP, SP+FS, SP+SGP+FS) enhance Zn bioavailability. PA and PA:Zn ratio vectors point in the opposite direction, meaning they are negatively correlated with Zn content. Higher PA and PA:Zn ratio values are seen to be associated with seedling priming (SGP) and control treatments, which tend to have lower bioavailable Zn. Kumkumashali performs better in Zn enrichment, while Annapoorna has higher PA and PA:Zn ratios, which might limit Zn bioavailability. This PCA analysis confirms that seed priming and seedling priming in combination and along with foliar spray (SP+SGP, SP+SGP+FS) significantly improve Zn biofortification, making these as the most effective strategies. The boxplot also illustrates the same in which control (C) group had the lowest Zn and bioavailable Zn content, while high PA and PA:Zn content. Combined treatments of seed and seedling priming along with foliar spray (SP+SGP+FS) showed the highest enhancement in Zn content, bioavailable Zn, while lowest content in PA and PA:Zn molar ratio (Fig. 22 & 23).

PA and PA:Zn ($r = 0.95$), this strong correlation suggests that phytate (PA) content directly influences the PA:Zn ratio, meaning an increase in PA leads to a higher PA:Zn ratio. PA:Zn and Zn ($r = 0.94$), a high PA:Zn ratio is closely associated with Zn levels, indicating that higher Zn content does not necessarily mean better bioavailability due to the presence of phytate. Zn and bioavailable Zn ($r = 0.61$), shows a moderate positive relationship, thus suggesting that not only the Zn content, other factors such as phytate levels also influence Zn bioavailability. Zn and PA ($r = -0.95$), shows a significant negative correlation, indicating that as phytate

content increases, Zn concentration decreases, highlighting the antagonistic role of PA in Zn accumulation. PA:Zn and bioavailable Zn (r = -0.66), exhibits a higher PA:Zn ratio, negatively affecting Zn bioavailability, reinforcing the role of phytate in limiting Zn absorption. The PA and bioavailable Zn (r = -0.60), suggests that increased phytate levels reduce bioavailable Zn, influencing the nutritional value of rice grains (Fig. 22).

4.6 Agronomic traits, grain zinc content, other essential elements, gene expression of *OsZIP*, *OsHMA* in rice under zinc deficient and sufficient conditions upon various treatments of zinc nitrate.

4.6.1 Agro-morphological, biomass and yield traits

4.6.1.1 Effects of zinc supply on agro-morphological, biomass and yield traits

The supply of Zn influenced the agro-morphological, biomass and yield traits under Zn deficient condition (0.05 μM), compared to the Zn-sufficient condition (1.5 μM). The entire rice growth and yield were impacted by Zn availability. Zn deficiency negatively affected all the yield-related traits. Under Zn deficient condition, the plant height was 117.53 cm, and under Zn sufficient condition, the plant height slightly increased to 121.2 cm. The highest root length was observed under Zn sufficient condition (29.92 cm). The Zn deficient condition reduced the shoot dry weight and root dry weight by 34 and 22% respectively, when compared to Zn sufficient condition. A 16 % increase in crown root number was observed under Zn sufficient condition. Zn deficiency led to a decrease in the number of tillers (27%), the number of reproductive tillers (85%), the length of the panicle (21%), grains per panicle (20%) and HI (15%). Overall, these results revealed that Zn deficiency was a severe constraint for rice growth, development, and yield compared to the Zn sufficient condition (Fig. 24-27).

4.6.1.2 Effects of different treatments of zinc nitrate on agro-morphological, biomass and yield traits under differential Zn supply

Seed priming with $ZnNO_3$ (0.5M, 18h) significantly enhanced rice plant growth and yield under varying Zn conditions. Seed priming had a significant impact on increasing the rice plant growth, biomass, and yield under both Zn

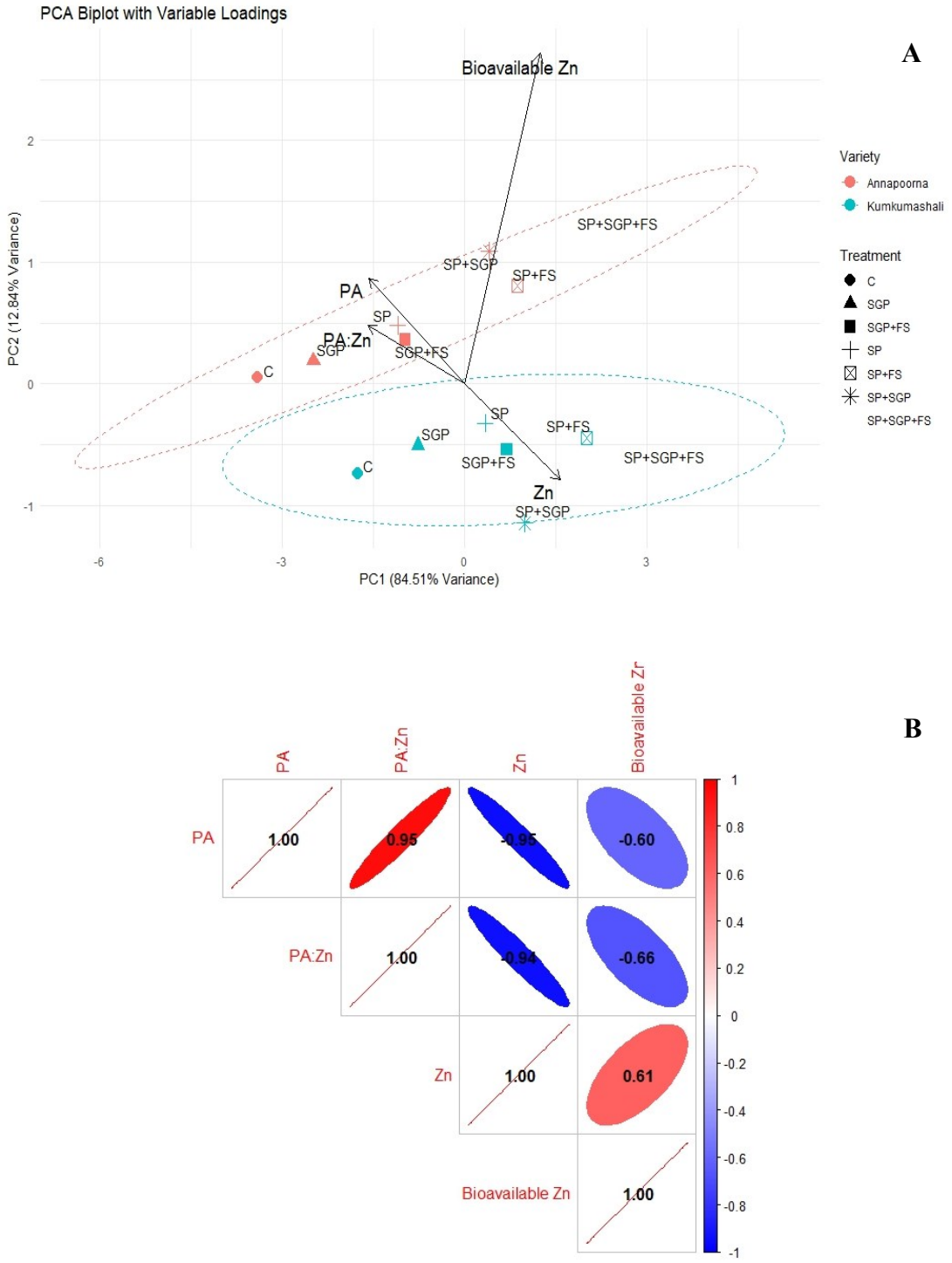


Figure 22. Principal component analysis (PCA) biplot representing effect of various $ZnNO_3$ priming treatments on grain Zn, phytic acid, phytic acid: Zn molar ratio and bioavailable Zn (A) correlation matrix (B) showing the relationships among grain phytic acid (PA), PA:Zn molar ratio, total Zn, and bioavailable Zn content studied in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. Ellipse shape and colour intensity represent the strength and direction of correlation (red = positive, blue = negative). C (control), SP (seed priming), SGP (seedling priming), SP + SGP (seed priming + seedling priming)

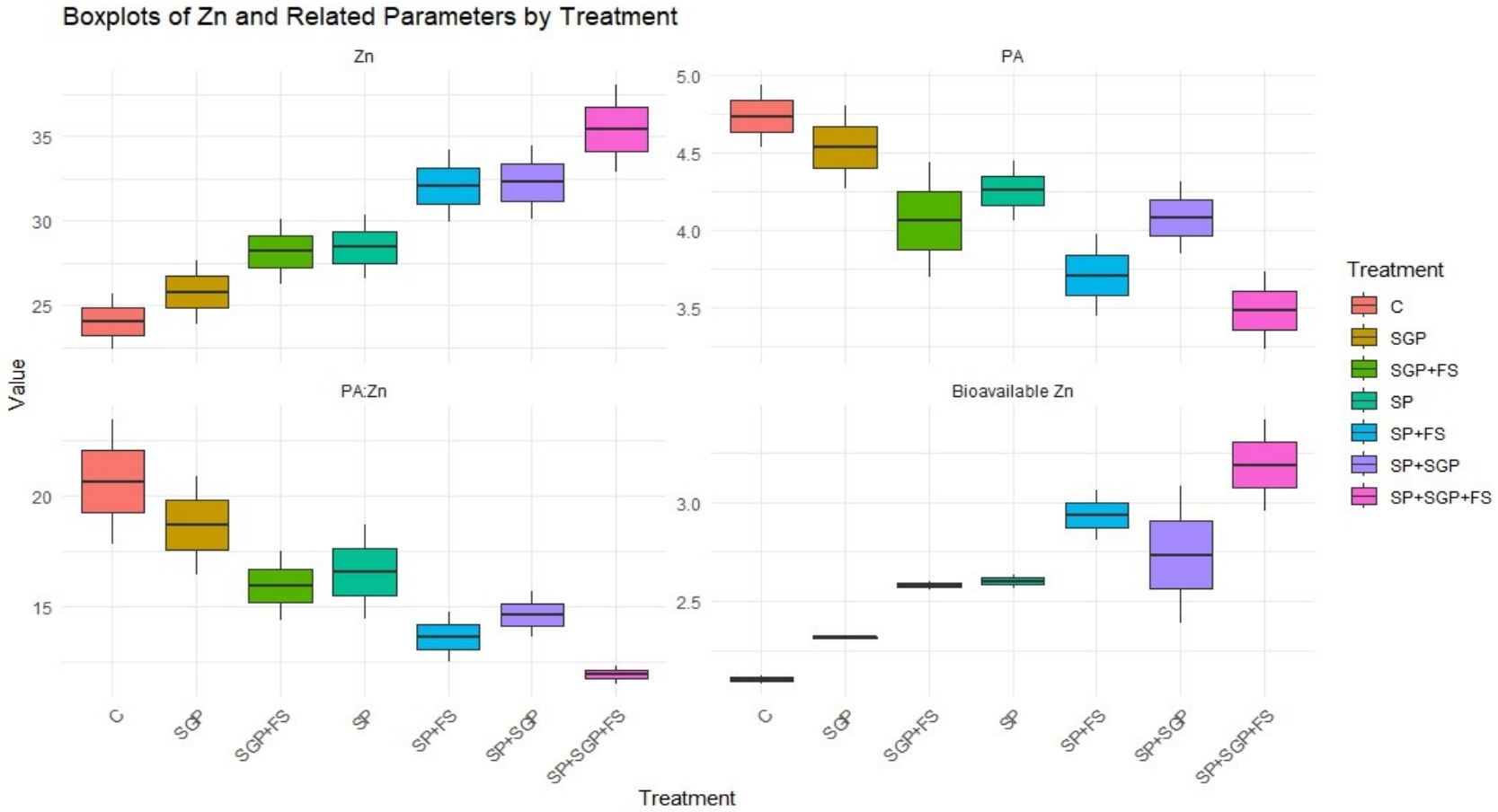


Figure 23. Boxplot for grain Zn content, phytic acid content, phytic acid to Zn molar ratio and bioavailable Zn content in Annapoorna and Kumkumashali subjected to various priming treatments with $ZnNO_3$. C (control), SGP (seedling priming), SGP + FS (seedling priming + foliar spray), SP (seed priming), SP + FS (seed priming + FS), SP + SGP (seed priming + seedling priming), SP + SGP + FS (seed priming + seedling priming + foliar spray)

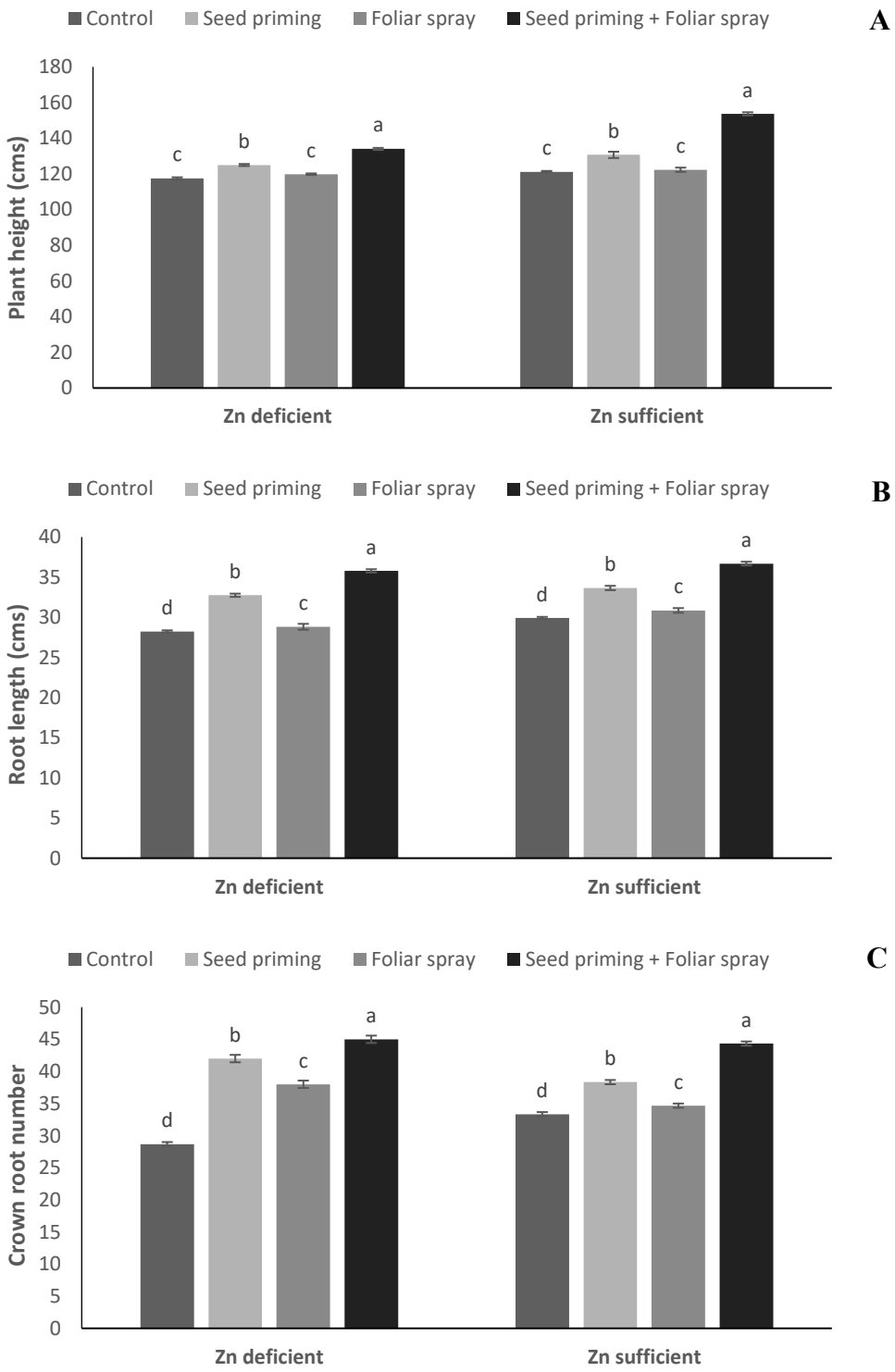


Figure 24. Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Plant height (A), Primary root length (B), Crown root number (C). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

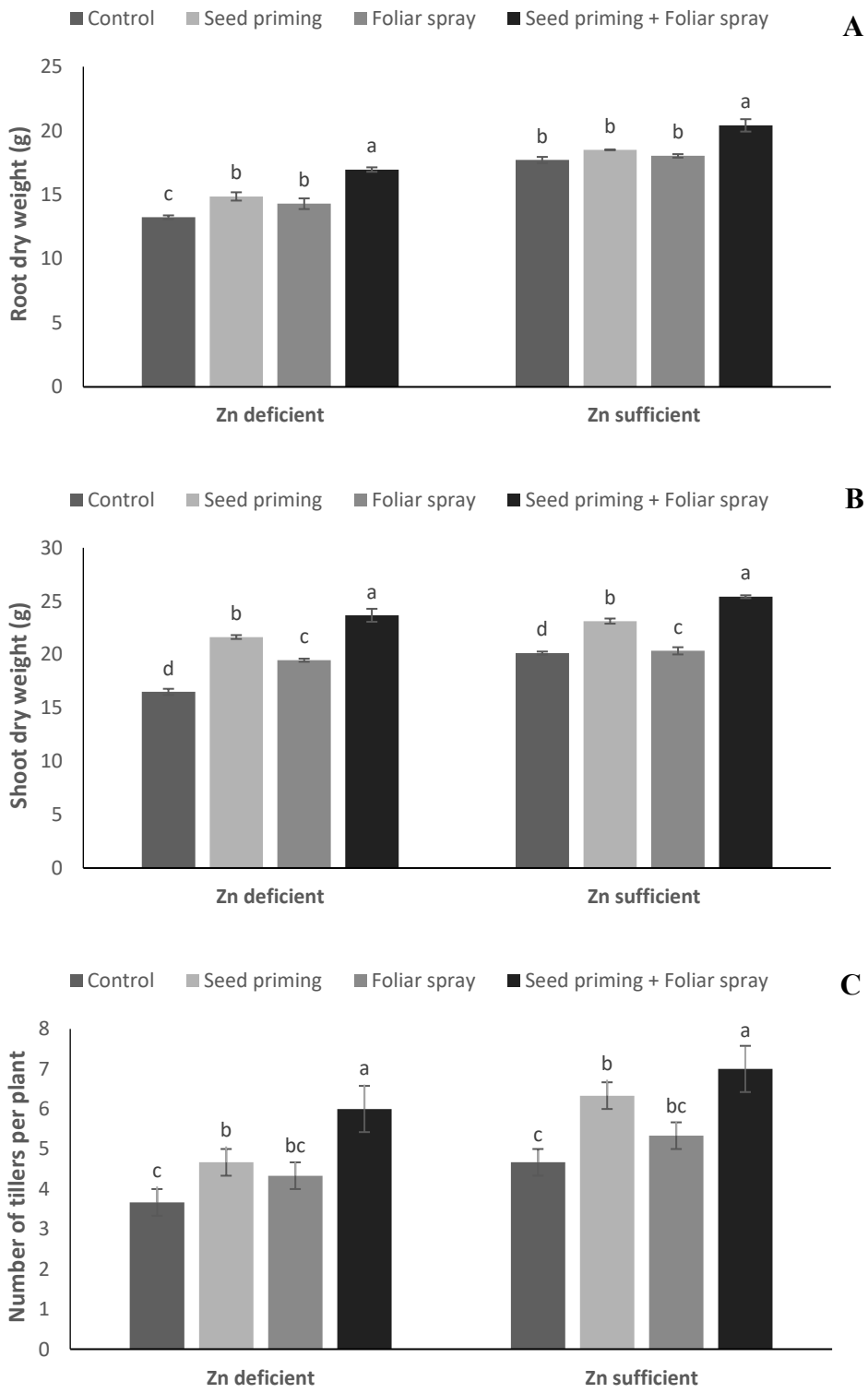


Figure 25. Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Root dry weight (A), Shoot dry weight (B), Number of tillers per plant (C). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

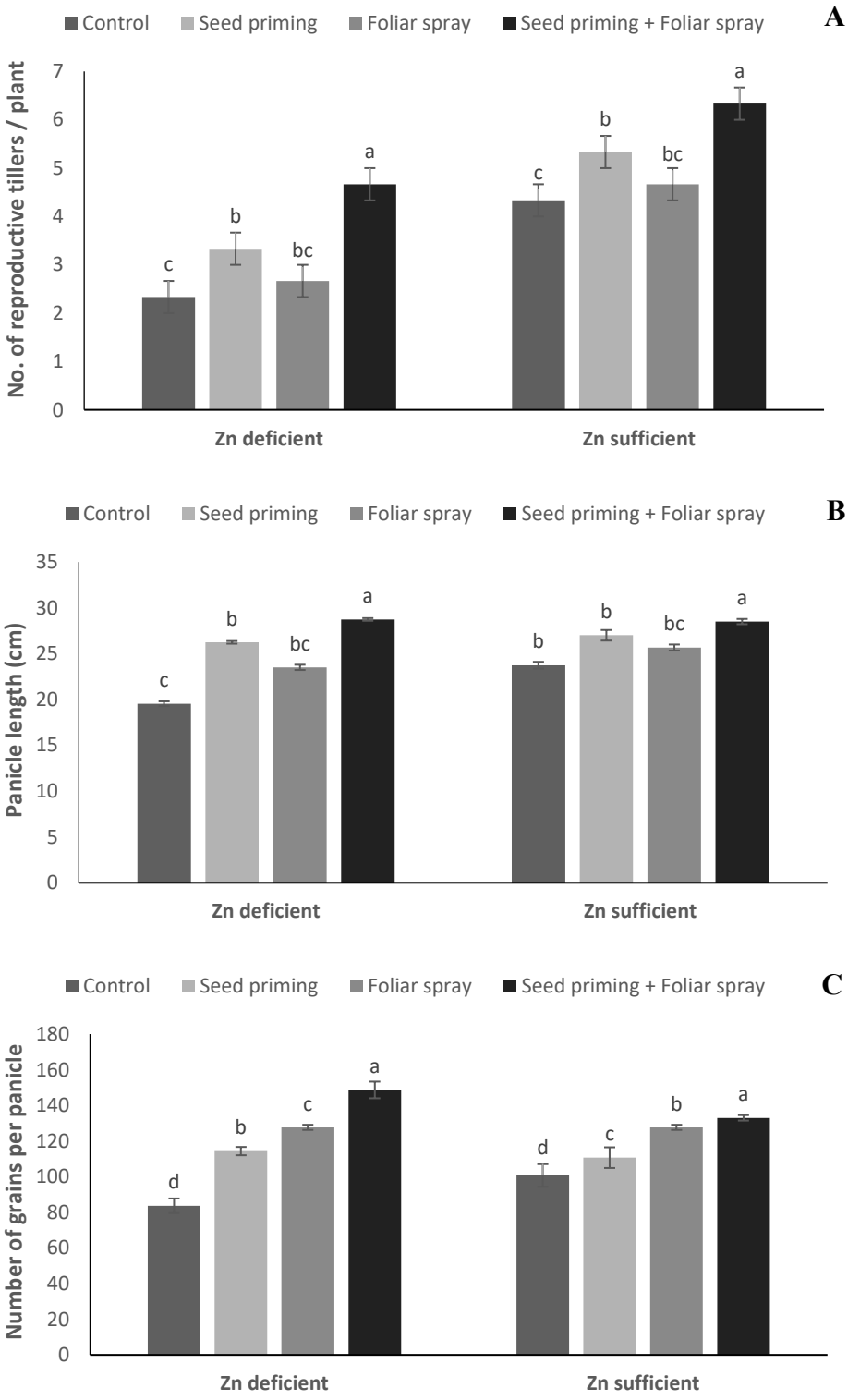


Figure 26. Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, Number of reproductive tillers / plant (A), Panicle length (B), Number of grains per panicle (C). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

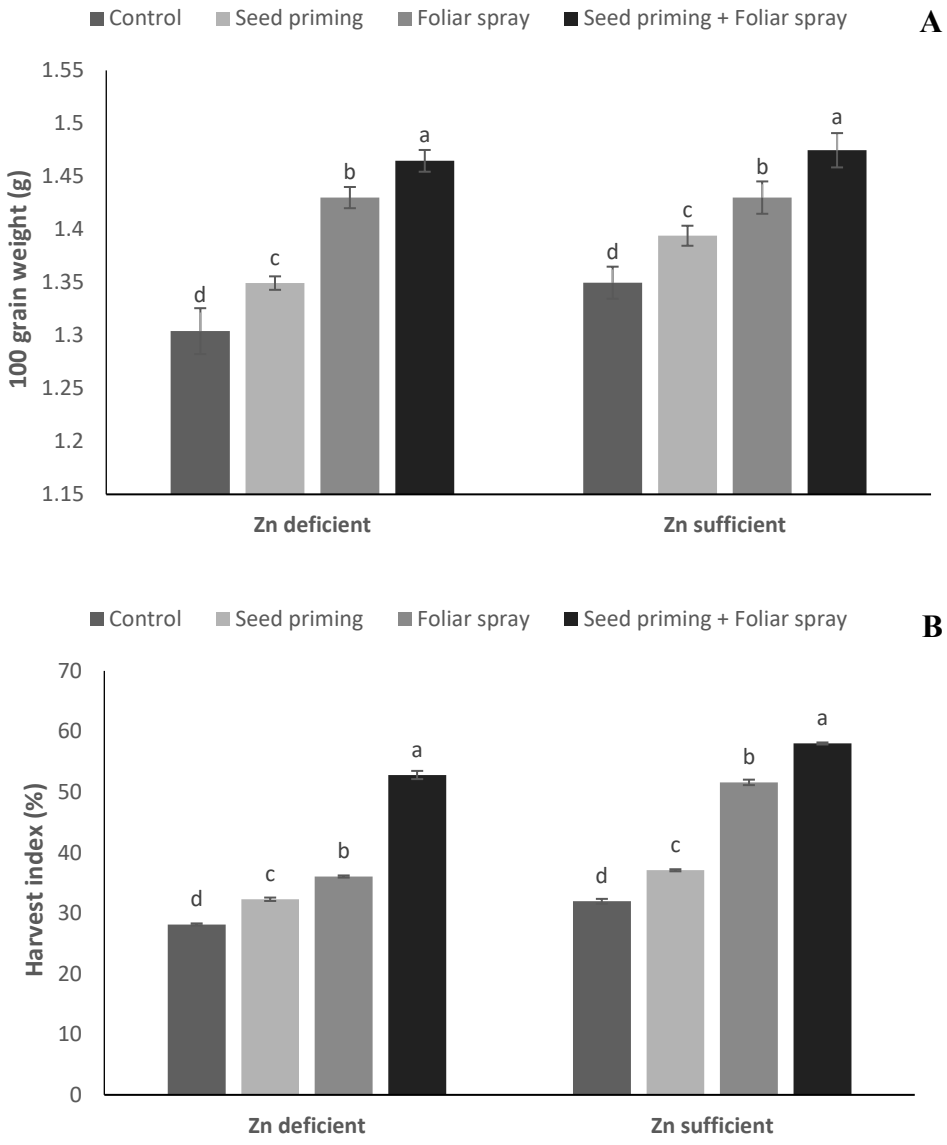


Figure 27. Agronomic and yield parameters in rice plants subjected to seed priming and foliar spray with $ZnNO_3$ under Zn deficient and sufficient conditions, 100 grain weight (A), Harvest Index (B). Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

deficient and sufficient conditions. Here the effect of various treatments with $ZnNO_3$, is compared with their respective controls. The plant height was 125 cm under the Zn deficient+ seed priming condition, and 130.67 cm at the Zn sufficient+ seed priming condition. The root length was increased by 16% under Zn deficient condition and 13% under Zn sufficient condition. The biomass of plants emerged from seed priming was also enhanced, when compared to the control plant. Both the root and shoot dry weight was enhanced by 5 to 31% under Zn deficient and Zn sufficient conditions. The effect of seed priming was profound in Zn deficient condition, when compared to the Zn sufficient condition and it helped the plant to overcome the Zn deficiency and attain growth and yield similar to those in the Zn sufficient condition. There was a significant enhancement in crown root number upon seed priming under Zn deficient (47%) and Zn sufficient condition (15%). Seed priming also enhanced the number of tillers/plant by 27% under Zn deficient condition and by 36% under Zn sufficient condition. The number of reproductive tillers/plant were increased by seed priming under both Zn deficient condition (43%) and Zn sufficient condition (23%). Upon seed priming, the panicle length was increased by 34% under Zn deficient condition, while in Zn sufficient condition it was by 14%. Seed priming enhanced the grains per panicle more during Zn deficient condition (37%), when compared to Zn sufficient condition (10%). Seed priming showed only a small change in 100-grain weight of the rice grains under both Zn deficient and sufficient conditions. Seed priming showed an enhancement in HI by 15% during Zn deficient condition and 16% during Zn sufficient condition (Fig. 24-27).

The foliar spray of $ZnNO_3$ (0.5%) during (booting, flowering, and milky stages) effectively enhanced the growth and yield of rice plants. The application of foliar spray increased crown root number by 33% under Zn deficient condition. However, foliar spray produced less crown root number in Zn sufficient condition. The number of tillers/plant and reproductive tillers/plant were more upon foliar spray under Zn deficient condition (18 and 14% respectively) and Zn sufficient condition (14 and 8% respectively). The panicle length was also enhanced under Zn deficient condition (20%) upon foliar spray application. Under Zn deficient

condition, a significant enhancement in grains per panicle (53%) was observed on applying foliar spray. While under Zn sufficient condition, grains per panicle was enhanced by 27%. There was an increment in 100-grain weight under Zn deficient condition (10%). More than 30% enhancement in HI was observed under Zn deficient and Zn sufficient conditions upon foliar application (Fig. 24-27).

The simultaneous application of seed priming (0.5µM for 18 hours) and foliar spray (0.5% at booting, flowering, and milky stages) with $ZnNO_3$ (seed priming + foliar spray) showed a synergistic effect on plant development and yield, in contrast to the separate treatments of seed priming and foliar spray alone. The height of the plant was enhanced by 14 and 27% under Zn deficient and Zn sufficient conditions respectively. There was a significant enhancement in root length upon seed priming + foliar spray under Zn deficient (27%) and Zn sufficient conditions (23%). At Zn deficient condition, the combined treatments of seed priming and foliar spray, enhanced root dry weight by 28% and shoot dry weight by 44% and at the same time in Zn sufficient condition this increase was only by 15 and 26% respectively. The crown root number on combined application of seed priming + foliar spray, was enhanced under Zn deficient (57%) and Zn sufficient (33%) conditions. The number of tillers/plant and reproductive tillers/plant were highly enhanced under Zn deficient condition (57and 100% respectively) than in Zn sufficient condition (50 and 46%). Seed priming + foliar spray enhanced panicle length under both Zn deficient (47%) and Zn sufficient conditions (20%). There was a significant enhancement in grains per panicle under Zn deficient (78%) and Zn sufficient conditions (32%). The 100-grain weight on combined treatments of seed priming and foliar spray was increased upto 12% under Zn deficient condition and 9% under Zn sufficient condition. The HI was enhanced by 88% during Zn deficient condition and 81% during Zn sufficient condition. Thus the effect of treatments was more in Zn deficient condition helping the plant to reach the growth and yield characteristics similar to Zn sufficient condition (Fig. 24-27).

4.6.2 Analysis of Zn and other elements levels in various tissues of rice with seed priming, foliar spray and combined treatments under differential Zn supply

4.6.2.1 Zinc

Zn deficiency and sufficiency influenced the accumulation of Zn in different rice tissues. There was a notable variation in Zn concentration in the root, node I, flag leaf, panicle, and grain under both Zn deficient and Zn sufficient conditions. The concentrations of Zn in the root, node I, flag leaf, panicle, and grain were 139%, 39%, 100%, 51%, and 50% higher respectively in positive control (Zn sufficient control), compared to negative control (Zn deficient control). The different treatments with Zn significantly enhanced the Zn concentration in the rice tissues examined. Under Zn deficient condition, seed priming elevated the Zn concentration by 119%, 32%, 88%, 39%, and 14%, while in Zn sufficient condition, it elevated the Zn concentration by 13%, 41%, 19%, 26%, and 20% in the root, node I, flag leaf, panicle, and grain, respectively. In Zn deficient condition, foliar Zn application resulted in 94%, 126%, 66%, 53%, 465%, and 122% increase of Zn content in the root, node I, flag leaf, panicle, husk, and grains, respectively. Conversely, under Zn sufficient condition, the increases were 61%, 36%, 33%, and 666% in node I, flag leaf, panicle and husk, respectively. The combined treatments of seed priming and foliar spray significantly increased the Zn concentration in different tissues of rice. In Zn deficient condition, the enhancements were 133% for roots, 152% for node I, 107% for flag leaves, 88% for panicles, 256% for husks, and 147% for grains. In Zn sufficient condition, the simultaneous use of seed priming and foliar spray improved Zn content in node I by 82%, flag leaf by 50%, panicle by 40%, husk by 764%, and grain by 56% (Fig. 28 & 29).

4.6.2.2 Other microelements

The Fe contents in panicles were higher (25%), but lower in root, node I, flag leaf, and husk by 30, 150, 27 and 23%, respectively in control plants grown under the Zn sufficient condition when compared to the control plants grown under Zn deficient condition. In node I (20%), husk (28%) and grain (10%), the Fe content were increased upon seed priming under Zn deficient condition. The Fe content was

enhanced in grain (22%) but it was decreased in node I (21%) under the Zn sufficient condition compared to Zn sufficient control (positive control) upon seed priming. Foliar spray enhanced Fe content in roots (96%), node I (12%), panicle (20%), husk (41%) and grain (10%) under the Zn deficient condition. In the case of the Zn sufficient condition, it enhanced Fe content in root (16%), node I (12%), panicle (21%), husk (15%) and grain (43%) were enhanced compared to Zn sufficient control (positive control). The combined treatments of seed priming and foliar spray caused a significant enhancement in the Fe content in roots (61%), node I (23%), panicle (35%), husk (88%) and grain (28%) under the Zn deficient condition compared to Zn deficient control (negative control). And in the Zn sufficient condition, the seed priming + foliar spray of Zn enhanced Fe content in root (30%), panicle (58%), husk (21%) and grain (69%), at the same time decreased in node I (23%) compared to Zn sufficient control (positive control). In flag leaf, under Zn sufficient condition, the seed priming, foliar spray and seed priming + foliar spray reduced Fe content by 25, 22 and 11% respectively compared to Zn sufficient control (positive control) (Fig. 30 & 31).

The seed priming enhanced the Cu content in roots (22%), node I (51%), flag leaf (25%), and panicle (11%) under the Zn deficient condition. Under the Zn sufficient condition, seed priming enhanced Cu content in roots (16%), node I (48%), flag leaf (13%) and panicle (16%). The foliar spray enhanced Cu content by 32, 88, 41, 12, 19 and 9% in root, node I, flag leaf, panicle, husk and grain respectively under the Zn deficient condition compared to Zn deficient control (negative control). Foliar spray also enhanced Cu content in the root (20%), node I (52%), flag leaf (18%), panicle (41%), husk (9%) and grain (10%) under the Zn sufficient condition compared to Zn sufficient control (positive control). The combined application had an additive effect in increasing the Cu content in rice plants under both the Zn deficient and Zn sufficient conditions. Under the Zn deficient condition, it enhanced Cu content by 63% in root, 127% in node I, 65% in flag leaf, 37% in panicle, 29% in husk and 14% in grain compared to Zn deficient control (negative control). In addition, under +Zn condition also it enhanced Cu

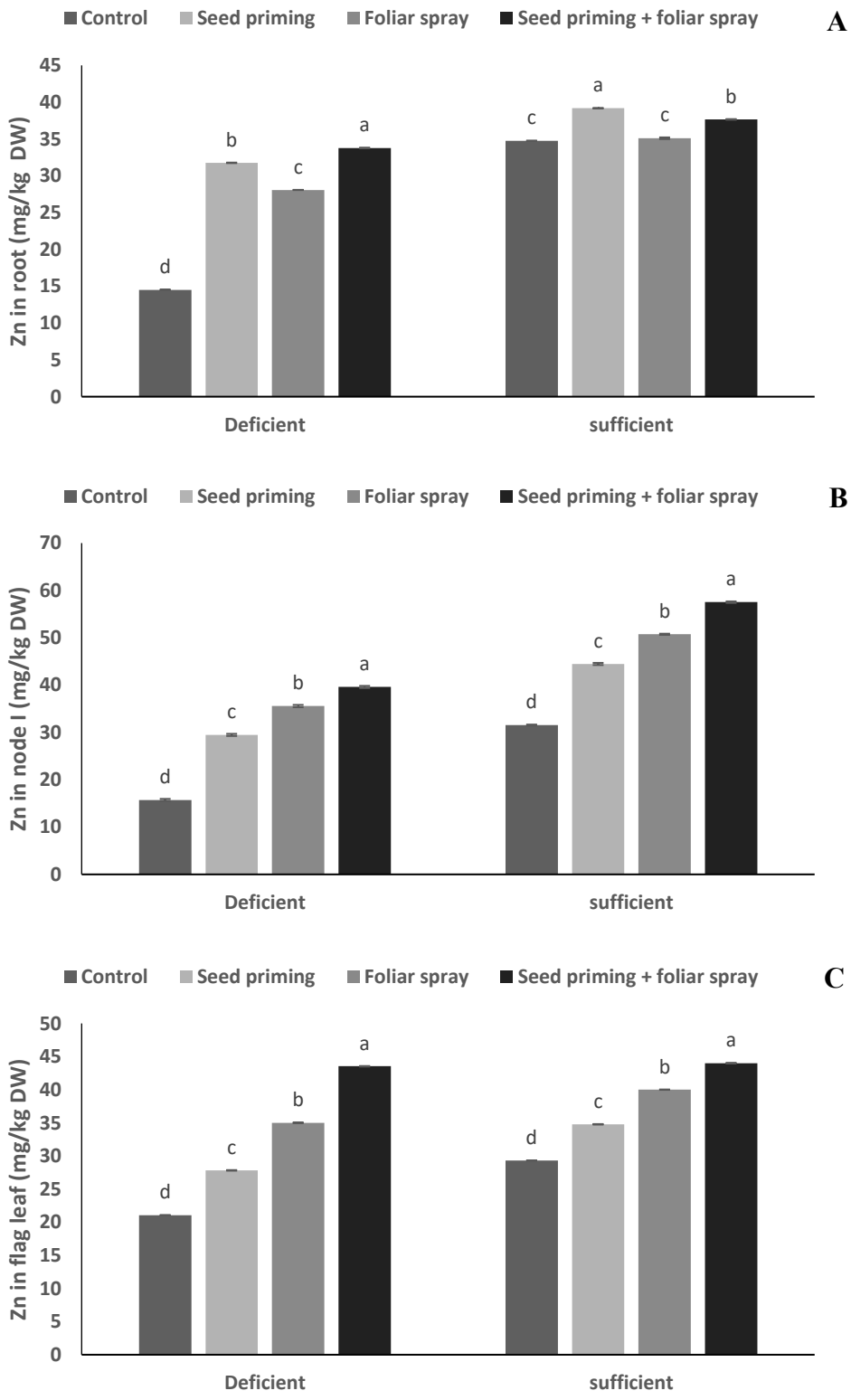


Figure 28. Zn content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

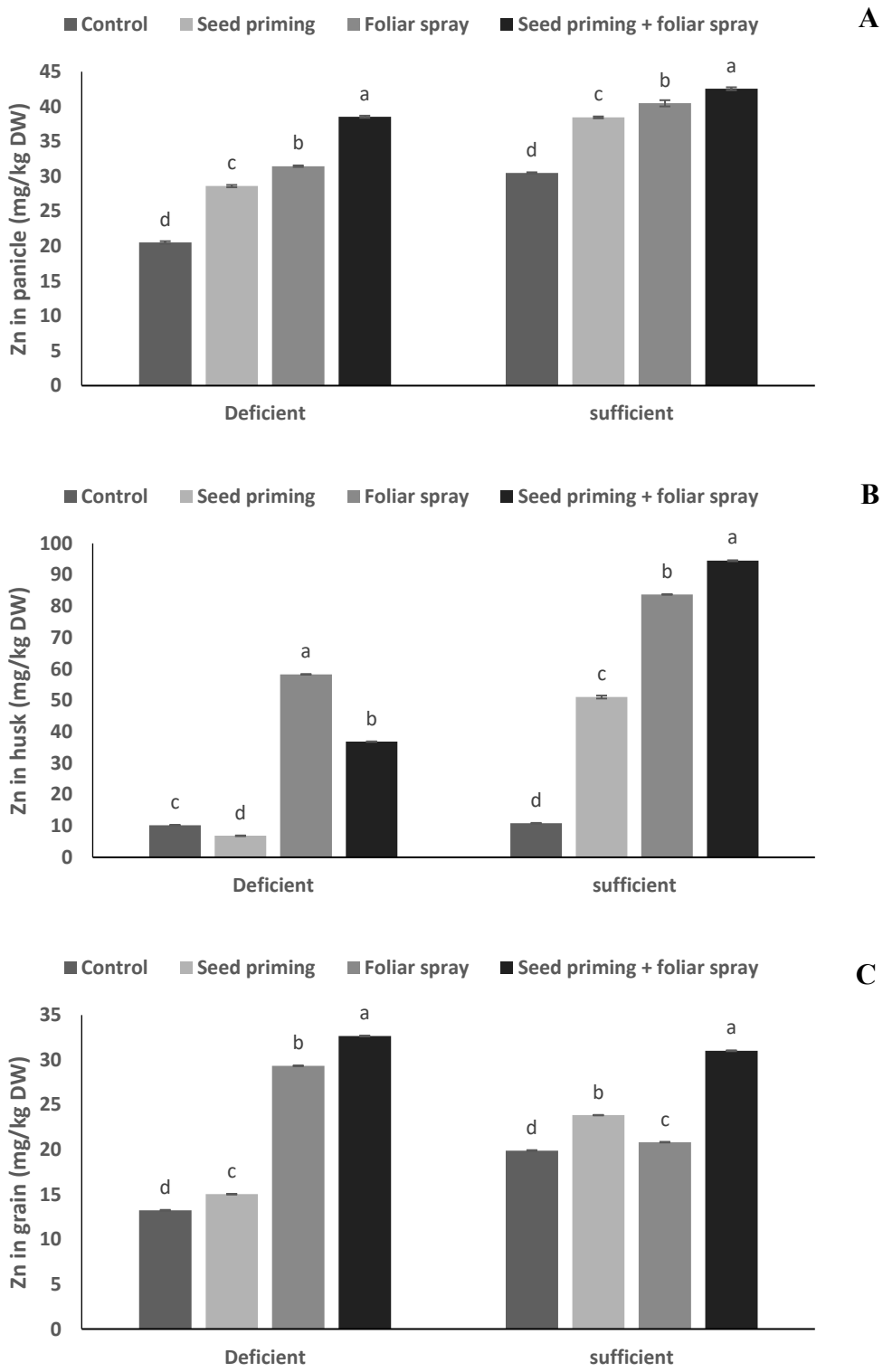


Figure 29. Zn content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

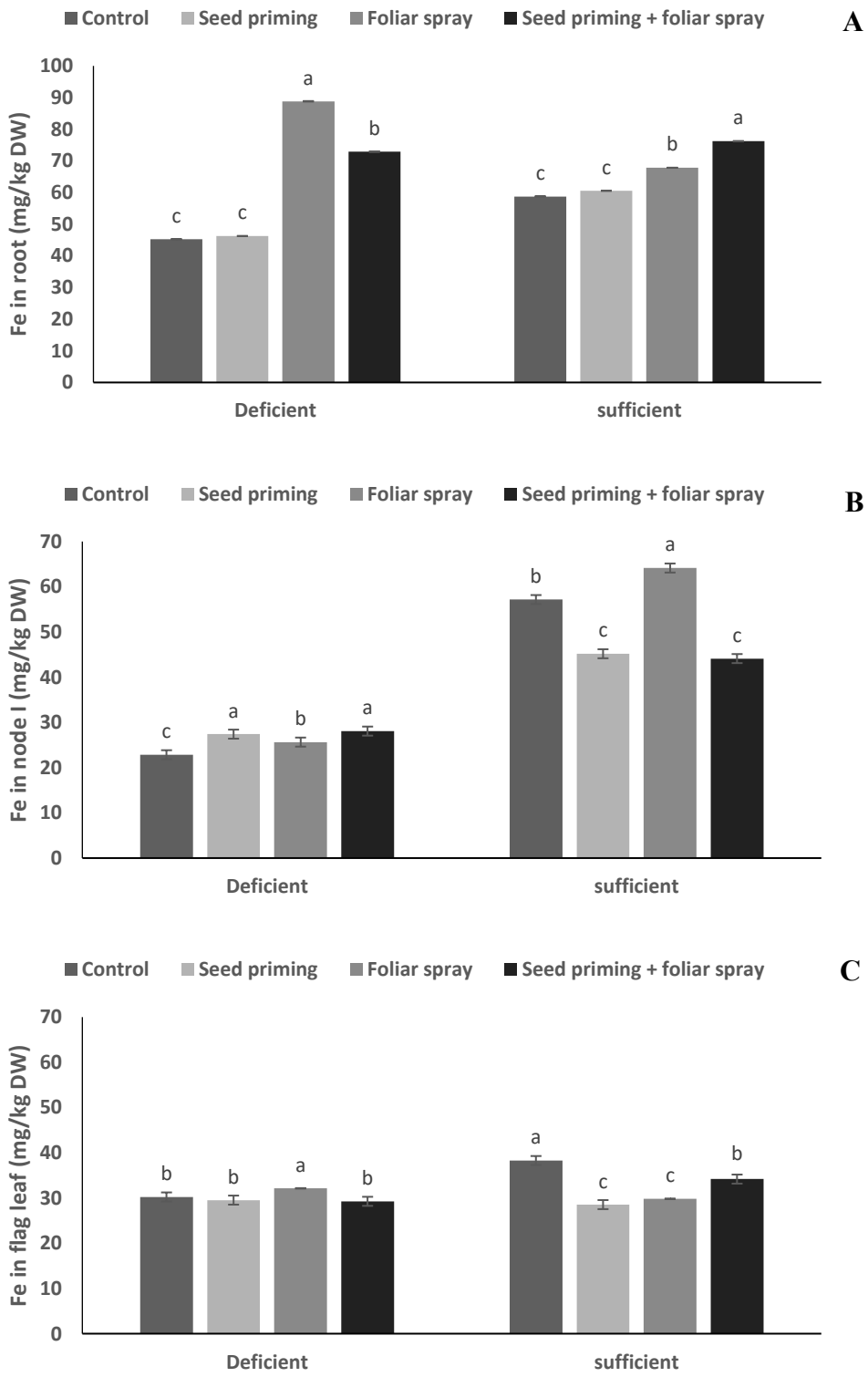


Figure 30. Fe content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

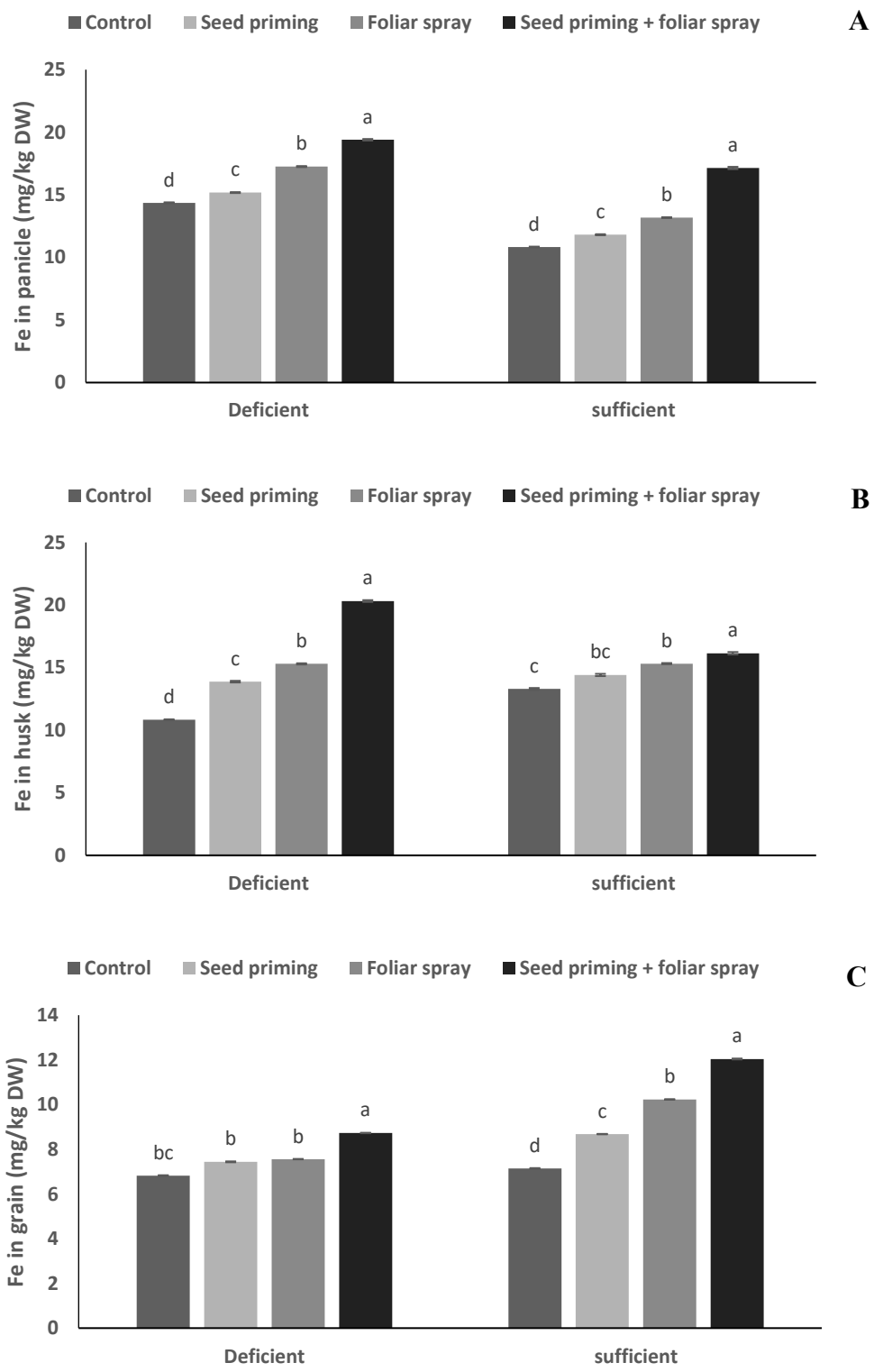


Figure 31. Fe content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

content in root (36%), node I (168%), flag leaf (64%), panicle (73%), husk (32%) and grain (13%) compared to Zn sufficient control (positive control) (Fig. 32 & 33).

The seed priming enhanced the Mn content in node I (16%), flag leaf (14%) and panicle (30%) under the Zn deficient condition. The Mn content in node I (14%), flag leaf (13%) and grain (13%) was enhanced upon seed priming under the Zn sufficient condition. The foliar spray enhanced the Mn content by 11, 16, 19, 38, 27 and 12% in the roots, node I, flag leaf, panicle, and grain, respectively under Zn deficient condition compared to Zn deficient control (negative control). During Zn sufficient condition it was enhanced by 11, 18, 38, 33, 50 and 24% in root, node I, flag leaf, panicle, and grain respectively compared to Zn sufficient control (positive control). The combined treatments of seed priming and foliar spray increased the Mn content in root (18 and 24%), node I (27 and 29%), flag leaf (24 and 49%), panicle (34 and 11%), husk (35 and 69%) and grains (19 and 134%) under Zn deficient and Zn sufficient conditions respectively compared to the respective Zn deficient and Zn sufficient controls (negative and positive controls) (Fig. 34 & 35).

4.6.3 Expression analysis of *ZIP* and *HMA* family genes in various tissues of rice grown under Zn deficient and sufficient conditions with different treatments of $ZnNO_3$

The expression of *OsZIP1–OsZIP10* and *OsHMA2* was analyzed in root, node I, flag leaf, and panicle tissues. Melt curve analysis showed single, sharp peaks for all genes, indicating specific amplification. No primer-dimer formation or non-specific products were observed (Fig. 36).

4.6.3.1 Root

OsZIP1 gene was found to be expressed in the root tissues under different treatments under Zn deficient and sufficient conditions. However, it was higher in the roots of plants raised from seed priming, under Zn sufficient condition than Zn deficient condition. Among the 10 *OsZIPs*, *OsZIP2* was highly induced in the Zn deficient control root than other ZIPs. In contrast, when plants were subjected to different treatments such as seed priming, foliar spray and seed priming + foliar

spray, the expression of *OsZIP2* was decreased in the root under the Zn deficient condition. Whereas, *OsZIP2* expression level was higher in roots of plants raised from seed priming and combined treatment of seed priming and foliar spray under Zn sufficient condition. Like *OsZIP2*, the expression of *OsHMA2* was decreased under the Zn deficient condition compared to the Zn sufficient condition in the roots of plants raised from seed priming and combined treatment of seed priming and foliar spray. Contrastingly, the expression of the same gene increased upon foliar spray in the roots under the Zn deficient condition compared to the Zn sufficient condition. *OsZIP10* showed higher expression in root tissues under the combined treatment of seed priming and foliar spray in the Zn sufficient condition, and this was the only gene that was induced at higher levels in roots compared to other *ZIP* family genes (Fig. 37).

4.6.3.2 Node I

Only a few ZIP family genes were expressed in node I tissues under all treatments. Three genes (*OsZIP2*, *OsZIP3*, and *OsZIP4*) showed increased expression at higher and moderate levels in all treatments under both Zn deficient and Zn sufficient conditions compared to other *OsZIP* genes. *OsZIP3* was highly expressed in the node I grown under the Zn sufficient condition without any treatments. Similarly, *OsZIP4* was found to be highly expressed in node I grown under Zn deficient condition without any treatments. Thus could be identified as a key gene involved in Zn transport in node I under Zn deficient condition (Fig. 38).

4.6.3.3 Flag leaves

In flag leaves, only few *OsZIP* were expressed, and they showed higher expression levels under the Zn sufficient condition compared to the Zn deficient condition. Among control treatments, the expression level of *OsZIP2* and *OsZIP10* was moderately higher in the flag leaves under Zn sufficient control compared to Zn deficient control. Similarly, the expression of *OsZIP8* increased in the flag leaves of control plants upon seed priming under the Zn sufficient condition compared to different treatments at Zn deficient condition. However, on analysing the expression pattern of ten *ZIP*s in rice, subjected to different treatments in the Zn deficient

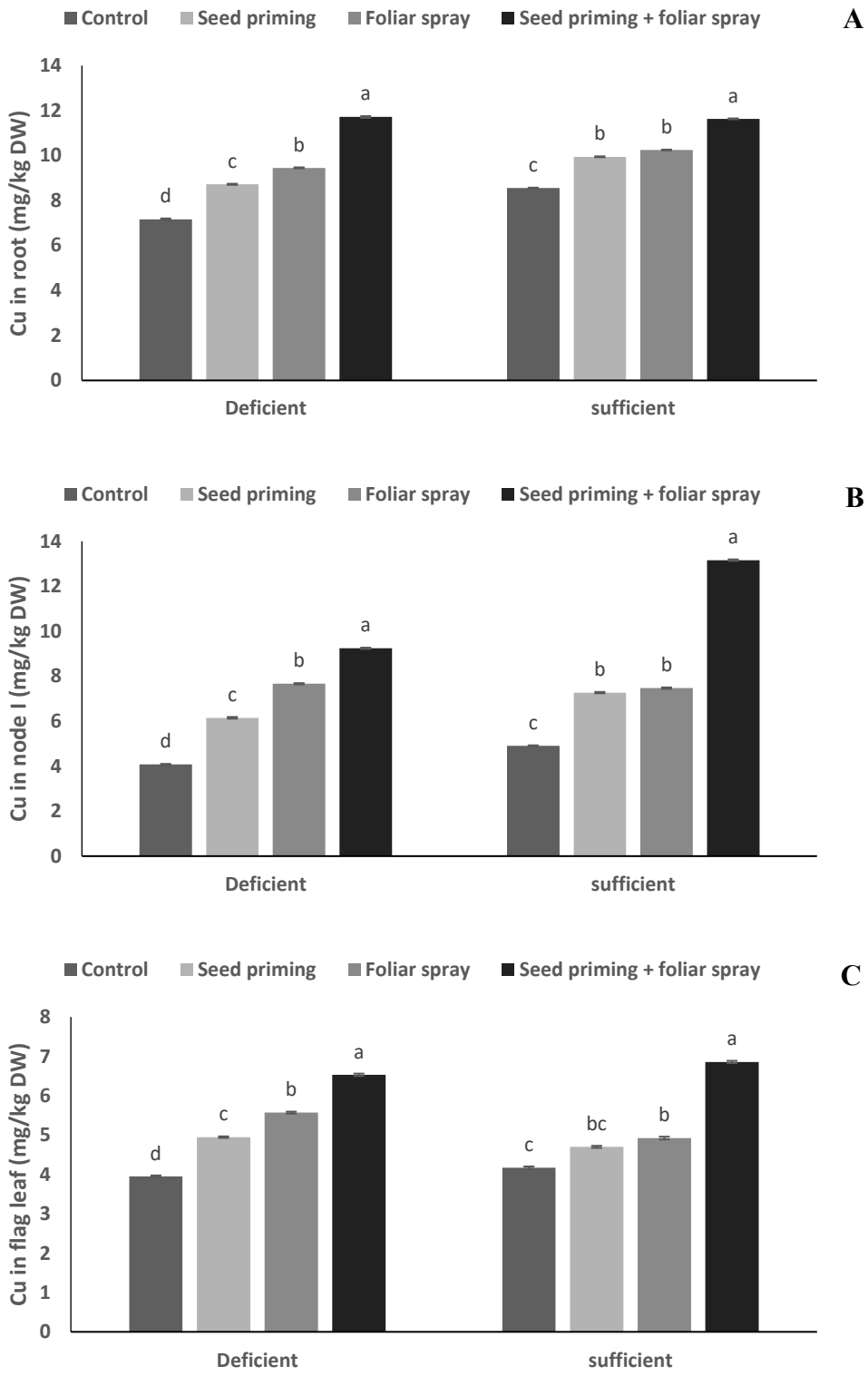


Figure 32. Cu content in various tissues, root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

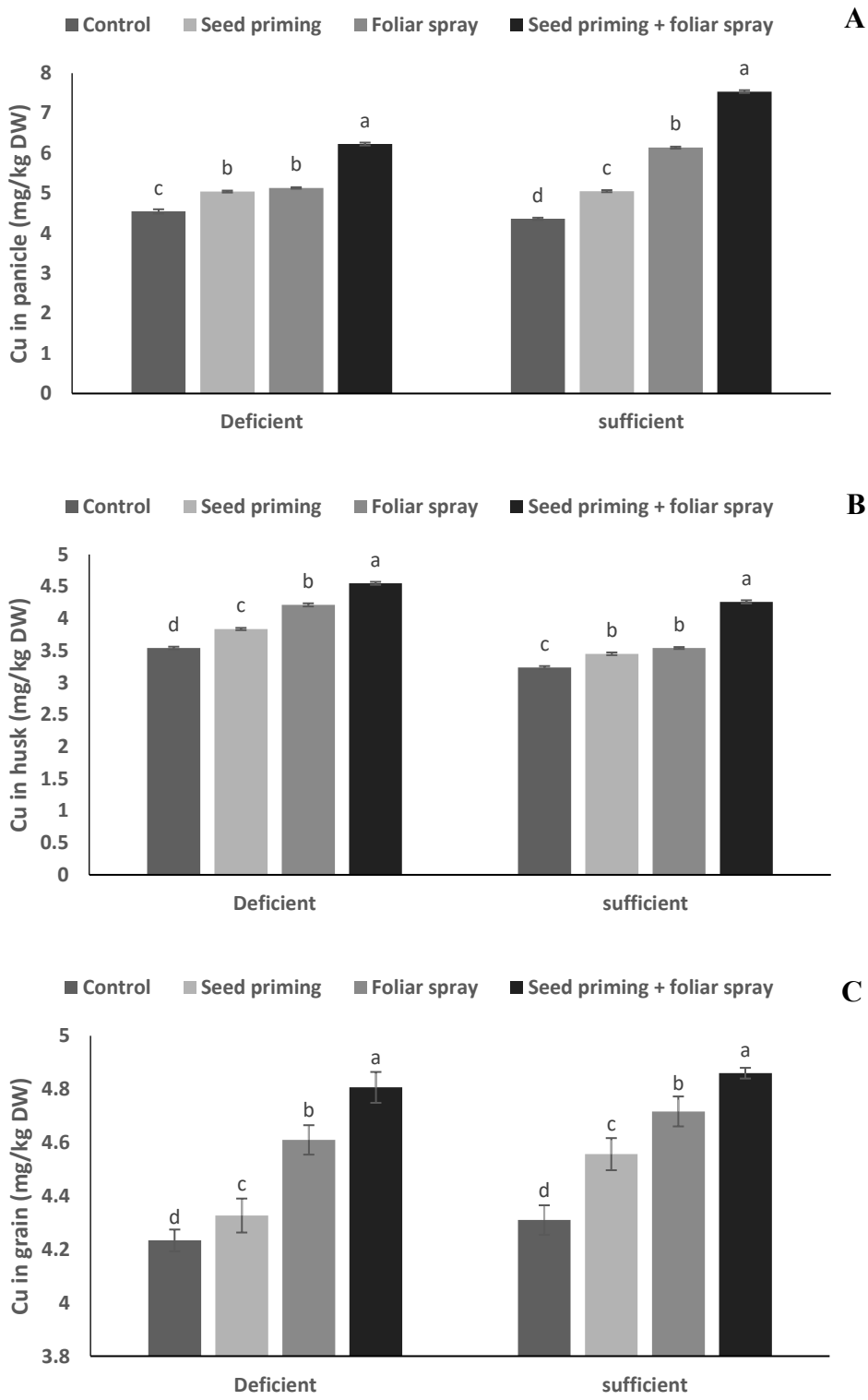


Figure 33. Cu content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

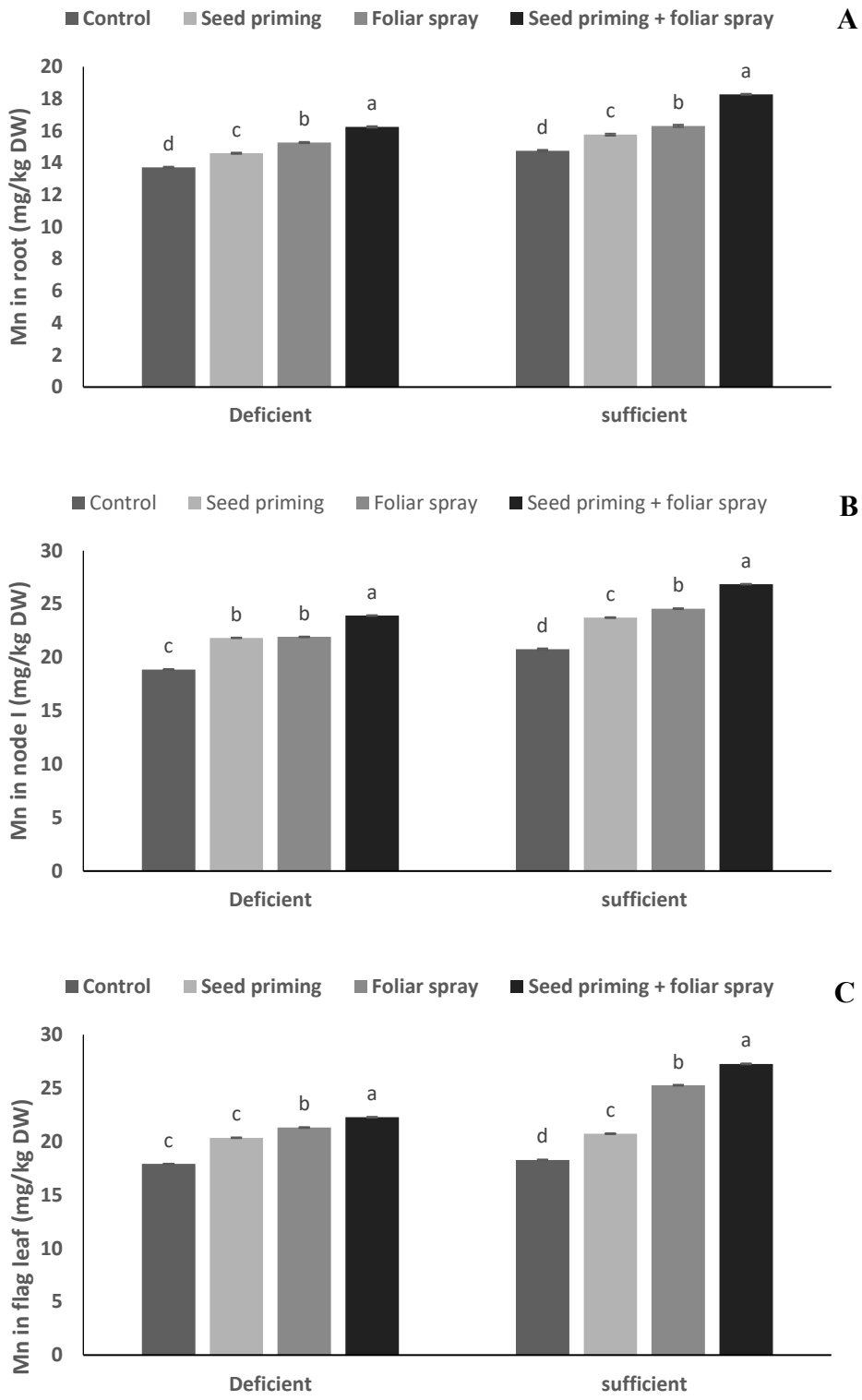


Figure 34. Mn content in various tissues root (A), node I (B) and flag leaf (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

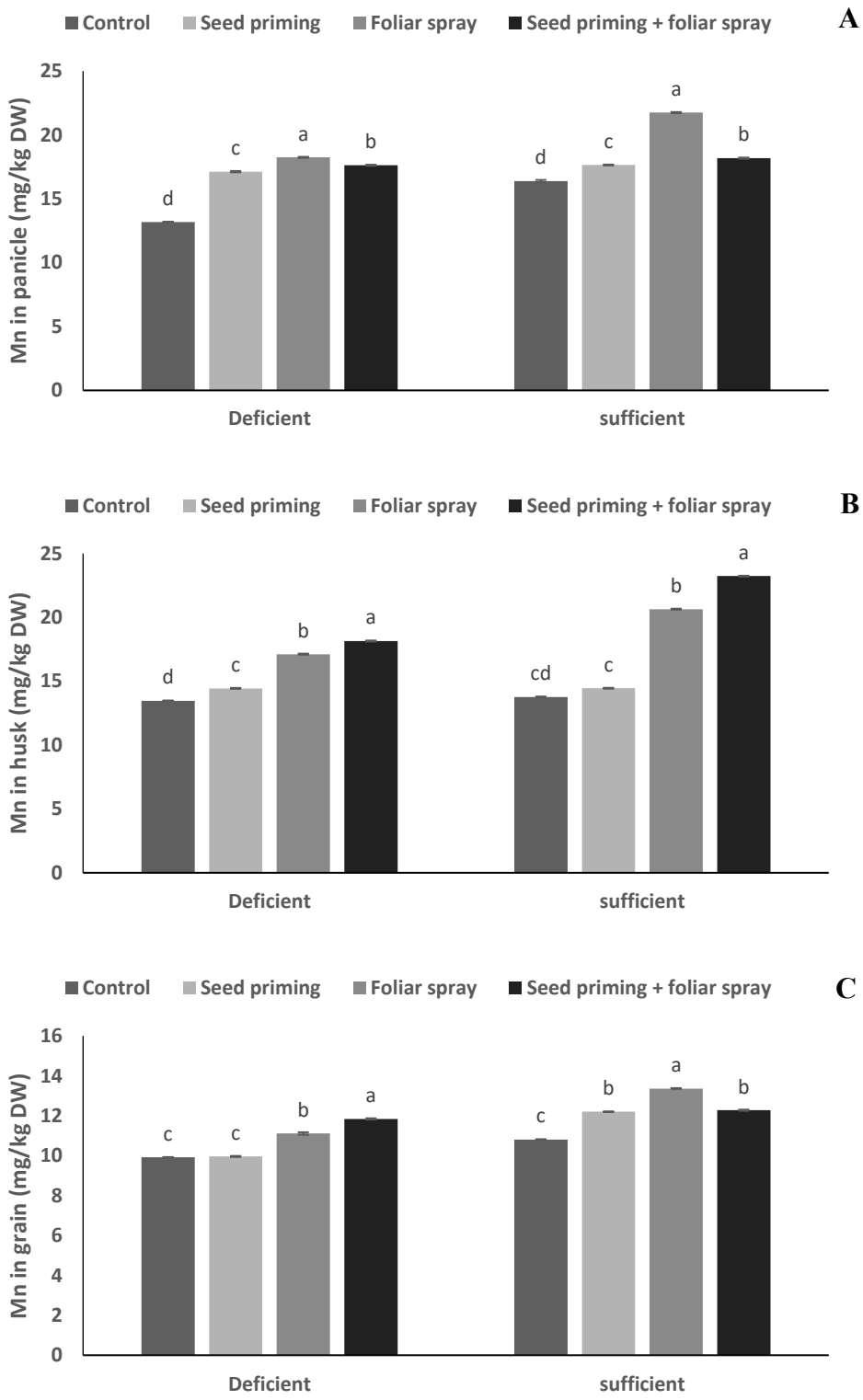


Figure 35. Mn content in various tissues, panicle (A), husk (B) and grain (C) upon seed priming, foliar spray and seed priming + foliar spray under Zn deficient and sufficient conditions. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$)

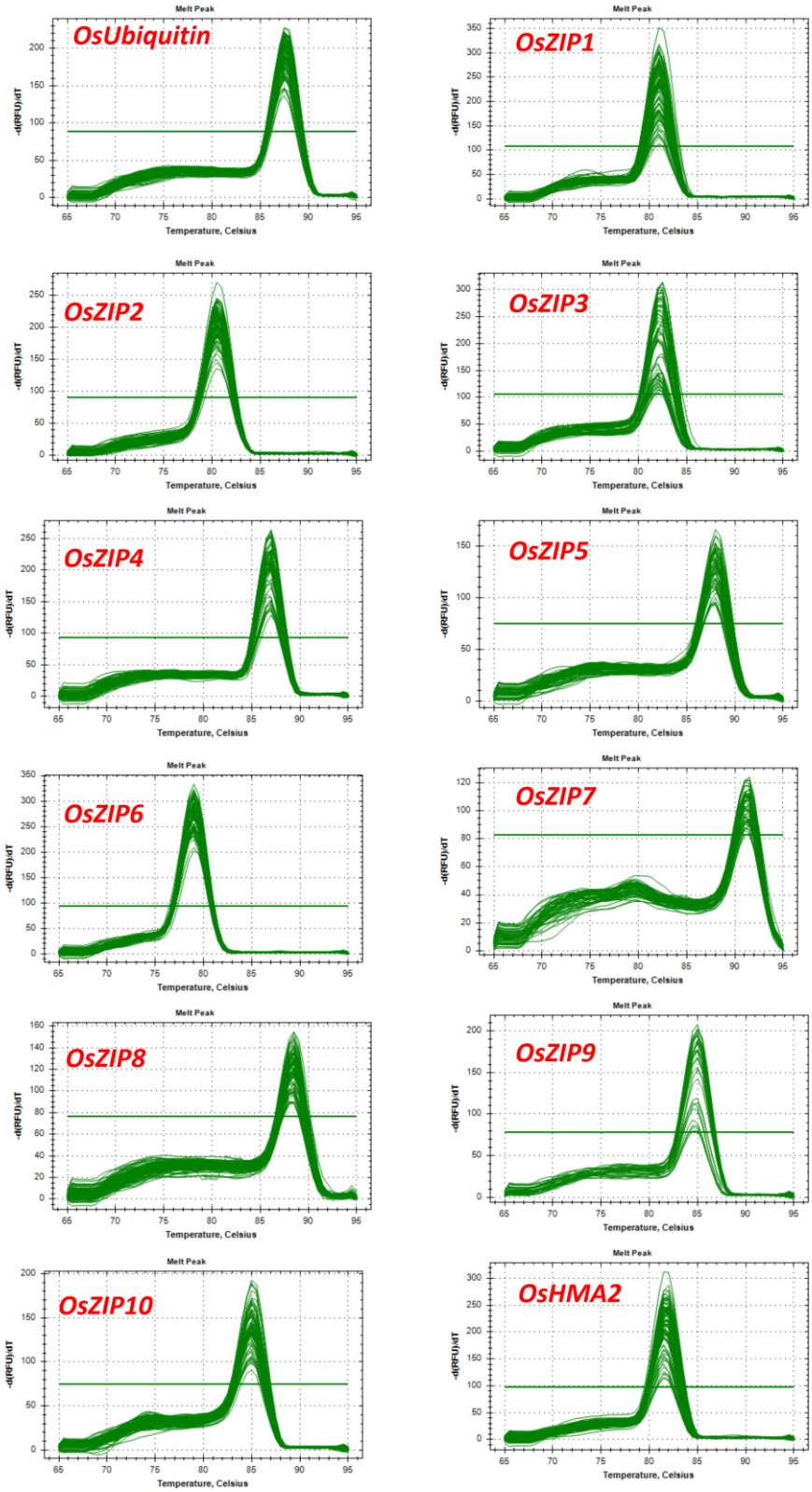


Figure 36. Image of melt curves of genes in qRT-PCR analysis. The height of the melt peak having the corresponding melting temperature of the cDNA

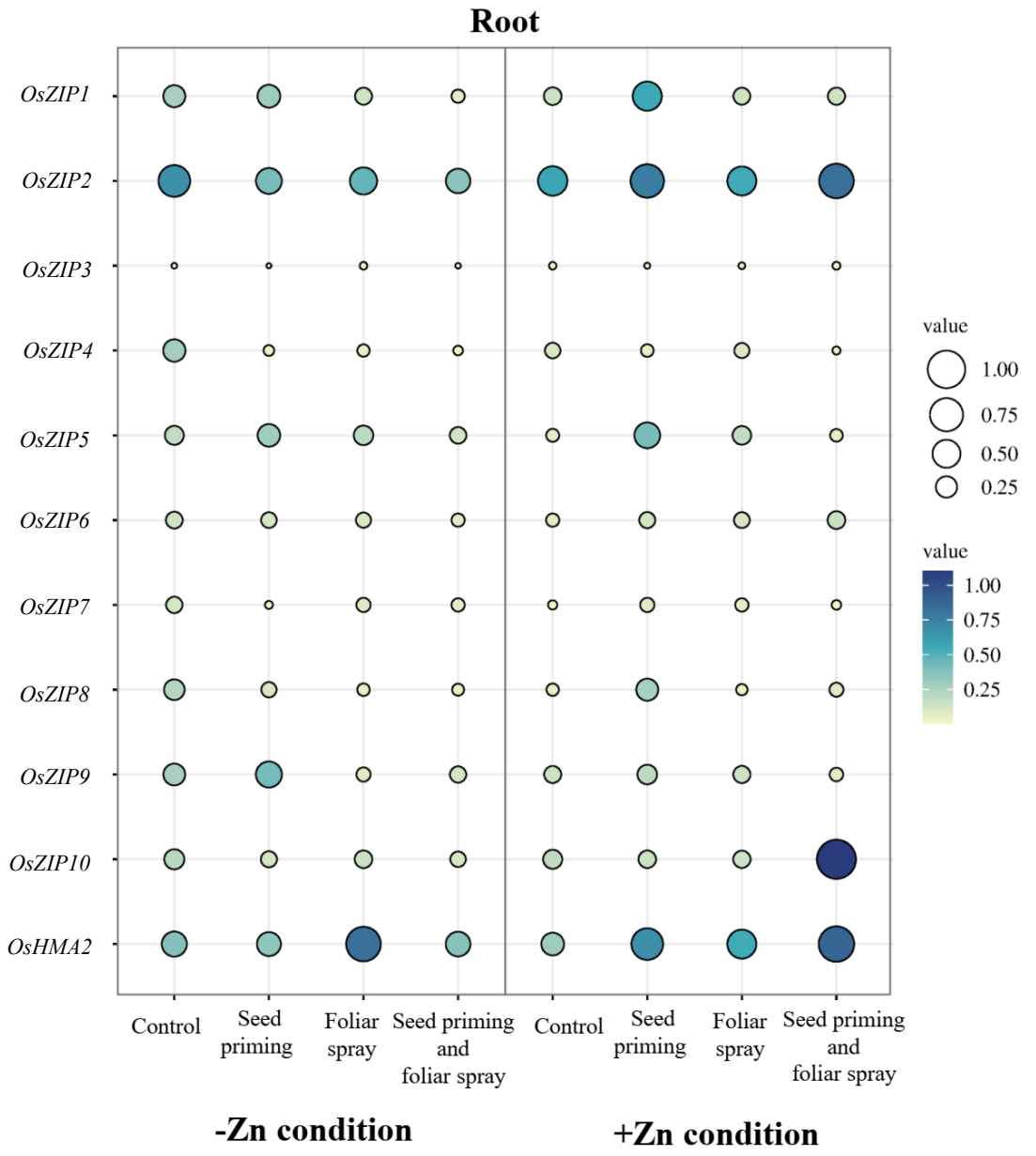


Figure 37. Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in root upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions were analysed. Os*Ubiquitin* was used as a constitutive gene to normalize the expression level of each gene in this study

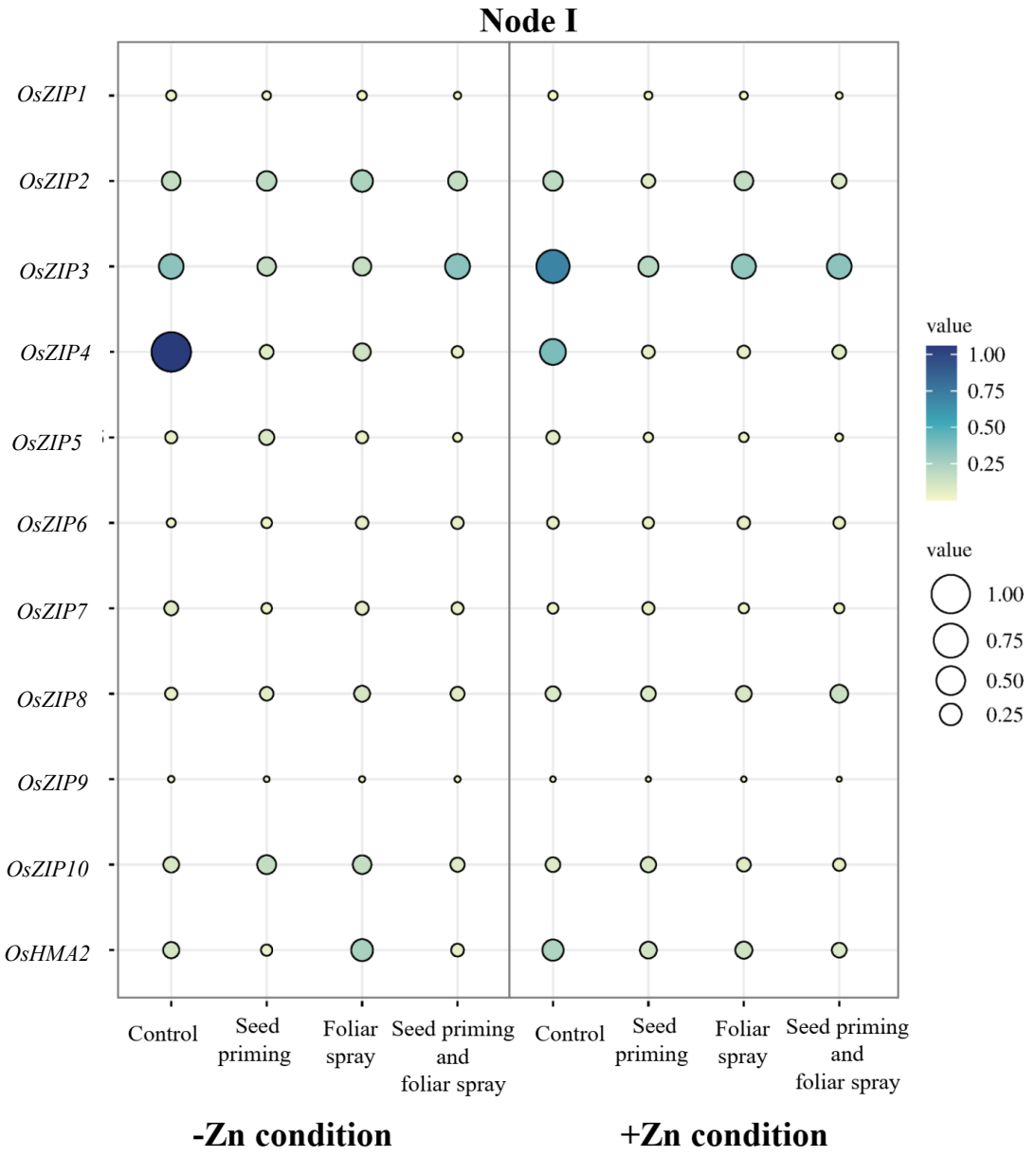


Figure 38. Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in node I upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions were analysed. Os*Ubiquitin* was used as a constitutive gene to normalize the expression level of each gene in this study

condition, *OsZIP2* and *OsZIP8* showed moderate expression levels in flag leaves of plants treated with seed priming and foliar spray, when compared to control plants and combined treatment of seed priming and foliar spray (Fig. 39).

4.6.3.4 Panicle

Only a minimal number of *OsZIP* genes was expressed in the panicle. The *OsZIP2* expression was found in the panicle in all treatments under both Zn deficient and Zn sufficient conditions. However, it was highly expressed in panicles of plants raised from seed priming under the Zn deficient condition. The same gene had moderate expression in the panicle during the foliar spray treatment under the Zn sufficient condition. *OsZIP10* expression was higher in the control under Zn sufficient condition. When comparing the expression levels of *OsZIP10* between treatments under Zn deficient conditions, seed priming had moderate expression. Seed priming alone and foliar spray alone treatments showed higher expression of *OsHMA2* under the Zn deficient and Zn sufficient conditions, respectively (Fig. 40).

4.6.4 Principal component analysis of various traits under study

The PCA plot illustrates the proportion of variance accounted for by each principal component. PC1 represents 42.7% of the variance, while PC2 accounts for 16.94%. Collectively, they account for almost 59.64% of the overall variance. The PCA biplot emphasises the effects specific to treatments in both Zn deficient and Zn sufficient conditions. The experimental treatments consist of a Zn deficient control (negative control), Zn sufficient control (positive control), seed priming, foliar spray and combined treatments of both seed priming and foliar spray. The plot shows a clear separation between Zn deficient (red) and Zn sufficient (blue) conditions. This indicates that the two Zn conditions differ substantially in their overall treatment effects. Under both Zn deficient and Zn sufficient conditions, treatments are distributed in distinct clusters, suggesting that each treatment contributes differently to the variation in traits. The control treatments (red and blue circles) are positioned at the extremes for each condition, further supporting the differential impact of treatments. The combined seed priming + foliar spray (square symbols) is the most

effective in differentiating the two conditions, suggesting its potential in enhancing the studied traits, particularly under Zn deficient conditions (Fig. 41).

4.6.7 The effect of Zn priming being carried over into the subsequent generation

This study examined the second-generation effects of seed priming on plants raised from seeds acquired in the first generation. For the current experiment, seeds were selected from controls (Zn deficient and Zn sufficient and combined seed priming and foliar application [Zn deficient (seed priming+ foliar spray) and Zn sufficient (seed priming+ foliar spray)]. The seeds were either non-primed or primed in the second generation, and evaluation of their yield and grain Zn content were carried out under the same soil and environmental conditions. The present study focused, significant attention on understanding the benefits of repriming second-generation seeds obtained from previously primed plants, so as to understand whether any priming imprints remained in primed plants, and it was compared with control non-primed plants (Table 25).

4.6.7.1 Agronomic and yield traits

The plants raised from seeds that were primed in the previous generation were taller than those plants that were raised from seeds that are non-primed in the previous generation. For instance, plant height was 108.17 cm in plants raised from seeds of Zn deficient control in the previous generation and non-primed in current generation and it was 136.28 cm in plants raised from seeds of Zn deficient seed priming+ foliar sprayed plants in the previous generation and non-primed in present generation. Re-priming also led to additional gains in plant height. The length of the panicles varied among all the groups. Plants raised from seeds of plants grown in Zn sufficient control in the previous generation, that are non-primed in this generation had a 12% increase in panicle length compared to plants raised from seeds of plants that were grown under Zn deficient condition without priming in the previous generation and also non-primed in the current generation. When compared to the plants raised from seeds of plants that were grown under both Zn deficient and sufficient conditions without priming in the previous generation (control), the plants

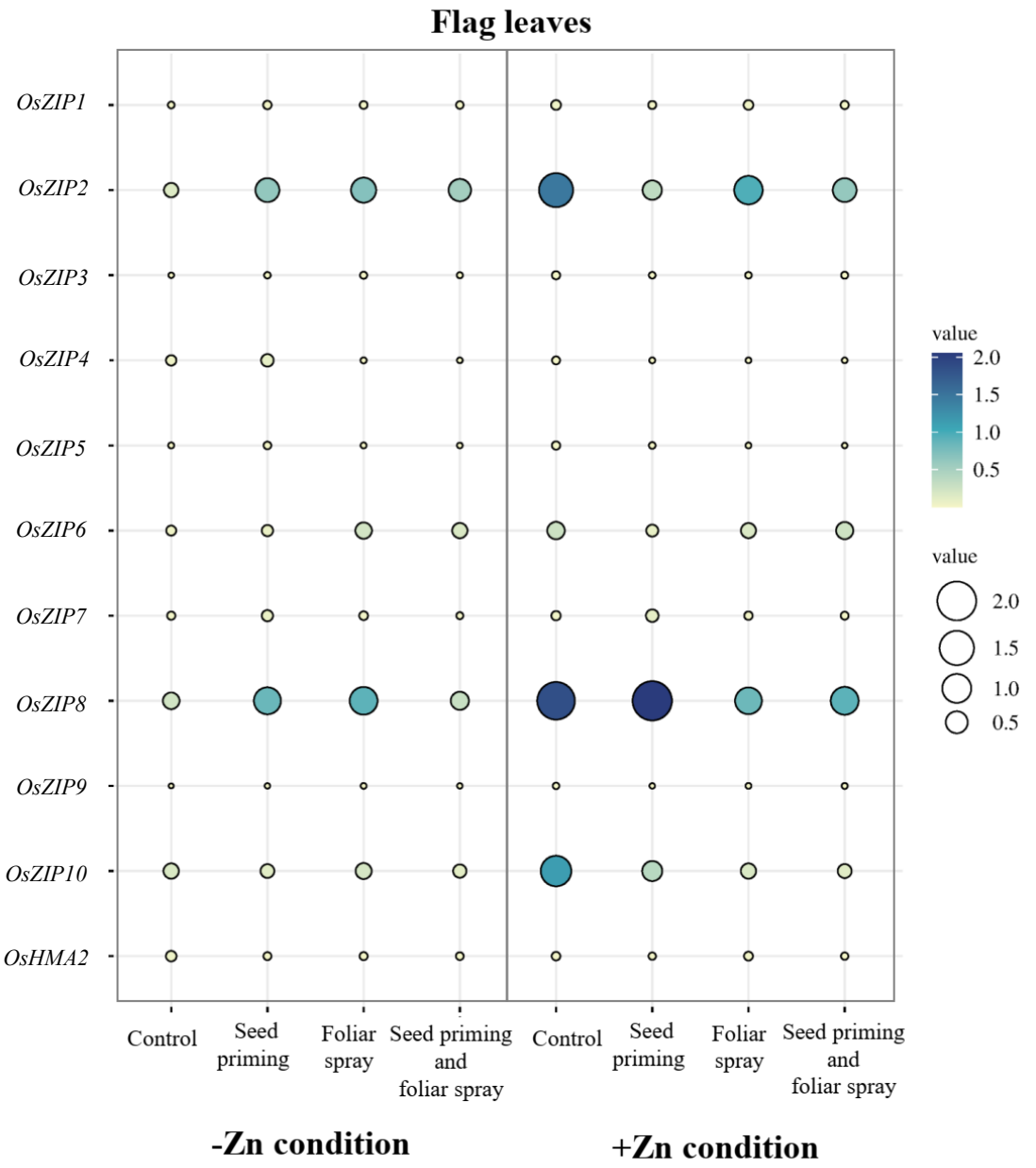


Figure 39. Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in flag leaf upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions were analysed. Os*Ubiquitin* was used as a constitutive gene to normalize the expression level of each gene in this study

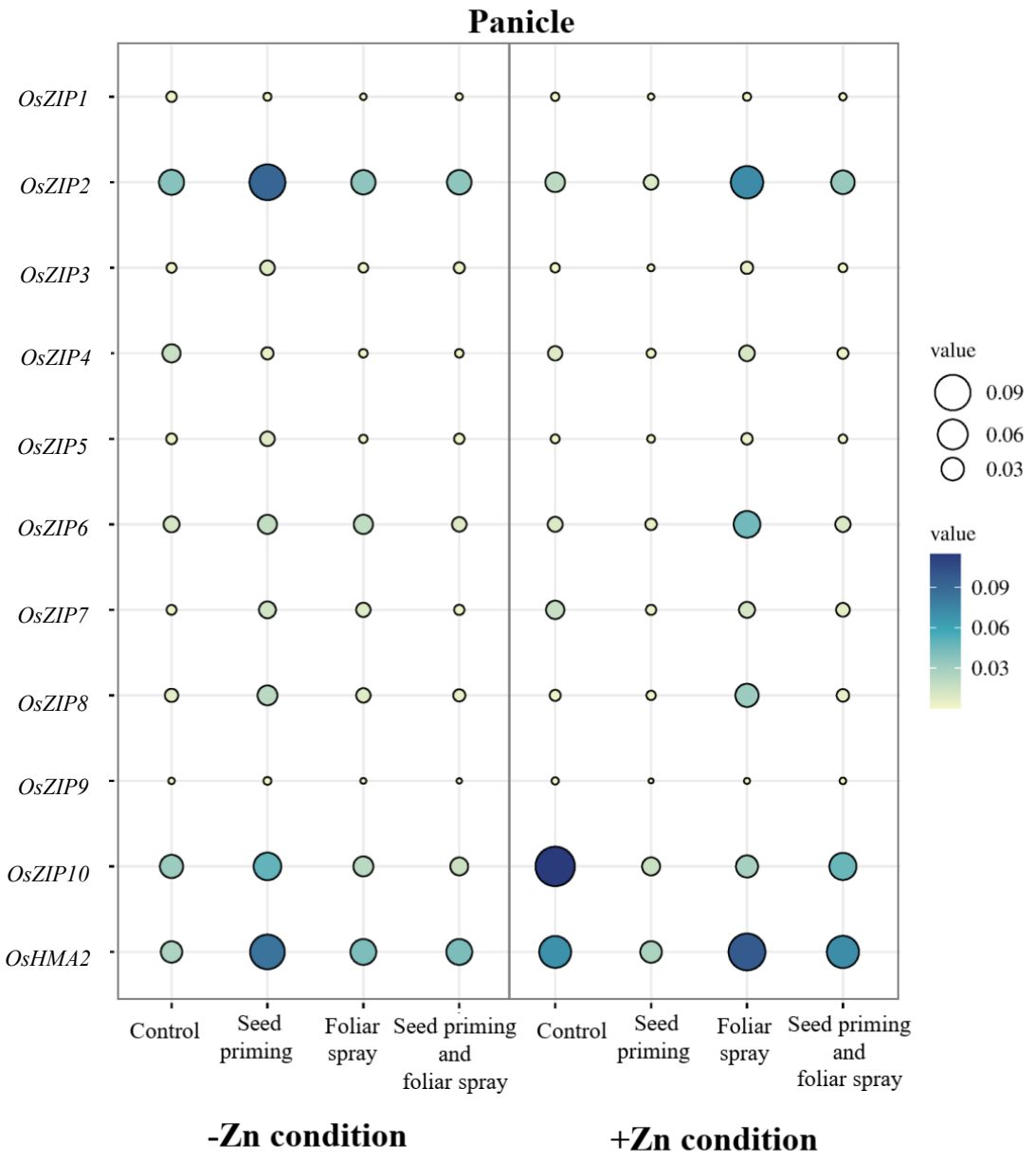


Figure 40. Expression analysis of *ZIP* and *HMA* family genes in different tissues of rice grown under various treatments, under Zn deficient and sufficient conditions. The expression of 10 *ZIP* family genes and 1 *HMA* gene in panicle upon various treatments of $ZnNO_3$ under Zn deficient and sufficient conditions were analysed. Os*Ubiquitin* was used as a constitutive gene to normalize the expression level of each gene in this study

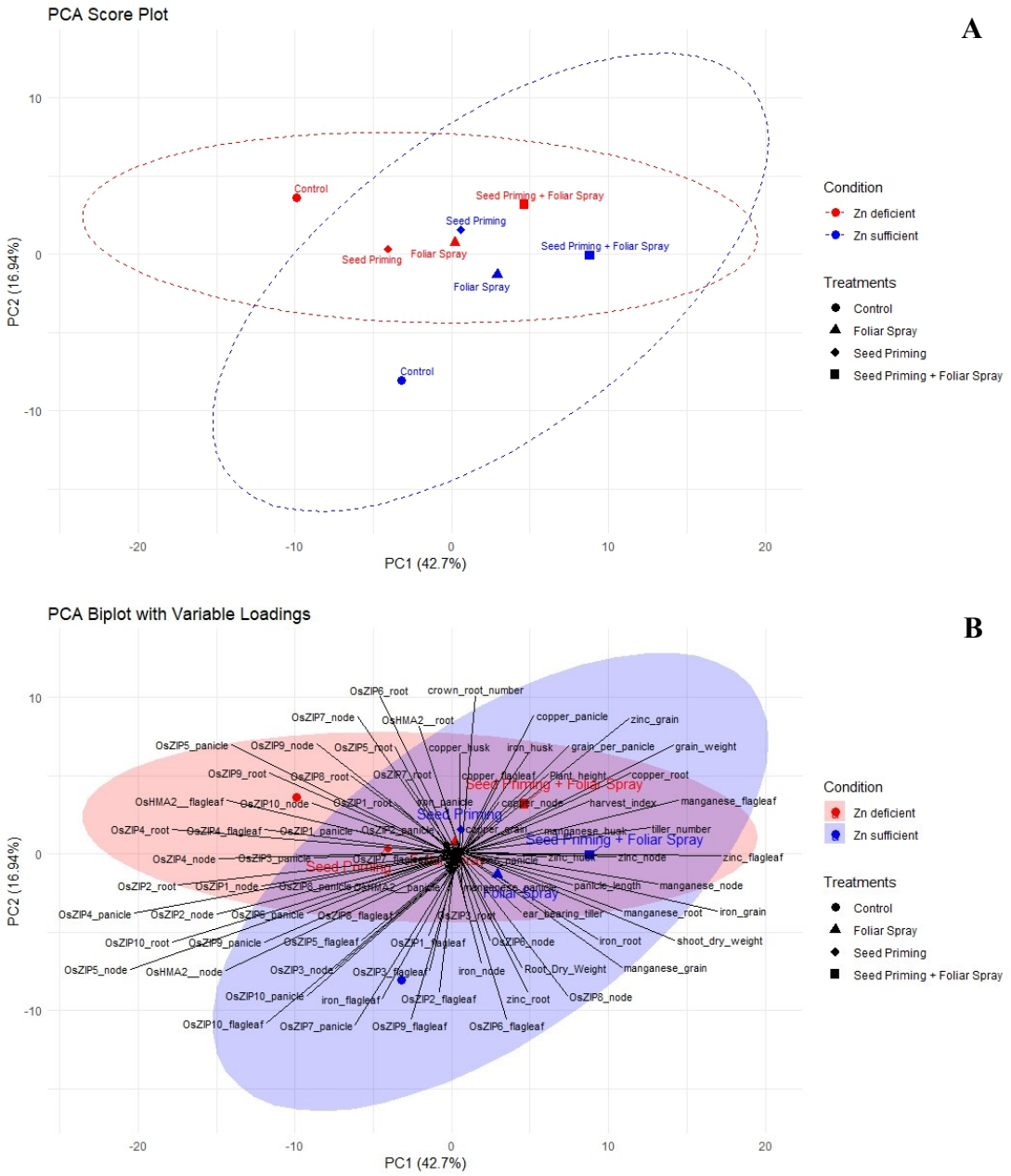
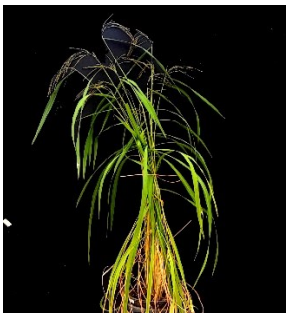


Figure 41. Principal Component Analysis (PCA) biplot (A) depicting the distribution of treatments (control, seed priming, foliar spray, seed priming + foliar spray) under Zn deficient and sufficient conditions based on gene expression, yield traits, and elemental content. PCA biplot (B) showing the correlation between gene expression, yield traits, and elemental content under Zn deficient and sufficient conditions. Arrows represent variable loadings, indicating the contribution of different parameters to the principal components. Different shapes represent treatments (control, seed priming, foliar spray, seed priming + foliar spray), and colours distinguish Zn conditions.

Table 25. Agronomic and yield parameters and grain Zn content in Kumkumashali in subsequent generation. Values are expressed as mean ± SE. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

ZDC NP 	Plant height (cm)	108.17 ± 1.26^{c}
	Panicle length (cm)	19.13 ± 0.55^{d}
	Grains per panicle	79.00 ± 2.00^{d}
	100 grain weight	1.38 ± 0.01^{c}
	HI %	33.94 ± 1.03^{d}
	Grain Zn content (mg/kg)	12.30 ± 0.15^{d}
ZDC P 	Plant height (cm)	118.17 ± 1.35^{b}
	Panicle length (cm)	22.50 ± 0.40^{c}
	Grains per panicle	83.33 ± 1.53^{c}
	100 grain weight	1.44 ± 0.01^{b}
	HI %	38.25 ± 0.28^{c}
	Grain Zn content (mg/kg)	16.50 ± 0.29^{c}
ZD SP+FS NP 	Plant height (cm)	136.28 ± 1.10^{a}
	Panicle length (cm)	23.48 ± 0.67^{b}
	Grains per panicle	108.33 ± 1.53^{b}
	100 grain weight	1.43 ± 0.02^{b}
	HI %	43.28 ± 1.20^{b}
	Grain Zn content (mg/kg)	27.37 ± 0.15^{b}
ZD SP+FS P 	Plant height (cm)	138.31 ± 1.50^{a}
	Panicle length (cm)	24.45 ± 3.51^{a}
	Grains per panicle	120.67 ± 1.00^{a}
	100 grain weight	1.48 ± 0.04^{a}
	HI %	45.18 ± 0.78^{a}
	Grain Zn content (mg/kg)	30.37 ± 0.26^{a}

ZSC NP 	Plant height (cm)	124.27 ± 1.63^{c}
	Panicle length (cm)	21.47 ± 0.45^{d}
	Grains per panicle	91.67 ± 1.53^{d}
	100 grain weight	1.40 ± 0.01^{d}
	HI %	41.00 ± 0.89^{c}
	Grain Zn content (mg/kg)	19.17 ± 0.44^{d}
ZSC P 	Plant height (cm)	134.00 ± 1.00^{b}
	Panicle length (cm)	22.37 ± 0.32^{c}
	Grains per panicle	99.33 ± 1.53^{c}
	100 grain weight	1.43 ± 0.01^{c}
	HI %	43.77 ± 0.41^{ab}
	Grain Zn content (mg/kg)	25.33 ± 0.18^{c}
ZS SP+FS NP 	Plant height (cm)	137.00 ± 1.32^{a}
	Panicle length (cm)	23.53 ± 0.15^{b}
	Grains per panicle	109.33 ± 1.53^{b}
	100 grain weight	1.45 ± 0.02^{b}
	HI %	43.02 ± 0.82^{b}
	Grain Zn content (mg/kg)	27.33 ± 0.30^{b}
ZS SP+FS P 	Plant height (cm)	139.03 ± 1.00^{a}
	Panicle length (cm)	24.50 ± 0.50^{a}
	Grains per panicle	121.67 ± 1.53^{a}
	100 grain weight	1.50 ± 0.01^{a}
	HI %	44.92 ± 0.41^{a}
	Grain Zn content (mg/kg)	30.27 ± 0.18^{a}

ZDC NP [Zinc-deficient, control (previous), non-primed (second stage)], ZDC P [Zinc-deficient, control (previous), primed (second stage)], ZD SP + FS NP [Zinc-deficient, seed primed + foliar sprayed (previous), non-primed (second stage)], ZD SP + FS P [Zinc-deficient, seed primed + foliar sprayed (previous), primed (second stage)]. ZSC NP [Zinc-sufficient, control (previous), non-primed (second stage)], ZSC P [Zinc-sufficient, control (previous), primed (second stage)], ZS SP + FS NP [Zinc-sufficient, seed primed + foliar sprayed (previous), non-primed (second stage)], ZS SP + FS P [Zinc-sufficient, seed primed + foliar sprayed (previous), primed (second stage)]

raised from seeds of plants that were primed and foliar sprayed in the previous generation grown under both deficient and sufficient conditions of Zn had increased panicle length by 23 and 10% respectively. This emphasize the benefits of seed priming and foliar spray for subsequent generations. Priming enhanced the panicle length in plants raised from seeds of Zn deficient control plants from previous generation, when primed in the current generation by 18% when compared to control plants, which are not primed in the previous generation grown under same condition. The re-priming of seeds from the Zn deficient seed priming + foliar sprayed plants from the previous generation increased the panicle length compared to the respective non-primed ones, indicating the benefit of the re-priming of seeds (Table 25).

The number of grains/panicle was increased by 16% in plants raised from seeds of plants that were grown under Zn sufficient condition without priming in the previous generation, when compared to plants raised from seeds of plants grown under Zn deficient condition without priming in the previous generation. The priming in the current generation enhanced the number of grains/panicle in plants raised from seeds of Zn deficient control in the previous generation by 6 to 8%, when compared to plants grown from seeds from the control plants of previous generation grown under both conditions of Zn. When compared to the plants that are raised from previously non-primed plants grown under both Zn deficient and sufficient control conditions, the plants that were primed and foliar sprayed with $ZnNO_3$ in the previous generation, but not primed in the current generation showed a marked increase in number of grains/panicle by 37% and 19%, respectively. In addition, re-priming in these plants in the current generation also enhanced number of grains/panicle by 11%. When compared to other variables, the 100-grain weight was least affected. The plants raised from seeds of Zn sufficient control (non-primed in the previous and current generations), showed a 21% increase in HI compared to plants raised from seeds of Zn deficient control plants (non-primed in the previous and current generations). The plants raised from seeds of previously primed plants grown under Zn deficient condition in the previous generation also showed enhancement in HI by 28% compared to the plants that are raised from seeds that

was non-primed and grown under Zn deficient condition in the previous generation. This again ascertain the positive effect of priming in the subsequent generation of plants, even though it is not primed in the current generation. Priming also boosted the HI, increasing it by 13% in plants raised from seeds of Zn deficient non-primed plants in the previous generation, that are primed in the current generation, compared to plants raised from seeds of Zn deficient non-primed plants in the previous generation. Re-priming of earlier primed plants (Zn deficient, seed priming + foliar spray and Zn sufficient, seed priming + foliar spray) also showed further enhancement in HI (Table 25).

4.6.7.2 Grain Zn content

The priming and re-priming caused differences in Zn content among various experimental groups. The plants raised from non-primed Zn deficient control and Zn sufficient control plants showed a difference of 56% in Zn content. In plants raised from Zn deficient, seed priming + foliar spray, there was 27.36 mg/kg DW of grain Zn content, while in plants raised from Zn deficient control, non-primed had only 12.3 mg/kg DW. There was an increase of 43% in grain Zn content for plants raised from seeds of seed priming + foliar sprayed plants grown under Zn sufficient condition in the previous generation and non-primed in the current generation, when compared to plants raised from seeds of control plants grown under Zn deficient condition, in the previous generation, which are non-primed in the current generation. Re-priming increased the grain Zn content in plants raised from Zn deficient control (previous generation), which are primed in the current generation (34%) and Zn sufficient control (previous generation), which are primed in the current generation (32%) compared to their non-primed counterparts (Zn deficient and sufficient control, non-primed plants). While in plants raised from seeds of Zn deficient, seed priming + foliar sprayed, and Zn sufficient, seed priming + foliar sprayed plants from the previous generation, re-priming increased grain Zn content by 11% and 12% respectively on comparison with Zn deficient, seed priming + foliar sprayed, and Zn sufficient, seed priming + foliar sprayed, plants in the previous generation, which are non-primed in the current generation (Table 25).

4.6.7.3 Principal component and correlation analysis

PC1 (91.8%) accounts for most of the variation in the data, while PC2 (5.52%) captures a smaller fraction of the variability. Grain Zn content, plant height, and grain number strongly contribute to PC1, meaning that treatments positioned in this direction tend to have higher values for these traits. Specifically, treatments such as priming in the first generation and re-priming in the subsequent generation align with these traits, suggesting their effectiveness in enhancing Zn accumulation and yield-related parameters. In contrast, non-primed plants, particularly those in the first generation grown under Zn-deficient conditions, are positioned farther from these traits, indicating lower performance in yield and Zn content. The strong positive correlations between grain Zn content and yield-related traits (grain number, panicle length, plant height, and harvest index) further suggest that biofortification strategies, such as seed priming, its memory and re-priming contribute to both improved Zn accumulation and higher yield (Fig. 42).

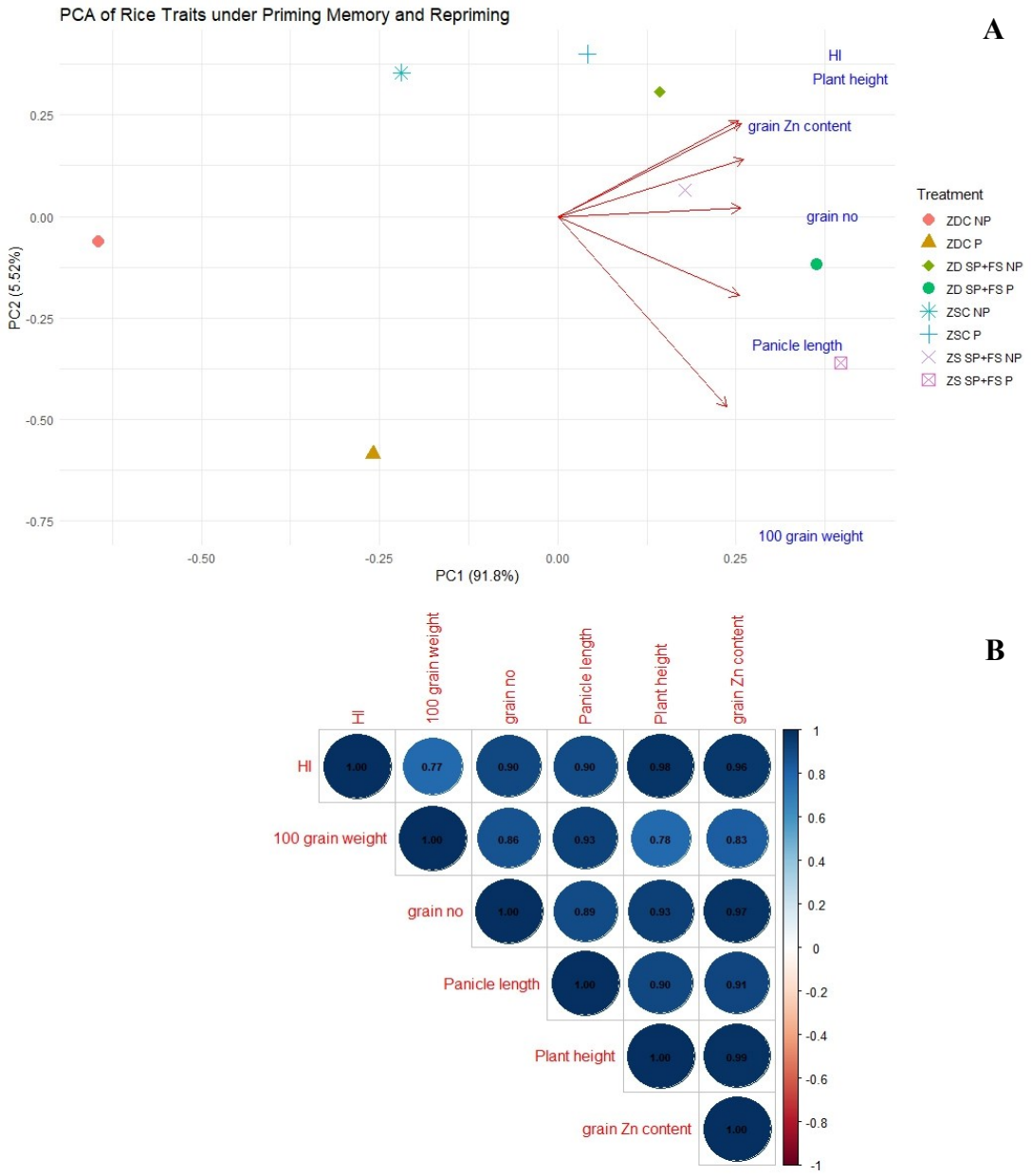


Figure 42. Principal component analysis (PCA) biplot of rice traits under various treatments involving priming memory and repriming (A). Correlation matrix (B) of rice traits under various treatments. The strength of the correlation between traits is represented by both the colour intensity and the size of the circles. ZDC NP [Zinc-deficient, control (previous), non-primed (second stage)], ZDC P [Zinc-deficient, control (previous), primed (second stage)], ZD SP + FS NP [Zinc-deficient, seed primed + foliar sprayed (previous), non-primed (second stage)], ZD SP + FS P [Zinc-deficient, seed primed + foliar sprayed (previous), primed (second stage)], ZSC NP [Zinc-sufficient, control (previous), non-primed (second stage)], ZSC P [Zinc-sufficient, control (previous), primed (second stage)], ZS SP + FS NP [Zinc-sufficient, seed primed + foliar sprayed (previous), non-primed (second stage)], ZS SP + FS P [Zinc-sufficient, seed primed + foliar sprayed (previous), primed (second stage)]

DISCUSSION

5.1 Screening of rice varieties for biofortification with Zn

The micronutrient composition of rice samples serves as a measure of its quality. Zinc (Zn) deficiency in rice-based diets represents a critical global issue, highlighting the need for the development of Zn-enriched rice varieties via biofortification. A crucial step in this process involves the systematic screening of various rice genotypes to identify those with enhanced Zn uptake, translocation, and accumulation in grains. This study screened 30 rice varieties, comprising 15 landraces and 15 elite varieties, to assess their potential for Zn biofortification. Landraces exhibit significant genetic diversity and demonstrate resilience to abiotic stresses, particularly in their capacity to acquire and accumulate micronutrients efficiently. Many traditional landraces cultivated in nutrient-deficient soils are therefore important genetic resources for improving Zn-use efficiency. Conversely, elite varieties are developed for enhanced yield and agronomic performance, yet they exhibit differing abilities for Zn accumulation (Senguttuvel et al., 2023). Through the screening of landraces and elite varieties, this study seek to identify genotypes that exhibit high Zn accumulation alongside favourable agronomic traits, thereby ensuring that biofortified rice is both nutritionally rich and high-yielding.

Deficiencies in Zn and Iron (Fe) contribute to the phenomenon of micronutrient malnutrition in human populations. The rice varieties exhibiting optimal concentrations of these minerals represent promising candidates for biofortification efforts. Biofortification represents a sustainable approach aimed at improving the nutritional quality of staple crops by augmenting their micronutrient content via genetic selection, agronomic practices, or biotechnological methods. The objective is to tackle prevalent micronutrient deficiencies, especially in areas where diets predominantly consist of staple foods that possess low intrinsic nutrient value. Biofortification has the capacity to enhance human health by mitigating malnutrition-related disorders and offers a sustainable, cost-efficient solution for

nutrient security, particularly in resource-constrained environments (Zulfiqar et al., 2024).

The Zn concentration in the analysed samples was found to be less than the concentrations observed in native aromatic rice varieties from West Bengal, which ranged from 50.21 to 91.64 mg/kg DW (Ghosh and Roychoudhury, 2020), as well as in the Gangetic Plains of India, where Zn levels varied from 14.5 to 281 mg/kg (Tyagi et al., 2020). Nonetheless, the Zn content was comparable to the concentrations found in landraces from Arunachal Pradesh, India (15.5 to 33.4 mg/kg DW) (Longvah and Prasad, 2020). Rao et al. (2020) have indicated that brown rice containing 35 mg/kg of Zn or higher are appropriate for biofortification purposes. The findings of this study will aid in identifying superior candidates for biofortification initiatives, as the Zn concentrations in the landraces (of the 15 varieties examined, 3 exhibited levels exceeding 35 mg/kg DW) and hybrid varieties (of the 15 varieties examined, 3 also showed levels above 35 mg/kg DW) surpassed this threshold. The iron concentrations in the analysed samples were found to be lower than those documented in other regions of India, such as West Bengal, where values ranged from 4.01 to 87.7 mg/kg DW (Ghosh and Roychoudhury, 2020; Longvah and Prasad, 2020; Rao et al., 2020).

Manganese (Mn) is a crucial trace element that serves as a cofactor for numerous enzymes and plays a vital role in the functionality of the oxygen-evolving complex associated with photosystem II. Nevertheless, in contrast to Zn and Fe, Mn deficiency is infrequently observed in humans. The Mn concentration in the analysed rice samples was comparable to that found in rice samples from the Gangetic Plains of India, specifically ranging from 9.6 to 32.7 mg/kg DW (Longvah and Prasad, 2020). Boron (B) and Molybdenum (Mo) are crucial micronutrients that plants require for optimal growth and productivity. This study reported B concentrations ranging from 0.44 to 1.27 mg/kg DW, with an average Mo concentration of 0.68 mg/kg DW. Li et al. (2013) studied the content of B in rice grains obtained from nine regions in China and found that the range of B varied from 0.203 to 0.690 mg/kg DW Even though this study was in concurrent with the

present study, the mineral profiling conducted in Indian rice showed more B compared to Chinese rice. In addition, a mineral profiling study in 1100 rice genotypes from International Rice Gene Bank of International Rice Research Institute, Philippines recorded the maximum amount of Mo in the range 1 to 2 mg/kg DW (Tiozon et al., 2024), which is on par with our study. The maximum concentration of Ni was 0.22 mg/kg DW, whereas the minimum was 0.12 mg/kg DW. In Chinese rice, the amount of Ni varied between 0.074 to 0.250 mg/kg DW (Li et al., 2013), which indicate a similar profile with our study. Nickel (Ni) serves as a vital micronutrient for both flora and fauna, required in minimal quantities.

The concentrations of macro elements were examined to assess the nutritional efficiency of the rice samples. Potassium (K) ions play a crucial role in the functioning of numerous enzymes, serving as cofactors and contributing significantly to the maintenance of osmotic potential (Ragel et al., 2019). Moreover, this is very essential for human system to regulate nervous system, endocrine system, fluid balance and blood pressure (Lindinger and Cairns, 2021). In the current study, the mean concentration of K was 1.8 g/kg DW of grain. In Indian rice germplasm, the average content of K was recorded as 254 mg/100g DW of grain tissue (Longvah et al., 2021). In another study conducted in the north east Indian plain belt, the K content in rice grain varied from 249.35 to 503.20 mg/kg DW (Nath et al., 2022). Magnesium (Mg) serves as an essential element for the functioning of enzymes that play critical roles in photosynthesis, respiration, and nucleotide synthesis. It serves as a vital element of the pigment chlorophyll, essential for the process of photosynthesis (Tränkner et al., 2018). The Mg concentrations measured in this study were comparable to those found in rice varieties from Arunachal Pradesh, India, ranging from 0.79 to 1.49 g/kg DW (Longvah and Prasad, 2020). Calcium (Ca) ions serve as a crucial co-factor for various enzymes and play a vital role in the development and preservation of healthy bones in humans. In the studied rice samples, the mean content of Ca is 107 mg/kg DW of grain.

The majority of physicochemical characteristics of starch are influenced by the content of amylose present. The starch present in rice can be categorised

according to its amylose content into several types: waxy (0 to 2%), very low (5 to 12%), low (12 to 20%), intermediate (20 to 25%), and high (over 25%). The culinary characteristics of rice are influenced by its amylose concentration, with grains exhibiting a high amylose to amylopectin ratio offering enhanced health advantages (Breyton et al., 2021). Rice grains characterised by elevated amylose levels exhibit increased resistant starch, leading to a reduction in the glycaemic index. This mechanism helps to mitigate the swift elevation of blood glucose levels following the consumption of carbohydrate-dense foods, thereby decreasing the risk of developing type II diabetes (Sun et al., 2017). In certain rice varieties grown in India, the reported concentrations of amylose varied between 3% and 16% (Singh et al., 2005). A research investigation involving 10 indigenous rice germplasm samples from Assam, India, revealed that the amylose content varied between 12% and 29% (Govindaraju et al., 2022). The findings from this study hold considerable importance, particularly since a majority of the rice samples analysed exhibit elevated levels of amylose content. The amylose contents determined in this experiment ranged from 17% to 37%. Among the studied landraces, Cheattuveliyan, Jeerakashala, Kannichennellu, Kumkumashali, Kuruva, Mullankayama, Paalthondivella, Urunikayama, were characterised by high amylose contents. The hybrid varieties, Aiswarya, Akshaya, Kanchana, Supriya, Ponmani, Neeraja, Varsha, Vytila 6, Veliyan, Swetha, Uma, Samyuktha and Vaishakh, had high amounts of amylose. Kumkumashali landrace showed the highest amylose content. This aids in the careful selection of rice varieties for human consumption, thereby minimising the risk of developing health disorders.

The pharmaceutical formulations employed to manage hyperglycaemia can elicit numerous side effects, thus the exploration of natural sources exhibiting carbolytic activity presents a promising alternative. The coloured grains of rice are increasingly attracting global interest, attributed to their antihyperglycaemic properties. These effects stem from the presence of phenolic compounds that play a role in regulating postprandial hyperglycaemia. While numerous medicinal plants exhibit antiglycation properties, their cost-effectiveness is often limited, making them impractical for regular dietary inclusion. The presence of phenolic compounds,

such as flavonoids and anthocyanins, has been shown to mitigate postprandial hyperglycaemia through a reduction in the glycaemic index (GI). This occurs through the inhibition of carbolytic enzymes, including pancreatic alpha-amylase and the membrane-bound alpha-glucosidase found in the intestinal epithelium (Krishnan et al., 2021). These compounds have demonstrated the ability to inhibit non-enzymatic glycation and enhance glucose uptake through glucose transporter protein (GLUT) (Krishnan et al., 2020). Consequently, the rice varieties examined in this experiment were analysed for the presence of these compounds and it was revealed that the total phenolic content was markedly elevated in the pigmented varieties compared to their non-pigmented counterparts, which align with earlier research findings (Krishnan et al., 2020). In addition to their antiglycation properties, these compounds, such as flavonoids and anthocyanins, exhibit significant antioxidant capabilities characterised by robust free radical scavenging activity (Proestos et al., 2006). Therefore, incorporating them into the diet is crucial to effectively manage hyper glycaemia.

Based on these observations, four hybrid elite varieties, Annapoorna, Jyothi, Ponmani, and Uma along with four landraces, namely Adukkan, Gandhakashala, Kumkumashali, and Mullankayama, were selected for further studies. Adukkan and Kumkumashali are pigmented rice, whereas Gandhakashala and Mullankayama are recognized for their aromatic qualities. The varieties demonstrated a significant correlation with bioactive compounds, including phenols and anthocyanins, as well as favourable cooking quality, particularly regarding amylose percentage. Their mineral profiles were also found to be highly varied, thereby giving a scope for Zn biofortification.

5.2 Standardization of dosage and duration of priming agents

In order to prime the seeds and seedlings of the eight rice varieties that were chosen (Adukkan, Annapoorna, Gandhakashala, Jyothi, Kumkumashali, Mullankayama, Ponmani, and Uma), initially the concentration and duration of Zn based priming agents ($ZnSO_4$ and $ZnNO_3$) was optimised. It is a well-known fact that Zn is essential for photosynthesis and membrane stability (Mousavi et al.,

2024), Thus, the assessment was done based on physiological markers, such as the levels of total chlorophyll, carotenoid, and malondialdehyde (MDA) content, an indicator of lipid peroxidation. Among the different concentrations (0.25, 0.5, and 0.75M) and durations (6, 12, 18, and 24 hours), seed priming with 0.5M $ZnSO_4$ and $ZnNO_3$ for 18 hours resulted in a marked enhancement of total chlorophyll and carotenoid levels in 14 d old seedlings, while also showing a significant decrease in MDA content when compared to the untreated control plants. The findings suggest enhanced photosynthetic performance and diminished oxidative stress in the primed samples. At the one-leaf stage, seedling priming with a 0.5% foliar spray of $ZnSO_4$ and $ZnNO_3$ (out of 0.25, 0.5, and 0.75%) produced similar increases in chlorophyll and carotenoid content and a significant drop in MDA levels in 14 d old seedlings when compared to control plants. The findings across all eight rice varieties examined, in terms of increased photosynthetic pigment content and reduction in MDA content highlights the strength and effectiveness of the optimal concentration of $ZnSO_4$ and $ZnNO_3$ in enhancing the physiological health of the plants. The noted enhancements are consistent with other studies showing Zn's critical function in improving the physiological health of plants. Optimal levels of Zn plays a crucial role in maintaining the integrity of cell membranes, mitigating lipid peroxidation, and serving as a cofactor for enzymes that are essential in the processes of chlorophyll biosynthesis and carotenoid accumulation. Earlier research have shown that the application of optimal concentration of Zn mitigates oxidative stress by boosting the function of antioxidant enzymes like superoxide dismutase and catalase, potentially accounting for the decreased MDA levels noted in this investigation (Gupta et al., 2024).

5.3 Agronomic, yield characteristics and grain Zn content in selected rice varieties

The selected eight varieties of rice (Adukkan, Annapoorna, Gandhakashala, Kumkumashali, Jyothi, Mullankayama, Ponmani, and Uma) were treated with the same dosage of priming agents ($ZnSO_4$ and $ZnNO_3$). The seeds were primed with 0.5M for 18 hours and the seedlings were primed with 0.5% of both Zn compounds.

All rice varieties exhibited positive responses to the various treatments, with notable enhancements in plant height, number of tillers, and grain Zn content when compared to the control group. The findings of the investigation indicated that the use of priming agents markedly improved both the growth and Zn content in the rice varieties examined. Furthermore, the observed growth in plant height and tiller number, suggests a beneficial effect on the yield potential of the crops. The results indicate that the application of seed priming and seedling priming using $ZnSO_4$ and $ZnNO_3$ can serve as beneficial approaches to enhance both the yield and nutritional quality of various rice cultivars. When compared to $ZnSO_4$, $ZnNO_3$ treatment brought about superior results. To the best of our knowledge, this is the first instance of utilising $ZnNO_3$ as a priming agent for biofortification of Zn in crop plants. The study suggests that the use of $ZnNO_3$ as a priming agent can significantly improve rice crop yield and nutritional content, thereby promoting sustainable agriculture.

The diverse methods of Zn application evidently improved all yield parameters in the plants observed. The application of Zn compounds through priming resulted in an increase of plant height, harvest index (%), 100 grain weight, and seed setting (%). Zn plays essential roles in processes such as cell division, expansion, gene expression, chromatin regulation, and the metabolism of carbohydrates, lipids, and proteins (Noulas et al., 2018). Consequently, these processes are meticulously controlled by the ambient concentrations of Zn at different growth stages, thereby contributing to the overall growth and yield of the rice plant. The primed plants exhibited a heightened seed setting percentage, attributed to their effective flowering process. This phenomenon may primarily result from the influence of Zn on the Zn finger transcription factors (TFs). The TFs play a crucial role in the development and expansion of floral organs, including the androecium, gynoecium, pollen grains, and the nutritive tissue known as tapetum (Hafeez et al., 2013). They are also vital for processes such as pollen grain germination, elongation, and the interaction between pollen and stigma (Pandey et al., 2006). The combined application of seed and seedling priming with Zn compounds has significantly influenced the reproductive success and, consequently, the yield performance of the genotypes. In comparison to $ZnSO_4$, the application of

$ZnNO_3$ resulted in a greater enhancement of the yield parameters. Nitrogen (N) serves as a vital nutrient for the growth of plants and is fundamental for achieving optimal crop yield. The primary form of nitrogen absorbed by plants is as nitrate (NO_3^-). Consequently, the utilisation of $ZnNO_3$ has led to effective movement of NO_3^- to the flag leaves, subsequently enhancing their photosynthetic efficiency and overall productivity (Gao et al., 2022; Aluko et al., 2023).

The documented increase in grain yield is supported by the results of Choukri et al. (2022), wherein they noted a 47% rise in grain yield in silage cone after treating seeds with 0.5% $ZnSO_4$. Harris et al. (2007) documented increases in yield between 24 to 44% after subjecting seeds to priming with 1% $ZnSO_4$ solution for a period of 16 hours. A significant number of studies have recorded the beneficial effects of seed priming on yield in various crops, such as chickpea (Harris et al., 2008), and wheat (Reis et al., 2018). The priming of seeds with Zn and endophytic bacterium, improved water relations and increased grain yield in wheat crop. It was demonstrated that the implementation of seed priming using endophytic bacteria led to an increase in grain yield ranging from 25 to 27% over the two years (2013 to 2015) of observation (Rehman et al., 2018). In common bean, the application of seed priming with Zn-aminoacid complexes enhanced grain Zn content by 8 to 34% (Tabesh et al., 2020). The grain Zn concentrations was increased by 32% upon seed priming with endophytic bacteria in wheat (Rehman et al., 2018). Seed priming with 0.01M $ZnSO_4$ enhanced grain Zn content by 60% in mung bean (Haider et al., 2020).

The application of $ZnSO_4$ and $ZnNO_3$ to the seeds as seed priming, along with the technique of seedling priming, resulted in an elevated Zn concentration within the grains of the rice plants. The concentration of Zn in the grains of the rice plant was significantly elevated compared to the control treatments. $ZnNO_3$ performed effectively in each of the eight chosen rice varieties, and the combined application was the most successful than the individual treatments. Among the eight rice varieties studied, Annapoorna, and Kumkumashali exhibited significant improvement in Zn content enhancement as seen in the principal component

analysis (PCA) plot. From the PCA plot it is clearly seen that these two varieties aligned closely with vectors representing grain Zn content, grain per panicle (GPP), harvest index (HI), and reproductive tillers (RT), indicating a strong association between Zn accumulation and key yield parameters. Consequently, these varieties were selected for further investigations. By choosing genotypes that have demonstrated potential for increased Zn content in grains, it could facilitate in enhancing public health status.

5.4 The effect of seed priming and seedling priming in photosynthetic features, antioxidant system, oxidative damage, membrane stability and metabolites.

The impact of various treatments of $ZnNO_3$ was studied on two selected rice varieties from the previous experiment, Annapoorna and Kumkumashali. There were three different treatments along with an untreated control. The treatments were seed priming with $ZnNO_3$, seedling priming with $ZnNO_3$, combined treatments of seed and seedling priming with $ZnNO_3$.

These varieties and the priming agent was selected based on the comprehensive examination of the correlation matrix, PCA biplot, and Zn boxplot across priming treatments. The correlation matrix indicated a strong positive correlation between Zn and harvest index (r = 0.77), suggesting that Zn enrichment substantially enhances yield efficiency. A moderate correlation between Zn and seed setting (r = 0.27) further substantiates the importance of Zn in augmenting grain production potential without compromises. The PCA biplot analysis distinctly indicated that Kumkumashali and Annapoorna are situated near the vectors for Zn, harvest index, seed setting, and reproductive tillers (r = 0.02), so affirming their strong correlation with advantageous agronomic and nutritional characteristics. The boxplot of Zn content across several treatments revealed that combined treatments of seed and seedling priming with $ZnNO_3$ exhibited the highest Zn concentration, succeeded by seed priming and seedling priming with $ZnNO_3$. Treatments utilising $ZnSO_4$ showed substantially diminished efficacy. Consequently, $ZnNO_3$ was identified as the optimal priming agent, and was chosen for further analysis.

5.4.1 Photosynthetic performance

The priming of seeds and seedlings with $ZnNO_3$ in rice positively influences primary photochemistry, leading to an enhancement in the photosynthetic performance of the treated plants, as demonstrated by the results. The enhancement observed in primary photochemistry and the increase in photosynthetic pigment content can be attributed to the influence of applied Zn on the photosynthetic systems of primed plants. Chl *a* fluorescence offers valuable information regarding the efficiency of light-dependent processes in photosynthesis, revealing potential improvements in energy transfer and utilisation in plants that have been primed with Zn. The IRGA study provides important insights into the mechanisms of gas exchange and CO_2 assimilation, enhancing our understanding on the impact of Zn priming on net photosynthetic rate, stomatal conductance, and transpiration.

Following the priming process, the PSII and thylakoid membranes exhibited enhanced stability, leading to an improvement in the primary photochemical activity in the Zn-primed plants. The performance index (PI) serves as a multiparametric expression that provides a sensitive assessment of a plant's health. It incorporates three crucial factors: reaction centre density, trapping efficiency, and electron transport efficiency, all related to PSII. This index quantifies the functional activity and structural stability of PSII effectively. An elevation in $PI_{(abs)}$ indicates a rise in PSII activity and an enhancement in the electron transfer rate to PSI via PQ (Strasser et al., 2004; Pavia et al., 2019). The different quantum yields ($PHI(D_0)$, $PHI(P_0)$) exhibited considerable variation across the treatments. The maximum quantum yield of primary photochemistry ($PHI(P_0)$) reflects the ability of absorbed photons to facilitate the movement of electrons beyond QA^- into the electron transport chain, and this capacity was enhanced following various Zn treatments. Consequently, the $PHI(D_o)$, representing non-photochemical quenching showed a reduction, suggesting an alteration in the efficiency of PSII (Strasser et al., 2000; Singh et al., 2022).

The process of photosynthesis serves as the fundamental pathway by which plants convert light energy into chemical energy, significantly influencing their growth and overall productivity. Zn can enhance the efficiency of photosystem II by

playing a direct role in the repair process mediated by Zn finger proteins. The process of PSII repair and reassembly entails the breaking and formation of disulphide bonds within the complex. The low quantum yield of photosystem II1 (LQY1) is a Zn finger protein exhibiting disulphide isomerase activity, primarily located in stroma-exposed thylakoids, where essential processes of PSII repair take place. This entity engages with the photosystem II (PSII) core complex and contributes to the repair of photodamaged PSII complexes (Lu et al., 2011). This process is essential in the light reaction, as it aids in the transfer of surplus energy from photosystem II (PSII) to photosystem I (PSI). A number of chloroplast proteins exhibit the ability to bind Zn, including several metalloproteases that assist the chloroplast in regulating its functions. Furthermore, Zn has the capacity to enhance the expression of multiple ribosomal genes that govern transcription and translation within chloroplasts, thereby increasing the expression of genes associated with both light and dark reactions (Tang et al., 2016).

Zn plays a crucial role in preserving membrane stability, thereby ensuring the structural and functional integrity of the thylakoid membrane. Additionally, it contributes to the regeneration of PSII and the synthesis of chlorophyll (Kaur et al., 2023). Furthermore, investigations into the transcriptome and proteome of maize have highlighted the critical role of Zn in preserving the integrity and functionality of chloroplasts. The genes and enzymes crucial for chlorophyll biosynthesis, including magnesium chelatase (subunit ChlH chloroplastic) and magnesium protoporphyrin IX methyltransferase (chloroplastic), are subjected to significant regulation by Zn. The membrane organisation and thylakoid density in chloroplasts are also influenced, as well as the gene and proteins for protein assembly of photosystem I i.e. Photosystem I assembly factor 2 (PSA2) (chloroplastic), which is crucial for the organisation of the thylakoid membrane. The expression of all these genes at both the transcription and translational levels is influenced by the availability of Zn, and under conditions of low Zn, these genes are downregulated. In this investigation, it was observed that an increase in chlorophyll levels alongside enhanced photochemical activity in the plant, suggests that the elevated Zn content positively regulates these processes (Zhang et al., 2019). Recent findings indicate

that the stability of isolated chlorophyll can be enhanced through the application of Zn (Hu et al., 2022). This aligns with the observations in this study, where it was noted that the stability of chlorophyll increased following the priming of seeds and seedlings with Zn compounds. Moreover, the findings align with previous research, demonstrating that seed priming with Zn has improved the photosynthetic pigments in wheat (Rai-Kalal and Jajoo, 2021; Chattha et al., 2022; Rehman et al., 2022).

The priming of seeds and seedlings with Zn can improve the leaf gas exchange, which is positively associated with an increase in photosynthetic activity. The presence of ambient Zn in mesophyll cells has the potential to increase stomatal conductance through its effects on potassium ions (K^+) uptake and the modulation of stomatal movement. Sharma et al. (1995), observed that in conditions of Zn deficiency, the guard cells of the cauliflower plant exhibited reduced levels of K^+ ion, resulting in a diminished stomatal aperture. However, upon the reintroduction of Zn, these physiological changes were reversed. The direct influence of Zn on stomatal opening and the uptake of K^+ ions has been demonstrated, both of which are essential for stomatal conductance. With an increase in CO_2 intake, there is a corresponding rise in the net photosynthetic rates of the plant being examined. Furthermore, the function of carbonic anhydrase (CA), which increases the concentration of HCO_3^- around Rubisco through the conversion of CO_2 to HCO_3^-, can be positively influenced by the presence of Zn (Santos et al., 2021). Furthermore, it was observed that seed priming with $ZnNO_3$ has improved the photosynthetic efficiency in wheat by elevating both stomatal conductance and the rate of photosynthesis (Rehman et al., 2022). The presence of Zn exhibits numerous beneficial characteristics that may lead to an enhancement in the net photosynthetic rate of plants that have developed from Zn-primed seeds and/or have undergone seedling priming. Both of the varieties showed enhancement in all the photosynthetic traits under study in which Kumkumashali was more efficient. The results also emphasize the simultaneous application of seed and seedling priming in enhancing the photosynthetic process in plants, subjected to priming, when compared to individual application methods.

5.4.2 Antioxidant system

The regulation of antioxidant homeostasis is crucial for maintaining cellular redox balance and protecting plants from oxidative stress. Evaluating the levels of antioxidants provides essential insights into the plant's ability to respond towards environmental stressors. Superoxide dismutase (SOD) is an enzyme that contains Zn and is essential for the cellular antioxidant defence system. The enzyme facilitates the conversion of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), effectively reducing oxidative stress and safeguarding cellular structures including proteins, lipids, and DNA from potential harm. Within chloroplasts, SOD plays a crucial role in protecting the photosynthetic apparatus from photooxidative harm by inhibiting the build up of reactive oxygen species (ROS) (Mathpal et al., 2015). Earlier research on rice has shown that applications of Zn, whether through soil or foliar methods, lead to a notable enhancement in SOD activity, with increases documented between 10 and 30%. Moreover, the utilisation of ZnO nanoparticles (NPs) in the context of Cd stress has demonstrated an enhancement in SOD activity ranging from 25 to 50% (Li et al., 2021). Sharma et al. (2021) noted an increase exceeding 30% in SOD activity following the application of ZnO NPs. In wheat, the application of seed priming with $ZnSO_4$ or ZnO NPs significantly enhanced SOD activity when subjected to drought stress conditions (Singhal et al., 2021; Pandya et al., 2024). Furthermore, the application of Zn to the leaves increased SOD activity in soybean subjected to salinity stress (Yaghoubian et al., 2021) and in *Vicia faba* subjected to salinity stress (Mogazy and Hanafy, 2022).

Peroxidases (PODs) are found in the cytosol, vacuole, cell wall, and extracellular space. They utilise guaiacol as an electron donor and H_2O_2 as a substrate to facilitate the oxidation of a range of organic and inorganic molecules. In the current study, the various application methods of $ZnNO_3$ has enhanced the activity of guiacol peroxidase (GPOXs) by 14 to 56%. In other studies too, there was enhancement in the activity of peroxidases by the application of Zn. The application of ZnO NPs under Cd stress led to an increase of over 30% in peroxidase activity in rice (Li et al., 2021; Sharma et al., 2021). Comparable results were

observed in wheat, indicating that seed priming with ZnO NPs enhanced POD activity (Pandya et al., 2024). The application of Zn resulted in an increase of peroxidase activity in soybean subjected to salinity stress (Yaghoubian et al., 2021) and in *Vicia faba* plants after foliar application (Mogazy and Hanafy, 2022). Ascorbate peroxidase (APX) serves a crucial role in the detoxification of hydrogen peroxide (H_2O_2) within the glutathione-ascorbate cycle, employing ascorbate as an electron donor in this biochemical process. The application of Zn has been shown to significantly increase APX activity in various crops, indicating its crucial function in reducing oxidative damage. In the current research, it was noticed that the treatments with $ZnNO_3$ increased the APX activity in rice plants by 9 to 48%. In a recent research, it was noticed that the seed priming with Zn has enhanced the APX activity in the groundnut and thus helped the plant to better germinate and establish well (Ashwini et al., 2024). It was noticed that maize plants raised from seeds primed with combination of Zn and Se enhanced the APX activity by 48%, when compared to untreated control plants (Nawaz et al., 2021). In *Aloe vera* under salinity stress, the application of $ZnSO_4$ in soil enhanced the APX activity by 67%, thus reduced the damage caused by salinity stress (Kavian et al., 2022). Zn finger transcription factors are reported to regulate the expression of the APX gene in the cytoplasm. Zat12 in *Arabidopsis*, PeSTZ1 in poplar, and ZFP36 in rice are C2H2-type Zn finger transcription factors that interact with the promoter region of APX genes, facilitating their increased expression (Yoshimura and Ishikawa, 2024).

Catalase (CAT) serves as a vital enzyme within the antioxidant defence system, facilitating the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. This enzyme plays a crucial role in the processes of photorespiration and the β-oxidation of fatty acids. In the current research seed priming and combined treatments of seed and seedling priming, enhanced the CAT activity in rice plants by 16 to 37%. The application of ZnO NPs resulted in a notable increase in catalase activity of rice, as documented by Li et al. (2021) and Sharma et al. (2021), with enhancements exceeding 30% was observed. Comparable patterns were noted in soybean subjected to salinity stress (Yaghoubian et al., 2021), and in wheat following ZnO NP seed priming (Pandya et al., 2024), and in *Vicia faba* plants

treated with foliar Zn application (Mogazy and Hanafy, 2022). In a recent research through various spectroscopic, molecular docking and enzyme activity assay, it was noticed that Zn can enhance the activity of catalase enzyme by binding closely to the active site of this enzyme (Qi et al., 2024). In the studied rice varieties, the priming of seeds and seedlings with Zn compounds significantly increased the activities of essential antioxidant enzymes, such as SOD, POD, APX, and CAT. This enhancement highlights the effectiveness of Zn application, especially via seed and seedling priming, in strengthening the antioxidant defence system. The documented elevations in these enzymatic functions to alleviate oxidative stress underscore the potential of Zn based interventions, as a viable approach for improving crop performance and resilience to stressors.

The application of seed priming and seedling priming with Zn compounds enhanced the increase of nonenzymatic antioxidants, specifically ascorbate and glutathione, in both the rice varieties examined. Ascorbate serves as the predominant antioxidant within plant cells, found throughout all intracellular compartments and in the apoplast space. Ascorbate exhibits a direct interaction with hydroxyl radicals, superoxide, and singlet oxygen, and it has the capacity to regenerate the oxidised forms of α-tocopherol (Ahmad et al., 2010). Ascorbate is essential in the AsA-GSH cycle, where it regulates reactive oxygen species through its ability to donate electrons (Hasanuzzaman et al., 2020). The AsA-GSH cycle, also known as the Asada-Halliwell cycle, serves as the primary antioxidant defence mechanism in plant cells for the detoxification of H_2O_2. In the AsA-GSH cycle, H_2O_2 is eliminated through the direct action of ascorbic acid and the indirect action of glutathione (Aazami et al., 2021). The result of the present study corroborated with the results of other studies in which the application of Zn treatment effectively enhanced the amounts of ascorbate and glutathione in foxtail millet by 23 and 10%, respectively (Saleem et al., 2023). The ascorbic acid content in lupine plants treated with Zn NPs exhibited increases of 23 to 71% in ascorbate content when compared to the control plants (Latef et al., 2017). In rice plants experiencing Zn deficiency, a reduction in ascorbic acid levels was observed. It was found that activity of phosphomannose isomerase (PMI) a Zn-dependent enzyme within the mannose/l-galactose pathway

(the biosynthetic pathway for ascorbate) was decreased due to the downregulation of the gene under Zn deficiency (Höller et al., 2014). There could be a possible upregulation of this gene resulting in an increase of AsA, in $ZnNO_3$ primed plants when compared to control plants.

The combined ZnO NP priming and spraying treatments increased glutathione levels by 37% in tomato plants under salinity (Aazami et al., 2021). This was almost at par with our studies in which the combined treatments of seed and seedling priming enhanced glutathione content by 28%. The application of Zn in soybeans subjected to salt stress facilitated the mitigation of salinity stress through the activation of the glutathione-mediated antioxidant defence system. The integration of Zn priming and spraying treatments resulted in a 37% elevation of glutathione levels in the presence of salinity (Al-Zahrani et al., 2022). Under As stress in rice, there was a significant reduction in glutathione levels, leading to a compromised antioxidant system. The application of Zn resulted in a two fold increase in glutathione levels within the roots and shoots of rice plants, aiding in the mitigation of As induced stress in the plants (Choudhury et al., 2022). Under Cd stress, the glutathione content decreased by 33%, leading to oxidative stress in *Ceratophyllum demersum* plants. The supplementation of Zn in plants subjected to Cd stress resulted in a restoration of glutathione levels, with an enhancement of 36% (Aravind and Prasad, 2005). Glutathione serves as a prominent non-enzymatic antioxidant, effectively neutralising reactive oxygen species (ROS). Glutathione is a small, widely distributed tripeptide (γ-glutamyl-cysteinyl-glycine; γ-Glu-Cys-Gly) characterised by its long hydrophilic groups, present in high (millimolar) concentrations across all aerobic life forms. It is located within the cytosol, endoplasmic reticulum, vacuoles, mitochondria, chloroplasts, peroxisomes, and the apoplast. The application of exogenous Zn effectively restored thiols, essential for maintaining the redox state of cells and the proper functioning of proteins. The introduction of Zn effectively suppressed the oxidation processes of AsA and GSH, thereby preserving the equilibrium of the redox state. This occurs by effective restoration and enhanced activities of the enzymes needed for the functioning of AsA-GSH cycle (Hasanuzzaman et al., 2017).

The seed priming, seedling priming, and their combined treatments resulted in an increase of total phenolics (22 to 34%), which are significant antioxidant compounds in plants, across the two selected rice varieties when compared to the control plants. The observed elevation in total phenolics may enhance the plants' ability to combat oxidative stress and withstand environmental challenges. The results suggest that the application of both seed and seedling priming methods in rice cultivation could enhance antioxidant capacity and promote overall plant vitality. The application of $ZnSO_4$ for seed priming resulted in an increase of total phenolic content in bitter gourd. The analysis of phenolic compounds indicated that priming resulted in elevated levels of syringic acid, sinapic acid, vanillic acid, ferulic acid, coumaric acid, and benzoic acid in the leaves, which contributed to the enhanced antioxidant properties of the plant. Therefore, it can be inferred that Zn exhibits a metabolic connection with the phenylpropanoid pathway, which plays a role in the upregulation and downregulation of certain genes related to the synthesis of phenolics as previously discussed (Bukhari et al., 2021). The application of Zn nutrient priming resulted in an elevation of total phenolic compounds and enhanced antioxidant activity in pea plants, as reported by Poudel et al. (2023). The priming of ZnO NPs in bitter gourd results in an increase of total phenolic content (Mazhar et al., 2023). The presence of salinity led to a decrease in the total phenolic content observed in both the shoot and root systems of the spinach plant. However, the application of seed priming and foliar application of Zn NP significantly increased total phenolics (70 to 120%) in conditions of salinity stress (Ahmad et al., 2024).

The application of Zn increased the activity of phenylalanine ammonia lyase (PAL), which is an important enzyme acting in first step of the polyphenol pathway. The activities of cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), and cinnamyl alcohol dehydrogenase (CAD) enzymes also exhibited notable enhancement (Zhang and Liu, 2015).The CAD multigene family is responsible for encoding proteins that facilitate the reduction of aldehyde derivatives, leading to the production of various metabolites such as phenylpropanoids. In *Arabidopsis thaliana*, the two isoforms, AtCAD5 and AtCAD4, exhibit the highest catalytic activity and play a crucial role in the biosynthesis of guaiacyl/syringyl lignins.

AtCAD5 and AtCAD4 are characterised as dimers, each containing two Zn ions per subunit (Youn et al., 2006; Ferrer et al., 2008). The influence of Zn in these different pathways are well clear from the above description and this may be the reason for the observed enhancement in total phenolics in the current study. To validate this, further studies has to be done in this direction.

The application of seed priming, seedling priming, and the combined treatments of seed and seedling priming with $ZnNO_3$ resulted in an increase of flavonoid in both the rice varieties examined by 16 to 47%. The application of seed priming with Zn in bitter gourd resulted in an enhancement of total flavonoid content ranging from 16 to 30% (Waqas et al., 2024), while the fruit exhibited an increase of 36% (Mazhar et al., 2023). Research outcome have shown that several genes involved in the flavonoid synthesis pathway, including flavonol synthase (FLS), chalcone synthase (CHS), flavanone 3′-hydroxylase (F3′H), and flavanone 3-hydroxylase (F3H), exhibited increased expression by the influence of Zn (Wang et al., 2023). Analysis of the transcriptome and metabolome in *Acanthopanax senticosus*, following foliar application of Zn demonstrated an upregulation of genes associated with phenylpropanoid biosynthesis, terpenoid biosynthesis, and flavonoid biosynthesis alongside transcription factors including ERFs, WRKYs, bHLHs, NACs, and MYBs (Sun et al., 2023). Transparent testa1 (TT1) is a Zn finger protein that possesses a WIP domain and plays a crucial role in the biosynthesis of flavonoids. The researchers observed that silencing of BnTT1 genes in *Brassica napus* caused downregulation in the expression of genes responsible for encoding flavonoid biosynthetic enzymes, including *PAL, C4H, 4CL, CHS, CFI, F3H, F3'H, DFR, ANR, and TT19* (Lian et al., 2017).

Studies have shown that flavonoids complexed with metals such as Zn exhibit enhanced antioxidant properties, increased ROS scavenging capabilities, and improved cell protective activity compared to their uncomplexed forms (Ikeda et al., 2015). As Zn stabilises the flavonoid structure, it may interact ROS more effectively and protect cells from oxidative damage (Halevas et al., 2021). Therefore, priming with Zn could lead to the formation of such complexes within plant cells, enhancing

their tolerance to stress. The capacity of Zn to stabilise flavonoid structures could be crucial in augmenting their efficacy as antioxidants, thereby increasing the stress tolerance of plant cells. From this, we can deduce that Zn plays a significant role in the biosynthesis of flavonoids. The findings underscore the significance of elucidating the molecular mechanisms that govern flavonoid biosynthesis and the potential influence of Zn in augmenting their antioxidative potential.

Different applications of Zn compounds resulted in varying levels of anthocyanin content. Although the two rice varieties in this study showed different responses and it was observed that the combined application of $ZnNO_3$ significantly increased the anthocyanin content in primed plants relative to control plants. Numerous studies have shown that the application of Zn can significantly increase the anthocyanin levels in a range of plant species. In *Eruca sativa*, treating seeds with Zn increased anthocyanin levels during drought conditions, which helped the plant to survive (Hussain et al., 2024). Treating maize seeds with lignin-ZnO NPs also led to higher anthocyanin levels (Del et al., 2022). The application of Zn-lys to wheat seeds resulted in a 38% increase in anthocyanin concentrations and contributed to the mitigation of Cd stress (Hussaan et al., 2021). It was found that treating *Hibiscus sabdariffa* seeds with Zn increased the amount of anthocyanin by more than 100% (Hassanein et al., 2021). In red *Perilla frutescens*, the amount of anthocyanin increased by 38% on treatment with ZnO NPs (Salachna et al., 2021). The Zn finger TFs are crucial for regulating anthocyanin production in plants (Lu et al., 2024). Zn finger TFs of *Arabidopsis thaliana* (ZAT6) is a key TF involved in anthocyanin synthesis, enhancing the expression of genes involved in early stages of biosynthesis (Shi et al., 2018). MdZAT17 is yet another TF in *Malus domestica,* regulating anthocyanin accumulation in response to salinity stress (Wang et al., 2022c).

5.4.3 Oxidative stress

The superoxide anion radical ($O_2^{\cdot-}$) forms through the one electron reduction of molecular oxygen. Superoxide participates in interactions with nitric oxide, ascorbate, and the iron constituents of proteins that feature [Fe–S] clusters.

Superoxide is generated within multiple intracellular compartments, including mitochondria, chloroplasts, and the apoplastic/cell wall interface of the plasma membrane (Karpinska and Foyer, 2024). In both the rice varieties under study the superoxide content was decreased upon various Zn treatment methods. Comparable investigations have demonstrated the significance of Zn in diminishing the superoxide levels within plant systems. In a study examining rapeseed seeds cultivated in saline conditions, the application of Zn NPs during priming was observed to improve stress tolerance and promote enhanced growth by decreasing superoxide levels (El-Badri et al., 2021). The application of Zn NPs for seed priming in maize subjected to Co stress resulted in a reduction of superoxide content (Salam et al., 2022). Rice plants that underwent Zn seed priming demonstrated a reduction in superoxide levels and alleviated the stress effects when cultivated under As stress (Choudhury et al., 2022). Earlier research has indicated that in bean, cotton, and tomato, Zn deficiency has led to an increased accumulation of superoxide content in the roots (Cakmak and Marschner, 1988).

The application of Zn in wheat plants increased the activity of SOD, leading to a decrease in $O_2^{\cdot -}$ (Singh et al., 2019). In this study, an increase in the activity of SOD was observed as detailed in section 4.4.2.1. Consequently, it can be inferred that the introduction of Zn by priming led to an increase in the activity of SOD and a decrease in $O_2^{\cdot -}$ production. The results indicate that Zn is essential for the regulation of superoxide production and the activity of SOD in plants. Zn is essential for the structural integrity and function of Cu/Zn-SOD, a key antioxidant enzyme that detoxifies harmful superoxide radicals. Although Zn is not directly involved in redox reactions, its presence stabilizes the SOD enzyme, enabling efficient scavenging of ROS. Under Zn deficiency, the activity of SOD declines, resulting in increased oxidative stress and cellular damage (Cakmak and Marschner, 1988; Cakmak and Marschner, 1993; Cakmak, 2000). Hence, adequate Zn nutrition is critical for enhancing oxidative stress tolerance via regulation of SOD activity. The findings suggest that sufficient Zn availability enhances the plants' ability to cope with oxidative stress and sustain their overall vitality.

Hydrogen peroxide is generated through the reaction involving superoxide and the enzyme SOD. This process can occur in various organelles, such as the electron transport chains in chloroplasts and mitochondria, as well as in the apoplast, involving plasma membrane NADPH oxidases and apoplastic oxidases. It can also be produced in the peroxisome, glyoxysome (peroxisomal oxidases, type III peroxidases), as well as in the vacuole and nucleus (Smirnoff and Arnaud, 2019). Consequently, it is generated under typical physiological circumstances and can be amplified in response to stressors. H_2O_2 is integral to various signalling pathways and defence mechanisms in plant systems. Under typical physiological conditions, it functions as a signalling molecule that modulates various cellular processes. Under conditions of environmental stress, including drought, elevated light intensity, or pathogen invasion, there is a marked increase in the production of H_2O_2, which plays a crucial role in enabling plants to adapt and endure these challenges. The dual role of H_2O_2 underscores its significance in the realm of plant biology and the mechanisms of stress responses.

In the present study, the H_2O_2 content reduced upon priming with $ZnNO_3$ in the primed plants, similar to the observed decrease in superoxide content when compared to control plants and this aligns with previous investigations. Okra seeds that were primed with ZnO NPs derived from *Salvadora persica* leaf extract exhibited a reduction of up to 80% in H_2O_2 levels when compared to the control plants (Ramzan et al., 2024). In *Vigna mungo* the treatment with As showed an enhancement of H_2O_2 content by more than 200%. In these plants, the priming of seeds with Zn has been shown to assist in alleviating As stress by decreasing H_2O_2 levels by 15 to 67% (Banerjee et al., 2023). In tomato, the foliar application of Zn effectively reduced the H_2O_2 content by 60% to mitigate drought stress (El-Zohri et al., 2021). However, at lower concentrations, H_2O_2 can function as a signalling molecule, modifying the expression of genes, thereby contributing to the development of a robust antioxidant system in plants. For example, the rice Zn finger protein ZFP36 functions as a crucial regulator in the H_2O_2 signalling pathway. This protein undergoes oxidative post-translational modifications in rice, particularly highlighting the H_2O_2 induced oxidation of its Cys residues. The

oxidation of ZFP36 promotes an increase in the expression and functionality of genes that code for protective antioxidant enzymes (Ji et al., 2024). This is one of the ways by which H_2O_2 plays a major role in reducing the oxidative stress in plants.

However, higher rate of ROS production can adversely affect cellular components. One of their targets is lipids in the membrane. ROS interact with polyunsaturated fatty acids (PUFAs) like arachidonic acid, leading to the formation of MDA and other byproducts, a process known as lipid peroxidation. One of the commonly utilized indicators for oxidative stress is the assessment of MDA content in cells (Tsikas, 2017; Zhang et al., 2024b). Recent research indicated that the application of Zn in soil to wheat resulted in a reduction of MDA content and lipid peroxidation, thereby enhancing plant productivity (Shehzadi et al., 2024). The application of ZnO NPs for seed priming in *Pisum sativum* demonstrated a reduction in lipid peroxidation when subjected to salinity stress (Mustafa et al., 2024). In lettuce plants subjected to Cd stress, the application of Zn improved their survival rates by decreasing the MDA content (Behtash et al., 2024). The application of Zn amino acid complex to wheat cultivated under salinity stress resulted in a reduction of MDA content (Mirbolook et al., 2024). Seed priming with Zn decreased the MDA content in Faba bean, thereby enhancing tolerance under drought conditions (Farooq et al., 2021). Sorghum plants that were subjected to Zn seed priming exhibited enhanced tolerance to salinity, as evidenced by a reduction in MDA content (Hassan et al., 2024). These results provide more evidence in favour of the present findings that the MDA content of the rice varieties under investigation was reduced by various methods of priming with $ZnNO_3$.

The finding of this study underscores the effectiveness of the priming technique with $ZnNO_3$ in mitigating oxidative stress and preserving cellular integrity in rice, espectially why preserving the membranes. There are multiple locations for Zn binding in the membrane, with the interior site being the most significant. The presence of Zn at these sites is essential for cellular functions, as it can form tetrahedral coordination bonds, predominantly with aspartate or glutamate, while cysteine and histidine serve as the primary ligands. The higher affinity of these

ligands for Zn as opposed to Fe indicates that the presence of exogenous Zn within the cell inhibits the generation of free radicals that would arise from interactions between these ligands and Fe. Consequently, Zn has the capacity to form complexes with the phospholipids and sulfhydryl groups present in proteins and lipids within the membrane, serving as a protective agent for biomolecules against oxidative damage (Cakmak, 2000; Broadley et al., 2012).

One of the mechanisms through which Zn exerts its antioxidant activity in reducing lipid peroxidation is by occupying regions in the lipid bilayer that could otherwise be occupied by Fe or Cu ions. The redox reactions facilitated by these ions can thus be inhibited (Zago and Oteiza, 2001). As previously noted, Zn can safeguard the sulfhydryl groups (thiol groups) through direct binding, which creates steric hindrance that prevents other molecules from attaching. Additionally, it may induce a conformational change, thereby acting antagonistically to redox-active metals and offering protection to these groups. Moreover, in the present investigation, various priming treatments with $ZnNO_3$ elevate the activity of antioxidant enzymes, including SOD and catalase, thereby further boosting the defence mechanisms against oxidative stress, which is detailed in section 4.4.2.1. This underscores the essential function of Zn in sustaining cellular integrity and safeguarding against the detrimental impacts of free radicals.

Electrolyte leakage is significant under stress conditions and efflux of K^+ ions occurs from the cellular environment. The production ROS has been observed to activate the genes responsible for K^+ selective channels, including the guard cell 'outward-rectifying' K^+ channel (GORK), the stelar K^+ 'outward rectifying' channel (SKOR), and annexin. Additionally, non-selective cation channels (NSCCs) are also activated, resulting in the efflux of K^+ (Demidchik et al., 2014). Zn can diminish this electrolyte leakage by initially decreasing the production of ROS, as previously noted, thereby inhibiting the subsequent processes. Zn also has the capacity to inhibit NSCCS, thereby diminishing electrolyte leakage. Earlier research has indicated that in *Arabidopsis*, the NSCCs present in the roots were inhibited by Zn (Demidchik and Tester, 2002; Britto et al., 2010). The current investigation

demonstrated that the application of seed priming, seedling priming, and combined treatments of seed and seedling priming with $ZnNO_3$ effectively decreased electrolyte leakage in the rice plants examined. Similar studies have also documented a decrease in electrolyte leakage following the application of Zn. The exposure of wheat plants to salinity stress resulted in an increase of electrolyte leakage exceeding 80%. However, the application of seed priming and foliar spray of Zn NP mitigated this increase by 30 to 57% (Chattha et al., 2022). In soybean experiencing drought stress, the application of a foliar spray containing Zn compounds resulted in a decrease of electrolyte leakage (Shirvani-Naghani et al., 2024).

In wheat, seed priming with ZnO NP resulted in an improved membrane stability index (MSI) (Pandya et al., 2024). The application of Zn through foliar application resulted in a 34% increase in the MSI in wheat subjected to drought conditions (Moitazedi et al., 2023), while under salinity in proso millet by 50 to 150% (Mushtaq et al., 2023). In the present investigation, priming with Zn resulted in an enhancement of the MSI across the two rice genotypes examined. Thus, the investigation provides valuable understanding regarding the function of Zn in promoting membrane stability. A positive correlation was observed between the increase in antioxidants and the reduction of oxidative stress markers. Moreover, the thiol groups present in membrane lipids and proteins, along with their interaction with Zn, contributed to a reduction in lipid peroxidation, thereby improving membrane stability (Saleem et al., 2022). Through the enhancement of membrane stability and the reduction of lipid peroxidation, Zn priming could enable plants to more effectively endure environmental stressors, thereby promoting overall growth and productivity.

5.4.4 Metabolites

The application of seed priming and foliar spray utilizing $ZnSO_4$ and Zn-amino acid complex resulted in an increased soluble sugar content in *Phaseolus vulgaris*, as reported by Tabesh et al. (2020). In faba beans, seed priming with $ZnSO_4$ resulted in an increase of soluble sugar content by 54% (Farooq et al., 2021).

Li et al. (2021) demonstrated that in aromatic rice, the application of Zn NPs can enhance α amylase activity. This enhancement correlates with an increase in soluble sugar content in the primed plants, attributed to the efficient breakdown of starch into soluble sugars. Transcriptome analysis in kiwi identified a Zn finger TF, DNA binding with one finger (AdDof3), which plays a crucial role in starch degradation. It has the capacity to activate AdBAM3L (β-amylase), thereby promoting the degradation of starch (Zhang et al., 2018). The utilization of $ZnSO_4$ as a foliar spray on the loquat plant resulted in a significant enhancement in enzymes involved in soluble sugar metabolism, including sucrose phosphate synthetase (SPS), sucrose synthase (SS), hexokinase (HK), and fructokinase (FK) (Ali et al., 2023). This underscores the significance of Zn in the metabolic breakdown of starch.

Another study revealed that seed priming and foliar application of Zn significantly increased the expression levels of sucrose transporters (SUTs) in wheat (Zarea and Karimi, 2023). In *Phaseolus vulgaris*, external application of Zn was observed to enhance the expression of the sugar transporter 13 (STP 13) protein. This investigation in *Phaseolus vulgaris* further substantiates the hypothesis that the application of Zn can enhance the expression of sugar transporter proteins, which in turn may result in an elevated concentration of soluble sugars (Urwat et al., 2021). In summary, the collective evidence suggests that Zn has the potential to increase the soluble sugar content in plants, thereby supporting the validity of current research findings, wherein it was noticed that priming with $ZnNO_3$ enhanced the soluble sugars in both the rice varieties (19 to 35%).

The application of seed priming and foliar spray utilizing Zn amino acid compounds in common beans resulted in an increase in total protein content ranging from 16 to 42% (Tabesh et al., 2020). Another research has shown that the application of Zn increases the total protein content in rice (Adhikary et al., 2022) and *Eruca sativa* (Hussain et al., 2024). In rice, the application of $ZnSO_4$ to both the soil and foliage resulted in an increase of protein content in the grains by 10 to 25% (Mathpal et al., 2015). The application of a Zn amino acid complex to the foliage resulted in an increase of total protein content in rice (Ghasemi et al., 2013).

Approximately 10% of plant proteins are categorized within the Zn proteome, indicating that Zn serves as a structural or functional cofactor in these proteins. The distinctive Zn-binding domains and motifs are described, highlighting that the predominant binding ligands are cysteine and histidine. In certain proteins that exhibit regulation by Zn, particularly where activity is inhibited by this metal ion, only a transient binding interaction with Zn is detectable. Currently, it is recognized that Zn is found in protein-protein interfaces due to its involvement in protein-protein interactions (Clemens, 2022). In tobacco plants lacking Zn, a significant reduction in total protein content and the quantity of 80S ribosomes was noted. The reduction in ribosome levels further supported the diminished total protein content (Obata and Umebayashi, 1988). Therefore, an adequate quantity of Zn is essential for the optimal protein content and thereby the functioning of cells. This provides evidence for the observed increase in total protein content in rice plants that have been primed with $ZnNO_3$.

Zn plays a crucial role in preserving structural integrity of ribosomes, and thereby support its function. In bacterial system, the ribosomal proteins, specifically S4, S14, S18, L28, L31, L32, L33, and L36, possess Zn-binding motifs characterized by the CXXC pattern, which are essential for the proper functioning of ribosomes. In the absence of Zn, ribosome remodelling takes place, and a continued reduction in Zn levels ultimately leads to a state of hibernation or inactivity (Li et al., 2018; Akanuma, 2021). TFs serve as regulators of gene expression, with nearly 50% exhibiting binding affinity for Zn ions. Although the modes of Zn coordination vary among them, the majority bind to DNA through their Zn finger domain. Zn has the capacity to directly influence chromatin accessibility and the binding of TFs to DNA, thereby regulating transcription (Damon et al., 2023).

The role of Zn in modulating gene expression via its interaction with TFs is essential for the optimal functioning of cellular processes. The interaction of Zn with TFs is crucial for regulating chromatin accessibility and transcriptional activity, underscoring the significance of Zn in sustaining cellular processes. Therefore, these factors may elucidate the observed increase in total protein levels when primed with

$ZnNO_3$ in both the rice genotypes examined in this study. The increase in protein content in the same plants can be linked to the increase of free amino acids caused by $ZnNO_3$ in rice genotypes. By stimulating the enzyme tryptophan synthetase, Zn plays a crucial role in the synthesis of tryptophan (Han et al., 2024). The shikimate pathway genes required for the production of aromatic amino acids are regulated by C2H2-type Zn finger TFs (Maeda and Dudareva, 2012). The increased amount of free amino acids in $ZnNO_3$ primed rice plants could be attributed to this. The addition of $ZnSO_4$ to the hydroponic solution for cabbage growth resulted in an increase of total free amino acids content, rising to the extend of 32 to 42%. HPLC analysis revealed an increase in the levels of essential amino acids, including phenylalanine (Phe), tryptophan (Trp), isoleucine (Ile), leucine (Leu), histidine (His), and lysine (Lys), as well as other amino acids such as arginine (Arg), glutamine (Gln), alanine (Ala), aspartate (Asp), and glutamate (Glu) in plants supplied with Zn, in comparison to the untreated control group. It was observed that the levels of enzymes in the shikimic acid pathway, specifically 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) and shikimate dehydrogenase (SKDH), were increased by more than 100%. The enhancement of these can be associated with the elevated levels of aromatic amino acids, specifically phenylalanine and tryptophan (Medina et al., 2017).

5.4.5 Synergistic effect of seed and seedling priming on increasing the physiological parameters and grain Zn content in rice

The combined treatment of seed priming and seedling priming had a synergistic impact, resulting in significant improvements in physiological parameters and grain Zn content compared to the individual treatments of seed or seedling priming. Elevated photosynthetic measurements, including increased photosynthetic pigment content, enhanced chl *a* fluorescence parameters, photosystem activities and improved stomatal conductance, were noted, signifying that the combined priming method enhanced photosynthetic efficiency and overall plant vitality. The combined seed and seedling priming treatment significantly enhanced antioxidant enzyme activities (SOD, CAT, GPOX), non-enzymatic antioxidant activities, reduced

oxidative stress markers (MDA and H_2O_2), and enhanced metabolite accumulation. These physiological advantages may have improved the absorption, transport, and assimilation of Zn into the growing grains. It was also noteworthy that the combined treatments of seed and seedling priming enhanced grain Zn content more than that of individual treatments. Seed priming functions at the initial phase of plant development by activating pre-germinative metabolic pathways, hence augmenting seed vigour, enhancing germination rates, and equipping the seedling with improved resilience. This first metabolic stimulation guarantees coordinated and homogeneous seedling emergence, resulting in a more robust physiological basis for future growth. Subsequently, seedling priming further enrich the seedlings raised from priming by improving nutrient absorption efficiency and promoting the formation of a more widespread and effective root system. This dual-phase priming technique guarantees ongoing physiological support, commencing at germination and persisting into the early vegetative phase, which is essential for Zn uptake and translocation.

5.4.6 Correlation between the various physiological parameters under study

The combined PCA and correlation analysis generated important insights into the interactions among photosynthetic traits, antioxidant defence mechanisms, oxidative stress parameters, and metabolite accumulation under various treatments of Zn in the two rice varieties, Annapoorna and Kumkumashali. The correlation heat map displayed distinct clusters signifying strong correlations among particular traits. A significant positive correlation was identified among essential photosynthetic parameters, including Fv/Fo, Fm, TRo/CSm, ETo/CSm, PSI activity, PSII activity, Pn, and gs indicating that enhanced photochemical efficiency and gas exchange are closely linked under various treatments of Zn. Photosynthetic pigments, including total chlorophyll and carotenoids, exhibited a positive correlation ($r = 0.83$ to 0.99) with enzymatic antioxidants such as SOD, CAT, and APX, suggesting that Zn enhanced antioxidant defence can maintain chloroplast integrity and facilitate efficient photosynthesis.

Antioxidants including SOD, CAT, APX, and GPOX demonstrated strong positive correlations ($r = 0.89$ to 1) with glutathione, phenolics, and flavonoids,

suggesting that enzymatic and non-enzymatic antioxidant systems function synergistically to mitigate oxidative damage. The negative correlations between antioxidant enzymes and oxidative stress markers, including H_2O_2 (r = –0.79 with SOD), MDA (r = –0.99 with CAT), and electrolyte leakage (EL), further substantiate the role of antioxidants in alleviating lipid peroxidation and membrane damage. Furthermore, photosynthetic efficiency measurements including Fv/Fo, ETo/CSm, and PHI(Eo) showed a positive correlation with ascorbate and glutathione, essential components in the ascorbate-glutathione cycle, thereby establishing a connection between energy capture and utilization with antioxidant capability. This synergism highlights the integrative function of photosynthesis linked with the preservation of cellular redox homeostasis.

The PCA biplot distinctly identified the combined treatment (SP+SGP) in Kumkumashali within the positive quadrant of PC1, where high loading vectors for photosynthetic and antioxidant traits were clustered, signifying that this treatment most effectively improved photosynthetic performance and diminished the oxidative stress markers. Conversely, oxidative stress markers (MDA, H_2O_2, SO) exhibited an opposing trend, as analysed from the position in a separate quadrant. This underscores the antagonistic connection between oxidative stress and physiological efficacy. The data collectively indicate that Zn treatments, particularly combined treatments of seed and seedling priming, markedly enhance photosynthetic efficiency and antioxidant defence, while diminishing oxidative stress, with the most profound and cohesive response noted in Kumkumashali. These findings establish a strong correlation between antioxidant status, metabolic flexibility, and photosynthetic competence, enhancing plant resilience through Zn biofortification strategies.

The correlation study revealed a robust positive relationship between photosynthetic parameters and essential metabolites, indicating a synchronised metabolic adjustment to enhance energy production and redox equilibrium under various treatments of Zn. The net photosynthetic rate (Pn), and stomatal conductance (gs), demonstrated positive correlation (r = 0.71 to 0.98) with soluble

sugars, total protein, and free amino acids, indicating that elevated photosynthetic activity facilitates metabolite accumulation through improved carbon and nitrogen assimilation. The results indicate that metabolite biosynthesis is predominantly dependent on effective photosynthetic activity, which is enhanced under various treatments of Zn, particularly with combined treatments of seed and seedling priming.

5.5 The effect of seed priming, seedling priming and additional foliar spray at reproductive stages (booting, flowering, milky stages) on Zn, phytic acid, phytic acid to Zn molar ratio and bioavailable Zn

The amount of Zn in grain cannot reveal the bioavailability of Zn present in them. Therefore, the bioavailability of Zn in the grain was assessed after additional foliar spray was done at the critical reproductive stages (booting, flowering, milky stages) of rice plants. The processes of seed priming, seedling priming, and foliar spray applied at various phenological stages had a significant impact on the grain Zn content, PA content, the molar ratio of PA to Zn, and the estimated bioavailable Zn in rice grain. The seed priming treatment utilising Zn compounds enhanced the Zn content in brown rice to the level of 35 mg/kg, aligning with the targeted Zn concentration deemed appropriate for biofortification processes (Rao et al., 2020). The application of Zn to the plant during the reproductive phase, particularly post-flowering, was efficiently moved into the developing grain tissues. Research indicates that rice plants have the capability to translocate approximately 40% of the Zn provided to them into the grains following the flowering stage (Saha et al., 2017). Ren et al. (2023) indicate that the milky stage represents a period characterised by the active accumulation of Zn in rice grain. The findings align with these observations, indicating that the concentration of Zn in grains increased following foliar spray applied during the flowering and milky stages of the plant's development. Currently, numerous studies have elucidated the significance of mineral profiling in rice, especially in the grains, so as to address the challenge of micronutrient deficiencies (Abdelsalam et al., 2025).

The bioavailability of minerals such as Zn is influenced by antinutritional compounds including PA, phenols, and fibres. The correlation analysis provides crucial insights into the influence of PA in increasing the bioavailability of Zn in rice grains. A strong negative correlation between PA and Zn ($r \approx -0.9$) was observed, suggesting that increased PA levels significantly inhibit Zn bioavailability. In plant-based sources, particularly in cereals like rice, the bioavailability of Zn is significantly hindered by the presence of PA, attributed to the polyvalent characteristics of PA. It comprises six negatively charged phosphate groups that can effectively interact with Zn, thereby influencing its bioavailability. Thus, the molar ratio of PA to Zn reflects the bioavailability of Zn in the dietary context (Kumar et al., 2023). In the current investigation, the integrated approach of seed priming, seedling priming, and foliar application resulted in a reduction of the molar ratio of PA: Zn, suggesting an enhancement in the bioavailability of Zn.

The suggested daily consumption of Zn is influenced by factors such as age, gender, and the physiological condition of the individual. The suggested intake for an adult is approximately 10 to 11 mg daily (FAO, 2004). The assessment of bioavailable Zn within a specific dietary context has been enhanced through the tri-variate methodology introduced by Miller et al. (2007). The present study revealed that the different methods of Zn application significantly elevated the Zn concentration while reducing the PA levels, thereby improving the bioavailability of Zn in rice grains. Current findings aligned closely with those reported by Saha et al. (2017) in rice and by Rehman et al. (2018) in wheat. It was noted that in rice, the application of Zn to the leaves improved the bioavailability of Zn in the grain by decreasing the presence of PA and at the same time elevating Zn levels. In wheat, seed priming with Zn resulted in improved productivity and mineral biofortification, characterised by elevated Zn levels and decreased phytic acid content. Based on the results of the present study, it was found that the landrace Kumkumashali was performing better than Annapoorna in terms of enhanced grain Zn content, reduced PA content and increased bioavailable Zn and hence this rice variety was taken for further studies.

5.6 The effect of various treatments of $ZnNO_3$ on agronomic traits, grain Zn content, other essential elements, gene expression of *OsZIPs*, *OsHMAs* in rice under Zn deficient and sufficient condition

The present study involves the effect of seed priming ($ZnNO_3$) and foliar application of $ZnNO_3$ at booting, flowering and milky stages of Kumkumashali plants. Zn is a crucial micronutrient necessary for the growth and metabolic functions of plants. The Zn deficiency represents a significant issue that affects diverse geographical regions and nearly all types of crops. About half of the world's rice fields are subjected to Zn deficiency, often leading to diminished productivity and quality in the rice crops grown in these nutrient-deficient soils (Krithika and Balachandar, 2016). A number of researchers observed that deficiency of Zn suppressed physiological traits and modified the biochemical functions of the plants. Optimal concentrations of Zn play a crucial role in fostering healthy plant growth and enhancing agricultural productivity (Zulfiqar et al., 2020; Rehman et al., 2022). The present study have primarily focused on the impact of seed priming and foliar spray with $ZnNO_3$ in rice under normal growth conditions, detailing on how these treatments influence various physiological traits under varying Zn availability i.e. Zn deficient and Zn sufficient conditions. Overall, this study provides strong evidence that Zn seed priming and foliar spray are promising strategies for Zn biofortification in rice, ensuring both yield stability and nutritional enhancement not only effective in addressing Zn deficiency stress, but also beneficial in Zn-sufficient environments, supporting their application in diverse agricultural scenarios.

5.6.1 Agronomic traits

The current investigation reveals that rice plants grown in Zn deficient condition (0.05 μM) exhibited adverse impacts on agro-morphological characteristics, biomass accumulation, and yield metrics. The observed levels of plant height, primary root length, root dry weight, shoot dry weight, and crown root number in plants grown in Zn deficient condition were found to be reduced in comparison to the state of sufficient Zn availability. In another study, Zn deficiency (0.005 μM) resulted in a reduction of plant height, primary root length, root dry

weight, shoot dry weigh and total dry matter by over 30% in rice when compared to Zn sufficient condition (1.5 μM) (Impa et al., 2013). In yet another study, rice grown in Zn deficient soil reduced shoot and root development than in Zn sufficient soil (Ismail et al., 2007). Similar to this, it was found that Zn deficient condition (0.05 μM) reduced the growth, biomass and yield of seven millets by more than 30% compared to Zn sufficient (0.5-1 μM) condition (Krishna et al., 2023a). And also, the Zn deficient condition (0.05 μM) greatly reduced the dry weight of sorghum shoot and root by more than 75% compared to Zn sufficient condition (1 μM) (Maharajan et al., 2023). All these results clearly showed that a sufficient amount of Zn is important for increasing plant growth and biomass traits. The results unequivocally indicate that an adequate supply of Zn plays a crucial role in enhancing plant growth and biomass characteristics. Under the Zn sufficient condition, the current research observed an increase in the number of crown root number, exceeding 30, while the Zn deficient condition yielded only 28 crown root number. The findings indicate that Zn deficiency led to a reduction in crown root number, potentially exerting a more significant influence on the necessity for Zn uptake compared to the Zn sufficient condition.

In the current study, the Zn deficiency exhibited a significant influence on tiller number, reproductive tillers, panicle length, grains per panicle, and the weight of 100 grains. For instance, the reproductive tillers demonstrated a decrease of 46%, whereas grains per panicle experienced a reduction of 17%. This observation underscores the essential function of Zn in the reproductive vitality of the plant. A significant number of studies have documented the decline in both yield and quality of different crops due to Zn deficiency, including rice (Chen et al., 2008), mung bean (Samreen et al., 2017), maize (Wang and Jin, 2005), and sorghum (Maharajan et al., 2023). To address the challenges posed by Zn deficiency, agricultural practitioners must rely on the use of fertilisers. This introduces an additional obstacle, particularly the increased cost of fertilisers makes it unaffordable for many farmers. Secondly, the indiscriminate application of fertilisers can adversely affect soil health, consequently diminishing crop growth and productivity (Khan et al., 2022). This process may also lead to the phenomena of bioaccumulation and

bioaugmentation. Consequently, seed priming serves as an economical, secure, and environmentally friendly approach to improve plant productivity (Farooq et al., 2018). The results of the present investigation demonstrate that the utilisation of seed priming with $ZnNO_3$ markedly enhances plant growth, elevates biomass, and improves yield under both Zn deficient and Zn sufficient conditions. The results of the present study also reveals that seed priming under Zn deficient condition significantly increased primary root length, crown root number, shoot and root dry weight with values surpassing 10-32%, and crown root number by 31%.

The findings of the current research indicate that the application of $ZnNO_3$ as seed priming, foliar spray and combined treatments of seed priming and foliar spray significantly promotes plant growth, increases biomass, and boosts yield under both deficient and sufficient conditions of Zn. The synergistic use of seed priming alongside foliar spray markedly enhanced all these traits, when compared to the application of each treatment alone. The seed priming aided the plant in mitigating Zn deficiency, thereby enhancing germination and establishment during the early phases, while also fostered increased root development, which allowed the plant to effectively uptake nutrients and translocate them to the aboveground parts, particularly the leaves. The foliar spray further helped by functioning as a mid-to-late stage nutritional supplement that directly supplied Zn to metabolically active tissues throughout essential growth periods. This dual strategy guaranteed a consistent and sufficient supply of Zn throughout the plant's life cycle, especially during critical demand phases such as tillering, flowering, and grain filling. The foliar applied Zn circumvented the limitations associated with root uptake, such as pH induced immobilisation and inadequate root uptake efficiency, which are prevalent in Zn deficient condition, hence maintaining adequate Zn levels during deficiency.

Similar to the present study, priming and foliar application of Zn enhanced yield in various plants. In rice, different combinations of nitrogen and Zn seed priming along with foliar spray resulted in a 30% increase in yield (Tuiwong et al., 2022). Seed priming using $ZnSO_4$ led to a 27% increase in yield of maize grown in

calcareous Zn deficient soil (Harris et al., 2007), a 40% enhancement in rice (Farooq et al., 2018), a 47% boost in silage corn (Choukri et al., 2022), and a 35% rise in mung bean yield (Haider et al., 2020). Foliar application of Zn-amino acid chelates (ZnAAC) resulted in a 15% increase in wheat yield (Ghasemi et al., 2013). The foliar application of 0.05% $ZnSO_4$ to *Pisum sativum* resulted in an enhancement of flower, pod, and seed yields. This externally applied Zn was observed to mitigate premature floral abscission, enhance pollen fertility, and elevate stigmatic esterase activity, which finally reflected in increased yield (Pandey et al., 2013).

5.6.2 Zn and other essential elements

The results of the present study indicate that a Zn deficient condition markedly diminishes Zn levels in various tissues of the rice plant. The levels of Zn within the root, which serves as the main site for absorption, as well as in node I, the area from where Zn is primarily transported to the above-ground structures, including the flag leaf and panicle is crucial, as it determines the amount of Zn that is loading into the grain. Excluding the husk, the examined rice tissues such as flag leaf, panicle, husk, and grain exhibited > 25% decrease in Zn content under Zn deficient condition compared to Zn sufficient condition. The decrease in Zn levels had a negative impact on plant growth, biomass accumulation, and yield parameters. As a result, one can delineate a correlation between the Zn levels present in various tissues and the overall productivity of the plant. The findings also revealed a significant decrease in grain Zn content (58%) under conditions associated with Zn deficient condition. This poses a considerable concern, as the consumption of rice grains that are deficient in Zn will inevitably lead to Zn deficiency and the related impacts on human health.

The present research findings suggest that employing seed priming, foliar spray, or a synergistic approach of applying both techniques simultaneously can markedly increase the Zn levels in the root, node I, flag leaf, panicle, husk, and grain when juxtaposed with the control group. This observation remained consistent irrespective of the variations in Zn concentration, whether deficient or sufficient Zn. The current observations are consistent with previous studies. The Zn concentration

in common bean plants exhibited an increase after the seeds were primed and the leaves were treated with $Zn(His)_2$ and $Zn(Met)_2$) (Haider et al., 2020). Nayak et al. (2023) noted that the introduction of Zn into the soil and its application through FS on wheat plants at the critical phases of peak tillering and flowering led to an increased content of Zn in both the straw and the grain. Our study distinctly combines seed priming and foliar spray at the booting, flowering, and milky stages, employing $ZnNO_3$ as the biofortification agent, while earlier investigations have examined biofortification of Zn with other compounds used as priming agents. The integration of seed priming with foliar spray has markedly enhanced the uptake of Zn along with other microelements in the root system, promoting their translocation across various tissues. As a result, these findings could have significant implications for food security and the sustainability of agricultural practices in regions where Zn deficiency is prevalent. Moreover, its significance under both deficient and sufficient Zn conditions enhances its applicability across diverse agricultural scenarios. The findings indicate that the impact of various Zn application methods were more prominent under Zn deficient condition than Zn sufficient, rendering this approach a more dependable strategy for Zn depleted soils. The results of this study also clearly revealed that plants translocate the Zn to the panicle by redistributing from flag leaves only when needed. Additionally, plants store Zn in their roots and do not translocate it once sufficient Zn levels reach the panicle. Hence under Zn sufficient condition, seed priming, foliar spray, and combined treatment resulted in higher concentration of Zn in roots. This indicates the possibility of a saturation effect, resulting in limited translocation of Zn to the upper parts and holding the excess in the roots. It was observed that even the little excess going into the panicle is sequestered in the husk and thus limiting the excess entry of Zn into the grains. Therefore, the various application methods experimented in this study ensures safety by limiting the quantity of Zn that is entering the plant tissues, especially the grains, during Zn sufficient.

The present study indicate that in Zn deficient condition, there is an increased concentration of Fe in the panicle, as well as elevated levels of Cu in both the panicle and husk, and also increased Mn in root, node I, panicle and grain. This

observation pose a hypothesis that the elevated levels of Fe, Cu and Mn in Zn deficient condition could serve as a compensatory mechanism for the function of Zn. It is a well-established fact that, the ZIP and HMA family transporters translocate these divalent ions (Amini et al., 2022). Hence, given the condition of deficiency of Zn, the chances of uptake of other micronutrients may increase due to reduced competition with the former. Thus the reason for the observed increase in Fe, Cu, and Mn levels in Zn deficient plants, as compared to those in Zn sufficient plants, is justified. Given the presence of Zn, the transporters may exhibit a preference for Zn over other elements. This indicates a competitive relationship among these micronutrients, with the presence of Zn potentially hindering the absorption of Fe, Cu and Mn (Rai et al., 2021). Future investigations on these observations may delve into the underlying intricate mechanisms of various mineral transportation processes ensuring overall plant health and nutrient equilibrium.

5.6.3 *ZIP* and *HMA* transporter gene expression

ZIP family transporters play an important role in the uptake and transport of Zn in plants (Milner et al., 2013). We selected 10 *ZIP* family genes based on previous research to determine what effects they have on different tissues of rice (roots, node I, flag leaves and panicles) when grown from primed seeds subjected to foliar spray treatments under Zn deficient and Zn sufficient conditions. In this study, *OsZIP2* had the highest expression level in root tissues of control plants under Zn deficient condition compared to the control plants grown under Zn sufficient. When comparing Zn contents between Zn deficient and Zn sufficient conditions, a reduction of Zn contents was observed in almost all tissues of rice plant by more than 40% under Zn deficient condition. Less availability of Zn induced *OsZIP2* family genes in Zn deficient condition. In *Arabidopsis*, the *AtZIP2* has been reported as a high-affinity transporter (Grotz et al., 1998). However, the role of *OsZIP2* has not yet been functionally characterized in rice. Therefore, further functional characterization of this gene in rice will help to understand the specific role of this gene under Zn deficient condition. In contrast, the expression level of the same *OsZIP2* was reduced in various tissues of rice plants on application of $ZnNO_3$

as seed priming, foliar spray and seed priming + foliar spray. This result clearly indicate that *OsZIP2* responded as per the Zn status in the root tissues and was responsible for the elevation of Zn content at conditions of different treatments mentioned above.

OsZIP2 expression was higher in the panicle of plants raised from seed priming under Zn deficient condition. The Zn contents in the panicle of seed primed plants increased by 28% compared to control treatment under Zn deficient condition. Based on this result we hypothesize that *OsZIP2* may play an important role in the translocation of Zn from the shoot/flag leaf tissues to panicle during seed priming under Zn deficient condition. In rice and other plants, the precise role of *ZIP2* in Zn transport is not well known. Hence, targeting this gene in rice by CRISPR/Cas tools would pave a way to identify the exact role of this gene under Zn deficient condition along with seed priming treatment.

Previously, *OsZIP4* has been reported to be involved in translocation of Zn within rice plants (Ishimaru et al., 2011). Mu et al. (2021) reported that *OsZIP4* was involved in translocation of Zn to the phloem of diffused vascular bundles in the nodes for subsequent distribution to tiller buds and other developing tissues. Our study found that Zn deficient condition strongly induced *OsZIP4* in the node I when Zn content was more than 40% lower compared to Zn sufficient condition. This also indicates the response of *OsZIP4* in node I as per the Zn status. This gene may be involved in Zn translocation from nodes to developing tissues as well as reproductive parts during Zn deficient condition. This is a very key gene identified in node I from this study, so further characterization and targeting of this gene by genome editing tool (e.g. CRISPR/Cas tools) would pave a way to elucidate its role under Zn deficient condition and mobilization of Zn to the panicle. Therefore, further research on this gene may help to improve rice growth and productivity during Zn deficient conditions.

It is important to note that Zn deficiency affected the accumulation of Zn in all tissues of rice. However, seed priming and foliar spray treatments increased Zn contents in all tissues of rice under Zn deficient condition. Foliar spray treatments

increased the Zn content by 48% in the root compared to control under the Zn deficient condition. *OsHMA2* was highly expressed in root tissues of rice grown under foliar spray treatment under Zn deficient condition. In rice, *OsHMA2* plays an important role in root-to-shoot translocation of Zn under Zn deficient conditions (Takahashi et al., 2012a). From this finding, we predict that foliar spray may induce the expression of the *OsHMA2* in root tissue to uptake Zn for plant functions, especially in shoots during Zn deficient conditions.

OsZIP8 was moderately expressed in flag leaves of rice grown under seed priming and foliar spray treatments compared to other genes under Zn deficient condition. However, the same gene showed higher expression in flag leaves under Zn sufficient condition. Researchers have previously reported that *OsZIP8* is a plasma membrane located transporter involved in the uptake and distribution of Zn (Lee et al., 2010). Based on this result, we propose that OsZIP8 may be involved in translocation of Zn to the flag leaf and re distribution of Zn from the flag leaf to other tissues when seed priming and foliar spray treatments are carried out in the Zn deficient condition. This transporter could be playing a significant role in increasing of Zn levels in the flag leaves and other above ground parts of rice plants under seed priming + foliar spray treatment in the Zn deficient condition.

Overall, this study reveals the expression pattern of *ZIP* family and HMA genes under foliar spray and seed priming during Zn deficiency. Further, we need to characterize the *ZIP* and *HMA* family genes by knock-out mutants and other studies under Zn-deficient conditions supplied with seed priming and foliar spray for a better understanding of its role. Through the results of this study, it was found that *ZIP* (especially *OsZIP2*) and *HMA* (*OsHMA2*) family genes are induced by seed priming and foliar spray, which helps to increase Zn content in rice during Zn deficient conditions. Targeting ZIP transporters under Zn deficiency conditions with the treatments of seed priming and foliar spray is a beneficial strategy to improve Zn content in plants.

The PCA biplot indicated a positive correlation between the expression of ZIP family genes (including *OsZIP1, OsZIP5, OsZIP9,* and *OsZIP10*) and *OsHMA2*

with Zn content in critical tissues such as the root, node, flag leaf, and panicle. These genes had elevated expression specifically under the seed priming and seed priming + foliar spray treatments, facilitating increased Zn absorption, translocation, and remobilization to the grain. Concurrently, yield parameters such as plant height, panicle length, tiller number, 100-grain weight, root and shoot dry weight, and harvest index exhibited a high correlation with these treatments.

5.7 The effect of Zn priming being carried over into the subsequent generation

This research illustrates the impacts of Zn seed priming, highlighting its capacity to enhance agronomic and yield characteristics, along with increasing grain Zn concentration in subsequent generation of rice. The documented advantages of seed priming, along with foliar application, continued into the next generation and were further amplified by re-priming. The results emphasize the role of seed priming with Zn compounds as an eco-friendly biofortification approach that may yield enduring benefits in subsequent generations.

Seed priming had a beneficial effect on plant height, panicle length, grain count, and harvest index (HI) in the subsequent generation. Plants that were cultivated from primed seeds demonstrated enhanced growth in comparison to non-primed controls, with further advantages noted following re-priming. For instance, the re-priming treatment of seeds obtained from the rice grain in Zn deficient, seed priming + foliar spray, primed plants (ZD-SP+FS-P) produced the tallest progenies, measuring 138.31 cm, underscoring its capacity to enhance growth in Zn-deficient environments. The recorded increases in plant height under Zn-deficient (ZD) condition indicate that priming alleviates the negative impacts associated with Zn deficiency. In a similar manner, the length of the panicle exhibited a notable increase, especially in plants that were grown from seeds that underwent both priming and foliar application in the previous generation. The observed 23% increase in panicle length for Zn deficient, seed priming + foliar spray, non-primed plants (ZD-SP+FS-NP) in comparison to Zn deficient control, non-primed plants (ZDC-NP) highlights the cumulative advantages of this combined treatment. The number of grains per panicle showed a 37% enhancement in ZD-SP+FS-NP relative

to ZDC-NP. This indicates that priming enhances reproductive characteristics, probably by increasing nutrient availability and mobilization during essential growth phases. The re-priming process resulted in 11% increase in grain number, suggesting its capacity to support elevated productivity levels. In Zn deficient condition, priming (non-primed in this generation, plants raised from seed primed and foliar sprayed plants in previous generation) had enhancement in HI by 28% in the subsequent generation, when compared to non-primed plants raised from control plants grown under Zn deficient condition in the previous generation (ZnD-CP). While re-priming enhanced the HI by 33%. This suggests that Zn priming improves the plant's capacity to allocate photosynthate effectively to grains, which is a vital aspect for enhancing yield in nutrient-deficient environments.

The notable enhancements in grain Zn content documented in this study underscore the effectiveness of seed priming and foliar application in improving Zn biofortification. Plants raised from Zn deficient, seed priming + foliar spray, non-primed (ZD-SP + FS-NP) seeds exhibited a grain Zn concentration of 27.36 mg/kg, which is more than double that of the ZDC-NP control (12.3 mg/kg). This notable enhancement indicates that priming promotes the uptake and movement of Zn to the grains, even in conditions where Zn is deficient. The process of re-priming resulted in a notable increase of 11% in grain Zn content in Zn deficient, seed priming+foliar spray, primed (ZD-SP+FS-P) when compared to the non-primed counterparts, illustrating the additive advantages of multiple priming cycles. The findings indicate that priming may establish a physiological "memory" within seeds, which could improve the efficiency of Zn uptake and utilization in future generations. The notable disparity in grain Zn content between Zn sufficient control-non-primed (ZSC-NP) and Zn deficient, control, non-primed (ZDC NP) (56%) highlights the critical role of sufficient Zn availability throughout the process of seed development.

The results of the present study indicate that the priming of seeds with Zn, especially when combined with foliar application, significantly improves the Zn concentration and overall yield in rice crops. The carryover effects into the subsequent generation indicate that priming imprints could provide a viable

approach for enhancing crop nutrition across various growing cycles. Re-priming further amplifies these advantages, suggesting its capacity to support biofortification initiatives in areas experiencing chronic Zn deficiencies. Future investigations ought to delve into the fundamental physiological and molecular processes that govern the transgenerational impacts of priming. Investigating the mechanisms by which priming "memory" affects nutrient uptake and allocation may yield significant insights for enhancing this strategy in various crops and ecological contexts.

Seed priming triggers physiological and molecular alterations that enhance plants' ability to respond to environmental stresses more efficiently. This mechanism establishes a "memory imprint," allowing plants to retain the priming stimulus and demonstrate improved responses when faced with analogous conditions in the future (Ganie et al., 2024). This type of memory can be passed down to future generations, a process referred to as transgenerational epigenetic inheritance. For example, seed priming has demonstrated an enhancement in stress tolerance among offspring. Research on wheat revealed that seeds gathered from plants subjected to drought stress showed enhanced tolerance to salt stress, exhibiting superior water relations and yield when compared to progeny from plants that received adequate watering. The process of osmopriming significantly improved stress tolerance, suggesting that the imprints from priming can be passed down to the following generation and provide benefits in stressful environments (Tabassum et al., 2017).

The intricate molecular processes that contribute to these memory imprints encompass alterations in gene expression, modifications in protein activity, and shifts in metabolic pathways. Epigenetic modifications, including DNA methylation and histone modification, are essential for the establishment and maintenance of this memory. The modifications observed can exhibit stability and heritability, enabling the primed state to be maintained through successive generations (Louis et al., 2023). Sen et al. (2022) demonstrated that rice seedlings could establish 'priming imprints,' enhancing their tolerance to stress at maximum potential in subsequent generations. The effective defence mechanism observed in UV-B-primed rice seedlings of the F1 generation can be attributed to the retention of the priming

imprint, which enhances tolerance to PEG stress. The re-priming observed in the second generation led to an enhancement of innate tolerance potential, suggesting that this approach has the potential to optimize the stress tolerance capacity of a plant. Through this mechanism, UV-B seed priming enhances the stress defence capabilities, which may be advantageous for subsequent generations facing adverse environmental conditions.

The findings of the present study indicate that the improved agronomic characteristics and elevated grain Zn levels seen in the offspring of primed plants imply that priming effects were effectively established and passed on to subsequent generations. The supplementary advantages noted with re-priming lend further credence to the concept that these memory imprints can be strengthened, resulting in progressive enhancements in plant performance. Investigating the underlying mechanisms of priming-induced memory imprints can provide insights into strategies aimed at improving crop resilience and productivity. Utilizing these inherent plant memory mechanisms could lead to the advancement of sustainable agricultural methods that enhance crop performance across diverse environmental scenarios. The increases in yield and grain Zn content observed through priming and repriming in this study could be linked to epigenetic mechanisms influencing the passing of traits to subsequent generations.

Epigenetics encompasses heritable alterations in chromatin architecture, such as DNA methylation, histone modifications, and chromatin remodeling, all occurring without any changes to the DNA sequence itself. The alterations govern essential cellular mechanisms and stress reactions, thereby affecting gene expression (Brito et al., 2020). Zn serves a crucial function as a cofactor for various epigenetic enzymes, affecting DNA methyltransferases (DNMTs), histone acetyltransferases (HAT), histone deacetylases (HDAC), and additional chromatin-modifying proteins. For example, DNMTs, which play a crucial role in DNA methylation, possess several Zn-binding sites that are vital for their activity. Histone acetyltransferases employ Zn ions within their Zn finger motifs to bind to DNA, whereas histone deacetylases depend on Zn in their active sites to facilitate enzymatic functions

(Brito et al., 2020; Yusuf et al., 2021). These Zn-dependent enzymes function as "writers," "erasers," and "readers" of epigenetic marks, engaging in a meticulously coordinated process to modulate the epigenome.

Zn deficiency interferes with these processes, frequently resulting in DNA hypomethylation due to diminished methionine synthetase (MTR) activity and lowered DNMT activity. Furthermore, a deficiency in Zn affects the structural integrity of Zn finger domains (ZnDs), which are essential for the recognition and binding of methylated DNA, consequently impacting gene expression (Noronha et al., 2022). Research has shown that a deficiency in Zn has an impact on histone acetylation. Fujisawa et al. (2023) demonstrated that a deficiency in Zn leads to a decrease in the enzymatic activity of histone acetyltransferase KAT7, resulting in reduced acetylation of histone H3 at Lys14 (H3K14ac) and subsequently inducing the expression of the Zn transporter ZIP10. These are clear cut evidences of the direct involvement of Zn in epigenetics based heritable alterations in chromatin architecture.

Shafiq et al. (2020) documented that Zn stress modifies the expression of Zn transporter genes (such as *ZIP1–ZIP8*) and epigenetic regulators (including DNMT1, MET3a, and HDACs) in maize, indicating a significant involvement of epigenetic mechanisms in Zn acquisition. Within the framework of this investigation, the processes of seed priming and re-priming could affect the expression of Zn transporters and epigenetic regulators, resulting in a "priming imprint" that improves Zn acquisition and translocation. This imprint may be formed through alterations in DNMTs, HDACs, and HATs, alongside variations in the expression of ZIP family transporters. Re-priming could potentially enhance these imprints, leading to progressive advancements in Zn homeostasis and yield. The transgenerational inheritance of epigenetic modifications in rice exposed to heavy metal stresses provides compelling evidence for the hypothesis that priming effects can endure through successive generations (Ou et al., 2012; Cong et al., 2019). This research contributes to the expanding evidence that priming can lead to heritable epigenetic modifications, emphasizing the potential for incorporating priming and

re-priming into sustainable Zn biofortification approaches. Additional investigations are essential to clarify the mechanisms governing Zn homeostasis in rice at the epigenetic level, especially under conditions induced by priming, and to pinpoint the critical Zn-binding proteins and transporters that play a role in this process.

SUMMARY AND CONCLUSION

A total of thirty rice varieties were selected for the first phase of the study. Fifteen landraces were collected from Padma Shri Cheruvayal Raman, Wayanad, Kerala, India, who had a good repository of landraces, which are very unique and poorly studied. Fifteen widely cultivated hybrid rice varieties were collected from regional rice research station (RARS) Pattambi, Palakkad, Kerala, India. Initially the thirty rice varieties were screened for the grain zinc (Zn) content present in them. In addition, other essential elements, total phenolics, flavonoids, anthocyanins and amylose content were estimated in these rice varieties. Based on these observations, four hybrid elite varieties, viz. Annapoorna, Jyothi, Ponmani, and Uma, and four landraces such as Adukkan, Gandhakashala, Kumkumashali, and Mullankayama were selected for further studies. In these varieties, standardisation of priming concentration for the priming agents $ZnSO_4$ and $ZnNO_3$ and duration of priming treatments were analysed for seed priming and seedling priming. The priming dosage and duration with the priming agents $ZnSO_4$ and $ZnNO_3$ was fixed as 0.5M, 18 h (from 0.25 M, 0.5 M, 0.75M, 1M concentrations and 6h, 12h, 18h, 24 h duration). The dosage of seedling priming was also standardized as 0.5% (from 0.25%, 0.5% and 0.75%).

A pot experiment was laid out to analyse the effect of seed and seedling priming with $ZnSO_4$ and $ZnNO_3$ on the growth, yield and Zn content of the seeds. From the results, it was evident that seed and seedling priming with $ZnNO_3$ had substantial impact on improving the growth, yield and grain Zn content of plant. Based on the results the two rice varieties Annapoorna and Kumkumashali were selected for further analysis. Various physiological, biochemical analysis were carried out in the primed and control plants. Supplementary application of Zn compound as foliar spray during reproductive phase was also carried out in these rice varieties and grain Zn content, phytate and bioavailability of Zn were analysed. Based on the results, Kumkumashali was selected and further studies were carried out on the expression of Zn transporter genes such as *OsZIP1* to *10* and *OsHMA2* in

various tissues (root, node I, flag leaf and panicle) under Zn deficient (0.05µM) and sufficient conditions (1.5µM). Along with this, the Zn content in the above-mentioned tissues, husk and grain was estimated and agronomic and yield traits were also recorded. The second-generation effects of seed priming on plants raised from seeds obtained from the previous experiment was also done. For this, seeds were selected from controls, Zn deficient (ZnD C) and Zn sufficient (ZnS C) and combined seed priming and foliar application in Zn deficient (ZnD SP+FS) and Zn sufficient (ZnS SP+FS) conditions. The seeds were either non-primed (NP) or primed (P) in the second stage, and evaluation was carried on the yield and grain Zn content under the same soil and environmental conditions.

The major conclusions derived from the present study are summarized below:

- The screening of 30 rice varieties for biofortification of Zn revealed that on average, 29.5 mg/kg of Zn was present in seeds of all the studied samples. The amount of other micro- and macro-elements present in the study samples were also analysed. The landraces had an enriched content of essential elements compared to the hybrid elite varieties. The presence of antioxidants and antihyperglycemic compounds in rice grains, such as total phenolics, flavonoids, and anthocyanin content were higher in pigmented rice varieties when compared to the non-pigmented ones.

- The eight varieties selected (Adukkan, Annapoorna, Gandhakashala, Jyothi, Kumkumashali, Mullankayama, Ponmani and Uma) were treated with various concentrations of priming agents. Based on the total chlorophyll and carotenoid content and malondialdehyde (MDA, lipid peroxidation) content, the dosage of the priming agents ($ZnSO_4$ and $ZnNO_3$) for seed priming was fixed as 0.5M for 18h duration. For seedling priming, the dosage of priming agents ($ZnSO_4$ and $ZnNO_3$) was fixed as 0.5%.

- The seed priming, seedling priming and combined treatments of seed priming and seedling priming with both the priming agents enhanced the agronomic, yield traits and grain Zn content in all the rice varieties under

study. Compared to all other treatments, the combined treatments of seed and seedling priming gave the highest enhancement in yield and grain Zn content. Based on the results, two rice varieties, Annapoorna, and Kumkumashali were selected for further analysis as they responded more to the various priming treatments. On comparing $ZnSO_4$ and $ZnNO_3$, the latter gave better results, thus this priming agent was selected for further treatments.

- The analysis of various physiological and biochemical parameters in both these rice varieties upon priming with $ZnNO_3$ revealed that combined treatment of seed priming and seedling priming showed highest enhancement in total chlorophyll content, PSI and PSII activities, chl *a* fluorescence parameters, leaf gas exchange parameters and chlorophyll stability index as compared to seed or seedling treatments.

- The enzymatic antioxidants (superoxide dismutase, guiacol peroxidase, ascorbate peroxidase and catalase) and non enzymatic antioxidants (ascorbate, glutathione, total phenolics, flavonoids and anthocyanin) were enhanced in both the rice varieties upon various treatments with $ZnNO_3$. This enhancement in antioxidant mechanisms were negatively correlated with the oxidative stress indicators such as MDA, superoxide, and hydrogen peroxide content and positively associated with improved membrane stability and reduced electrolyte leakage. Zn act as a co-factor for copper-zinc superoxide dismutase (Cu, Zn-SOD). Zn can enhance the activity of catalase enzyme by binding to the active site of the enzyme and Zn finger transcription factors are reported to regulate the expression of the APX gene in the cytoplasm.

- The levels of key metabolites, including total protein, soluble sugars, and free amino acids, were enhanced in both rice varieties, Annapoorna and Kumkumashali, upon priming with $ZnNO_3$. This enhancement may be attributed to the improved photosynthetic performance.

- Additional to seed priming, seedling priming and combined treatments of seed and seedling priming, additional foliar spray during the three

reproductive stages (booting + flowering + milky stage) were administrated in Annapoorna and Kumkumashali. It was observed that these treatments reduced the phytic acid (PA) content and PA to Zn molar ratio and at the same time enhanced the grain Zn content and bioavailable Zn in grain.

- Based on observations related to physiological traits, biochemical responses, yield traits and bioavailable Zn content, the rice variety Kumkumashali performed better compared to Annapoorna, and was therefore selected for further detailed studies.

- In Kumkumashali, studies were conducted under both Zn deficient and sufficient conditions to provide insights into how Zn priming and availability affects rice growth and yield, particularly under Zn deficient conditions. Furthermore, it is essential to investigate how the transporter genes *ZIP* (Zinc-regulated, Iron-regulated transporter-like Protein) and *HMA* (Heavy Metal ATPases) family genes (*OsZIP1* to *10* and *OsHMA2*) in various tissues (root, node I, flag leaf, and panicle) respond to different Zn treatments under conditions of Zn deficiency and sufficiency.

- The application of seed priming and foliar spray (at booting, flowering, and milky stages) significantly improved yield traits as well as Zn content in various rice tissues (root, node I, flag leaf, panicle, husk and grain) under both Zn deficient and sufficient conditions. Notably, under Zn deficient conditions, the combined treatment of seed priming and foliar spray enhanced these traits to levels comparable with or exceeding those seen under Zn sufficient conditions.

- Under Zn sufficient condition, seed priming, foliar spray, and combined treatment resulted in higher concentration of Zn in roots. This indicates the possibility of a saturation effect, resulting in limited translocation of Zn to the upper parts and holding the excess in the roots. It was observed that even the little excess going into the panicle is sequestered in the husk and thus limiting the excess entry of Zn into the grains. Additionally plants translocate the Zn to the panicle by redistributing from flag leaves only when needed.

This will ensure that Zn is not accumulated in the grain in excess, which will have serious repercussions, if consumed.

- Under Zn deficient condition, there is an increased concentration of Fe in the panicle, as well as elevated levels of Cu in both the panicle and husk, and also increased Mn in root, node I, panicle and grain. This pose a hypothesis that the elevated levels of Fe, Cu and Mn in Zn deficient condition could serve as a compensatory mechanism for the function of Zn. As the ZIP and HMA family transporters translocate these divalent ions, under condition of deficiency of Zn, the chances of uptake of other micronutrients may increase due to reduced competition with the Zn.

- Expression patterns of *ZIP* (Zinc-regulated, Iron-regulated transporter-like Protein) and *HMA* (Heavy Metal ATPases) family genes were analyzed in key rice tissues, including root, node I, flag leaf, and panicle. The treatments with $ZnNO_3$ induced the expression of several transporter genes, such as *OsZIP2*, *OsZIP8* and *OsHMA2*, particularly under Zn deficient conditions, indicating a crucial role for these transporters in improving Zn uptake, transport, and remobilization in rice.

- OsZIP4 is reported to be involved in translocation of Zn to the phloem of diffused vascular bundles in the nodes for subsequent distribution to tiller buds and other developing tissues. In the current study, Zn deficient condition strongly induced *OsZIP4* in the node I. This is a key gene identified in node I from this study so further characterization and targeting of this gene by genome editing tools would pave a way to elucidate its role under Zn deficient condition and mobilization of Zn to the panicle. This gene may be involved in Zn translocation from nodes to developing tissues as well as reproductive parts during Zn deficient condition. Therefore, further research on this gene may help to improve rice growth and productivity during Zn deficient conditions.

- *OsZIP2* expression was higher in the panicle of plants raised from seed priming under Zn deficient condition. Thus, it is hypothesized that *OsZIP2*

may play an important role in the translocation of Zn from the shoot/flag leaf tissues to panicle during seed priming under Zn deficient condition. In rice and other plants, the precise role of *ZIP2* in Zn transport is not well known. Hence, targeting this gene in rice by CRISPR/Cas tools would pave a way to identify the exact role of this gene under Zn deficient condition along with seed priming treatment.

- *OsZIP8* is a plasma membrane located transporter involved in the uptake and distribution of Zn in rice plants. From the results, this transporter could be playing a significant role in increasing of Zn levels in the flag leaves and other above ground parts of rice plants under seed priming + foliar spray treatment in the Zn deficient condition.

- *OsHMA2* plays an important role in root-to-shoot translocation of Zn under Zn deficient conditions. From this finding, we predict that foliar spray may induce the expression of the *OsHMA2* in root tissue to uptake Zn for plant functions, especially in shoots during Zn deficient conditions.

- The second-generation effects of seed priming on yield and grain Zn content in plants raised from seeds acquired in the first generation were analysed. Along with this, the effect of re-priming of seeds acquired from first generation was also examined. This research illustrates the impacts of Zn seed priming, highlighting its capacity to enhance agronomic and yield characteristics, along with increasing grain Zn concentration in subsequent generation of rice. The documented advantages of seed priming, along with foliar application, continued into the next generation and were further amplified by re-priming. The results emphasize the role of Zn seed priming as an eco-friendly biofortification approach that may yield enduring benefits.

The present study shows strong proof that the combined treatments of seed priming with foliar application of $ZnNO_3$ is an efficient approach for enhancing grain Zn content and yield in rice. Among the tested varieties, Kumkumashali responded best to these methods, highlighting the potential for targeted biofortification strategies to mitigate micronutrient deficiencies. The enhancement

of photosynthetic efficiency and an efficient antioxidant system led to a reduction in oxidative stress, which in turn resulted in increased metabolite production and grain yield. These findings not only deepen our understanding of Zn uptake and distribution mechanisms but also provide practical, cost-effective solutions for tackling hidden hunger in populations suffering from Zn deficiency. The advantageous effects of priming persisted into the next generation, resulting in increased grain Zn content and yield. This phenomenon illustrates its enduring and transgenerational potential for sustainable biofortification. This research suggests wide use of sustainable farming approaches for nutritionally rich rice production.

RECOMMENDATIONS

The biofortification of rice with Zn through nutripriming in rice was studied in detail. The present study proposes the following recommendations for the future research

1. **Field level trials** have to be conducted to explore the applicability of this technique in large-scale cultivation and yield.
2. To extend the study to ***invitro*** **model** such as Caco-2 cells to study the bioavailability of zinc by simulating the human intestinal environment.
3. **Genome editing studies** with CRISPR/Cas9 and other Cas derivatives to elucidate the role of genes (*OsZIP2*, *OsHMA2*) upon seed priming and foliar spray treatments to enhance Zn content in plants.
4. **Broadening the biofortification studies** to other micronutrients, such as Fe, Se and to other staple food crops, including an extensive study in landraces.
5. The **epigenetic mechanism** underlying the priming and related memory in the ensuing generations with respect to the carryover of the effects of nutri priming has to be studied in detail.

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PUBLICATIONS AND PRESENTATIONS

Articles

1. **Veena, M**., Sen, A., Faseela, P., & Puthur, J. T. (2025). Priming and foliar application as potent methods for agronomic biofortification, enhancing zinc bioavailability, photosynthetic traits and yield in rice. *Journal of Plant Nutrition*, 1-19. https://doi.org/10.1080/01904167.2025.2482095 (IF: 1.6)

2. Faseela, P., **Veena, M**., Sen, A., Anjitha, K. S., Aswathi, K. R., Sruthi, P., & Puthur, J. T. (2025). Elicitors fortifies the plant resilience against metal and metalloid stress. *International Journal of Phytoremediation, 27*(3), 372-389. (IF: 3.4)

3. **Veena, M**., Puthur, J. T., Stępień, P., & Kalaji, H. M. (2023). Minerals profile and nutraceutical factors in landraces and hybrid varieties of rice: A comparison. *Food Bioscience*, *53*, 102779. (IF: 4.8)

4. **Veena, M**., Sameena, P. P., Sarath, N. G., Noble, L., Aswathi, K. R., Amritha, M. S., & Puthur, J. T. (2023). Revelations on photosystem II, thermoluminescence, and artificial photosynthesis: a retrospective of Govindjee from fundamentals to applications. *Physiology and Molecular Biology of Plants*, *29*(9), 1225-1238. (IF: 3.4)

5. **Veena, M**., & Puthur, J. T. (2022). Seed nutripriming with zinc is an apt tool to alleviate malnutrition. *Environmental Geochemistry and Health*, *44*(8), 2355-2373. (IF: 3.2)

Book Chapters

1. Aswathi, K. P. R., Jisha, K. C., **Veena, M**., Sen, A., Sarath, N. G., & Puthur, J. T. (2025). GABA priming induced modulations in the redox homeostasis of plants under osmotic stress. In A. R. Sheteiwy, K. El-Maghraby, & J. T. Puthur (Eds.), *GABA in plants: Biosynthesis, plant development, and food security* (pp. 173–187). Wiley.

2. Shackira, A. M., Sarath, N. G., **Veena, M**., Johnson, R., Jeyaraj, S., Raj, K. P. A., & Puthur, J. T. (2023). Resistance identification and implementation: Genomics-assisted use of genetic resources for breeding against abiotic stress. In R. Kole, R. Kumar, & H. K. Jain (Eds.), Cereal crops (pp. 141–156). CRC Press.

3. Janeeshma, E., Sameena, P. P., Sarath, N. G., **Veena, M.,** & Puthur, J. T. (2022). Modulation of soil microbiome and related alterations in response to pesticides. In M. N. V. Prasad (Ed.), *Pesticides in the natural environment: Sources, health risks, and remediation* (pp. 223–243). Elsevier.

Papers presented

1. Presented a poster entitled "Seed priming with Zinc influences the Expression Pattern of Zinc and Heavy Metal Family Transporter Genes in Rice" held at the International Phytotechnology Conference (IPC-18) on Phytotechnologies for Sustainable Environment and Food Safety, Department of Botany, University of Calicut, Kerala, India, on October 22-24, 2024

2. Presented a poster entitled "Zinc nutripriming influences key physio-chemical processes in rice" held at Conference on Millets: Breeding, Physiology, Genomics, Biotechnology and Nutraceuticals-2023, (ICM-BPGBN-2023), Rajagiri College of Social Sciences, Kerala, on July 5-7, 2023

3. Presented a poster entitled "Wayanadan nellinangal, unexplored germplasms - a better option as functional foods" at the International Seminar on New Horizons in Plant Sciences (NHPS 2023)-Emergent and Innovative technologies in Plant Sciences held at Department of Botany, University of Kerala, Kariavattom, on March 21 - 23, 2023.

4. Presented a paper entitled "Rakthashali, a landrace is a good source of nutraceuticals as assessed by quality matrix" at the International Conference on Advanced Biology 2022 (ICAB 2022) held virtually at University of Kerala, on February 23-25, 2022.