

Biostimulant mediated modulations of physiological, nutritional and yield traits in *Oryza sativa* L. under nutrient deficiency: A metabolomic perspective

*Thesis submitted to
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the requirements for the degree of*

DOCTOR OF PHILOSOPHY IN BOTANY

By
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Certificate

This is to certify that the thesis entitled “**Biostimulant mediated modulations of physiological, nutritional and yield traits in *Oryza sativa* L. under nutrient deficiency: A metabolomic perspective**” submitted by **Riya Johnson** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Botany** of the **University of Calicut**, is a *bonafide* record of the research work undertaken by her in this department under my supervision and guidance and no part thereof has been submitted for the award of any other degree.

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Declaration

I, **Riya Johnson.**, do hereby declare that the PhD thesis “**Biostimulant mediated modulations of physiological, nutritional and yield traits in *Oryza sativa* L. under nutrient deficiency: A metabolomic perspective**” is a original research work accomplished by me under the guidance of **Dr. Jos, T. Puthur, Professor, Plant Physiology and Biochemistry Division, Department of Botany, University of Calicut**, and in partial fulfillment of the requirements for the award of **Doctor of Philosophy in Botany, University of Calicut**. I also declare that this has not been included in any other thesis submitted previously for the award of any degree, and it represents original work done by me. The content of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K Library, University of Calicut and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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ABSTRACT

The utilization of chemical fertilizers presents substantial risks to the ecosystem and novel biostimulant formulation serves as a multifaceted approach, for sustainable agricultural system. Characterizing new biostimulants, understanding how biostimulants work, and developing efficient methods for abiotic stress tolerance is very important. Two rice varieties (Hraswa and Manu ratna) were selected for evaluation based on their performance in nutrient-deficient conditions in response to the application of different biostimulants. The experimental design was structured across four developmental stages—seedling, tillering, booting, and ripening—to capture dynamic changes in physiology and metabolism. At each stage, parameters such as carbohydrate translocation, metabolite allocation, reactive oxygen species (ROS) generation, antioxidant enzyme activity, and chlorophyll *a* fluorescence based physiological aspects were systematically assessed. The stage-wise evaluation provided a comprehensive understanding of how biostimulant treatment modulated plant responses under stress at different stages of its lifecycle. Among the four stages, the booting phase emerged as particularly critical, showing pronounced changes in physiological performance and metabolic regulation. The booting stage marks a crucial shift from vegetative to reproductive growth in cereals like rice. The developmental sequences that ultimately determines spikelet fertility and yield potential occurs at this period, when the panicle primordia rapidly differentiate and elongate within the flag leaf sheath. Numerous studies have emphasized the physiological importance of this stage as plants are more vulnerable to abiotic stressors such as temperature extremes, water shortages, and nutritional deficiencies during this growth phase. Exposure to stress during booting can cause irreversible yield loss by interfering with pollen viability, assimilate partitioning, and floral organ development. Furthermore, the booting period marks the peak demand for mineral elements, especially potassium and nitrogen, which are essential for the transfer of carbohydrates and the synthesis of proteins. During this phase, lack of nutrients results in sterility and inadequate grain filling. Booting stage analyses may also be used as a diagnostic window to evaluate source-sink dynamics, translocation patterns, and nutrient absorption efficiency in both ideal and stressful conditions. Therefore, detailed analyses such as gas exchange measurements and ICP-MS-based nutrient profiling were carried out at this stage. Subsequently, yield traits and FTIR spectroscopy were employed to examine grain development and functional group

modifications of leaf metabolites subjected to biostimulant application revealed that among the both varieties studied, the var. Manu ratna appeared more sensitive to nutrient deficiency, however biostimulant treatment induced more structural and biochemical changes that improved its tolerance.

Based on the observations, Manu ratna was selected for further in-depth metabolomic investigations, since it displayed greater sensitivity to nutrient deficiency but also exhibited marked improvement when treated with the biostimulant. Advanced analytical approaches including GC-MS/MS, HR-LCMS, and amino acids profiling were employed to characterize metabolic shifts induced by biostimulant application. These analyses revealed clear alterations in the abundance of primary metabolites, secondary compounds, and amino acid pools, underscoring the capacity of biostimulant treatment to reprogram central metabolic pathways under nutrient deficient conditions. Metabolomic profiling of rice under control, stress, and biostimulant treatments provided crucial insights into the biochemical reprogramming that accompanies stress adaptation and the ameliorative effects of biostimulants. Using complementary platforms (GC-MS/MS, HRLC-MS and amino acids profiling), a wide range of metabolites—including amino acids, organic acids, fatty acids, sugars, and secondary metabolites—were identified, revealing distinct signatures across treatments. The integration of multivariate analyses (PCA, PLS-DA, VIP scores, SAM) and pathway enrichment further highlighted treatment-specific metabolic regulation, underscoring the importance of biostimulants in restoring metabolic balance under treatments. The findings underscore the potential of tailored biostimulant formulations to optimize nutrient release, enhance plant resilience, and improve agricultural sustainability. This study establishes a foundational framework for the strategic design and application of biostimulants across diverse crops and agroecological conditions, supporting innovative and sustainable agricultural practices.

Keywords: Biostimulants, Nutrient deficiency, Rice, Booting Stage, Metabolomics

സംഗ്രഹം

രാസവളങ്ങളുടെ ഉപയോഗം ആവാസവ്യവസ്ഥയ്ക്ക് ഗണ്യമായ അപകടസാധ്യതകൾ സൃഷ്ടിക്കുന്നു. സുസ്ഥിരമായ ഒരു കാർഷിക സംവിധാനത്തിന് ഒരു ബഹുമുഖ സമീപനമായി നവീന ബയോസ്റ്റിമുലന്റ് ഫോർമുലേഷൻ പ്രവർത്തിക്കുന്നു. പുതിയ ബയോസ്റ്റിമുലന്റുകളുടെ സ്വഭാവം വ്യക്തമാക്കുക, ബയോസ്റ്റിമുലന്റുകൾ എങ്ങനെ പ്രവർത്തിക്കുന്നുവെന്ന് മനസ്സിലാക്കുക, കാര്യക്ഷമമായ ഉപയോഗ രീതികൾ വികസിപ്പിക്കുക എന്നിവ വളരെ പ്രധാനമാണ്. പോഷകക്കുറവുള്ള സാഹചര്യങ്ങളിൽ വ്യത്യസ്ത ബയോസ്റ്റിമുലന്റുകളുടെ പ്രയോഗത്തോടുള്ള പ്രതികരണത്തെ അടിസ്ഥാനമാക്കി, വിലയിരുത്താനായി രണ്ട് നെല്ല് ഇനങ്ങൾ (ഹ്രസ്വ, മനരത്ന) തെരഞ്ഞെടുത്തു. ഫിസിയോളജിയിലും മെറ്റബോളിസത്തിലും ചലനാത്മകമായ മാറ്റങ്ങൾ മനസ്സിലാക്കുന്നതിനായി നാല് വികസന ഘട്ടങ്ങളിലായി പരീക്ഷണാത്മക രൂപകൽപ്പന ചെയ്തിട്ടുണ്ട്. സീഡ് ലിംഗ് (10 - 12 ദിവസം പ്രായമായ തൈകൾ), ടില്ലറിംഗ് (കൂടുതൽ മുളകൾ വരുന്ന സമയം), ബൂട്ടിംഗ് (ചെടികൾ വളർച്ചയുടെ പാതി പിന്നിട്ട അവസ്ഥ), റൈപ്പനിംഗ് (നെൽവിത്തുകൾ വന്ന് പാകമാകുന്ന സമയംവരെ). ഓരോ വളർച്ചാഘട്ടങ്ങളിലായി കാർബോഹൈഡ്രേറ്റ് ട്രാൻസ്‌ലോക്കേഷൻ, മെറ്റബോളൈറ്റ് അലോക്കേഷൻ, റിയാക്ടീവ് ഓക്സിജൻ സ്പീഷീസ് (ROS) ജനറേഷൻ, ആന്റിഓക്സിഡന്റ് എൻസൈം പ്രവർത്തനം, ക്ലോറോഫിൽ ഫ്ലൂറസെൻസ് അടിസ്ഥാനമാക്കിയുള്ള ഫിസിയോളജി തുടങ്ങിയ പാരാമീറ്ററുകൾ വ്യവസ്ഥാപിതമായി വിലയിരുത്തി. ബയോസ്റ്റിമുലന്റ് ടീറ്റ്‌മെന്റ് നെൽച്ചെടിയുടെ ജീവിതചക്രത്തിന്റെ വിവിധ ഘട്ടങ്ങളിൽ സമ്മർദ്ദത്തിന് കീഴിൽ സസ്യ പ്രതികരണങ്ങളെ എങ്ങനെ മോഡ്യുലേറ്റ് ചെയ്തു എന്നതിനെക്കുറിച്ചുള്ള സമഗ്രമായ പഠനം ഘട്ടംഘട്ടമായി തിരിച്ച് വിലയിരുത്തൽ നടത്തി. നാല് ഘട്ടങ്ങളിൽ, ബൂട്ടിംഗ് ഘട്ടം പ്രത്യേകിച്ചും നിർണായകമായി ഉയർന്നുവന്നു, ഫിസിയോളജിക്കൽ പ്രകടനത്തിലും ഉപാപചയ നിയന്ത്രണത്തിലും പ്രകടമായ മാറ്റങ്ങൾ കാണിക്കുന്ന ഘട്ടമാണ് ഇത്. നെല്ല് പോലുള്ള ധാന്യങ്ങളിൽ സസ്യജന്യ വളർച്ചയിൽ നിന്ന് പ്രത്യുൽപാദന വളർച്ചയിലേക്കുള്ള നിർണായക മാറ്റത്തെ ബൂട്ടിംഗ് ഘട്ടം അടയാളപ്പെടുത്തുന്നു. സ്‌പൈക്കിന്റെ ഫലഭൂയിഷ്ഠതയും വിളവ് സാധ്യതയും ആത്യന്തികമായി നിർണ്ണയിക്കുന്ന വികസനക്രമങ്ങൾ ഈ കാലഘട്ടത്തിലാണ് സംഭവിക്കുന്നത്. പ്ലാഗ് ലീഫ് പോളുള്ളിൽ പാനിക്കിൾ പ്രൈമോർഡിയ വേഗത്തിൽ വ്യത്യാസപ്പെട്ട് നീളുന്ന ഘട്ടമാണിത്. വളർച്ചാ ഘട്ടത്തിൽ താപനില തീവ്രത, ജലക്ഷാമം, പോഷകാഹാരക്കുറവ് തുടങ്ങിയ അജൈവ സമ്മർദ്ദങ്ങൾക്ക് സസ്യങ്ങൾ കൂടുതൽ ഇരയാകുന്നതിനാൽ ഈ ഘട്ടത്തിന്റെ വളർച്ചാപ്രാധാന്യത്തെക്കുറിച്ച് നിരവധി പഠനങ്ങൾ ഊന്നിപ്പറഞ്ഞിട്ടുണ്ട്. ബൂട്ടിംഗ് സമയത്ത് സമ്മർദ്ദത്തിന് വിധേയമാകുന്നത് പൂമ്പൊടിയുടെ പ്രവർത്തനക്ഷമത, സ്വാംശീകരണ വിഭജനം, പുഷ്പ അവയവ വികസനം എന്നിവയെ തടസ്സപ്പെടുത്തുന്നതിലൂടെ മാറ്റാനാവാത്ത വിളനഷ്ടത്തിന് കാരണമാകും. കൂടാതെ, ബൂട്ടിംഗ് കാലഘട്ടം കാർബോഹൈഡ്രേറ്റുകളുടെ കൈമാറ്റത്തിനും പ്രോട്ടീനുകളുടെ സമന്വയത്തിനും അത്യാവശ്യമായ ധാതു മൂലകങ്ങൾ, പ്രത്യേകിച്ച് പൊട്ടാസ്യം, നൈട്രജൻ എന്നിവയുടെ ഏറ്റവും ഉയർന്ന ആവശ്യകതയെ അടയാളപ്പെടുത്തുന്നു. ഈ ഘട്ടത്തിൽ, പോഷകങ്ങളുടെ അഭാവം വന്ധ്യതയ്ക്കും ധാന്യങ്ങളുടെ അപര്യാപ്തതയ്ക്കും കാരണമാകുന്നു. അനുയോജ്യമായതും സമ്മർദ്ദകരവുമായ സാഹചര്യങ്ങളിൽ ഊവിട-സിങ്ക് ഡൈനാമിക്സ്, ട്രാൻസ്‌ലോക്കേഷൻ പാറ്റേണുകൾ, പോഷക ആഗിരണം കാര്യക്ഷമത എന്നിവ വിലയിരുത്തുന്നതിനുള്ള ഒരു ഡയഗ്നോസ്റ്റിക് വിൻഡോയായും ബൂട്ടിംഗ് ഘട്ട ഗവേഷണങ്ങൾക്ക് ഉപയോഗിക്കാം.

അതിനാൽ, ഗ്യാസ് എക്സ്പെൻഡിചർ അളവുകൾ, ഐസിപി-എംഎസ് അടിസ്ഥാനമാക്കിയുള്ള പോഷക പ്രൊഫൈലിംഗ് തുടങ്ങിയ വിശദമായ വിശകലനങ്ങൾ ഈ ഘട്ടത്തിൽ നടത്തി. തുടർന്ന്, ബയോസ്റ്റിമുലന്റ് പ്രയോഗത്തിന് വിധേയമാക്കിയ ഇല മെറ്റബോളൈറ്റുകളുടെ വികസനവും, FTIR സ്പെക്ട്രോസ്കോപ്പിയും ഉപയോഗിച്ച് പരിശോധിച്ചിരുന്നു. പഠിച്ച രണ്ട് നെൽ ഇനങ്ങളിലും, മനുരത പോഷകക്കുറവിനോട് കൂടുതൽ സെൻസിറ്റീവ് ആയി കാണപ്പെട്ടു, എന്നിരുന്നാലും ബയോസ്റ്റിമുലന്റ് ട്രീറ്റ്മെന്റ് ഈ ഇനത്തിൽ കൂടുതൽ ഘടനാപരവും ജൈവ രാസപരവുമായ മാറ്റങ്ങൾക്ക് കാരണമായി, അത് നെൽച്ചെടിയുടെ വളർച്ചയെ മെച്ചപ്പെടുത്തി.

നിരീക്ഷണങ്ങളുടെ അടിസ്ഥാനത്തിൽ, മനുരത എന്നയിനത്തെ കൂടുതൽ ആഴത്തിലുള്ള മെറ്റബോളിക് പഠനങ്ങൾക്കായി തെരഞ്ഞെടുത്തു. ഈ ഇനം പോഷകക്കുറവിനോട് കൂടുതൽ സംവേദനക്ഷമത രേഖപ്പെടുത്തിയെങ്കിലും, ബയോസ്റ്റിമുലന്റ് ഉപയോഗിച്ച് ട്രീറ്റ്മെന്റ് നടത്തുമ്പോൾ ശ്രദ്ധേയമായ പുരോഗതി കാണിച്ചു. ബയോസ്റ്റിമുലന്റ് പ്രയോഗത്താൽ പ്രേരിതമായ മെറ്റബോളിക് ഷിഫ്റ്റുകളെ പഠിക്കുവാൻ GC-MS/MS, HR-LCMS, അമിനോ ആസിഡ് പ്രൊഫൈലിംഗ് എന്നിവയുൾപ്പെടെയുള്ള നൂതന വിശകലന സമീപനങ്ങൾ ഉപയോഗിച്ചു. ഈ വിശകലനങ്ങൾ പ്രാഥമിക മെറ്റബോളൈറ്റുകൾ, ദ്വിതീയ സംയുക്തങ്ങൾ, അമിനോ ആസിഡ് പൂങ്കുരു എന്നിവയുടെ സമൃദ്ധിയിൽ വ്യക്തമായ മാറ്റങ്ങൾ വെളിപ്പെടുത്തി. പോഷകക്കുറവുള്ള സാഹചര്യങ്ങളിൽ മുഖ്യ മെറ്റബോളിക് പാതകളെ പുനർനിർമ്മിക്കാനുള്ള ബയോസ്റ്റിമുലന്റ് ട്രീറ്റ്മെന്റിന്റെ ശേഷി അടിവറയിടുന്നു. ഈ പഠനം നെല്ലിന് ബയോസ്റ്റിമുലന്റ് ട്രീറ്റ്മെന്റിന് വിധേയമാക്കുമ്പോൾ മെറ്റബോളോമിക് പ്രൊഫൈലിംഗ്, സമ്മർദ്ദത്തിനോടുള്ള പ്രതികരണം, ബയോകെമിക്കൽ റീപ്രോഗ്രാമിംഗ് എന്ന മേഖലകളെക്കുറിച്ച് നിർണായക ഉൾക്കാഴ്ചകൾ നൽകി. പൂരക പ്ലാറ്റ്ഫോമുകൾ (GC-MS/MS, HRLC-MS, അമിനോ ആസിഡ് പ്രൊഫൈലിംഗ്) ഉപയോഗിച്ച്, അമിനോ ആസിഡുകൾ, ഓർഗാനിക് ആസിഡുകൾ, ഫാറ്റി ആസിഡുകൾ, മെറ്റബോളൈറ്റുകൾ എന്നിവയെ തിരിച്ചറിയാൻ സഹായിച്ചു. മൾട്ടിവേരിയേറ്റ് വിശകലനങ്ങളുടെയും (PCA, PLS-DA, VIP സ്കോറുകൾ, SAM) പാത സമ്പുഷ്ടീകരണത്തിന്റെയും സംയോജനം ബയോസ്റ്റിമുലന്റ് ട്രീറ്റ്മെന്റ് നിർദ്ദിഷ്ട മെറ്റബോളിക് നിയന്ത്രണത്തെ കൂടുതൽ എടുത്തുകാണിച്ചു. ട്രീറ്റ്മെന്റുകൾക്കിടയിൽ ഉപാപചയ സന്തുലിതാവസ്ഥ പുനഃസ്ഥാപിക്കുന്നതിൽ ബയോസ്റ്റിമുലന്റുകളുടെ പ്രാധാന്യം അടിവറയിടുന്നു. പോഷക പ്രകാശനം ഒപ്റ്റിമൈസ് ചെയ്യുന്നതിനും സസ്യ പ്രതിരോധശേഷി വർദ്ധിപ്പിക്കുന്നതിനും കാർഷിക സുസ്ഥിരത മെച്ചപ്പെടുത്തുന്നതിനും അനുയോജ്യമായ ബയോസ്റ്റിമുലന്റ് ഫോർമുലേഷനുകളുടെ സാധ്യതയെ കണ്ടെത്തലുകൾ അടിവറയിടുന്നു. വൈവിധ്യമാർന്ന വിളകളിലും കാർഷിക പാരിസ്ഥിതിക സാഹചര്യങ്ങളിലും ബയോസ്റ്റിമുലന്റുകളുടെ തന്ത്രപരമായ രൂപകൽപ്പനയ്ക്കും പ്രയോഗത്തിനും ഈ പഠനം ഒരു അടിസ്ഥാന ചട്ടക്കൂട് സ്ഥാപിക്കുന്നു. കൂടാതെ നൂതനവും സുസ്ഥിരവുമായ കാർഷിക രീതികളെ ഈ ഗവേഷണം പിന്തുണയ്ക്കുന്നു.

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ABBREVIATIONS

ABS/CSm	-	Absorption flux per cross section
ABS/RC	-	Absorption flux per reaction center
AGC	-	Ascorbate glutathione cycle
ALA	-	Aminolevulinic acid
APX	-	Ascorbate peroxidase
AsA	-	Ascorbate
Bl	-	Biostimulant with live yeast extract
Bs	-	Biostimulant with supernatant of yeast extract
B	-	Biostimulant with yeast extract (sigma Aldrich)
CA	-	Carbonic anhydrase
CAT	-	Catalase
Ci	-	Internal Carbon dioxide concentration
DCMU	-	3 (3,4dichlorophenyl) -1, 1-dimethyl ureasss
DCPIP	-	2,6-dicholrophenolindophenol
DHA	-	Dehydroascorbate
DHAR	-	Dehydroascorbate reductase
DIo/CSm	-	Dissipated energy flux per cross section
DIo/RC	-	Dissipated energy flux per reaction center
DTNB	-	5-dithio-bis-2-nitrobenzoic acid
DW	-	Dry weight
DW%	-	Dry weight percentage
EDTA	-	Ethylene diamine tetra acetic acid
ETo/CSm	-	Electron transport flux per cross section
ETo/RC	-	Electron transport flux per reaction center
FAA	-	Formalin acetic acid
Fm	-	Maximum Chl <i>a</i> fluorescence
Fo	-	Initial Chl <i>a</i> fluorescence
Fv	-	Variable Chl <i>a</i> fluorescence
Fv/Fm	-	Potential or maximum quantum yield of PSII

FW	-	Fresh weight
GPOX	-	Guaiacol peroxidase
GR	-	Glutathione reductase
gs	-	Stomatal conductance
GS	-	Glutathione synthetase
GSH	-	Glutathione
GSSG	-	Oxidized glutathione
HEPES	-	[N-(2-Hydroxyethyl)piperazine-N-(2-Ethanesulphonic acid)
IBA	-	Indole-3-butyric acid
iWUE	-	Intrinsic water use efficiency
LEA	-	Late Embryogenesis Abundant
LHC	-	Light harvesting complex
LMW	-	Low molecular weight
μS	-	Micro second
mS	-	Milli second
MDA	-	Malondialdehyde
MDHA	-	Monodehydroascorbate
MDHAR	-	Monodehydroascorbate reductase
MV	-	Methyl viologen
NAR	-	Net assimilation ratio
NBT	-	Nitroblue tetrazolium
NRAMP	-	Natural resistance-associated macrophage protein
OP	-	Osmotic potential
pBQ	-	Parabenzoquinone
PCs	-	Phytochelatin
PI _{ABS}	-	Performance index on absorption basis
POD	-	Peroxidase
Pn	-	Photosynthetic rate
PS I	-	Photosystem I
PS II	-	Photosystem II

PVPP	-	Poly vinyl poly pyrrolidone
QAC	-	Quaternary ammonium compounds
ROS	-	Reactive oxygen species
RC/CSm	-	Reaction center per cross section
RC/CSo	-	Number of reaction centres per excited cross section
RWC	-	Relative water content
SOD	-	Super oxide dismutase
SuSy	-	Sucrose synthase
SPS	-	Sucrose phosphate synthase
SWU	-	Seed water uptake
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
TF	-	Translocation factor
Tr	-	Transpiration rate
TRo/CSm	-	Trapping flux per cross section
TRo/RC	-	Trapping flux per reaction center
TW	-	Turgid weight
VPD	-	Vapor pressure deficit
WUE	-	Water use efficiency

1. INTRODUCTION

1.1 Global challenges in agriculture

Humanity's primary goal continues to be addressing the serious problems of malnutrition, food instability, and world hunger. About 800 million people are malnourished, and more than 2 billion people lack sufficient access to food (OECD-FAO 2024). Various biotic and abiotic stressors significantly impact plant growth, leading to 50-70% reduced crop productivity and economic losses (Tchoukouang et al. 2024). Abiotic stressors such as temperature, drought, salt, and nutritional deficits impair essential physio-chemical processes in plants. Likewise, biotic variables, such as pests and diseases, provide additional challenges to agricultural sustainability. These combined factors present a significant challenge to attaining the anticipated 70% rise in food production necessary to support a worldwide population of 9.7 billion by 2050 (Sida et al. 2025).

Plants require necessary minerals for growth and development, which are mostly absorbed from the soil by their roots at optimal levels. Nutrient scarcity has an adverse influence on plant growth, and excess has an adverse effect on soil. In modern agricultural practices, soil degradation and nutrient depletion have emerged as major concerns due to overexploitation and inadequate replenishment of essential nutrients. The decline in soil fertility, coupled with the excessive use of chemical fertilizers, has led to environmental concerns, necessitating the exploration of sustainable alternatives. Currently, 24 billion metric tons of fertile soil are lost annually, and one-third of all terrestrial areas on Earth are somewhat degraded. It is estimated that 50 million people may be displaced in the next decade as a consequence of land degradation (Jones 2020; Rosa 2022). Therefore, in order to restore productive soil in the upcoming years, immediate action is required. Nutrient deficiency, particularly of major nutrients (N, P, and K), is one of the most limiting factors along with drought, salinity, soil alkalinity, and extreme temperatures, on the performance of plants in their natural habitats and agricultural environments (Rahim et al. 2025). The macro and micronutrients play pivotal roles in plant metabolism,

acting as key components of cellular structures, osmotic regulators, and energy-transfer molecules. The appearance of deficiencies of micronutrients in different soils is mainly due to enhanced cropping intensity, high-yielding cultivars of various crops, increased use of NPK fertilisers with very less micronutrients contents, or due to very less application of organic manures (Vejan et al. 2021). Nutrient scarcity not only limits plant productivity but also compromises crop quality, reducing food availability and economic stability. Soils with micronutrients deficiencies produce crops with low concentration of micronutrients (Manzeke et al. 2019). The use of foods derived from these edible crops results in detrimental health effects for both animals and humans due to micronutrient deficiency. However, effective management of micronutrients, namely soil and foliar application of micronutrients, in various soil–crop situations could help in alleviating micronutrients deficiency in soils and crops, enhancing crop production and crop quality and reducing micronutrients malnutrition in animals and human being (Shukla and Behera 2020).

1.2 Importance of rice cultivation

Over 2.7 billion people worldwide rely on rice as their primary food, making it the most significant food crop cultivated worldwide (Fig. 1). It occupies 7.46 percent of the total cropped area of the world (Abeysekara and Rathnayake 2024). Since 1980s, the area under rice cultivation has been declining at an alarming rate. The paddy area decreased from 8.82 lakh hectares in 1974–75 to 1.96 lakh hectares in 2015–16. An estimated 2.6 thousand kg of rice per acre were produced in the nation during the 2019 fiscal year. This is not surprising because rice is a staple grain in India and its production is comparable to China's. Rice is an annual grass characterised by round, hollow, jointed stems; narrow, flat, sessile leaf blades attached to leaf sheaths by collars; prominent, hairy, sickle-shaped auricles; small acute to acuminate or bifid ligules; and terminal panicles (Fig. 2). The life cycle of rice cultivars spans from 110 to 150 days, commencing from germination to maturity, contingent upon the variety and environmental conditions. Rice requires between 3 to 6 months to develop from seeds to fully grown plants, contingent upon the variety and environmental circumstances. The growth stages are: seedling, vegetative, reproductive, and ripening (Fig. 3). Rice varieties can be categorized into

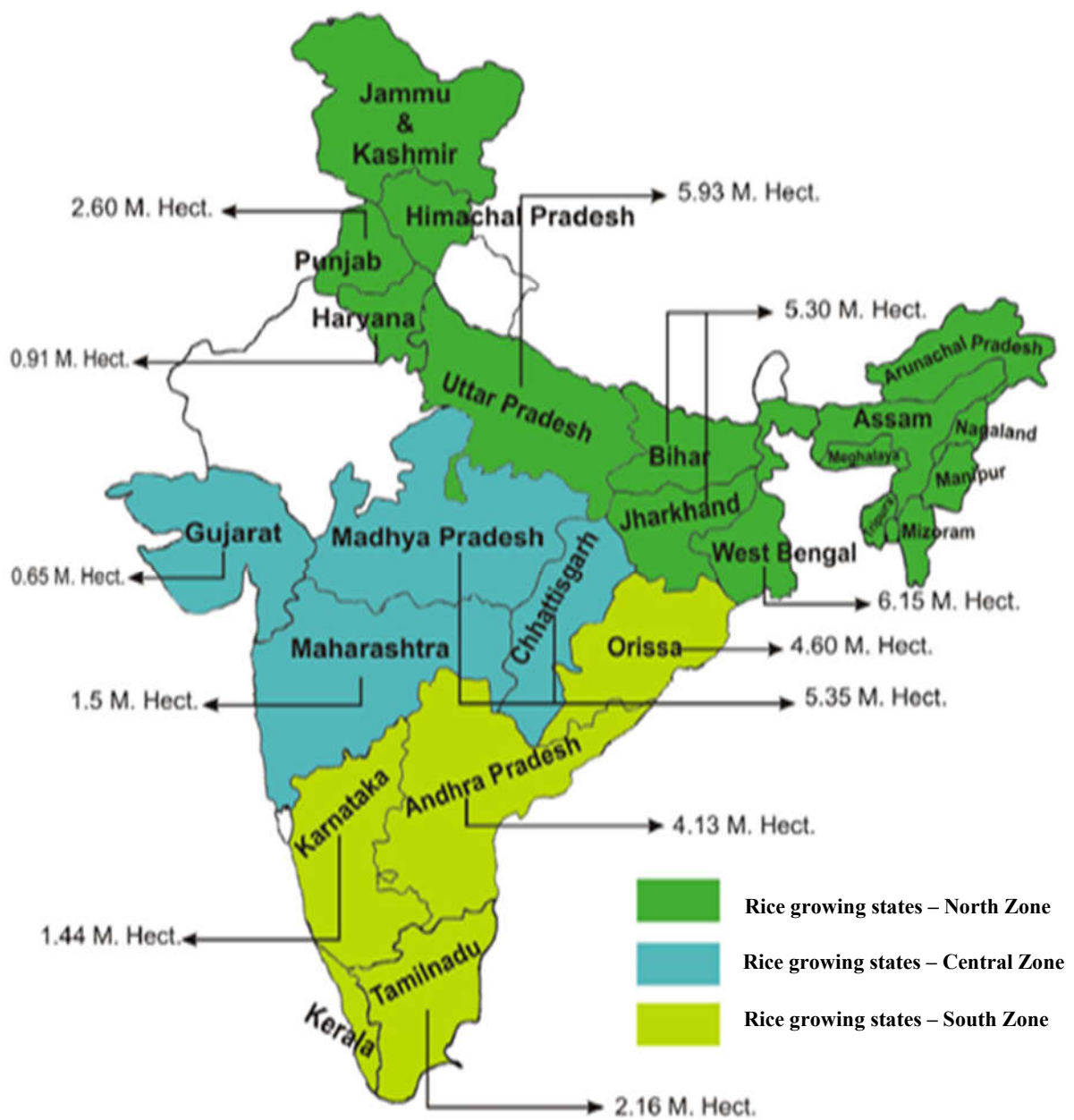


Fig. 1: Rice producing states in India

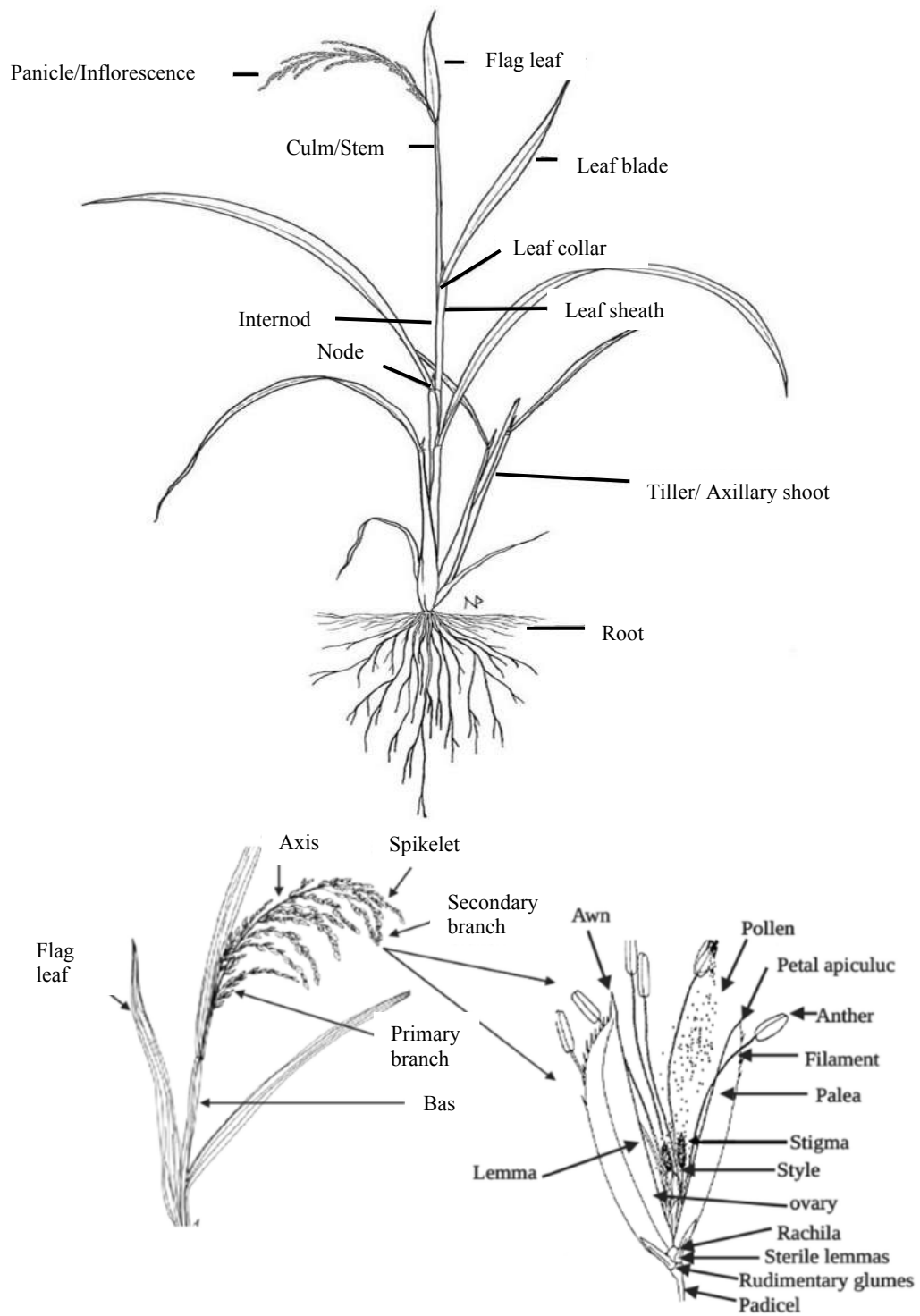


Fig. 2: Morphology of the rice plant showing the flag leaf, panicle and floral structures

two stages: short duration (100) and long duration varieties (120). A 120 day variety requires 60 days in vegetative phase and 30 days in reproductive and 30 days in ripening phases. Rice plant growth can be divided into three agronomic stages of development:

1. Vegetative (germination to panicle initiation)
2. Reproductive (panicle initiation (PI) to heading)
3. Grain filling and ripening or maturation (heading to maturity).

According to Dwiningsih (2020), stages influence the three yield components: number of panicles per unit land area, the average number of grain produced per panicle and the average weight of the individual grains. The grain yield is determined by these three factors. The growth phases of rice plants are depicted in the following descriptions and diagrams.

1. Vegetative stage

The vegetative growth phase is marked by vigorous tillering, a progressive rise in plant height, and the periodic appearance of leaves. The duration of this stage significantly dictates the growth period of varieties. Certain early-maturing cultivars exhibit a truncated vegetative development phase, whereas others display reductions in both vegetative and reproductive growth phases. Panicle initiation (PI) may transpire prior to achieving the maximum tiller count in very-short season and certain short-season types. Heading in these cultivars may be offset due to subsequent tillers that generate panicles. In mid-season types, following the peak tiller count, a vegetative lag phase ensues prior to panicle start. The following distinct steps occur during the vegetative stage:

1. Germination phase transpires after the seed coat has absorbed sufficient water. The seed coat becomes pliable and flexible. The coleorhiza, which encases the radicle or embryonic primary root, elongates marginally, penetrating the seed coat, while the radicle subsequently breaches the coleorhiza and secures itself in the soil. The coleoptile or primary leaf extends. Consequently, in dry-seeded or

aerobic circumstances, the radicle precedes the emergence of the coleoptile. In submerged or anaerobic environments, the coleoptile may arise prior to the root.

2. Seedling emergence transpires when the initial internode, known as the mesocotyl, elongates and propels the apex of the rice coleoptile above the soil surface. The mesocotyl length differs among cultivars.
3. Pre-Tillering is the time between the first and fourth leaf development. 15 to 25 days are needed for this stage. The first four leaves emerge, the secondary or lateral roots grow, and the seminal roots continue to develop during this period. A new leaf appears following seedling emergence, signalling the start of the tillering stage.
4. Tillering is the process that typically starts at the fifth leaf stage, when the first tiller appears from the axillary bud of the culm's second leaf. The second tiller originates from the axillary bud of the third leaf, and tillering continues until the sixth leaf appears. With the n th leaf of the main culm and tiller developing from the axillary bud of the $(n-3)^{\text{th}}$ leaf, tillering proceeds in a synchronised way. The secondary roots spread downward till flooding occurs during this time. These roots grow horizontally and vertically after flooding. The availability of O_2 at the soil-water interface is most likely the cause of the lateral growth. As the main culm enters the booting stage, new leaves appear more quickly during active tillering.
5. Until the maximum tiller number is attained, tillering rises in a sigmoidal pattern. It could be challenging to tell the main culm from the tiller at this point. Rice plants typically generate 2 to 5 panicle-bearing tillers per plant in direct-seeded rice fields with a normal plant population (10 to 20 plants per square foot), as opposed to 10 to 30 tillers per plant in transplanted rice where there is more room between plants. No more efficient tillers are produced until maximal tillering has taken place. The impacts of competition will usually cause some of the late tillers to die. At this point, the first yield component—potential panicles per unit area—is calculated.

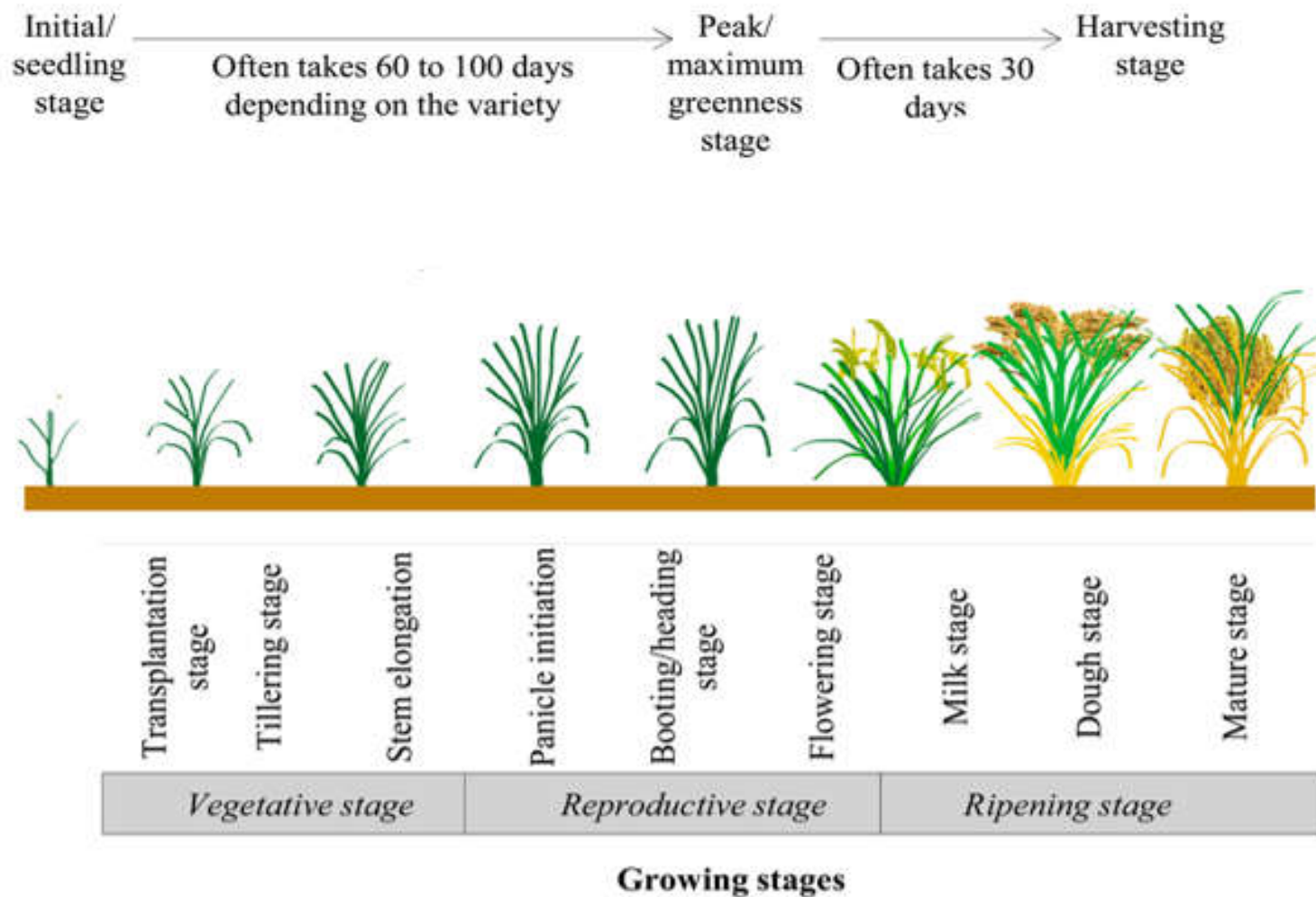


Fig. 3: Diagrammatic representation of different growth phases in rice

2. Reproductive stage

The reproductive phase is marked by culm elongation, a reduction in tiller count, booting, flag leaf emergence, heading, and blooming. The reproductive phase typically endures for around 30 days in the majority of types. This phase is occasionally termed the internode elongation or jointing phase and exhibits modest variations according to the variety and climatic conditions. The following processes occur during the reproductive stage:

1. Panicle Initiation (PI) – is the time when the panicle premordia initiate the production of a panicle in the uppermost node of the culm. At this point, the panicle is not visible to the naked eye. It is sometimes referred to as the green ring stage in rice. A thin green band is visible just above the top node, and represents the very beginning of internode elongation and is evident for only a couple of days.
2. Internode Elongation – begins about the time panicle initiation is occurring, continues until full plant height is reached and is followed by heading. The top five internodes may elongate. This stage is also referred to as the “jointing stage”. Stem internodes can be distinguished from root internodes by the green color in the stem wall.
3. Panicle Differentiation (PD) – is closely associated with “jointing” or the internode elongation stage. PD is roughly equivalent to 1/2 to 3/4 inch internode elongation. At this point, the panicle is 1 to 2 mm in length and the branching of the panicle is visible. This is a critical stage during rice plant development. At this stage, the environment can have a major effect on rice plant development. The second yield component, number of potential grains per panicle, is set by the time this development stage occurs.
4. Booting – this stage is loosely defined as that period characterized by a swelling of the flag leaf sheath which is caused by an increase in the size of the panicle as it grows up the leaf sheath. Full or late boot occurs when the flag leaf has completely extended. Booting is the stage in which meiosis

occurs. Environmental stress during this stage may reduce rice grain yield. Late boot occurs about 6 days prior to heading.

5. Heading – Heading refers to the period when the panicle begins to exert from the boot. Heading may require a duration of 10 to 14 days due to discrepancies among tillers within the same plant and between different plants in the field. Agronomically, "heading date" refers to the moment when 50 percent of the panicles have partially emerged from the boot. Certain panicles may fail to fully emerge from the boot.
6. Anthesis or flowering denotes the period between the opening and shutting of the spikelet (floret), lasting 1 to 2 hours. Flowering typically commences with panicle exertion or the subsequent day, and is therefore seen as equivalent with heading. The procedures involved are: a) The apices of the lemma and palea (hulls) separate. b) The filaments extend. c) The anthers protrude from the lemma and palea. d) As the lemma and palea expand more, the tips of the delicate stigma become discernible. e) The filaments extend beyond the tips of the lemma and palea. f) The spikelet collapses, exposing the anther externally. Anther dehiscence or pollen release typically transpires immediately before or concurrently with the opening of the lemma and palea. Pollen grains remain viable for around five minutes post-emergence from the anther, but the stigma may retain fertility for three to seven days. Rice is a self-pollinating species, as it typically undergoes pollination prior to the opening of the lemma and palea, which release pollen into the atmosphere. The fertilisation of the ovary by the pollen grain typically concludes within five to six hours post-pollination.

3. **Ripening stage**

The grain filling and maturation stage occurs post-ovary fertilisation and is defined by grain development. Throughout this phase, the grain expands in size and mass as starch and sugars are transported from the culms and leaf sheaths where they have been stored. The grain transitions from green to golden or straw hues at maturity, while the leaves of the rice plant commence senescence. Light intensity is

crucial during this period, as 60 percent or more of the carbohydrates allocated for grain filling are photosynthesised at this time. This period is adversely influenced by temperature. The ultimate factor, individual grain weight, is ascertained during the ripening phase. While grain weight is largely consistent for a certain variety, it is subject to environmental influences. Elevated temperatures typically diminish the grain filling duration and may decrease grain weight. Reduced temperatures typically prolong the duration necessary for grain filling and maturation. The ripening process may terminate following a substantial frost. Steps in the ripening process are:

1. Milk stage – During this phase, the nascent starch grains within the kernel are pliable, and the kernel's interior is filled with a white liquid like to milk.
2. Soft dough stage – The starch in the grain commences to solidify while remaining pliable.
3. Hard dough stage – The entire grain is robust during this phase and nearly prepared for harvest. The moisture content remains above 22%.
4. Maturity - The whole grain is firm and prepared for harvest, with a moisture level of 20 to 22%.

1.3 Role of nutrients in rice physiology

The proper growth and development of rice depend on the sufficient supply of critical nutrients such as nitrogen (N), phosphorus (P), and potassium (K). Inadequate levels of these nutrients are a considerable limitation on global rice production (Kubitza et al. 2024). Nitrogen is a crucial element of amino acids and proteins, and it modulates the enzymatic activity necessary for energy metabolism, encompassing photosynthesis and respiration (Liu et al. 2025). Phosphorus is an essential component of critical cellular components (including ATP, nucleic acids, and phospholipids) and plays a crucial role in energy conservation and metabolism (Nicholls et al. 2023). Similarly, potassium supply is essential for optimal plant growth and development, playing a crucial role in cellular homeostasis by

facilitating osmotic adjustment, charge balancing, and enzyme catalysis (Anil et al. 2024; Aina et al. 2024).

Nutrient deficit can induce oxidative stress by excessive formation of reactive oxygen species (ROS), resulting in protein degradation, alterations to DNA, and membrane lipid peroxidation (Juan et al. 2021). Identifying the causes and consequences of oxidative metabolism under such conditions is challenging. Therefore, assessing oxidative markers such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) as primary indicators of ROS, along with malondialdehyde (MDA) as a marker of lipid peroxidation, becomes essential. Antioxidant system has been found to be of paramount importance in the response and tolerance of rice to environmental stresses (Aina et al. 2024). In addition, nutrient deficiency can disturb the plant's antioxidant system, as nutrients are needed for antioxidant biosynthesis (Chen et al. 2024). Plants have a long-distance mobile signal that enables them to react to famine and stress signals via their vascular system. The main symptoms of nutrient deficiency are stunted growth, chlorosis, necrosis and a disproportionately high allocation of biomass to the root system (Yadav et al. 2024). Many genes play a central role in the acquisition and distribution of nutrients, including many protein-coding genes as well as microRNAs (miR395, miR399, miR398, miR397, and miR408) (O'Brien et al. 2018). To enhance yield characteristics, the overuse of chemical fertilizers and pesticides has become a prevalent approach, leading to environmental and health issues and driving the transition towards sustainable agricultural practices.

1.4 Approaches to enhance stress tolerance in rice

Various techniques were used to modify plants to become more resilient to different conditions. These include both traditional breeding techniques like hybridization and selection as well as more contemporary techniques like genetic engineering, polyploidy breeding, and mutation breeding. Limitations of conventional breeding techniques are the requirements of large man power, energy, etc. using selection-based breeding approaches generate plant varieties with better abiotic stress tolerance have proved mostly unsuccessful because of the well-

recognized complexity or multigenic nature of abiotic stress-tolerance traits (Parmar et al. 2017). The development of transgenic plants enables them to endure various forms of stress. Through genetic engineering, stress tolerance can be enhanced in a rapid and predictable manner; however, its application has been largely limited to the targeted incorporation of individual heterologous traits into elite crop varieties (Husaini 2022). The incorporation of transgenes into breeding programs is not an easy mechanism. Because of the effects of pleiotropy and gene silencing, it is not feasible to continue through the plant breeding process (Parmar et al. 2017). Additionally, these techniques are very expensive, and the introduction of transgenics into the field is hampered by biosafety laws and regulations (Hussain 2022).

Because of these limitations of the above-mentioned techniques, it has become crucial to adopt alternate solutions to impart tolerance to plants against various stresses which should be simple, cost effective and can be adopted by the farmers without any complication and at the same time it should be effective in tolerance (Hussain et al. 2022). There is a paradigm shift around the world post COVID-19 and an increase in demand for organic food among consumers as a preventive health measure both from developed and developing countries (Ciric et al. 2020; Brata et al. 2022; Wang et al. 2022).

With a growing global population and increasing concerns about environmental degradation there is a growing demand for sustainable agriculture practices and the adoption of GLOBALGAP (GLOBAL Good Agricultural Practices) policies (Mook and Overdeest 2021). Innovative farming methods and more effective, eco-friendly substances must be developed in order to lessen the widespread usage of fertilisers and pesticides. In this sense, compounds that cause plant metabolic reactions at low concentrations may be useful components to increase crop resilience and fitness. Modern farming methods that can satisfy the rising demand for food must be adopted in order to solve this problem effectively. To stop soil erosion and water contamination, these strategies must be sustainable (Cristofano et al. 2021).

1.5 Biostimulants as a sustainable alternative

Products known as plant growth regulators or biostimulants, regardless of their nutrient content, are used at modest dosages to promote nutrient absorption and assimilation, so as to enhance stress tolerance, or improve characteristics related to growth and yield. It is well established that plant growth promoting substances especially biostimulants control plant growth and development, increase plant metabolism, and improve nutrient uptake, all of which lead to healthier plants with higher yields. They work differently, though, to encourage plant development. Plant growth regulators are defined as synthetic compounds, phytohormone derivatives such as [2, 4-Dichlorophenoxy Acetic acid), alpha-Naphthalene Acetic Acid, 6-benzylaminopurine, thidiazuron and 6-furfurylaminopurine or kinetin] that mimic naturally occurring plant hormones [indole-3-acetic acid, trans-Zeatin, cis-zeatin, dihydrozeatin DZ, isopentenyladenine] (Gupta et al. 2023). Growth regulators do not hold nutritive value and function by either suppressing or boosting plant growth and development by directly influencing plant hormones at low doses (Sabagh et al. 2021).

On the other hand, biostimulants usually are complex mixtures containing organic (extracts of seaweed, vermicompost leachate, protein hydrolysates, humic substances, smoke-water), microbial (fungi and bacteria) and inorganic (Si, Se) constituents (Gupta et al. 2023; Sun et al. 2024). They enhance plant growth and health by stimulating natural processes at a minute quantity rather than directly controlling plant growth (Rouphael and Colla 2020a). Biostimulants have gained considerable attention as an eco-friendly and innovative approach to enhance crop resilience and productivity while offering a viable solution by enhancing nutrient use efficiency and mitigating stress-induced yield losses (Malik et al. 2020).

Biostimulants are natural or manufactured substances that, when applied to plants or soils, improve physiological processes, hence increasing nutrient uptake, stress tolerance, and total growth. In contrast to conventional fertilisers, biostimulants do not directly provide nutrients; instead, they improve the availability and utilisation of these nutrients by plants. According to European Union (2019), a

biostimulant is defined as any chemical, combination, or microbe that, when applied to plants or the rhizosphere, autonomously enhances nutrient absorption efficiency, increases soil nutrient availability, and improves stress resistance. They encompass several categories including humic and fulvic acids, protein hydrolysates, seaweed extracts, microbial inoculants, and beneficial fungi. Their capacity to regulate plant metabolism and enhance adaptive responses to environmental stress renders them a valuable asset in sustainable agriculture.

Biostimulants are categorised into microbial and non-microbial types. Recent studies indicate that both microbial and non-microbial biostimulants, such as seaweeds, phytoextracts, humic substances, protein hydrolysates, and beneficial bacteria or fungi, demonstrate significant potential in enhancing plants' resilience to various environmental stresses. Consequently, they may improve plant growth and various performance indicators, including root development, flowering, nutrient absorption, soil moisture retention, and microbial activity. Regardless of the quantity of nutrients available to the crop, plant biostimulants increase crop quality, tolerance to abiotic stresses, nutrient absorption and efficiency when applied to plants or the rhizosphere (Ricci et al. 2019).

Du Jardin (2015) defines a plant biostimulant as any substance or microorganism applied to plants, seeds, or the root environment to enhance natural processes that improve the utilisation of nutrients and tolerance to abiotic stresses, irrespective of the amount of nutrients it contains, or any mixture of such compounds and microbes intended for this purpose. Biostimulants, as defined by Yakhin et al. (2017), are "formulated products of biological origin that enhance plant productivity due to the novel or emergent properties of their complex constituents, rather than solely from the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds." Plant biostimulants are chemicals and/or microorganisms that, when applied to plants or the rhizosphere, enhance natural processes that improve crop quality, nutrient absorption efficiency, and abiotic stress tolerance. Applying a range of biostimulants, such as amino acids, humic acids, vitamins, and minerals, can reduce oxidative stress symptoms.

Biostimulants are primarily composed of amino acids, hormones, and peptide combinations degraded chemically or enzymatically from plant or animal sources (Rouphael and Colla 2020b). More recent studies are emphasising the use of biostimulants to promote the more prudent application of mineral fertilisers, which is essential for environmental preservation (Khoulati et al. 2025). In order to stimulate natural processes, biostimulants are frequently applied directly onto the soil (or another growth medium) or to seeds. Although most activities influenced by biostimulants occur within the plant, some may transpire in the soil surrounding the roots. The advantages of biostimulants cannot be equated with the effects of fertilisers, as they are utilised in far smaller quantities, despite potentially containing identical mineral constituents. Biostimulants are also known as phytostimulators, metabolic enhancers, and plant conditioners (Yakhin et al. 2017). They are often used by farmers to promote and expedite development, the synthesis of phytohormones, the activity of rhizosphere microorganisms and soil enzymes, as well as other biological processes during the life cycle of various plants (Hamid et al. 2021).

1.6 Mode of biostimulant application

Currently, biostimulants are utilised in many methods. Biostimulants are mostly utilised as priming agents, foliar applications, or soil amendments to enhance natural processes. Seed priming has become an essential method in recent years for developing plants that are resilient to diverse stresses. Priming was initially employed to denote defence against infections, but it subsequently came to refer to responses to abiotic environmental influences. Seed priming is a widely accepted technique established by researchers to enhance stress tolerance in plants (Canizares et al. 2025).

Plants exhibit increased resilience to subsequent stress when initially subjected to a triggering factor or condition. The priming process enhances many physiological and biochemical defence mechanisms, allowing seeds and seedlings to endure multiple environmental stresses. Priming treatment induces rapid and uniform seed germination and facilitates effective crop establishment under stress

circumstances (Farooq et al. 2019). It is a straightforward, cost-effective, and low-risk method for addressing various abiotic stresses in agricultural regions. Priming-mediated metabolic pathways in seeds and seedlings mitigate the adverse effects of diverse abiotic stress conditions on agricultural plants.

Priming is a form of pre-germinative metabolism that guarantees optimal seedling vigour and development by repairing seeds through the activation of DNA repair pathways and antioxidant systems. It increases the seed vigour, germination potential, enhanced stress tolerance and also ensures the seed quality and improved activities of antioxidant machinery for scavenging ROS and ensures appropriate seedling development and tolerance towards stress conditions (Rhaman 2025). The germination rate was increased and strengthened in primed seeds, which can improve stress tolerance at later stages of growth and eventually boost agricultural yields. Priming makes the seedlings adaptable to wider range of germination temperatures and also improves the capacity to compete with weeds and pathogens (Hussain et al. 2016).

Biostimulants, including algal extracts, enhance the physiological processes of seeds when incorporated into the soil, hence increasing successful germination and improving plant health. Consequently, by enhancing chlorophyll production, biostimulants can augment nutritional availability, boost plant water retention capacity, elevate antioxidant levels, and optimise metabolism. The term "biostimulant" has been expanded over the last few years to include new compounds and mechanisms of action. Various biostimulants are being explored by seed researchers in an effort to break seed dormancy, enhance seed germination, promote field emergence, speed up seedling growth till maturity, and preserve plant adaptability under favourable and abiotic stress circumstances (Campobenedetto et al. 2021). These benefits are achieved through multiple mechanisms, such as enhancing nutrient availability to the plant or minimizing energy expenditure during periods of stress. These impacts often exert a beneficial impact on yield, irrespective of nutrient content.

Certain biostimulants also have mineral components related to those present in fertilisers; however, their usage is so little that any benefits cannot be ascribed to fertiliser effects. The physiological effects of biostimulants appear to provide plant hormone-like phenotypes, which is not surprising given that many products are derived from natural materials or microbials, which synthesise hormones (Li et al. 2024). Seaweed derived biostimulants have been shown to enhance plant nutrient uptake and metabolic activity, while also increasing plant resistance to a diverse range of environmental stresses (Gandhi et al. 2024). Biostimulants can increase yield and quality as well as mitigate environmental stresses (Johnson et al. 2024). Biostimulants derived from seaweed may improve plant resilience to environmental challenges by influencing molecular priming processes and the normal stress response.

Finding novel compounds with the potential to function as biostimulants has grown in importance for both the seed industry and academia. Biostimulants are one of these novel items that may be important as treatments for treating seeds (Masondo et al. 2018). Contemporary biostimulants can be complex formulations derived from many source materials, including byproducts from the paper and food industries. They are considered safe for the environment and possess a broad spectrum of biological activities (Bulgari et al. 2019). In the last 25 years, plant biostimulants have received considerable attention since these innovative products offer a potentially novel approach for the modulation of physiological processes in plants to stimulate growth, to enhance stress tolerance, and to increase yield (Du Jardin 2015). Therefore, biostimulants have become significantly utilized in contemporary agricultural cultivation (Yakhin et al. 2017).

The application of biostimulants to mitigate stress has emerged as a crucial strategy for maintaining final crop output and yield (Bulgari et al. 2019). Yeast-derived biostimulants (*Saccharomyces cerevisiae*) encompass all essential nutrients, amino acids, vitamins, particularly those of the B group, and growth factors. Yeast (*Saccharomyces cerevisiae*) extracts are acknowledged bioactive substances that enhance growth performance and yield characteristics of crop plants in nutrient-deficient conditions. According to Sadak et al (2025), yeast extract is a natural

source of many growth substances (thiamine, riboflavin, niacin, pyridoxine and vitamins B1, B2, B3 and B12), cytokinins and many of the nutrient elements as well as organic compounds i.e., protein, carbohydrates, nucleic acid and lipids. El-Shazoly et al. (2025) reported that the application of yeast extract would bring about positive effects in imparting tolerance towards abiotic stress-induced effects in plants.

Yeasts act as natural, safety biofertilizer and is also rich source of phytohormones (especially cytokinins), sugar, vitamins, enzymes, amino acids and minerals (Dmytruk et al. 2025). It was reported that yeast has got stimulatory effects on cell division and enlargement, synthesis of protein and nucleic acid as well as chlorophyll formation (Nassar et al. 2015; 2016). The increase in plant growth, influenced by yeast is attributed to its stimulating effect on enzyme activity, production of some phytohormones, improving the uptake of nutrients and conversion of insoluble form of phosphorous (Mukherjee et al. 2020). Yeast extract also releases CO₂ which is reflected in improving net photosynthesis (Sadak and Dawood 2020). Improving growth and productivity of different plant species by application of yeast extract was recorded by many researchers (Mekki and Ahmed 2005; Khalil and Ismael 2010; Sarhan et al. 2011).

Multifunctional priming approach includes organic and inorganic components that are relatively more effective as compared to single component priming approach (Gohari et al. 2024). Yeast extract components exert stimulating effects on cellular processes including cell division and expansion, protein and nucleic acid synthesis, and the formation of chlorophyll, as described by Abo et al. (2017). Non-microbial include ascorbate (AsA) and cysteine where AsA is attributed to its stimulating effect on enzymatic activity, production of specific phytohormones, improving the uptake of nutrients and conversion of insoluble form of phosphorous (Wu et al. 2024). Cysteine, an amino acid containing sulfur, plays a pivotal role as a metabolic hub by contributing its sulfur for the production of diverse molecules including amino acids, vitamins, Fe-S clusters, glutathione (GSH), and proteins containing thiol groups (Francioso et al. 2020).

1.7 Advances in understanding biostimulant mechanisms

Scientific advancements in plant biotechnology using techniques such as omics have provided insights into the mechanisms through which biostimulants exert their beneficial effects. The experimental investigation of chemical reactions involving metabolites is known as metabolomics. Specifically, metabolomics is the ‘systematic study of the unique chemical fingerprints that specific cellular processes leave behind’, and the study of their small-molecule metabolite profiles (Mattoli et al. 2023). The metabolites, or small chemicals involved in metabolic processes, are profiled using a high-throughput technology known as metabolomics.

Metabolites are identified and quantified using mass spectrometry (MS) after optional separation by gas chromatography (GC), high-resolution liquid chromatography (HRLC-MS), and liquid chromatography-mass spectrometry (LC-MS). It is the first hyphenated technique to be developed with libraries, enabling identification of a metabolite according to this fragmentation pattern. In addition to being sensitive, MS can be highly particular. Additionally, there are some methods that employ MS as a stand-alone technology in which the sample is pumped straight into the mass spectrometer without first being separated, and MS offers enough selectivity to separate and identify metabolites. The metabolomics study of rice is used to ‘fingerprint’ different rice samples in order to identify strains that hold significant rice quality improvement and increased nutritional content (Wang et al. 2024). Crude extracts are typically employed for non-targeted metabolite profiling, necessitating chromatographic separation for the analysis of fractionated chemicals.

Studies have shown that biostimulants can activate stress-responsive genes, enhance antioxidant enzyme activity, and improve root architecture, thereby increasing plant resilience under adverse conditions. Moreover, its use cultivates an environmentally friendly farming system by diminishing reliance on chemical inputs, alleviating soil degradation, and enhancing beneficial microbial populations. The worldwide biostimulant industry has experienced tremendous growth, propelled by the desire for sustainable agriculture methods and environmental legislation restricting synthetic pesticide usage. Research and development endeavours persist

in refining biostimulant formulations, elucidating their relationship with plant physiology, and investigating their synergies with traditional fertilisers. Standardising biostimulant products, guaranteeing consistent performance, and incorporating them into conventional agricultural systems continue to be difficult tasks despite their encouraging promise. This study seeks to investigate the function of biostimulants in alleviating abiotic stress, enhancing nutrient usage efficiency, and augmenting agricultural output. This research aims to elucidate the physiological, biochemical, and metabolomic processes of biostimulant activity to advance sustainable agriculture techniques that harmonise productivity with environmental preservation.

OBJECTIVES:

1. Identification and characterization of different biostimulants to find a potential biostimulant.
2. Assessment of growth retardation in terms of visually observable morphological and physiological changes induced by nutrient deficiency.
3. To determine the optimal concentration and duration of biostimulants for effective priming.
4. To determine the optimal concentration of biostimulant for soil application.
5. Evaluating the effect of biostimulant application on various physiological changes in rice by
 - a) Analysing the carbohydrate profile
 - b) Estimating the extent of lipid peroxidation
 - c) Metabolite accumulation
 - d) Studying antioxidant defence mechanisms (enzymatic and non- enzymatic)
6. Studying the effect of biostimulant application on the photochemistry of rice.
7. Analysis of various yield-related quantitative and qualitative parameters as influenced by biostimulant.
8. Detailed study of elemental analysis or ionomics.
9. To study the untargeted metabolomics in rice influenced by biostimulant application.
10. To analyse the changes occurred in amino acid profile and nutritional content of biostimulant treated 1st generation seeds.

2. REVIEW OF LITERATURE

2.1 Introduction

According to Marino et al. (2025), a significant proportion of individuals experience moderate food insecurity, including inadequate access to healthy and nutrient rich food. The production of food crops must rise by 70% in order to support the 9.7 billion people that are expected to inhabit the planet by 2050. This situation underscores the imperative to enhance agricultural productivity in both optimal and adverse situations. Millions of hectares of fertile land remain fallow owing to severe environmental pressures, greatly constraining global food supply.

Stressful situations in agriculture result from both biotic and abiotic sources, causing significant crop output reductions. The primary variables adversely affecting crop growth and development are abiotic and biotic stresses. Abiotic stress factors include extreme temperatures (heat, cold and freezing), too high or too low irradiation, water logging, drought, inadequate mineral nutrients in the soil and excessive soil salinity while pathogens, pests, and weeds are examples of biotic stresses that can severely impair plant health and productivity (Shabbir et al. 2022).

Plants largely depend on nutrient supply available in the soil, water and sunlight for their physiological and biochemical processes. Nevertheless, soils have been utilised for crop cultivation for ages without significant attention to nutrient replenishment. This has resulted in soil depletion and weariness, leading to diminished yield. In particular, alterations in soil conditions such as imbalanced nutrient content, poor organic matter and changes in pH impart a direct effect on the nutrient absorption and transport in plants.

The interplay of environmental stress and soil deterioration is a twin problem for agriculture. Plants are subjected to harsh exterior circumstances, while the same medium that supports their growth i.e., soil degrades in its ability to provide necessary nutrients. Such circumstances necessitate prolonged interventions to rehabilitate soil fertility, enhance nutrient efficiency, and bolster

plant resilience to stress. Emerging solutions, such as the use of biostimulants, improved breeding methodologies, and climate-smart agronomic practices, are being investigated to solve these difficulties and assure long-term food security.

2.2 Nutrient deficiency and its effect on rice

Nutrient deficiency, especially of essential nutrients such as nitrogen, phosphorus, and potassium, is among the primary limiting factors, alongside drought, salinity, soil alkalinity, and extreme temperatures, affecting the growth and performance of plants in both natural habitats and agricultural settings (Oustric et al. 2019). Within this context, both an excess and a deficiency of essential macronutrients in soils represent common sources of stress, with nitrogen (N), potassium (K), and phosphorus (P) being the most critical. These macronutrients are directly involved in numerous metabolic pathways and physiological processes, serving as structural components of essential metabolites, playing a crucial role in osmotic regulation and cellular permeability, and being vital for appropriate growth and development (Osmolovskaya et al. 2019). Rice is the primary food commodity cultivated worldwide and constitutes a staple dietary component for more than 2.7 billion people globally. However, rice growth and development is controlled by optimum supply of nutrients like nitrogen (N), phosphorus (P), and potassium (K), and the suboptimal levels of these nutrients is the key limiting factor of rice production throughout the world (Kubitza et al. 2024). Nitrogen is an important component of amino acids and proteins, and it regulates the enzyme activities that are required for energy metabolism including photosynthesis and respiration (Liu et al. 2025). Phosphorus is the constituent of key cellular molecules (such as ATP, nucleic acids, and phospholipids) and has a pivotal role in energy conservation and metabolism (Nicholls et al. 2023). Likewise, K supply is inevitable for optimum plant growth and development, and it plays important role in plant cellular homeostasis by contributing to osmotic adjustment, charge balance and enzyme catalysis (Anil et al. 2024).

It is currently believed that the presence of rapid recycling processes does not restrict macronutrient availability for plant growth in wild, uncultivated

ecosystems. However, due to a wide spread overexploitation of soils, it might be the case in the modern agricultural practice (Pravalie 2021). In particular, deficiency of nitrogen (N) and potassium (K) is quite common in developing and least developed countries, especially of rice and wheat production in Asia, Africa and Central and South America (Francis et al. 2023). A lack of these nutrients will eventually make plants less productive and cause agricultural yields to drop. Visual manifestations of stress caused by a deficiency of individual macro- and microelements are well documented (Munner et al. 2024). The underlying key physiological processes, affected by mineral deficiencies, are well characterized and include photosynthesis, protein synthesis, primary and secondary metabolism and carbohydrate distribution between source and sink tissues (De Bang et al. 2021). Mild effects of these stress factors have the potential to up-regulate various genes and accumulate proteins and metabolites which are directly or indirectly involved in alleviating the deleterious effects of stress by adjustment of the cellular mechanism and plant tolerance (Hasanuzzaman et al. 2017; Muhammad et al. 2021).

To mitigate the adverse effects of stressful situations, plants have developed resilient defence systems, including physical adaptations and coordinated molecular and cellular responses, to endure abiotic challenges. Enhancing the stress tolerance of plants is a primary objective for plant scientists and biologists globally. Various methodologies for enhancing plant stress resistance have been continuously revised.

Exogenous chemical applications, traditional breeding, genetic engineering, and biotechnological approaches, among others, have all been used in the past, but their commercial success has been constrained by the genetically complex stress tolerance mechanisms of crops (Saleem et al. 2025). Today's agricultural production faces greater difficulties in satisfying increasing demands for high productivity and environmentally friendly crop management techniques. The use of chemical fertilisers and pesticides is restricted in organic agriculture hence, there is a need for various plant supplements suited for such production (Kalogiannidis et al. 2022). Thus, the use of biological products such as biostimulants is essential to improve crop output in order to sustainably and holistically feed the global population. People are paying more and more attention to natural biostimulants, how they work,

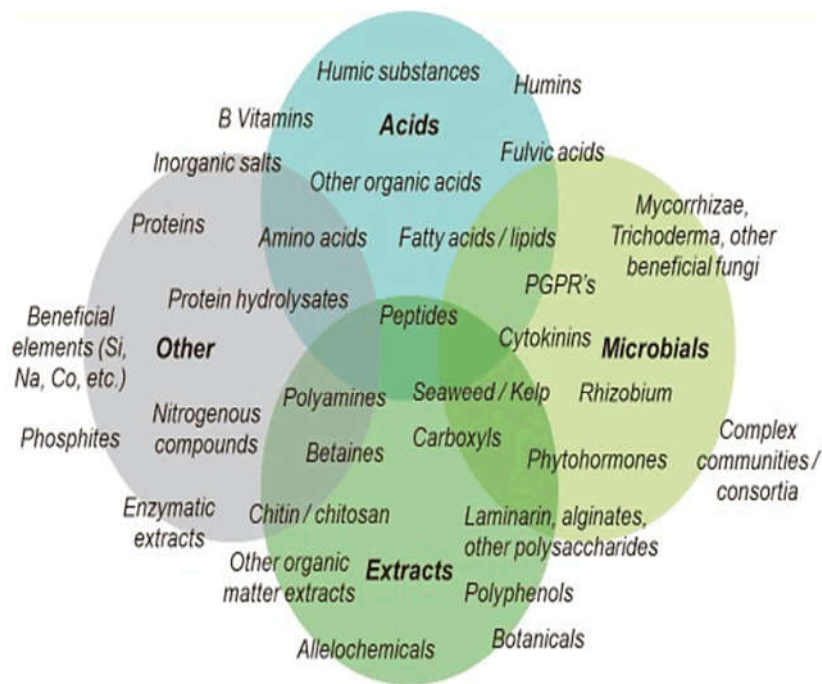
and how they can make soil healthier. They are also talking about how biostimulants might help crops deal with abiotic challenges.

2.3 Biostimulants: A key to plant stress resistance

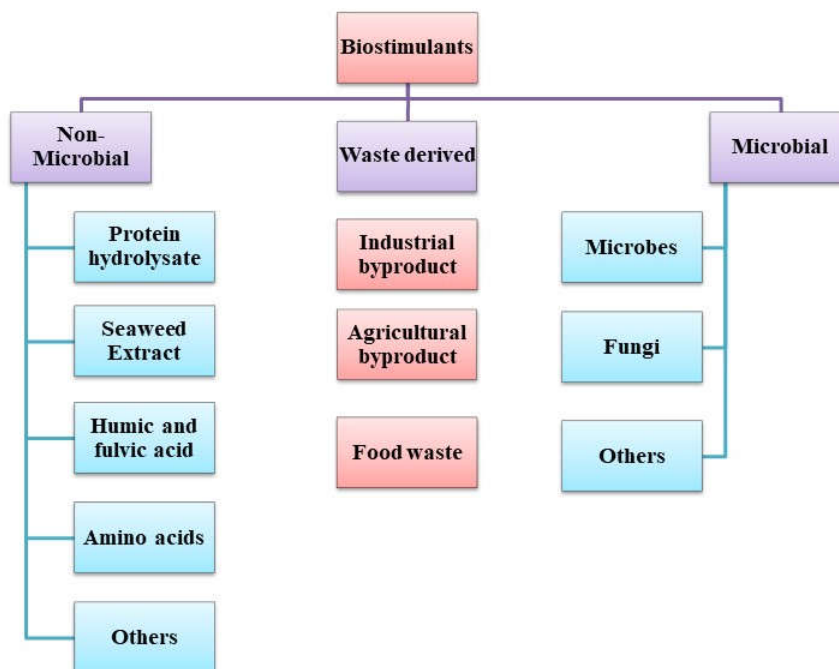
Biostimulants are organic, nutritional extracts that are easy to make and economical. They can be used instead of chemical fertilisers and other inorganic compounds. Yakhin et al. (2017) stated biostimulants as “a formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex constituents, and not as a sole consequence due to the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds”. Oxidative stress symptoms can be lessened by employing several biostimulants that comprise amino acids, humic acids, vitamins, and minerals. Biostimulants also lessen the requirement for fertilisers and are made up of a variety of different microorganisms and elements that support plant growth and development (Bhupenchandra et al. 2022). The functions of biostimulants in agriculture, their constituent parts, and their mechanism of action should all be highlighted in their description. Smaller quantities of biostimulants—which might be a single ingredient or a mixture of compounds—are required to improve crop quality features. The significance of this term is highlighted by the idea that individual molecules or their combinations can have a favourable impact on biological activities, even when their precise mechanisms of action are yet unknown. These substances work well in modest concentrations, improving nutritional effectiveness, abiotic stress resistance, boosting grain protein content, nutritional value, and shelf life (Bulgari et al. 2019).

2.4 Types of biostimulants

Based on the origin of the active ingredients, biostimulants are divided into three main categories: non-microbial biostimulants (chitosan, humic and fulvic acids, protein hydrolysates, phosphites, seaweed extracts, and silicon), microbial biostimulants (arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria, and *Trichoderma* spp) and byproduct derived biostimulants (Rouphael and Colla 2020a) (Fig. 4). Researchers have categorised biostimulants according on how they



A



B

Fig. 4: A) Diagrammatic representation of different types of biostimulants. B) Classification of biostimulants based on the mode of origin. The three categories include Non-microbial, microbial and waste-derived biostimulants. Waste derived biostimulants are recently incorporated by EU (2017)

impact plants or the physiological reactions of plants, rather than just their composition. It is hard to fully identify the components of biostimulants, and the stimulatory impact on plants is dependent on the synergistic activities of several substances rather than a individual component (Yakhin et al. 2017). In the preceding section, various biostimulant types are described according to their origin.

2. 24.1 Biostimulants derived from non-microbial agents

a) Protein hydrolysates

Biostimulants produced by protein hydrolysate are composed up of amino acids and soluble peptides. The term "mixtures of polypeptides, oligopeptides, and amino acids that are generated from protein sources through partial hydrolysis" refers to the class of plant biostimulants known as protein hydrolysates (PHs) (Martín et al. 2022). Protein hydrolysates are primarily made by enzymatically or chemically hydrolyzing entire protein sources with the suitable enzymes under controlled circumstances, then processing the resulting complex mixture of active and inactive peptides to separate out the powerful bioactive peptides (Nasri 2017). The structure, pH, and charge of amino acids have a big impact on the bioactivity of peptides. Their structure and arrangement also affect how they work in other ways. Tryptophan and other peptides that are constituents of PHs may have an impact on the balance of phytohormones and also on the development of plants (Colla et al. 2017). According to Halpern et al. (2015), the efficiency of water and nutrient (macro and micro elements) usage is increased when PHs are applied to plant leaves and roots. PH-treated plants are better at absorbing nutrients because there are more microbes and enzymes in the soil, micronutrients move and dissolve more easily, plant roots change shape, enzymes like nitrate reductase, glutamine synthetase, and Fe (III)-chelate reductase become more active, and plant lateral roots get longer, denser, and more numerous. Auxin- and gibberellin-like effects were also activated by the application of plant-derived PHs, which enhanced crop performance (Goyal et al. 2025). It has also been shown that using PH can cut down on or even remove output losses caused by poor soil conditions and environmental stresses.

b) Seaweed extracts

To improve crop quality traits, abiotic stress resistance, or nutrient utilisation, plants are treated with sea-based materials or microorganisms called seaweeds or microalgae. Based on colour, seaweeds are divided into three groups: Phaeophyta, Rhodophyta, and Chlorophyta. The three main classifications for brown seaweeds are *Ascophyllum*, *Fucus*, and *Laminaria*. Because the extracts can differ greatly based on the type of seaweed, the raw material's spatiotemporal source, and the processes utilised to produce plant biostimulants, seaweed biostimulants are a very diverse set of chemicals (Ali et al. 2021). Seaweed products are the second most common form of biostimulant on the market due to their ease of production and ability to boost plant biomass. The biochemical composition of seaweed products, which consists of minerals, vitamins, lipids, acids, antioxidants, colours, and hormones, is a significant component. The numerous interactions between the several bioactive compounds contained in a single extract make it difficult to understand their mode of action and frequently necessitate a multidisciplinary approach (Braun and Colla 2023). Seaweed extract act as a source of nutrients and hormonal effects and thus impart beneficial impacts on soil remediation, retention and microbiota (Du Jardin 2015).

c) Fulvic and humic acids

Non-renewable resources like coal and peat are used to manufacture most HS, which fuels the expansion of sustainable intensification. HS were once categorised as fulvic acids, humins, and humic acids based on their molecular weights and solubilities. A significant characteristic about these compounds is that they may combine and break apart in complicated ways to make supramolecular colloids. This is controlled by the secretion and hydrogen discharge from plant roots. So, plant roots, bacteria, and organic matter all work together to make humic chemicals and their complexes in the soil (Canellas et al. 2022). Biostimulants containing ingredients like fulvic and humic acids stimulate the roots, increasing nutrition absorption and tolerance to abiotic stresses (Turan et al. 2022). When

humic acids are used to aid in plants growth and crop yield, they need to connect with soil microbes as much as possible in order to get the results that are wanted.

2.4.2 Microbial extracts

A certain microbe or a group of bacteria from different genera —*Azotobacter* spp., *Rhizobium* spp., *Bacillus* spp., *Azospirillum* spp., Mycorrhizal fungi, *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., and cyanobacteria—make up a microbial plant biostimulant. In order to produce extracts from microorganisms, bacteria, fungus, and arbuscular mycorrhizal fungi are used as a source, that are generally present in organic resources such as soil, plants, composted manures, and other materials (Natesan et al. 2023). AMF and *Trichoderma* spp. provide as sources of functional microbial-based biostimulants that have been shown to enhance crop resilience and growth. The phylum Glomeromycota encompasses arbuscular mycorrhizal fungi (AMFs), which are directly involved in root colonisation and are crucial for enhancing stress tolerance and nutrient uptake. Likewise, *Trichoderma* spp. are well known for their role in improving soil health, nutrient solubilization, and biocontrol, all of which promote plant performance in the face of biotic and abiotic stresses. Furthermore, a very varied group of plant growth-promoting rhizobacteria (PGPR), including phyla like Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, have demonstrated the value of microbial extracts in boosting soil enzyme activity, adjusting plant hormone levels, and enhancing resistance to environmental stressors. Numerous fungi and microorganisms have been found to contribute to crop stress tolerance in addition to these well-studied groups, and many more species are still to be discovered. It has been shown that microbial-based biostimulants work together to improve photosynthetic efficiency, regulate hormonal balance, improve the transfer and assimilation of macro- and micronutrients, restructure root architecture, and modify microbial communities in the rhizosphere, all of which contribute to increased crop productivity and sustainability (Castiglione et al. 2021).

a) Fungal biostimulants

The mutualism-parasitism continuum hypothesis is helpful in explaining the diverse range of connections that have emerged over evolutionary time, as terrestrial plants and fungus have coevolved since the beginning of time. Some types of symbiotic relationships between fungus and plant roots are parasitism and mutualistic symbioses, where both species live together and help each other out. Fungus-based biostimulants can be used to treat plants to boost their crop growth, ability to take in nutrients, ability to handle stress, and quality of the product. There are signs that fungi cooperate to promote plant-to-plant communication, which might have important environmental and agricultural repercussions (Johnson and Graham 2013). With agricultural and horticultural plants, fungi of the Glomeromycota genus develop branching structures known as arbuscules by piercing root cortical cells and thus develop mycorrhizal association (Behie and Bidochka 2014). Arbuscular mycorrhiza provide crucial tripartite interactions between rhizobacteria and plants that benefit plant nutrition, water balance, and defence against biotic and abiotic stresses (Diagne et al. 2020). According to more recent evidence, hyphal networks exist that connect specific plants within a plant community as well as fungi and plants. Different from mycorrhizal species, some fungal endophytes can colonise roots, live at least a portion of their life cycle outside of the plant, and transport nutrients to their hosts by poorly known methods (Behie and Bidochka 2014).

b) Bacterial biostimulants

Bacteria often associate with plants through the soil, either briefly or for a long time, and can get into plant cells in the root system. By taking part in biogeochemical cycles, delivering nutrients, and enhancing nutrient absorption, certain bacteria can even propagate vertically through seeds and have an impact on plant life (Hamid et al. 2021). According to their functions and environments, rhizobium-type mutualistic endosymbionts and mutualistic rhizospheric PGPRs, which are also sometimes called "plant growth-promoting rhizobacteria," are two different types of biostimulants that bacteria can produce. Rhizobium and similar species are

commercially available biofertilizers, which are also called microbial inoculants. They help plants absorb nutrients. There are many scientific papers and books that go into great detail about the biology and farming uses of symbioses based on rhizobium. All elements of plant life, including nutrition and growth, morphogenesis and development, responses to biotic and abiotic stresses, and interactions with other species in agroecosystems, are impacted by PGPRs, which are used for a number of purposes (Basu et al. 2021). Some of these functions require the collaboration of various bacterial strains, although many are frequently carried out by a single species. The primary roles of bacteria are to decompose organic matter, which contributes to nutrient supply, increase nutrient use efficiency, improve plant resistance to pests and diseases, tolerate abiotic stress, and encourage the production of plant growth regulators (Du Jardin 2015; Sharma et al. 2018).

2.4.3 Waste derived biostimulants

The production of biostimulants from byproducts opens up a path for the recycling and reduction of waste, which benefits growers, the food sector, corporate registration, and distribution. Biostimulants can be produced by utilising a considerable amount of the waste that is generated by industries that have the highest requirements, such as the food industry and agriculture. Food waste, composts, manures, vermicompost, waste streams from aquaculture, and sewage treatment are all sources of the biostimulants produced from waste streams (Xu and Geelen 2018). The circular economy theory highlights the idea of sustainable utilisation and places a lot of attention on the valorisation process, which entails converting waste products and materials into new sources. Increased use of bio-based wastes as novel fertilisers is the goal of European Commission (2017). Things that are intended for disposal are referred to as "waste," although sometimes it is more accurate to refer to them as "by-products." One of the crucial components created during the manufacturing process is a by-product, which is not produced on purpose but can be used for particular applications and purposes without further processing. It follows that a waste is no longer a waste when it is used as a raw material for industrial or agricultural purposes (European Commission 2017). Vermicompost, sewage sludge, and composted urban trash are only a few of the

types of waste-derived biostimulants and raw organic material with biostimulant components that have been demonstrated to be successful in horticulture and agriculture. According to one of the most recent research, sugarcane vinasse, a byproduct of the sugar ethanol industry, has been studied as a source of nourishment for microorganisms that help with soil bioremediation by detoxifying and removing xenobiotics from the environment. Thus, making biostimulants from waste materials provides innovative solutions to prevent unintentional disposal and generates environmentally friendly ways to recycle waste.

2.5 Mode and mechanism of biostimulant application

2.5.1 Different modes of biostimulant application

Biostimulants are natural or synthetic substances that are administered to seeds, plants, and soil through foliar fertilisation, fertigation, or direct soil application to promote crop growth and development as well as crop quality (Van Oosten et al. 2017; Wozniak et al. 2020). Soaking the seeds, spraying the leaves with a mist, or adding powders, granules, or solutions straight into the soil are all common methods for applying biostimulants to help plants grow properly under normal circumstances (Fig. 5). Products applied to the foliage make up 78% of the global biostimulant industry, according to the European Biostimulants Industry Council (EBIC). This is significantly more than treatments given to the soil (almost 9%) and seeds (12%). Nevertheless, certain research indicates that foliar application of biostimulants presents issues due to the uncertainty regarding their impact on crops. The two primary challenges are the structural makeup of the leaves and the different components of biostimulants. The leaves alter their shape to exclude access from external elements in order to avert possible damage. Professor Goldbach in his talk at the World Biostimulant Congress (2022) called "Foliar Application of Biostimulants: How to make them reach the Apoplast" that biostimulants are capable of penetrating the outermost layer of the foliage. Plant and seaweed extracts are employed as foliar sprays, whereas humic chemicals and nitrogen-based biostimulants are frequently sprayed directly into the ground. Moreover, applying biostimulants on leaves purportedly enhanced the moisture status of pea plants cultivated under drought-like conditions.

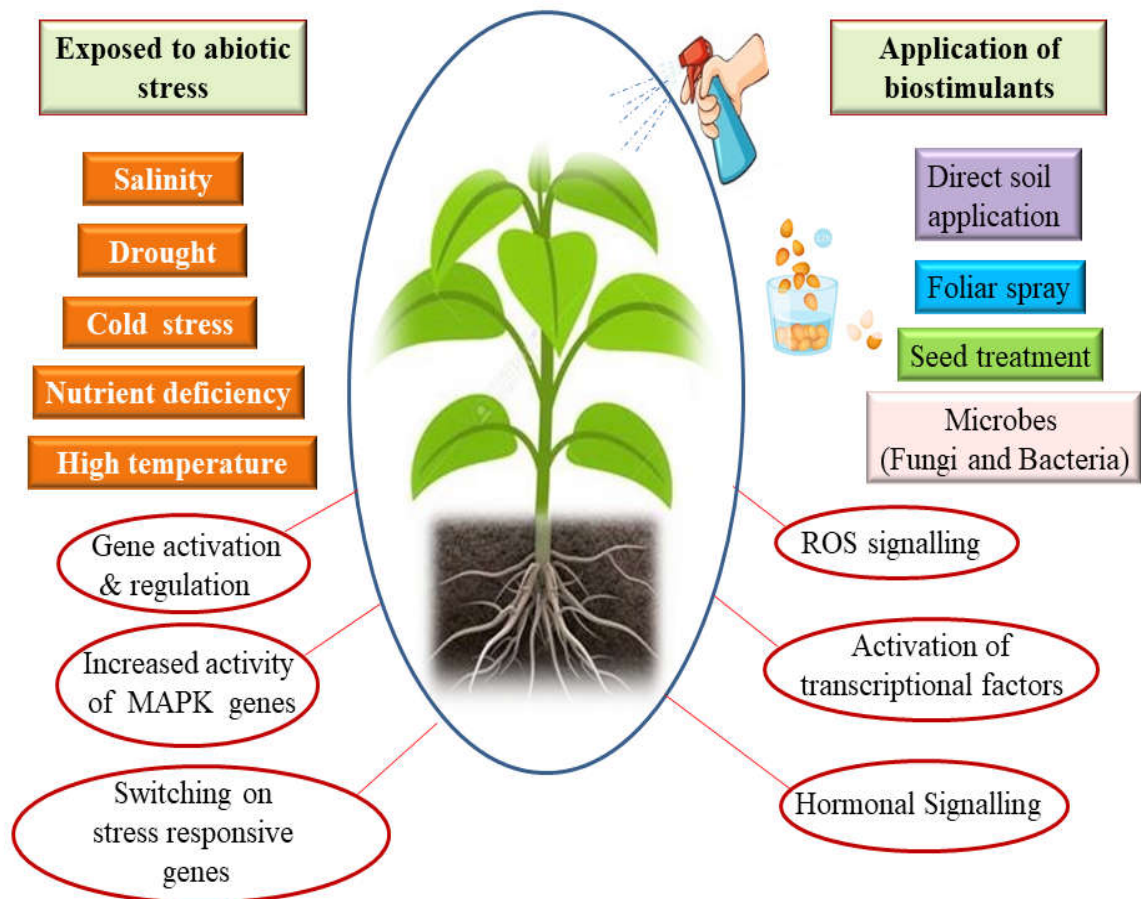


Fig. 5: Modes and mechanism of biostimulant application under abiotic stress condition

Since plants may immediately absorb biostimulants together with water, they can be introduced to the irrigation system. Most recent case studied is biostimulant Kelpak SL (*Ecklonia maxima* extract), that was sprayed as an aqueous solution over *Phaseolus vulgaris* for the duration of the vegetative phase. When this biostimulant was used during a frost, it caused an increase in the seed weight and quantity as well as the pod number (Kocira et al. 2018). The foliar application of the biostimulant outperformed the soil treatment. Following foliar application of a biostimulant based on sewage sludge, the quantity of macronutrients and micronutrients in maize leaves increased. With doses of 3.6 L/ha and 7.2 L/ha, respectively, the nitrogen content of maize leaves improved by 26% and 46% (Tejada et al. 2016).

Biostimulants are often used in priming procedures as ready-to-use extracts or powders that can dissolve in water. It has been reported that seed priming with biostimulants, such as humic substances, seaweed extracts, protein hydrolysates, and microbial inoculants, improves water uptake, stimulates antioxidant defense systems, and increases enzymatic activities involved in early germination (Paparella et al. 2015; Rouphael and Colla 2020b). Further, it has been demonstrated that microbial-based priming, specifically with *Trichoderma* spp., yeast extract and plant growth-promoting rhizobacteria (PGPR), induces systemic tolerance mechanisms, improving vigor and resilience under abiotic stresses like drought, nutrient deficiency and salinity (Yakhin et al. 2017; Rajput et al. 2019; Johnson and Puthur 2022).

Soil biostimulants often change the root's structure, increasing the root's ability to absorb nutrients. When utilising biostimulants, it is important to take into account the plants' circadian cycle. The best time to apply biostimulants is in the morning when the stomata are open and the absorption rate is highest (Goni et al. 2018). Moreover, biostimulants are sprayed directly onto selected fruits. After being exposed to the biostimulant, the fruits' resistance to mechanical damage and putrefaction enhanced, increasing their storage life and making them more suitable for consumption.

2.5.2 Mechanism of action of biostimulants

Although there are several reports on biostimulants, little or nothing has been discussed thus far regarding their modes and mechanisms of action (Fig. 6). A live

organism's physiological or anatomical changes are defined by its mode of action, whereas molecular changes are indicated by its mechanism. Biostimulants act directly by providing nutrients and growth promoting substances like phytohormones and organic acids (Hernandez et al. 2021). In most biostimulants, hormones such as IAA, cytokinins and amino acids play a special role to bring about major physiological changes (Bhupenchandra et al. 2022). While indirect mechanism indulges in improving soil health, promoting plant defense against stress and improving plant metabolism together with nutrient uptake (Joshi et al. 2021). These mechanisms also include the secretion of antifungal compounds, competition with pathogens, production of beneficial metabolites and activation of the plants own defense system (Zhang et al. 2020).

The most characterized products are extracts from protein hydrolysates and acids like humic acids. The effects of biostimulants have been suggested to be significantly influenced by the presence of hormones in the extracts, especially auxins. Although it is well known that the processing and storage methods of the extract can significantly alter its hormone content and profile, but limited research has examined the availability and effectiveness of these chemicals in formulated products, which appears to be crucial in figuring out the mode or mechanism of action. By analysing the investigations in *A. thaliana*, research has shed light on potential mechanisms of action for the usage of algal extract, which increases the capacity of plants to scavenge reactive oxygen species and enhance salt tolerance (Drira et al. 2021), soybean (Lewandowska et al. 2022), wheat (Merwad 2020), tomato (De stasio et al. 2018), and cucumber (Hassan et al. 2021). Overall it is described that plant biostimulants have an impact on the endogenous hormone balance, change the expression of genes involved in the transfer of nutrients across cell membranes, promote photosynthesis, and reduce stress-related reactions (De saeger et al. 2020). Ultimately, to better understand how algal samples work, functional mutants can be used, especially those that change how hormones are made.

Ertani and colleagues (2018) investigated how the effects of algae extract from *A. nodosum* and *Laminaria* spp. on maize (*Zea mays*) seedlings cultivated in a nutrient solution. Their objective was to establish a connection between the extracts' composition and the effects that were seen. For 48 hours, the seedlings were treated

with 0.5 mL L⁻¹ of a single algal extract by the researchers. Alginic and uronic acids were the most prevalent constituents in the algal extract, along with polyaromatic ring compounds, according to Fourier-transformed infrared (FT-IR) and Fourier-transformed Raman (FT-Raman) examinations of the extracts. Although both algal extracts differed among the functional groups, but hormones and phenolics were found in common. Based on these results, the authors concluded that the root morphological traits were formed by the extract that had the highest quantity of the hormone IAA. The authors hypothesised that the many ways that algae extracts can cause reactions in maize were probably related to their chemical composition, notwithstanding the heterogeneity among the extracts. The same justifications and conclusions are included in reports on the mechanism of action of protein hydrolysate as those for algal extract (Casadesus et al. 2019). The characteristics of the protein supply and processing techniques yield a diverse product composition, encompassing protein hydrolysate, which consists of an intricate combination of amino acids, oligopeptides, polypeptides, and other compounds. Recent research on protein hydrolysate showed that these products typically influence carbon and nitrogen metabolism, nutrient absorption, and hormonal equilibrium. Finally, it has been proposed that seed-coating may be interfering with the rhizosphere microbiota, which would be advantageous for plant development (Colla et al. 2017).

2.6 Biostimulant induced physiochemical and biochemical changes under abiotic stress condition

2.6.1 Biostimulants induced changes in metabolites

Biostimulants have generally been found to be more effective in promoting the accumulation of several metabolites, including proteins, amino acids, carbohydrates, and sugars. The reason behind this beneficial impact of biostimulants could be the biologically active substances generated by these biofertilizers, such as auxins, gibberellins, cytokinins, amino acids, and vitamins (De Vasconcelos et al. 2019). According to Santini et al. (2022), the best outcomes vary for different biostimulants. Reports indicate that when biostimulants are used at a standardized concentration of 1 g/L, can lead to reduction in cell turgor and this decline occurs as a result of relative water content, cell volume, and stomatal conductance suggesting

that the concentration might be inducing osmotic stress (Van Oosten et al. 2017). Thus, a dose-dependent assessment (0.1–2 g/L) is necessary to determine the optimal concentration in order to find out the range that improves physiological performance—such as increased chlorophyll content, biomass accumulation, and water retention—without compromising plant stability (Du Jardin 2015; Rouphael and Colla 2020a).

Biostimulants enhance the highest levels of overall sugar content and have a positive impact on the build-up of carbohydrates in the leaves of field beans (Tecklic et al. 2021). The positive effect of biostimulant on ROS, metabolites and chlorophyll *a* and *b* is in consistence with the result obtained in Brassicaceae family by Borhannuddin et al. (2020) and barley by Jarosova et al. (2016). Furthermore, the application of biostimulants may benefit a number of plant functions, including cell division and elongation, which would enhance the area of the leaves and, eventually, the amount of photosynthesis and photosynthates. Baqir et al. (2019) emphasised the significance and role of amino acids in abiotic stress tolerance, noting that this category of molecules includes certain amino acids, including proline, and quaternary ammonium compounds. These chemicals are believed to be essential for plants to react to osmotic stress by modifying their cytoplasmic osmotic balance.

The application of biostimulants, which stimulate the production of growth hormones, may contribute to the increased accumulation of proteins and free amino acids. As a natural supply of cytokinins, biostimulants promote cell growth and division as well as the production of proteins and nucleic acids (Mady 2009). By enhancing water status, stabilising membranes and protein complexes, and preventing denaturation of enzymes, biostimulants help to raise the level of sugars and free amino acids, which aids in the development of osmotolerance in salt-exposed maize plants (Singh et al. 2015). The increased quantity of carbohydrates and nitrogen containing compounds can also function as an energy source and a precursor of protein synthesis, to overcome impaired cellular metabolism and sustain plant development under salt stress. According to Abdel et al. (2019), priming with biostimulants in salt-stressed plants reduced proline accumulation,

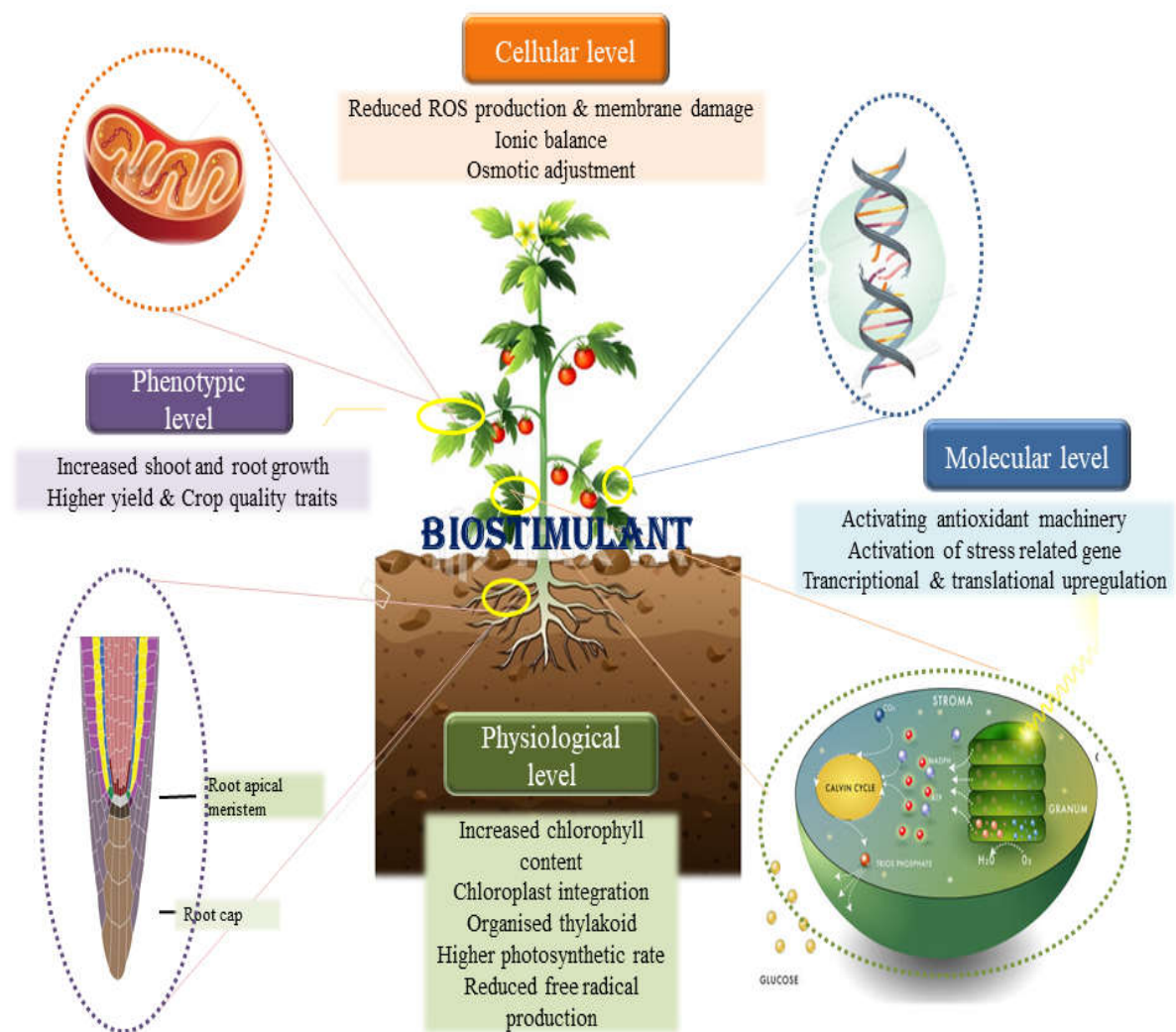


Fig. 6: Key changes induced by the application of biostimulant under abiotic stress condition

indicating that these biostimulants functioned independently of proline to lessen salt toxicity.

2.6.2 Biostimulants induced changes in ROS and antioxidant activity

Chemically reactive molecules that contain oxygen, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), are referred to as "reactive oxygen species" (ROS). Since ROS are a normal byproduct of many plant biological activities, an excessive accumulation of them can cause oxidative stress, which can damage cell components like DNA, proteins, and lipids. Plants produce free radicals that can damage their own cells when they are subjected to stressful situations like salt, drought, high temperatures, nutrient shortages, or heavy metals. However, the harmful effects of free radicals can be lessened by using biostimulants in conjunction with antioxidants. Through a variety of mechanisms, including ROS scavenging, enzyme activation, enhanced photosynthesis, hormone regulation, and signalling pathway modulation, biostimulants can interact with ROS and affect oxidative stress responses in plants. In a similar way, seed-priming with biostimulants led to improvements in growth, chlorophyll, carotenoid levels followed by decreased ROS due to higher antioxidant activity in rice under nutrient deficiency (Johnson and Puthur 2022).

Exogenous GSH and *Moringa oleifera* leaf extracts increased endogenous GSH and AsA levels, provided osmotic resistance, preserved membrane properties, and decreased electrolyte leakage (EL) and ROS level in wheat plants under salinity stress (Rehman et al. 2021). According to Hassanuzzaman et al. (2021), higher antioxidant levels are produced by plants under environmental stress, which is followed by improved root and shoot growth, a high-water content in the leaves. The amount of malondialdehyde (MDA) decreased in maize leaves, indicating that biostimulants helped to reduce ROS toxicity by promoting the activities of SOD, CAT, and APX in salt-stressed plants (Abdel et al. 2019). Plants exposed to salt and primed with biostimulants showed greater resistance to oxidative stress and a more robust antioxidant system. In tomato plants, AMF ameliorated the impacts of high temperatures (HT), noting that MDA and H_2O_2 contents increased under HT before

being lowered by roughly 40% as a result of increased antioxidant enzyme activity in both roots and leaves (Duc et al. 2018).

2.6.3 Biostimulants induced changes in hormones and nutrient accumulation

Hormone precursors, such as tryptophan, are among the substances that fall under the category of biostimulants. These precursors can generate hormones that either inhibit or promote growth. These precursors of biostimulants are responsible for controlling cell division, expansion, and metabolism in order to expedite the development of vegetation in plants. According to Rupawalla et al. (2022), the greatest levels of cytokinins, IAA, and GA were found in the microalgae-based biostimulant, which also confirmed that the uncharacterized microalgal strains generated pertinent PGRs. Due to their high auxin and cytokinin contents and also increased carbohydrate accumulation, biostimulants have been proposed to have a positive function during vegetative and reproductive growths by increasing flower formation and fruit set in specific plants (Shukla and prithiviraj 2021). Additionally, this chemical compound production improved cell division and nutrient status, which led to an increase in the number of leaves and the dry weight of leaves, stems, and roots.

Microbial biostimulants are helpful in the absorption of nutrients, the production of specific phytohormones, and the transformation of insoluble phosphorus into soluble phosphorus, which results in a greater amount of phosphorus being accessible to plants. Nutrient assimilation and acquisition of phosphorus, and calcium and magnesium contents were boosted by legume derived protein hydrolysate based biostimulants through foliar treatment in spinach (Carillo et al. 2019). When wheat plants that were treated with Delfan-a biostimulant, it exhibited improved plant growth. This could be due to the fact that Delfan-a has the capability to increase the division and expansion of cells as well as the creation of new tissues and organs. Flemming et al. (2019), reported on the positive impact of amino acids on crop growth characteristics. Additionally, spraying *Arabidopsis thaliana* with a biostimulant based on amino acids under water stress resulted in a significant improvement in all growth metrics with respect to control. The beneficial

effects of biostimulants enhanced vegetative growth and alleviated the adverse effects of moisture stress. The results align with those seen in maize under drought stress (Nephali et al. 2021) and in tomato under salt stress (Di Stasio et al. 2016).

2.6.4 Biostimulants a means to influence yield parameters

According to Caradonia et al. (2019), biostimulants are agents that improve crop quality attributes like yield and nutrient absorption and utilisation efficiency. Biostimulants influenced the degree of elemental uptake by roots, as well as the redistribution and concentration of these elements in leaves. Biostimulants may enhance yield and its components due to their composition of cytokinins, vitamin B12, and minerals that facilitate the guidance and translocation of substances from leaves to reproductive organs. Moreover, it could contribute to the production of protein and nucleic acids and reduce their breakdown, which might increase plant output (Koleska et al. 2017). Biostimulants showed a beneficial influence on minimising the deleterious consequences of abiotic stressors. Mannino et al. (2020), discovered that application of biostimulants on tomato had a positive impact on nutritional values, plant growth, yield, and was able to lessen the ripening time.

Seaweed extracts, humic compounds, hydrolyzed proteins, and microbes are some of the major categories of biostimulants. By having the ability to boost nutrient efficiency, which measures how well a nutrient is used by the plant once it is made accessible to it, biostimulants can improve production and quality as well as reduce environmental pressures. According to Popko et al. (2018), the benefits of biostimulants include an increase in yield components, which was mostly attributable to the impact of complex constituents, which can be extremely important in providing the plants with essential nutrients. Furthermore, the inclusion of vitamins, growth regulators, and macro and micronutrients in biostimulants may enable the plants to store dry matter. Numerous studies have convincingly shown that the utilisation of natural organic biostimulants, such as humic acid, enhances yield in wheat under both pot and field conditions (Izhar et al. 2020), as well as in pea (Nalia and Sengupta 2019), okra and cassava (Canellas et al. 2022), improving yield characteristics including fruit/plant biomass, straw weight, grain quality,

protein content, fruit size, and nutritional content. Protein hydrolysates affect nitrogen uptake and assimilation, regulate the activities of Krebs cycle enzymes, and also impact the interplay between carbon and nitrogen metabolism (Du Jardin 2015).

Halpern et al. (2015) assert that biostimulants enhance plant performance by optimising iron and nitrogen metabolism, therefore improving nutrition and water utilisation efficiency. They also enhance soil microbiological and enzymatic activities, hence promoting soil health. Moreover, the administration of biostimulants enhances root system development, resulting in greater number, density, and length of lateral roots, along with higher nitrate reductase activity. Moreover, protein hydrolysates (PHs) enhance the physicochemical quality of fruits by elevating the concentrations of carotenoids, flavonoids, polyphenols, aromatic compounds, and pungency (Sharma et al. 2018). In both pot and field trials, biostimulants comprising humic and/or fulvic acids enhanced the yield of pepper (*Capsicum annuum*) and wheat (Akladios and Mohammed 2018; Nasiroleslami et al. 2021). Research confirmed that the application of diverse biostimulants improves crop quality characteristics and yield in various agricultural plants, contingent upon their developmental stage and genetic potential.

2.7 Molecular signalling mechanisms induced by biostimulants against abiotic stress

Recognising stress signals and relaying them to initiate the proper chemical reactions that support survival is the first and most important step in protecting against abiotic stress. Receptors with receptor-like kinase/proteins (RLKs or RLPs) are typically responsible for plants' perception of abiotic stressors. According to recent studies, guard cell hydrogen peroxide-resistant1 (GHR1), calcium/calmodium-regulated cysteine-rich rlk (CRK36), proline-rich-extensin-like rlk4 (PERK4), and receptor-like protein kinase1 (RPK1) are involved in sensing abiotic stress and have been shown to control water stress signalling in plants. The process by which a signal is transported into different downstream signalling networks after being detected as a stress by receptor proteins on cell surfaces is known as signal transduction. The first step in the information transmission pathway

is perception, followed by the generation of secondary messengers. This triggers a series of phosphorylation which affects proteins that regulate genes involved in stress defence. Reactive oxygen species (ROS), cytosolic calcium (Ca^{2+}) increase, and the activation of the mitogen-activated protein kinase (MAPK) cascade are examples of early reaction signals (Wang et al. 2018).

The ability of biostimulants to both eliminate ROS and activate signalling pathways has been highlighted by their usage in priming plants. This priming technique not only prevented the build-up of ROS but also enabled the regulation of stress response through the control of Mg and Ca^{2+} ion channels (Johnson et al. 2024). Ca^{2+} ions are known to be a crucial secondary messenger in signal transmission during times of abiotic stressors, and their activation can be accelerated by using biostimulants for priming. Stress signals cause calcium ion channels to open, allowing Ca^{2+} ions to enter the cytosol. This increases the quantity of calcium in the cytosol and opens more Ca^{2+} ion channels, which in turn controls the proteins that alter the genome. As a result, the plant can withstand a variety of environmental stressors more effectively. By increasing sensitive protein and Ca^{2+} ion signalling, the usage of biostimulants led to the activation of the oxidative stress response system. The activation of stress hormones such as ABA is initiated by concentrations of Ca^{2+} that are even higher than those previously discussed within the cytoplasm. Through the process of biostimulant priming, these hormones exert indirect control over the tolerance mechanism that is responsible for combating abiotic stress.

2.8 Biostimulants and priming induced trans-generational plasticity

The term "trans-generational plasticity" refers to the capacity of living things to transmit particular characteristics or circumstances to their descendants by means of mechanisms that are not genetic in nature. The transfer of information from one generation to the subsequent generation is involved, and this information has the potential to exert an effect on the adaptability and phenotypic of the offspring. When biostimulants are administered to plants, they increase the plants' growth, development, and stress tolerance. This is due to their ability to regulate the

molecular pathways that occur within plants, which results in an enhanced expression of genes and proteins that are associated with stress. The plant's has built the ability to manage stress and to establish cross-tolerance, which is the capacity to build tolerance to other forms of stress after being exposed to one type of stress, can both be increased as a result of the activation of these stress response pathways. Stress memory and epigenetic alterations are two significant factors in the development of trans-generational plasticity and stress tolerance. There are adjustments known as "epigenetic" that occur in patterns of gene expression but do not involve any changes in the sequence of the DNA that is underlying them. These variations can include changes to important signalling molecules, transcription factors, and adjustments to the state of chromatin (the packing of DNA), all of which have the potential to impact the expression of genes. The modifications that have been made to the epigenome and the memory of stress that have been accumulated by the parent plant can be passed down to the progeny. This process has the ability to give adaptive advantages and resistance to stress. These modifications to gene expression and chromatin states can prepare the plants that are the offspring to react more effectively to stressful environments that they might encounter while growing and developing. According to the findings of recent research, humic acids contribute to the enhancement of the transcriptional activation of heat shock proteins in *Arabidopsis* during heat stress (Cha et al. 2021). In general, biostimulants have the ability to serve as mediators of stress tolerance by beginning and strengthening the processes that plants utilise to respond to stress. They have the ability to cause modifications in chromatin states and the expression of genes, both of which contribute to the flexibility of trans-generational memory and memory related to stress. Plants are able to endure adverse environmental circumstances and have a better total crop yield as a result of their improved capacity to deal with a wide range of stresses.

The physiochemical and molecular mechanisms controlling plant adaptation and cross tolerance that further increase crop yield have been clarified by recent studies on the abiotic stresses faced by crop plants. Compared to the offspring's of non-exposed plants, barley plants exposed to drought stress during their

reproductive stages were shown to have longer roots. This suggests that the parents' descendants inherited the primed state. During the grain filling stage, primed plant progeny exhibit cross-tolerance to thermal stress. These plants yield greater grain, exhibit reduced damage to their cell membranes, and possess enhanced photosynthetic capacity. In the offspring of primed plants, proteomics indicated an overexpression of proteins associated with signal perception, structural maintenance, and the accumulation of heat shock proteins (HSPs). This suggests that HSPs may serve as indicators for enhanced thermotolerance in progeny (Zhang et al. 2016). A potentially effective technique for mitigating excessive heat stress in wheat production is transgenerational thermotolerance. In the following generation, it may result in higher grain yields, improved retention of photosynthesis in the leaves, improved activity of antioxidant enzymes, and less damage to cell membranes during critical growth stages. Transcriptome study findings indicate that primed plants undergo heritable epigenetic alterations and signaling transduction pathways in response to elevated temperatures. These alterations result in a thermo-tolerance effect and induce adjustments in defense-related responses, including anti-oxidation, transcription, energy synthesis, and protein transport and storage in the progeny of these plants (Wang et al. 2018).

2.9 Biostimulant and Omics approach

In the quest of identifying, quantifying, and functionally characterising the cellular pool, separation techniques, data analyzing platforms, use of chemical libraries, and bioinformatics tools for visualisation strategies have emerged as essential tools. It has been found that omics techniques, such as transcriptomics, proteomics, metabolomics, and genomics, are very helpful in examining and determining the intricate molecular biochemical processes and events involved in the physiological effects of biostimulant formulations on plants. Biostimulants have been extensively investigated through modern omics methodologies to clarify their molecular mechanisms of action. Ertani et al. (2017) conducted a transcriptomics study utilising microarray and next-generation sequencing (NGS) technology to examine the effects of biostimulant application on the transcriptome of *Solanum lycopersicum*. The results emphasised particular biochemical mechanisms that

facilitate development, such as the enhancement of cytokinin metabolism, sugar transport, phloem loading, and the assimilation of nitrogen and phosphate. Briglia et al. (2019) indicated that in maize, biostimulant treatment stimulated pathways related to cytokinin metabolism and nutrient transport, while in soybean, improved growth was associated with the activation of nitrogen metabolism, sulphate reduction, metal and ion transport, and amino acid biosynthesis. These omics-based findings indicate that biostimulants modulate essential metabolic networks in a crop-specific manner, hence enhancing growth and production.

Transcriptomics research can support current scientific initiatives by offering basic research results that describe the mechanisms and processes of biostimulant action. This work possesses specific limitations, including the observation that not all translated proteins exhibit enzymatic activity and that elevated mRNA levels do not consistently correlate with increased protein levels. Due to these constraints, alterations at the transcriptome or proteome level may not match with modifications in biochemical phenotypes and may inadequately represent the plant's biochemical condition in response to biostimulants and abiotic stressors. The integration of transcriptomics and metabolomics levels in systems biology, as well as metabolic engineering studies, is therefore required to completely and thoroughly understand the molecular mechanisms of action that biostimulants use to mitigate abiotic stressors.

2.9.1 Metabolomics in relation to biostimulant application

The traditional definition of metabolomics is "a complete and integrated assessment of the whole complement of tiny molecular weight compounds, specifically metabolites (1500 Da in size) inside a biological system" (Varadharajan et al. 2025). Metabolomics is considered the most challenging and rigorous of the "omics" sciences due to the ontological intricacy of the metabolome, which encompasses the vast and varied molecular universe of cellular systems. The chemical space and language of metabolism, known as the metabolome, is thought to be more responsive to alterations in metabolic fluxes and enzyme activity compared to both the transcriptome and proteome. This is due to the fact that the

metabolome not only contains genetic and environmental markers, but is also the primary medium through which metabolic processes occur (Tugizimana et al. 2019). The metabolome's worldwide quantitative measurements can help to examine tiny cellular environments, uncover hidden patterns, and demonstrate the operational biochemistry and cellular physiology of a stressed system (Lephatsi et al. 2021). Consequently, employing this multidisciplinary omics research in the realm of plant biostimulants would yield a comprehensive understanding of the cellular and molecular mechanisms and actions of biostimulants.

There are both untargeted and targeted metabolomics techniques. Untargeted metabolomics seeks to objectively measure every detectable molecule in order to provide a thorough global overview of the metabolome. A limited, particular group of metabolites may be quantified using the focused technique. Furthermore, research often employs non-specific methods to produce hypotheses, but specific methods are frequently used to verify, expand upon, and interpret the initial results of studies that produce hypotheses. However, there is a lack of research on plant biostimulants that is based on metabolomics.

Currently, there are relatively few metabolomics studies examining the dual connection among biostimulants and plants, as well as a three-way interaction involving biostimulants, plants, and abiotic stress. Lucini et al. (2015) found that applying a biostimulant made from protein hydrolysate to lettuce resulted in increased levels of defense-related metabolites such as terpenes, amino acids, carbohydrates, and sterols. This increase lessened the plants' exposure to saline stress. El-Arroussi et al. (2018) investigated the effects of a biostimulant derived from seaweed extract (*Dunaliella salina* exopolysaccharides) on tomato plants in order to determine how the former reduced salt stress. According to metabolomic study, *D. salina* contains bioactive compounds called sulfated exopolysaccharides, which increased proline, phenolics, osmoprotective chemicals, and antioxidant enzyme activity, thereby mitigating the effects of salt stress. Furthermore, by inducing significant metabolic alterations in phytohormones, hydroxycinnamic acid amides, and lipids, including terpenes, membrane lipids, and sterols, the plant-derived protein hydrolysate improved drought tolerance in tomatoes, according to

the untargeted metabolomics analysis. As a result, cytokinins decreased and salicylates accumulated. Furthermore, through metabolic reprogramming, PH-based biostimulants enhanced stomatal conductance and the redox balance system (Paul et al. 2019a). Therefore, creating a theoretical foundation for biostimulant product development is essential. This involves acquiring a basic comprehension and awareness of the cellular and molecular mechanisms of action of the biostimulants.

3. MATERIALS AND METHODS

3.1 Preparation of biostimulants

Ten different biostimulants belonging to the categories of microbial and non-microbial biostimulants were used for the initial biostimulant selection. The microbial biostimulant formulations evaluated in this study were prepared using different extracts of *Saccharomyces cerevisiae* -live yeast extract (Yl), supernatant (Ys), and yeast in powdered form (Y). The non-microbial biostimulants include sulphur derived amino acid - L-cysteine (C), antioxidant based biostimulant - Ascorbic acid (A), Protein Hydrolysate (PH), Humic Acid (HA). The non-microbial biostimulants used in this study are commercial products available in the market. The combined form of microbial and non-microbial biostimulants were also prepared and labelled as Bl (Yl+A+C), Bs (Ys+A+C) and B (Y+A+C).

The microbial biostimulants used in this study is yeast extract.

Scientific name	<i>Saccharomyces cerevisiae</i>
Higher classification	Saccharomyces
Kingdom	Fungi
Class	Saccharomycetes
Family	Saccharomycetaceae
Division	Ascomycota

3.1.1 Preparation of yeast extract (Yl, Ys & Y)

Yeast (*Saccharomyces cerevisiae* WB 40) strain was obtained from Central University of Tamil Nadu, India. For making a 5g pellet, about 500 mL of O/N culture of yeast was needed. Five grams of yeast pellet were mixed with 15 mL of

Milli-Q water to make an aqueous solution. It was mixed again and put in an autoclave at 115°C for 10 minutes. The supernatant was frozen right away in an ice bath or at -20°C for 15 minutes. It was then centrifuged at 400–800g for 4°C. The supernatant was immediately frozen in ice bath or at -20°C for 15 minutes followed by centrifugation at 400-800g at 4°C. The supernatant was then loaded to a vacuum evaporator for making powder (YI) and also the supernatant enriched with microbial components was used directly as priming components (Ys). The commercially available yeast extract purchased from sigma aldrich was used in powdered form (Y).

3.1.2 Preparation of cysteine (Cys)

L-cysteine is an amino acid that contains Sulphur and is non-polar and uncharged. After dissolving a standardized concentration of L-cysteine (15 ppm) in 1N HCl, a very little amount of 0.5g emulsifier mix was added while stirring at room temperature. The mixture was then stored at 4°C.

3.1.3 Preparation of ascorbate (AsA)

In a dark amber bottle, a standardized concentration of AsA (20 ppm) was dissolved in deionised water and vortexed for five minutes. Kept at 4°C for further storage.

3.1.4 Preparation of biostimulant YCA

The concentration needed to prepare the biostimulant mixture is 25 ppm of yeast extract, 20 ppm of AsA, and 15 ppm of L-cysteine. Each prepared formulation was combined, vortexed, and filtered through 0.2mm filter paper. It was then diluted with 50 mL of distilled water and kept at room temperature in a dark bottle. The ideal concentration of this biostimulant formulation was applied to the soil prior to the booting stage of the primed seedlings. And the same concentrations were used for the combinations B1 (YI+Cys+A)-60 ppm, B2 (Ys+Cys+A)-60 ppm and B3 (Y+Cys+A)-60 ppm. The biostimulant YCA, employed for the study was patented with Indian Patent granted number 497761.

3.2 Plant material

Rice (*Oryza sativa* L.) belongs to the family Poaceae. The seeds of rice varieties (Hraswa and Manu ratna) were procured from Kerala Agricultural University, Mannuthy, Kerala, India. Hraswa-extra short duration (75-80), red, medium, bold, ideal as a contingent variety for areas where there is crop loss. It is Susceptible to leaf folder and raised only as a direct sown crop. This rice cultivar offers an alternative approach to conserve water and reduce labour cost over transplanting ones. Manu ratna-short duration (95-99 days), transplanting type, awless, red kernelled, medium sized grains, non-lodging, tolerant to stem borer, leaf folder and whorl maggot, suitable for normal wetlands of Kerala and kole areas of Thrissur district.

3.3 Experimental setup

For seed priming treatments, only healthy and consistent seeds from both rice varieties were chosen, and each seed was pre-washed for one minute with 0.25% Triton X-100 to get rid of any dirt. The washed seeds were immersed in standardized concentrations of priming agents, volumes of which were three times more of the seed weights. During seed priming treatment in an open beaker, the seeds along with the solution were swirled regularly to ensure proper aeration. The seed priming period for each variety of rice were determined through a series of standardization. In terms of shoot length, fresh weight, and dry weight of seedlings, the seed priming time that produced the greatest improvement in seedling growth was chosen. Following priming, the seeds underwent three rounds of two-minute distilled water washing before being surface-dried on absorbent paper. In order to recover the initial seed moisture prior to the priming treatment, the seeds were then spread out on a piece of filter paper and allowed to dehydrate in the shade at $25\pm 3^{\circ}\text{C}$. To make sure the seeds reached their initial dry weight, seed weights were measured several times at predetermined intervals. The control group consisted of the untreated seeds. Initially, seeds were planted in seed trays in a greenhouse with $24/16^{\circ}\text{C}$ day/night temperatures, a 16-hour photoperiod, $300\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ irradiance, and 60–70% relative humidity. Uniform seedlings that were ten days old

were placed on to 7.5-liter plastic pots. The potting mixture was sterilized by solar sterilization. For this, the potting mixture was spread over a polythene sheet spread out on a clean ground, where maximum penetration of sunlight was obtained. Following a week of acclimation, one set of plants was kept in nutrient-deficient conditions, while the other set was kept in typical, non-stressful condition (control). Primed as well as non-primed seeds were germinated in nutrient deficient soil. The soil sample which lack ~ 50% mobile nutrients were used as nutrient deficient soil and nutrient rich soil was considered as control for both varieties. The developmental and physiological characteristics of primed, non-primed, and control seedlings were documented on the day after transplantation i.e., 12th day after germination of rice varieties. The 12th day was selected based on the best performance of the seedlings (in terms of shoot length, fresh and dry weight of the seedlings).

For each variety, there were four treatments with three replicates. The four treatments include C (control- non-treated seeds without stress condition), C+B (biostimulant treated seeds without stress condition), S (nutrient deficient soil condition), S+B (biostimulant treated seeds subjected to nutrient deficient stress condition). Further physiological and biochemical parameters in rice seedlings emerging from primed and non-primed seeds were done on seedling (12th day), tillering (21th day), booting (40th day) and ripening stages (75th day). During the end of tillering stage, the soil was amended with the application of 250 ppm of YCA biostimulant for thrice in alternative days in C+B and S+B treatments. The control and stress treatments were amended with water, instead of the biostimulant.

3.4 Preparation of nutrient deficient soil condition

Soils used for cropping in 3 different seasons were continuously washed with distilled water to remove mobile nutrients and prepared by mixing soil and sand in different ratios such as 100:0, 75:25, 50:50, 25:75 and 0:100 respectively. The nutrient deficient soil was analysed by determining soil phosphorus, organic carbon, PH, electrical conductivity and nutrient content. The soil nutritional status of each mixture was analyzed in CWRDM, Kozhikode, Kerala (Table 5). The soil sample

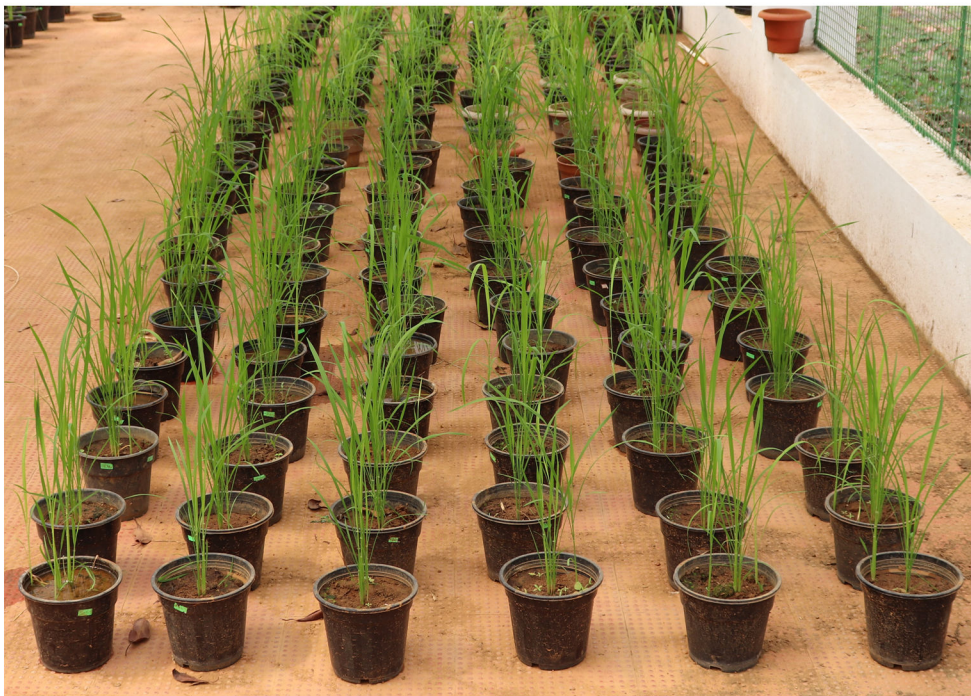


Fig. 7: Experimental Conditions

which is deficient of ~ 50% mobile nutrients were considered as nutrient deficient soil.

3.5 Cumulative stress responsive index (CSRI) for determining tolerant and sensitive rice variety

The CSRI was calculated to evaluate the physiological responses of both var. Hraswa and Manu ratna subjected to nutrient deficient condition for determining the tolerant and sensitive rice variety. It is the sum total of individual parameter responses at each treatment. The CSRI was calculated according to Dai et al. (1997)

3.6 Standardisation of biostimulant for seed priming and soil application

3.6.1 Determination of effective concentration of biostimulant for priming effects in rice

The seeds were immersed in different concentrations of biostimulant solutions (10, 15, 20, 25 and 30 ppm) prepared by dissolving in water. After pre-treatment for 12 hour, the seeds were surface dried on absorbent paper. To recover the original seed moisture, surface dried seeds were placed on clean filter paper and dehydrated for 12 hour in the shade at 25°C. The untreated seeds were used as the control. The primed and non-primed seeds were sown in soil.

3.6.2 Determination of effective concentration of biostimulants for soil application

The biostimulants were applied directly in nutrient deficient (ND) soil for standardizing the effective concentration. Different concentrations (200, 250 and 300 ppm) were applied in the ND soil and compared with ND and control condition. Soil enzymes were analysed to find out the optimum concentration.

3.7 Biostimulant characterization

3.7.1 pH and Electrical conductivity (EC)

The pH and conductivity (EC) of each formulation were measured using 1 % concentration of the original product for standardized dilution (Sharma et al. 2016).

3.7.2 CHNS

Carbon and nitrogen percentage of the formulations were analysed using CHNS analyser (Flash 200, Thermo Fisher Scientific, USA). Two milligram powdered samples were measured, enclosed in tin containers, and securely sealed to eliminate any air gaps, ensuring they reached a constant weight. It must be devoid of residual solvents and exhibit maximal homogeneity. The CHNS analyser employed in this work integrates two distinct single-furnace systems: one for Dynamic Flash Combustion and the other for High Temperature Conversion, into a unified analyser. Samples were introduced into the combustion chamber by the auto sampler. Post-combustion, the resultant gases (NO_2 , CO_2 , and H_2O) were transported by flowing helium gas, subsequently traversing a gas chromatography (GC) column that facilitated the separation of the combustion gases, and ultimately detected by a thermal conductivity detector (TCD). The integrated TCD and specialised Eager 300 software enable the FLASH 2000 HT analyser to function as an independent instrument for quantifying elemental weight percentages of N, C, H, O, and S. The total duration was approximately 12 minutes. The analysis was conducted in triplicate, and the average value for each element was given. Sulfanilamide ($\text{C}_6\text{H}_8\text{N}_2\text{O}_2\text{S}$) was used as the standard.

3.7.3 Powdered X-Ray diffractometer (XRD)

The crystallite size was analyzed for different biostimulant formulations using Powder X-ray diffractometer (Malvern PANalytical, X'Pert³ Powder, USA). This instrument was equipped with an X'Celerator solid state detector where $\text{Cu K}\alpha$ radiation ($\lambda = 1.5406\text{\AA}$) was used as the radiation source with 0.25° divergence slits. Diffraction patterns were obtained within the 2θ range of 20° to 80° , utilising a step size of 0.0167° and a scanning rate of 2° per minute. The operational voltage and current were established at 45 kV and 20 mA, respectively.

3.7.4 Fourier transform infrared spectroscopy (FTIR)

Liquid samples of different biostimulant formulations were used for deriving FTIR spectra. The FTIR spectra of the liquid samples and reference materials were

obtained using a spectrum one FTIR spectrometer (Jasco-4100, Shanghai, China) equipped with an attenuated total reflectance sampling device containing a diamond/ZnSe crystal. Spectra were collected at ambient temperature in transmission mode over the range of 400 to 4000 cm^{-1} , with a scan speed of 0.20 cm s^{-1} , employing 60 accumulations and a resolution of 2 cm^{-1} .

3.7.5 X-Ray fluorescence spectrometer (XRF)

Approximately 5 mL of various biostimulant formulation samples was transferred into a sample cup covered with a prolene film, which was then positioned on the holder of the SPECTRO XEPOS spectrometer (SPECTRO Analytical, GmbH, Kleve, Germany) for multi-elemental analysis of major, minor, and trace element concentrations, utilising the following specifications: Excitation Source: X-ray tube equipped with a thick binary Pd/Co alloy anode and an air-cooling system, Operating Conditions: Maximum power of 50 W and a maximum voltage of 50 kV, Excitation System: Adaptive excitation system equipped with optimised filtering components. A Highly Annealed Pyrolytic Graphite (HAPG) polarizer was used to improve sensitivity for low atomic number elements (Na–Cl), while a bandpass filter enhanced performance for elements ranging from K to Mn.

Detection was performed using a Silicon Drift Detector (SDD) with Peltier cooling. The detector had an active area of 20 mm^2 (total 30 mm^2), providing a spectral resolution of ≤ 130 eV for Mn $K\alpha$ lines. The system supported an input count rate up to 1,000,000 counts per second and was equipped with an automatic filter changer for active pile-up reduction. Measurements were performed under vacuum conditions to enhance sensitivity for light elements. Each sample was scanned over a broad energy range, and peak identification and quantification were achieved using the instrument's built-in software. All analyses were conducted in triplicate to ensure reproducibility, and mean values were reported.

3.8 Seed germination parameters

Seeds of rice var. Hraswa was selected for selection of one of the best performing biostimulant formulation based on seed germination parameters. Seeds

were placed into petri plates with double disks of Whatmann filter paper. The seed were kept in dark for germination at $23 \pm 2^{\circ}\text{C}$ (Upadhyaya et al. 2017).

3.8.1 Germination percentage

Germination percentage (GP) was measured according to the method of Bagawade and Jagtap (2018). Germinating seeds were counted based on 2mm radical emergence on the 3rd day (72 hour) of germination. The number of seeds germinated in each sample was noted every day which was continued until maximum number of seeds were found germinated.

$$\text{Germination percentage} = \left(\frac{\text{No. of seeds germinated}}{\text{total no. of seeds sown}} \right) \times 100$$

3.8.2 Seed water uptake (SWU)

The amount of water that rice seeds absorbed in during imbibition was measured for three sets of fifty seeds in each treatment. Weighed primed and non-primed rice seeds were soaked in distilled water and incubated at 25°C at an interval of 6 hour (Kummerova et al. 1997). The seeds were all taken out, dried with a paper towel, and measured. The amount of water taken per dry weight of the seed was used to show changes in weight due to imbibition. This can be found using the following formula:

$$\text{SWU}\% = \frac{(\text{Fresh weight of seed} - \text{Dry weight of seed})}{\text{Dry weight of seed}} \times 100$$

3.8.3 Study of various seedling growth parameters

The shoot and root lengths of seedlings of rice were measured on a graduated scale and defined in centimetres. After 8 days of germination, 10 seedlings per plate were randomly chosen to assess their fresh weight. For the measurement of fresh and dry weights, the seedlings were blotted and individually wrapped in pre-weighed, labelled aluminium foil. The fresh weight of the samples was ascertained by weighing them immediately after packaging. For dry weight measurements, the samples were maintained in a hot air oven at 100°C for one hour, followed by 60°C

overnight. Following a 48-hour period, the samples were placed in a desiccator, permitted to cool, and subsequently weighed. The samples were reweighed at regular intervals (every 24 hours) as previously described, until their weights stabilised (ISTA 2007). The proportion of dry weight was determined using the accompanying formula:

$$\text{Dry weight percentage} = \frac{\text{Dry weight}}{\text{Fresh weight}} \times 100$$

3.8.4 Seedling vigor index (SVI)

For measuring SVI, germination %, root length and shoot length were required. Root length (RL) and shoot length (SL) were measured using calibrated scale. SVI was measured according to the method of Orchard (1977).

$$\text{SVI} = \text{Germination \%} \times \text{Seedling length (root length + shoot length)}$$

3.8.5 α -amylase activity

α -amylase activity was determined in rice seeds after 72 hour of germination following the method described by Kataria et al. (2019). 200 mg of rice seeds were blended with 5 mL of cooled 80% acetone and centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 mL of 0.02M phosphate buffer (pH 6.4), followed by centrifugation at 15,000 rpm for 20 minutes at 4°C; the resulting supernatant was utilized as the amylase enzyme extract. The reaction mixture, with a total volume of 5 mL, comprised 0.02M sodium phosphate buffer (pH 6.4), 0.1% starch, 0.1N HCl, 0.1% I2KI, and 0.2 mL of enzyme extract. The absorbance was quantified at 660 nm. The enzyme activity was expressed as mg starch hydrolysed mg protein⁻¹h⁻¹.

3.8.6 Protease activity

Later 72 hour of seed germination, 1g of rice seeds were crushed in 5 mL of phosphate buffer (0.2M, pH 7.6), then using Whatman No.1 filter paper the homogenate was filtered and centrifuged at 12,000 rpm for 30 minutes at 4°C. The resultant supernatant was used for the enzyme assay following the method of Kunitz

(1947). One mL of supernatant was incubated with 1 mL of 1% casein solution in 0.2M carbonate buffer (pH 9.5) followed by 60 minutes incubation at 37°C incubation. The reaction was terminated using 10% TCA (Trichloroacetic acid). The proteolytic activity was measured at 660 nm in TCA soluble fraction after reaction with Folin Phenol reagent (Lowry et al. 1951). The enzyme activity was expressed as mg protein hydrolyzed g⁻¹ fresh weight.

3.8.7 Total soluble sugar content

The total soluble sugars was assessed following the method of Hedge and Hofreiter (1962), with few modifications. The 0.5g primed seeds after 72 hour germination were homogenized in 95% ethanol and centrifuged. 50µl supernatant obtained after centrifugation was added to 5 mL of anthrone. Following the cooling of the mixture, absorbance was measured at 620 nm. A standard curve was constructed using glucose to determine the quantity of sugar. A standard curve was prepared from glucose to calculate the sugar quantity.

3.9 Soil related enzyme activity

Extraction: Fresh rhizospheric soil was obtained and stored in a well-ventilated area for one week. The air-dried soil samples were subjected to sieving using a 20-mesh screen for further analysis.

3.9.1 Estimation of soil sucrase: The methodology of Yang et al. (2020) was used to quantify soil sucrase. A sample of two-gram air-dried soil was placed into a conical flask containing 15 mL of sucrose, 5 mL of phosphate buffer, and 0.5 mL of toluene. The solution was incubated at 37°C for 24 hours. Subsequent to filtration, 1 mL of filtrate and 3 mL of DNS (3, 5-Dinitrosalicylic acid) were introduced into the test tube. Sucrose was used as the standard.

3.9.2 Estimation of soil protease: The analysis methods of protease activity were described by Boyce et al. (2010). Two-gram air dried soil sample were transferred to conical flask containing 10 mL of casein solution with 0.5 mL of toluene and incubated at 30°C for 24 hour. Later, the filtrate (1 mL) was added to a test tube containing 0.5 mL sulphuric acid solution and 0.5 mL of sodium sulphate solution.

After centrifugation at 6000g for 15 minutes the OD was recorded at 560 nm. Tyrosine was used as the standard.

3.9.3 Estimation of soil dehydrogenase: The dehydrogenase activity in the biostimulant treated soil were analysed using the protocol of Garcia et al. (1997). Two-gram air dried soil sample were placed in a conical flask and add 0.02g CaCO₃ to buffer the soil. Now, add 1 mL of 3% TTC (2,3,5- triphenyltetrazolium chloride) and 2.5 mL of distilled water. Add 0.5 mL of 1% glucose to increase microbial activity. Seal the flask to limit oxygen diffusion and incubate at 37°C for 24 hour in darkness. After incubation, add 25 mL methanol as organic solvent, shake vigorously to extract TPF (Triphenyl formazon) from the soil into solution. Filter the extracts into volumetric flask. Read the absorbance at 485 nm.

3.9.4 Estimation of soil urease: The urease activity in the soil treated with biostimulants at different concentration were estimated following the method of Guan et al. (2011) by using citric acid buffer as the extracting medium. Two-gram air dried soil sample were transferred in a conical flask containing 1 mL toluene, 20% 10 mL urea with 20 mL citric acid buffer solution (pH-6.7) and incubated at 37°C for 2 hour. After incubation, filter the mixture to separate the soil and collect the supernatant. Now, measure a known volume of supernatant (1 mL) and add 4 mL sodium phenol and 3 mL of hypochlorite to determine the release of NH₄⁺-N. read the absorbance at 578 nm.

3.10 Physiological parameters

3.10.1 Leaf pigment composition

Extraction: The chlorophyll and carotenoid pigments in the leaves at different stages of rice were estimated following the method of Arnon (1949) and Lichtenthaler and Buschmann (2001) by using 80% acetone as the extracting medium. The newly harvested rice seedling leaves were thoroughly rinsed with distilled water and dried using filter paper. To ascertain pigment content, one hundred milligrams of fresh leaf sample were weighed, homogenised, and extracted in 80% acetone (v/v). The homogenate was centrifuged at 5000 rpm for 10 minutes

at 4°C, and the supernatant was retrieved. The residue was re-extracted with the extracting solution and centrifuged at 5000 rpm for 10 minutes. This method persisted until the particle became colourless.

Estimation: The final volume of the acetone extract was noted and the optical density was read at 663, 646, 750 and 470 nm against the solvent blank (80% acetone) using spectrophotometer (Multiskan, Thermo Fisher Scientific, Vantaa, Finland). The concentration of the total chlorophyll (Chla+b) and carotenoids contents were expressed in mg chlorophyll/carotenoids g⁻¹ dry weight of leaf sample.

$$\text{Chlorophyll } a = \frac{12.69(A_{663} - A_{750}) - 2.69(A_{646} - A_{750})}{\text{Fresh weight of the sample}} \times \text{Volume}$$

$$\text{Chlorophyll } b = \frac{22.9(A_{646} - A_{750}) - 4.68(A_{663} - A_{750})}{\text{Fresh weight of the sample}} \times \text{Volume}$$

$$\text{Chlorophyll } a + b = \frac{20.12(A_{646} - A_{750}) + 8.02(A_{663} - A_{750})}{\text{Fresh weight of the sample}} \times \text{Volume}$$

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

3.10.2 Analysis of carbohydrate composition

Rice leaves at various stages (seedling, tillering, booting, and ripening) were extracted with 80% ethanol, followed by 70% ethanol, then centrifuged at 10,000 rpm for 20 minutes. The pooled supernatants were placed in a water bath at 80°C for 20 minutes to evaporate the ethanol. The resulting solution was filtered and utilised to determine total soluble sugar, starch, reducing sugar, and sucrose.

3.10.2.1 Total soluble sugars (TSS)

The total soluble sugar was estimated using the method proposed by Dubois et al. (1956).

Extraction: A clean mortar and pestle were used to homogenise five hundred milligrams of leaf tissue in 80% ethyl alcohol. The homogenate was aliquoted into centrifuge tubes and subjected to centrifugation at 10,000 rpm for 10 minutes at 4°C.

The supernatant was obtained, and the pellet was re-extracted using 80% alcohol. The supernatant was obtained, and the total soluble sugars were quantified.

Estimation: A known volume of aliquot was obtained from the supernatant and added to the test tube to make up to 1 mL of distilled water. To this, 0.1 mL of 5% (v/v) phenol was added and thoroughly mixed. Using a burette, five millilitres of strong sulphuric acid were swiftly poured into the tube. After cooling, the optical density of the resulting solution was measured at 490 nm with a spectrophotometer. D-glucose was utilised as a standard.

3.10.2.2 Starch

The starch content was determined as per the method of Yoshida et al. (1976).

Extraction: Five hundred milligrams of rice leaf tissues were extracted with 80% ethanol, followed by 70% ethanol, then centrifuged at 10,000 rpm for 20 minutes. The pooled supernatants were placed in an 80°C water bath for 20 minutes to evaporate the ethanol. To the residual, add 5 mL of water and 6.5 mL of 52% perchloric acid. Centrifuge and pool the supernatants to make up to 100 mL.

Estimation: Pipette out 0.1 mL of the supernatant and make up to 1 mL of water. In each tube, add 4 mL of the anthrone reagent. Boil for eight minutes in a boiling water bath. Cool rapidly and measure the intensity of green to dark green colour at 630nm. D-glucose was employed as the standard. The measured glucose content was multiplied by a factor of 0.9.

3.10.2.3 Reducing sugar (RS)

RS were determined by the dinitrosalicylic acid reagent (DNS reagent) method described by Miller (1972)

Extraction: Weigh two hundred milligrams of sample and extract with hot 80% ethanol twice (5 mL each time). Centrifuged at 10000 rpm for 20 min. Separate the supernatant and evaporate it by placing it in a water bath at 80°C. Introduce 10 mL of water and dissolve the sugars.

Estimation: Transfer 0.3 mL and dilute to 3 mL with purified water. Introduce 3 mL of DNS reagent into each tube. Submerge the tubes in a hot water bath for five minutes. Add 1 mL of 40% Rochelle salt solution when the contents of the tubes are still warm. Measure the intensity of dark red hue at 510 nm. The standard curve was established using D-glucose, and non-reducing sugars were determined by subtracting reducing sugars from total sugars.

3.10.2.4 Sucrose

The sucrose content was determined as per the method of Roe (1934).

Extraction: Weigh 200 milligrams of the material and extract with 80% ethanol at elevated temperature twice (5 mL each extraction). Centrifuged at 10,000 rpm for 20 minutes. Take the supernatant and evaporate it by placing it in a water bath at 80°C. Pour 10 mL of water and dissolve the sugars.

Estimation: To ascertain sucrose concentration, 0.1 mL of sugar extract was subjected to boil with 6% KOH, followed by the incorporation of 0.1% resorcinol and 30% HCl, and thereafter incubated in a water bath for 10 minutes at 80°C. The absorbance was measured at 460 nm. The standard curve was prepared with D-sucrose.

3.10.3 Sucrose metabolizing enzymes

3.10.3.1 Sucrose synthase (SuSy)

SuSy activity were determined by the method outlined by Hubbard et al. (1989) with slight modifications.

Extraction: Leaves at various developmental phases (seedling, tillering, booting, and ripening) of rice were crushed in a solution of 50 mmol/L HEPES-NaOH buffer (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L sodium EDTA, 2.5 mmol/L dithiothreitol, 0.5 mg/mL bovine serum albumin, and 0.05% Triton X-100. The resultant extracts were further centrifuged at 15,000g for 20 minutes at 4°C.

Estimation: The SuSy assay reaction mixture comprised 150 mmol/L fructose, 20 mmol/L uridine diphosphate glucose (UDPG) in a 40 mmol/L HEPES-NaOH buffer

(pH 7.5), and 0.2 mL of the enzyme extract. After a 30 minutes incubation at 37°C, 0.1 mL of 30% KOH was introduced, and the mixture was subjected to a boiling water bath for 20 minutes to remove free fructose. Subsequently, 1 mL of resorcinol and 3 mL of 30% HCl were introduced, and after comprehensive mixing, the mixture was incubated at 80°C for 10 minutes, with absorbance recorded at 490 nm. For the SPS assay, the identical technique was employed, with fructose 6-phosphate substituted for fructose.

3.10.3.2 Sucrose phosphate synthase (SPS)

SPS activity were determined by the method outlined by Hubbard et al. (1989) with slight modifications.

Extraction: Leaves at various developmental phases (seedling, tillering, booting, and ripening) of rice were crushed in a solution of 50 mmol/L HEPES-NaOH buffer (pH 7.5), 5 mmol/L MgCl₂, 1 mmol/L sodium EDTA, 2.5 mmol/L dithiothreitol, 0.5 mg/mL bovine serum albumin, and 0.05% Triton X-100. The resultant extracts were further centrifuged at 15,000g for 20 minutes at 4°C.

Estimation: The SPS assay reaction mixture comprised 150 mmol/L fructose 6-phosphate, 20 mmol/L uridine diphosphate glucose (UDPG) in 40 mmol/L HEPES-NaOH buffer (pH 7.5), and 0.2 mL of the enzyme extract. After a 30-minute incubation at 37°C, 0.1 mL of 30% KOH was introduced, and the mixture was subjected to a boiling water bath for 20 minutes to remove free fructose. Subsequently, 1 mL of resorcinol and 3 mL of 30% HCl were introduced, and after comprehensive mixing, the mixture was incubated at 80°C for 10 minutes, with absorbance assessed at 490 nm.

$$\text{Enzyme activity (nmol/min/g Fw)} = \frac{C \times V_t \times \text{OD (absorbance)}}{F_w \times T \times V_s}$$

where,

C = Amount of glucose from standard curve (μg)

T = Reaction time

V_t = Total volume of enzyme extracted (mL)

V_s = Aliquot volume (mL)

F_w = Fresh weight (g)

OD = Absorbance

3.10.4 Determination of leaf osmotic potential

Leaf osmotic potential was measured according to Hura et al. (2007) by using a vapour pressure osmometer (Wescor 5520, USA). The chamber was calibrated utilising standard solutions of 100, 290, and 1000 mmol/kg (Wescor). Cell sap from the seedling was obtained using the freeze-thawing technique. Ten leaf discs, each with a diameter of 10mm, were collected from the central regions of the leaves and combined in an Eppendorf tube. For the estimate, leaf samples were thawed at ambient temperature, and the sap exuding from the leaf discs was collected using a 10µl pipette and promptly transferred to the Whatman filter paper disc situated in the osmometer's disc chamber, where the results were documented.

3.10.5 Chlorophyll *a* fluorescence measurement

Chl *a* fluorescence parameter was analyzed by using Plant Efficiency Analyzer (Handy PEA; Hansatech Ltd., King's Lynn, Norfolk, UK), which is a portable fluorometer having high resolutions (Strasser et al. 2004). All measurements were performed depending upon the stages. During the seedling stage, measurements were conducted on the upper surfaces of the initial leaves, while in the tillering, booting, and ripening stages, measurements were taken on the flag leaf (photosynthetically active) following a 20-minute dark adaptation using leaf exclusion clips, subsequently illuminated with high-intensity continuous red light (3000 µmol m⁻² s⁻¹). All measurements were documented for 1 second with a data acquisition frequency of 10 µs for the initial 2 ms and at 1 ms thereafter.

Subsequently, the diverse fluorescence parameters were assessed, including maximal fluorescence (F_m), the activity of the water-splitting complex on the donor side of PSII (F_v/F_o), the PSII structure-function index (SFI), performance index

(PI), electron transport quantum yield (F), the time at which maximum fluorescence is attained [Tf(max)], and the yield of electron transport per trapped exciton ($\Psi_o E_o$). The phenomenological energy fluxes were calculated using the energy pipeline leaf model: the concentration of active reaction centres per cross-section (RC/CSO), the number of photons absorbed per cross-section (ABS/CSO), the maximum exciton trapping rate measured per cross-section (TR/CSO), the electron transport flux per cross-section (ET/CSO), and the dissipation rate per cross-section (DI/CSO). PSII energy fluxes per reaction center: flux of absorption per reaction center (ABS/RC), trapping flux per reaction center (TR/RC), electron transport flux per reaction center (ET/RC) and dissipated energy flux per reaction center (DI/RC)] was figured as specific membrane model. Data were analyzed; energy pipeline and specific membrane models were deduced using Biolyzer HP3 software (Chl fluorescence analyzing program by Bioenergetics Laboratory, University of Geneva, Switzerland).

3.10.6 Photosynthetic gas exchange measurements

Leaf photosynthetic gas exchange parameters were analyzed 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 of booting stage, with readings taken between 09.00 am to 12.00 pm in the morning under room temperature and ambient CO₂ conditions. The internal light source in the LI-6400 was adjusted to an intensity of 1500 $\mu\text{mol m}^{-2} \text{S}^{-1}$ to maintain consistent and uniform lighting throughout all measurements. Leaf gas exchange parameters, including net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), internal CO₂ concentration (Ci), vapour pressure deficit (VPD), water use efficiency (WUE) and intrinsic water use efficiency (iWUE) were measured in rice leaves. Pn, gs, Ci, Tr, VPD, WUE and iWUE were computed utilizing the equations established by Caemmerer and Farquhar (1981).

3.11 Biochemical parameters

3.11.1 Total free amino acids

Total free amino acids were determined by following the method of Moore and Stein (1948).

Extraction: Five hundred milligrams of fresh samples were mixed together with 80% (v/v) ethanol in a clean mortar and pestle. At 4°C, the extract was spun at 10,000 rpm for 15 minutes. Then, 10 mL of 80% ethanol was added to the supernatant.

Estimation: One millilitre of the supernatant that was left over was mixed with one millilitre of the ninhydrin solution in a test tube. Tubes were maintained in a boiling water bath for 20 minutes before being filled with 5 mL of diluent, which was made up of equal parts water and n-propanol. The mixture was left to sit at room temperature for 15 minutes. After that, it was tested using a UV-VIS spectrophotometer against a reagent blank to see its intensity at 570 nm. The results were given as mg g⁻¹ Fw. A standard curve was made by putting different amounts of leucine in a 0.1M citrate buffer with a pH of 5.

Preparation of reagent: Reagent solution was prepared by dissolving 20g of ninhydrin and 3g of hydrindantin in 750 mL of methyl cellosolve. The solution was stirred carefully to avoid air bubbles formation in the solution. 250 mL of sodium acetate buffer (pH 5.5) was added to this solution and the resulting reddish reagent solution was immediately transferred to a 1 litre dark glass bottle. The reagent was used freshly without storage.

3.11.2 Total protein

The total protein content of the rice leaves was determined using the Folin-Ciocalteu reagent, as described by Lowry et al. (1951).

Extraction: Two hundred milligrams of leaf tissue were homogenized in 5 mL of phosphate buffer using a pre-chilled glass mortar and pestle. After pipetting a known volume of the homogenate into a centrifuge tube, the same volume of 10% TCA was

added. For 1 hour, this mixture was refrigerated at 4°C to promote flocculation. The protein precipitate was obtained through centrifugation at 5000 rpm for a duration of 10 minutes at 4°C. The supernatant was drained off. The residue was washed twice to remove starch with 2% cold TCA, followed by treatment with 30% perchloric acid. The lipids were extracted using diethyl ether, and the pigments were removed with 80% acetone.

Estimation: The centrifuged pellet was dried and then digested in 5 mL of 0.1N sodium hydroxide by boiling in a water bath for 10 minutes. After cooling, the suspension was centrifuged at 5000 rpm for 10 minutes at 4°C, and the supernatant was collected. A known volume of aliquot was pipetted and filled up to 1 mL with distilled water. 5 mL of alkaline copper reagent was added to each aliquot and thoroughly shaken. After 10 minutes, add 0.5 mL of 1N Folin-Ciocalteu's phenol reagent and immediately shake thoroughly. The tubes were left for 30 minutes for colour development. The optical density of the solution was measured at 700 nm with a UV-VIS spectrophotometer (Systronics 2201). BSA fraction V powder was employed as a standard. Total protein was expressed as mg of protein g⁻¹ dry weight of plant tissue.

3.11.3 Proline

Proline content in the rice seedlings was assessed according to the method of Bates et al. (1973).

Extraction: Two hundred milligrams of both experimental and control plant tissues were measured and crushed in 10 mL of 3% (w/v) aqueous sulfosalicylic acid using a clean glass mortar and pestle. The homogenate was placed in centrifuge tubes and centrifuged for 10 minutes at 10,000 rpm. Subsequently, the supernatant was obtained, and the proline content was determined using acid ninhydrin..

Estimation: Two mL of supernatant was taken in test tubes in triplicate and equal volume of glacial acetic acid and 2.5% acid ninhydrin (1.25 g of ninhydrin dissolved in a mixture of 30 mL of glacial acetic acid and 20 mL of 6M ortho phosphoric acid) were added to it. The tubes were then heated in a boiling water bath for 1 hour and

then the reaction was terminated by placing the tubes in an ice bath. Four mL of toluene was added to the reaction mixture and stirred well using a vortex mixer. The chromophore-toluene layer was separated carefully and the optical density of the separated solution was measured at a wavelength of 520 nm using spectrophotometer. L-proline was used as the standard.

3.11.4 Rate of lipid peroxidation

The malondialdehyde content (MDA) estimation was done according to the method of Heath and Packer (1968).

Extraction: Two hundred milligrams of rice tissue were measured and crushed in 5 mL of 5% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12,000 rpm for 15 minutes. The supernatant was collected and utilized to estimate MDA

Estimation: The estimate of MDA content was conducted in accordance with Heath and Packer (1968). Two mL of the supernatant were combined with 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA), and the mixture was heated at 95°C for 24 minutes, subsequently cooled, and centrifuged at 3000 rpm for 2 minutes. The absorbance of the supernatant was quantified at 532 and 600 nm relative to a reagent blank using a UV-VIS spectrophotometer (Systronics 2201). The absorbance at 532 nm was adjusted for non-specific turbidity by deducting the absorbance at 600 nm. Then the MDA content was calculated using its extinction coefficient of $155 \text{ mM}^{-1}\text{cm}^{-1}$.

3.11.5 Superoxide anion

Superoxide content was determined as per the method of Doke (1983).

Extraction: Two hundred milligrams of rice leaf tissue were weighed, cut into small pieces, and submerged in 0.01 M potassium phosphate buffer (pH 7.8) with 0.05% nitroblue tetrazolium chloride and 10mM sodium azide.

Estimation: For fifteen minutes, the mixture was incubated at 85°C in a water bath. The mixture was promptly moved to an ice bath to reduce the temperature after

incubation. A spectrophotometer was used to measure the mixture's absorbance at 580 nm after it had cooled. The standard was sodium nitrate (NaNO_2).

3.11.6 Hydrogen peroxide content

Hydrogen peroxide content was determined according to Junglee et al. (2014) protocol.

Extraction: Two hundred milligrams of rice leaf tissue was weighed and homogenized with 5 mL of 0.1% ice cold trichloroacetic acid (TCA). For fifteen minutes, the homogenate was centrifuged at 12,000 rpm. The amount of hydrogen peroxide was measured using the recovered supernatant.

Estimation: 1 mL of 1M potassium iodide was added to 500 mL of supernatant and 0.5 mL of potassium phosphate buffer (pH 7). At 390 nm, the mixture's absorbance was measured. Hydrogen peroxide was used as the standard.

3.12 Non-enzymatic antioxidants

3.12.1 Ascorbate (AsA) content

For the estimation of AsA content, the method of Chen and Wang (2002) was adopted.

Extraction: An electronic balance was used to weigh five hundred milligrams of rice leaf tissue, which were then homogenised with 5 mL of 5% TCA. After being moved to a centrifuge tube, the homogenate was centrifuged for 15 minutes at 4°C at 12,000 rpm. The supernatant was collected and its AsA content was estimated.

Estimation: An aliquot of 0.1 mL of the supernatant was mixed well with 0.3 mL of 200 mM NaH_2PO_4 . To this mixture, 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) was added. The solution was incubated at 42°C for 15 minutes. The absorbance was recorded promptly upon incubation at 524 nm, and the AsA content was determined using a standard curve generated with various amounts of AsA.

3.12.2 Glutathione (GSH) content

The GSH content estimation was done as per the protocol of Chen and Wang (2002).

Extraction: Two hundred milligrams of leaves of rice tissue was weighed using an electronic balance and homogenized in 5 mL of 5% TCA (w/v). The homogenate was filtered through a filter paper and centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was collected and used for the estimation of reduced glutathione content.

Estimation: To an aliquot of 0.5 mL of the supernatant, 2.6 mL of 150 mM NaH₂PO₄ buffer (pH 6.8) and 0.18 mL of 3 mM 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) were added (DTNB was dissolved in 100 mM phosphate buffer, pH 6.8) and kept for 5 minutes. The absorbance was measured at 412 nm, and the GSH content was determined using a standard curve utilising different amounts of reduced glutathione.

3.12.3 Total phenolics

Total phenolics was estimated in accordance with Folin and Denis (1915) method.

Extraction: After being weighed and homogenised with 80% ethanol (v/v), 500 milligrams of rice leaf tissue were centrifuged for 20 minutes at 10,000 rpm, and the supernatant was collected. The homogenate was centrifuged once more after the pellet was extracted in 80% ethanol. Five millilitres of distilled water were used to dissolve the residue after the supernatant was collected and dried in an oven.

Estimation: 50 µl aliquots of the supernatant were pipetted out, made up to 2 mL with distilled water, and then an equivalent volume of Folin-Denis reagent was added. After three minutes of thorough mixing, two millilitres of 1N sodium carbonate were added. This combination was thoroughly mixed, and then it was incubated for one hour to develop its colour and optical density was measured at 700 nm. Tannic acid was used as a standard to determine the total phenolic content.

3.13 Enzymatic antioxidant system assay

3.13.1 Superoxide dismutase (SOD, E C 1.15.1.1)

Estimation of SOD activity in the leaves of rice at different stages was done as per the modified protocol of Giannopolitis and Ries (1977).

Extraction: Using a pre-chilled mortar and pestle, 500 milligrams of rice leaf tissue were weighed and gently homogenised in 50 milligrams of phosphate buffer with a pH of 7.8. The homogenate was centrifuged for 15 minutes at 4°C at 16,000 rpm. The enzyme assay was performed using the supernatant.

Enzyme Assay: The ability of SOD to prevent the photochemical reduction of nitro blue tetrazolium chloride (NBT) was used to inhibit its activity. 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, 0.3 mL of 0.63 mM NBT, and 0.1 mL of enzyme extract made up the reaction mixture. Phosphate buffer (50mM, pH 7.8) was used to increase the reaction mixture to 3.0 mL. Various assay systems, including test samples, light-control, and dark-control, were established. Test tubes with just the test mixture and no enzyme extract were exposed to a fluorescent lamp for 30 minutes (light-controls). Test samples (tubes containing assay mixtures with enzyme extract) were also illuminated and other set (tubes containing assay mixtures with enzyme extract) was kept in dark (dark-control). The formazan accumulation in different tubes was quantified using UV-VIS spectrophotometer (Systronics 2201) by recording the absorbance of the developed blue colour at 560 nm against the blank (reaction mixture without NBT). Results were expressed as units SOD mg⁻¹protein⁻¹. One unit of SOD was defined as the enzyme activity that inhibited the photo reduction of NBT to blue formazan by 50%.

3.13.2 Catalase (CAT, EC 1.11.1.6)

CAT activity in the leaves of rice at different stages was measured according to the protocol of Kar and Mishra (1976).

Extraction: Five hundred milligrams of leaves of rice tissue was weighed and homogenized using a mortar and pestle in an ice-cold phosphate buffer 50 mM (pH7.0), and 200 mg of polypyrrolidone as phenolic binder. The homogenate was filtered using two layers of muslin cloth, then phosphate buffer was added to get it up to 10 mL. The filtrate was centrifuged for 15 minutes at 4°C at 16,000 rpm. The enzyme assay was performed using the obtained supernatant.

Enzyme assay: One mL of 50mM phosphate buffer (pH 7.0), two mL of enzyme extract, and one mL of 30mM hydrogen peroxide made up the enzyme assay combination. 2.4 mL of 50mM phosphate buffer (pH 7.0), 0.3 mL of enzyme extract, and 0.3 mL of 30mM hydrogen peroxide were all included in the enzyme assay combination. In a test tube, the phosphate buffer and enzyme extract were pipetted out and thoroughly combined. The addition of hydrogen peroxide to this combination started the enzyme's activity. The enzyme activity was immediately measured at 240 nm for 90 seconds following the addition of hydrogen peroxide. Every 15 seconds, readings were taken. The activity of CAT was determined in terms of μmol hydrogen peroxide oxidised per min per gram fresh weight. The CAT activity was expressed in terms of decrease in absorbance at 240 nm for 1 minute following the decomposition of hydrogen peroxide. One unit of the enzyme was defined as μmoles hydrogen peroxide decomposed per min per mg protein.

3.13.3 Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity in the fresh samples was assayed by following the method of Nakano and Asada (1981).

Extraction: Leaf extraction for enzyme assay was prepared as per the protocol of Zhang and Kirkham (1996). Using a pre-chilled mortar and pestle, five hundred mg of rice leaf tissue was homogenized in a 10 mL of extraction medium. The extraction buffer consisted of 50 mM sodium phosphate buffer (pH 7.0), containing 0.33M sorbitol, 1mM MgCl_2 , 2mM EDTA, 10mM NaCl, 0.5mM KH_2PO_4 and 1mM ascorbate. The homogenate was filtered through two layers of muslin cloth and centrifuged at 4°C for 4 minutes at 2000 rpm. The pellet was discarded and the

supernatant was centrifuged again at 15000 rpm for 15 minutes at 4°C and the supernatant was used further as the cytosolic fraction for APX assay.

Enzyme Assay: Cytosolic APX activity was determined by measuring the drop-in absorbance at 290 nm caused by AsA oxidation. The 3 mL assay apparatus contained 0.5mM AsA and 0.1mM EDTA in 50mM sodium phosphate buffer (pH 7.0). After adding 20µL of cytosolic enzyme extract to the buffer, 10µL of 100mM H₂O₂ was added to initiate the enzyme reaction, resulting in a final concentration of 0.1mM H₂O₂ in the reaction mixture.

3.13.4 Guaiacol peroxidase (GPOX, EC 1.11.1.7)

GPOX activity in the leaves of rice at different stages was measured by adopting the method of Gaspar et al. (1975).

Extraction: A cold mortar and pestle was used to weigh and gently homogenize five hundred milligrams of rice leaf tissue in 50mM Tris-HCl buffer (pH 7.5). Two layers of muslin cloth were used to filter the extract. The filtrate was put into a centrifuge tube and spun in a Thermo Scientific X1R refrigerator centrifuge set at 15,000 rpm for 15 minutes at 4°C. The supernatant was used for the enzyme assay after being moved to a test tube and kept in an ice bath.

Enzyme Assay: GPOX activity was measured following the H₂O₂ dependent oxidation of guaiacol (extinction coefficient 26.6 mM⁻¹cm⁻¹) at 420 nm. 2.86 mL of 100mM phosphate buffer (pH 7.8), 30µL of 1% guaiacol, and 100µl of enzyme extract made up the three mL assay mixture. In place of the enzyme extract, 50mM Tris-HCl (pH 7.5) was added to the reaction mixture to create the blank. After carefully combining all the ingredients, 12µL of H₂O₂ was added to start the enzyme activity. Immediately after the addition of H₂O₂, the increase in absorbance due to oxidation of guaiacol was measured at 420 nm using a UV-VIS spectrophotometer (Systronics 2201) for 3 minute at intervals of 30 seconds. One unit of GPOX activity was defined as the amount of enzyme that caused the formation of 1µM of tetraguaiacol per min.

$$\text{Enzyme activity (Units/ml)} = \frac{Vx(\text{change in absorbance/ min})}{\epsilon. d. v.}$$

where,

V = total volume (mL)

ϵ = extinction coefficient of substrate (that disappears) or the product (which appears).

d = path distance of cuvette in cm (1 cm)

v = aliquot volume (mL)

The value calculated above represent the activity of 1 mL enzyme extract in ‘units’ as the total number of moles of product formed or substrate disappeared in one min. Since, one enzyme unit represents one micromole of product formed or substrate disappeared in 1 minute, one mL of enzyme solution would contain ‘units’ equivalent to that calculated from 1 mL extract.

Specific activity was calculated after determining soluble protein concentration according to Bradford (1976).

$$\text{Specific activity} = \frac{\text{Activity in Units}}{\text{mg protein/ml enzyme extract}}$$

3.14 Mineral analysis

A total of 28 elements were analysed using the inductively coupled plasma mass spectrometry (Agilent 7800, Merck, Darmstadt, Germany). A 0.2g leaf, grain and biostimulant-treated soil sample was transferred to a Kjeldahl flask, and 5 mL of concentrated HNO₃ was added and heated in a heating mantle until the solution was colourless. Upon completion of digestion, the solution was cooled, filtered, and diluted to a final volume of 50 mL using distilled water. The ICP-MS apparatus was calibrated using an internal multi-element standard from Merck, Germany. The operational parameters during the measurement were as follows: Radio Frequency

Power: 1550 W; plasma gas flow rate: 15.0 l min⁻¹; auxiliary gas flow rate: 0.9 l min⁻¹; nebuliser gas flow rate: 1.01 l min⁻¹; scan mode: peak hooping.

3.15 Yield and its attributes

At the time of harvest, thirty spikes were randomly selected from each pot to assess various characteristics, including panicle height, the number of grains per spike, grains' weight per spike (measured in grams), and the weight of 100 grains, following the methodology outlined by Kishore et al. (2015). Yield-related parameters were recorded, including grain yield and its components such as plant height, panicle density, panicle length, number of filled grains per panicle, and 1000 grain weight. Plant height, panicle density, the number of tillers bearing panicles per plant, and the number of filled and unfilled grains per panicle were all measured in five randomly selected plants for each entry in each replication. But, grain yield was measured per pot. In contrast, data for 100-grain weight were gathered from a randomly selected sample of grains from each pot in each entry and replication. Additional parameters such as the number of spikes per square meter, straw yield, harvest index (HI), and biological yield were calculated from each plant per pot using the method described by Panwar and Ali (2007). Yield response percentage was calculated according to the equation of Slafer et al. (2014). Actual yield is the yield observed or measured during treatment; baseline yield is the yield used for comparison or control treatment. Here the yield response % of each treatment was compared with the hydropriming and biostimulant available in market i.e., Syngenta Quantis (SQ).

$$\text{Yield response \%} = \frac{\text{Actual yield} - \text{Baseline yield}}{\text{Baseline yield}}$$

3.16 Metabolite profiling using GC-MS

3.16.1 Sample extraction

For the metabolome analysis, the youngest fully expanded (flag) leaves of booting stage were sampled between 10:00 to 11:00 am. The leaves were immediately frozen in liquid nitrogen. Each frozen tissue sample (three biological

replicates) was grounded into a fine powder using liquid nitrogen. 100mg of flag leaf were transferred to centrifuge tubes and extracted with ice-cold methanol and acetonitrile solution (2:1) and centrifuged at 12000 rpm for 10 minutes at 4°C. Thereafter, 60µL of ribitol (internal standard) was added to the supernatant. The supernatant from each sample was dried with speed vac. The samples were chemically derivatized using 40µl methoxyamine for oximation (30 mg methoxyamine in 1 mL pyridine) and shaking at 30°C for 90 minutes followed by 80µl N-trimethylsilyl trifluoro acetamide (MSTFA) for silylation.

3.16.2 GC-MS analysis

The derivatized extracts were analyzed with Agilent 7010C Triple Quadrupole GC/MS System (7010C GC/TQ, 8890 GC). Aliquots of each sample (1 µL) was injected into a HP-88 (100 m × 250 µm × 0.25 µm) column in the splitless mode. The temperature of the injector was maintained at 250°C. The initial oven temperature of GC was 40°C and hold time was 5 minutes and after injection it was increased to 300°C to hold for 5 minutes and total run time was 61 minutes. Helium was used as a carrier gas with the flow rate at 1 mL/min. Detection was achieved by using MS detection in electron impact mode and full scan monitoring mode (m/z 40–600). The temperature of the ion source was set at 250°C and energy voltage (eV) to 70. Quadrupole mass spectrometer was used as detector. The detected mass spectra peaks were integrated using the GCMS solution software (agilent), and identified peaks were annotated using NIST and Mass hunter mass spectral libraries.

3.16.3 Metabolomic data analysis

The metabolites detected in at least three replicates were selected. For GC-MS, the metabolites were normalized using ribitol as an internal standard followed by differential expression ($\log_2FC \geq +1$ or ≤ -1 and $FDR \leq 0.05$) analysis using metaboanalyst. Normalization of the obtained data was carried out to remove systematic as well as replicates variation within the samples. For this purpose, the peak areas derived from each specific metabolite (analysed compounds were normalized) by the peak area derived from an internal standard within the same

chromatogram, such as ribitol. This step consequently results in response ratios for all the compounds analysed. The normalized value of the identified metabolites was used for correlation and clustering analyses using MetaboAnalyst 5.0. Pearson's correlation was set as a default setting (www.metaboanalyst.ca/). To further remove data variables, the data was filtered by normalization by sum, followed by log transformation and Pareto-scaling.

3.17 High resolution liquid chromatograph mass spectrometer (HRLC-Orbitrap-MS) analysis

3.17.1 Sample extraction

Two grams of powdered leaves of rice samples were grounded in liquid nitrogen and extracted with 10 mL methanol: acetonitrile (1:1) followed by thorough mixing at 4°C for 30 minutes and filtered through 0.22-µm PTFE membrane filter. Samples were then placed at -20°C for 30 minutes and centrifuged at 12,000 rpm for 10 minutes at 4°C and the supernatant obtained was dried in a speed vac. The pellet was re-solubilized with 120µl acetonitrile: water (1:1) and sonicated again for 15 minutes, followed by another centrifugation at 10,000 g for 15 minutes. The supernatant was transferred to the HRLC-MS vials for injection. Three independent extractions were conducted on each tissue to serve as biological replicates (n = 3).

3.17.2 HRLC-MS analysis

Q-Exactive Plus Biopharma- High Resolution Orbitrap was used for the identification and separation of non-volatile components. Direct infusion mass ESI and APCI interface was operated in both positive and negative mode. HRLC-Orbitrap-MS was performed using a Thermo Fischer Scientific (Thermo, USA) mass spectrometer, equipped with a C18 analytical column. The mobile phase consisted of 1.0% HCOOH in H₂O (A) and MeOH (B), with a flow rate of 0.4 mL/min and an injection volume of 1.0µL was used. The column temperature was maintained at 35°C. The following non-linear gradient was applied: 0 minute, 5% B; 0–8 minute, 15%B; 8–11 minute, 25% B; 11–13 minute, 32%; 13–20 minute, 40% B; 20–26 minute, 55% B; 26–32 minute, 95% B; 32–34 minute, 95% B. The

compounds were identified by comparing their retention time (RT), mass, abundance area and m/z cloud with the stored Metlin Library available at IIT Bombay. Thermo compound discoverer 3.2 was used for the mass spectrometric analysis.

3.17.3 Qualitative determination of metabolites

Tentative annotation has been performed based on database matching and analysis of mass spectra. Based on the detected m/z values, the characterization of metabolites has been performed via METLIN (https://metlin.scripps.edu/batch_search.php) and XCMS. The group compounds were identified for the obtained metabolites from the online databases KEGG (<http://www.genome.jp/kegg/>), Chempider, PUBCHEM (<https://pubchem.ncbi.nlm.nih.gov/>) and HMDB (<https://hmdb.ca/>).

3.18 Amino acid profiling

3.18.1 Sample extraction and analysis

Hundred milligrams of powdered leaves of booting stage and seeds of 1st generation rice samples were hydrolysed with 10mL of 6N HCl at 110°C for 24 hour. From the above solution 20µl of samples were taken and evaporated by speed vac. Then reconstituted by 50µl of 0.1N HCl. The amino acid profiling were analysed at the Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay using HRLCMS in conjunction with an Agilent 6550 iFunnel Quadrupole-Time of Flight (Q-TOF) LC/MS system (Agilent technologies, USA). The system utilized dual Agilent jet stream electro spray ionization (AJS ESI) as the ion source. The ZORBAX Eclipse Plus-C18 column, measuring 150 × 2.1 mm and 5 microns (Agilent), was utilized for the separation process. The operating parameters were set with a pressure restriction of 1200 bar and a stop time of 35 minutes. The auto MS2 acquisition mode was configured to operate within the range of 120–1200 m/z. For the separation and identification of chemical constituents, a mixture of mobile phases was utilized. Solvent A consisted of 0.1% formic acid in Milli-Q water, while

solvent B was 100% acetonitrile. Data acquisition and processing were conducted utilizing Agilent Mass Hunter Qualitative Analysis B.06 software.

3.19 Statistical analysis

All tests were performed in triplicate and repeated three times ($n = 3 \times 3$). The statistical analysis was carried out using SPSS. The comparisons were undertaken using one-way ANOVA, followed by Tukey's HSD test, with a significance level of 0.05. All the data are given as mean $\pm$ standard deviation with letters of significance (mean values that does not share a common lowercase superscript differ substantially at $P < 0.05$ according to Tukey's HSD test).

All metabolites were evaluated using Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) for a supervised examination of the datasets. A selection of metabolites was identified as having variable significance in the projection (VIP) according to PLS-DA analysis. Metabolites with a fold change more than 2 and a p-value less than 0.05 were deemed statistically significant. The hierarchical clustering analysis and heat map were produced using MetaboAnalyst 5.0. Data were analyzed using fold change and T-test methodologies. Fold Change (FC) was derived by variation fold analysis, and p-values were adjusted using the False Discovery Rate (FDR) to get q-values. Venn diagrams were utilised to illustrate the quantity of differential metabolites and were generated utilizing VENNY 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>). The pathways including substantially regulated metabolites underwent pathway and enrichment analysis.

3.20 Chemicals

Analytical reagent (AR) or guaranteed reagent (GR) chemicals were used and was purchased from Merck, SRL, Qualigens, BDH, GMBH, Spectrochem and Himedia companies. Riboflavin, glutaraldehyde, bovine serum albumin (BSA), methyl viologen, 3- (3, 4-dichlorophenyl)-1,1- dimethyl urea (DCMU), sodium

azide and L-ascorbate were purchased from Sigma chemicals, USA. Chemicals for GC and HRLC-MS such as Methanol, acetonitrile, ribitol, O- Methylhydroxylamine hydrochloride and N-Methyl-N-trimethylsilyltrifluoroacetamide were purchased from SRL and TCI companies.

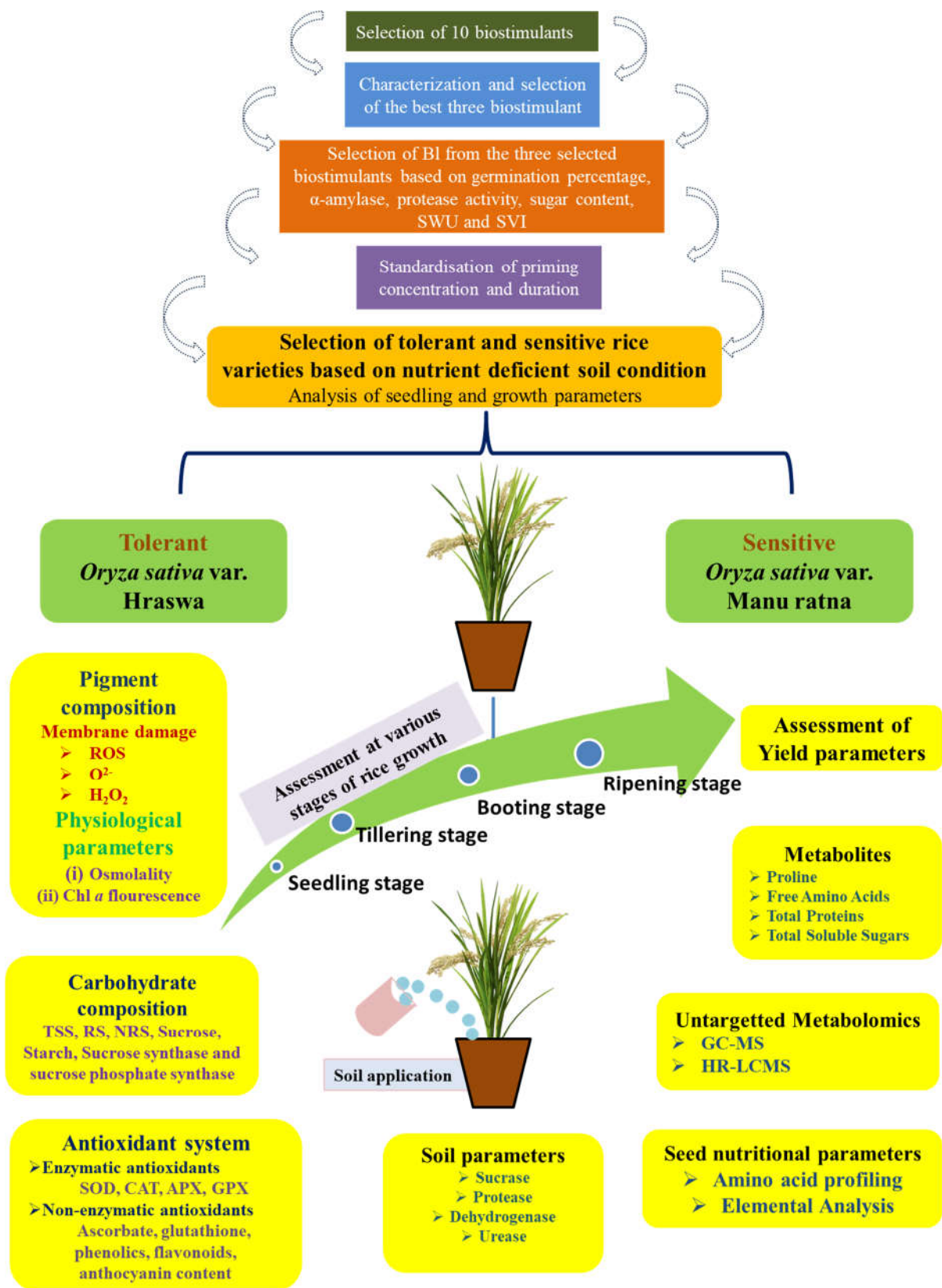


Fig. 8: Schematic representation of the work flow



Fig. 9: Effect of biostimulant application on the seedling stage of var. Hraswa
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

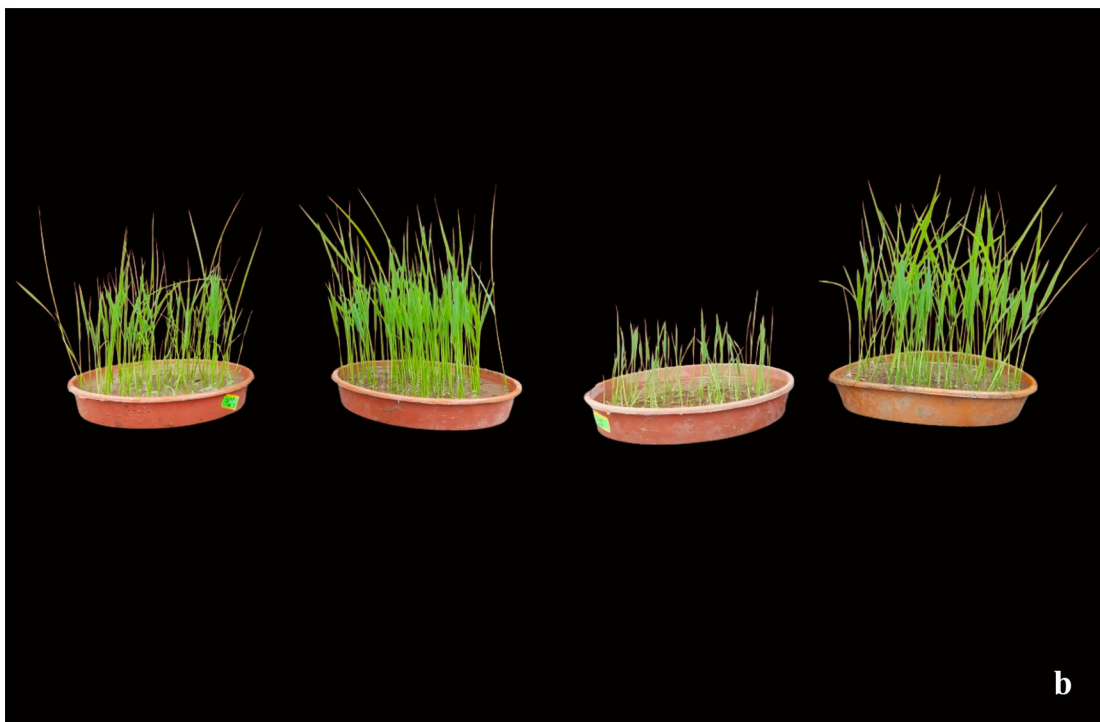


Fig. 10: Effect of biostimulant application on the seedling stage of var. Manu ratna
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

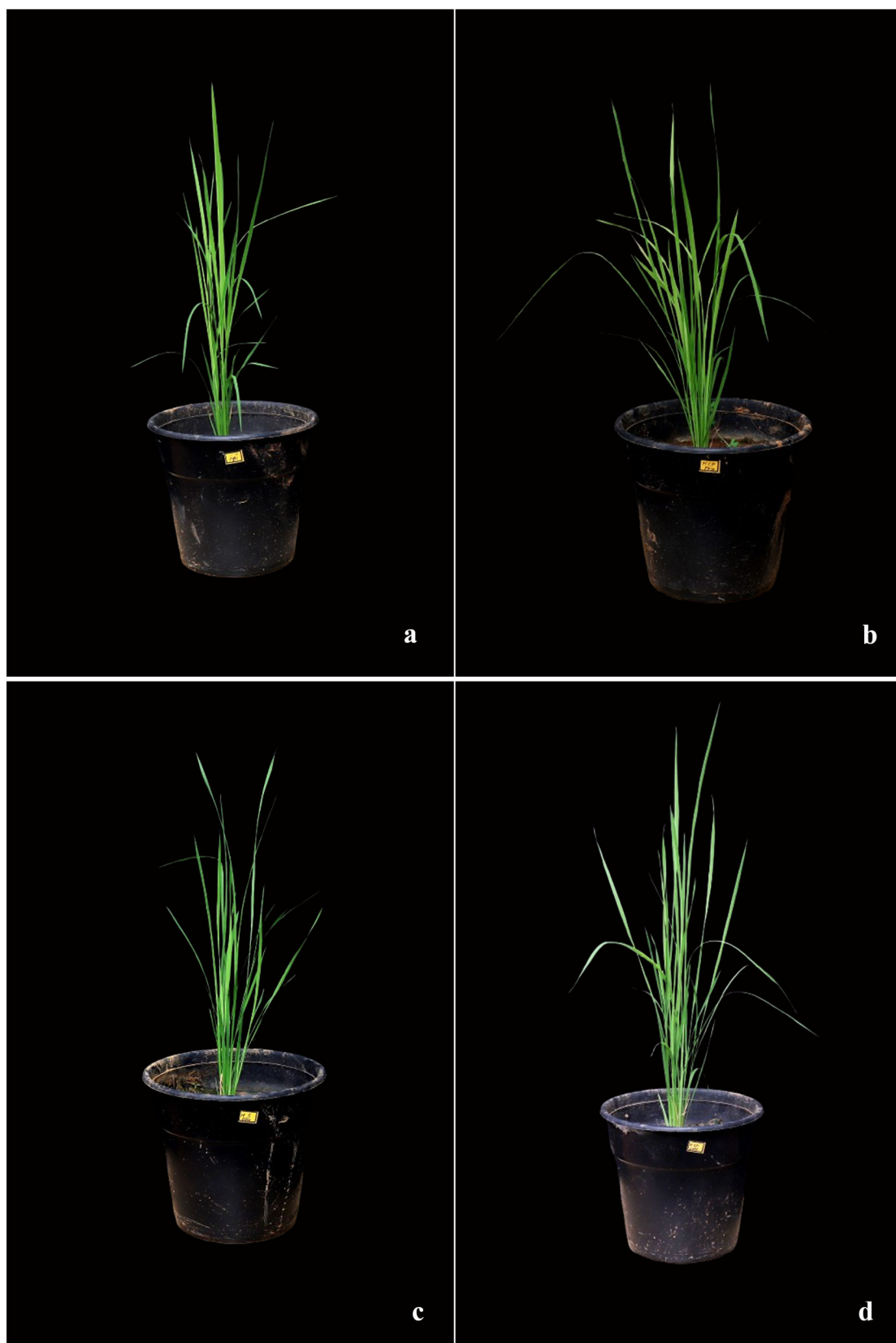


Fig. 11: Effect of biostimulant application on the tillering stage of var. Hraswa
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

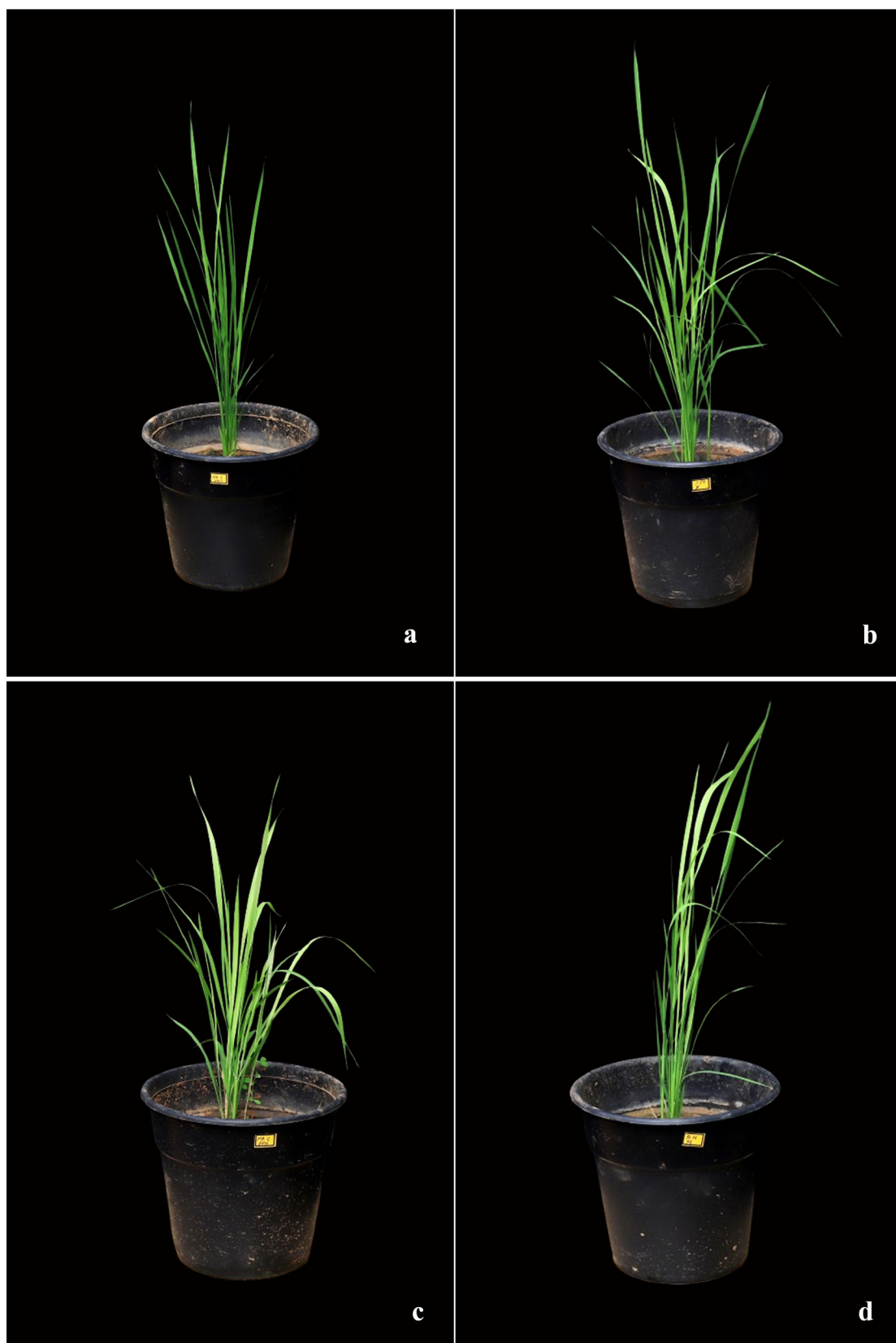


Fig. 12: Effect of biostimulant application on the tillering stage of var. Manu ratna
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).



Fig. 13: Effect of biostimulant application on the booting stage of var. Hraswa
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

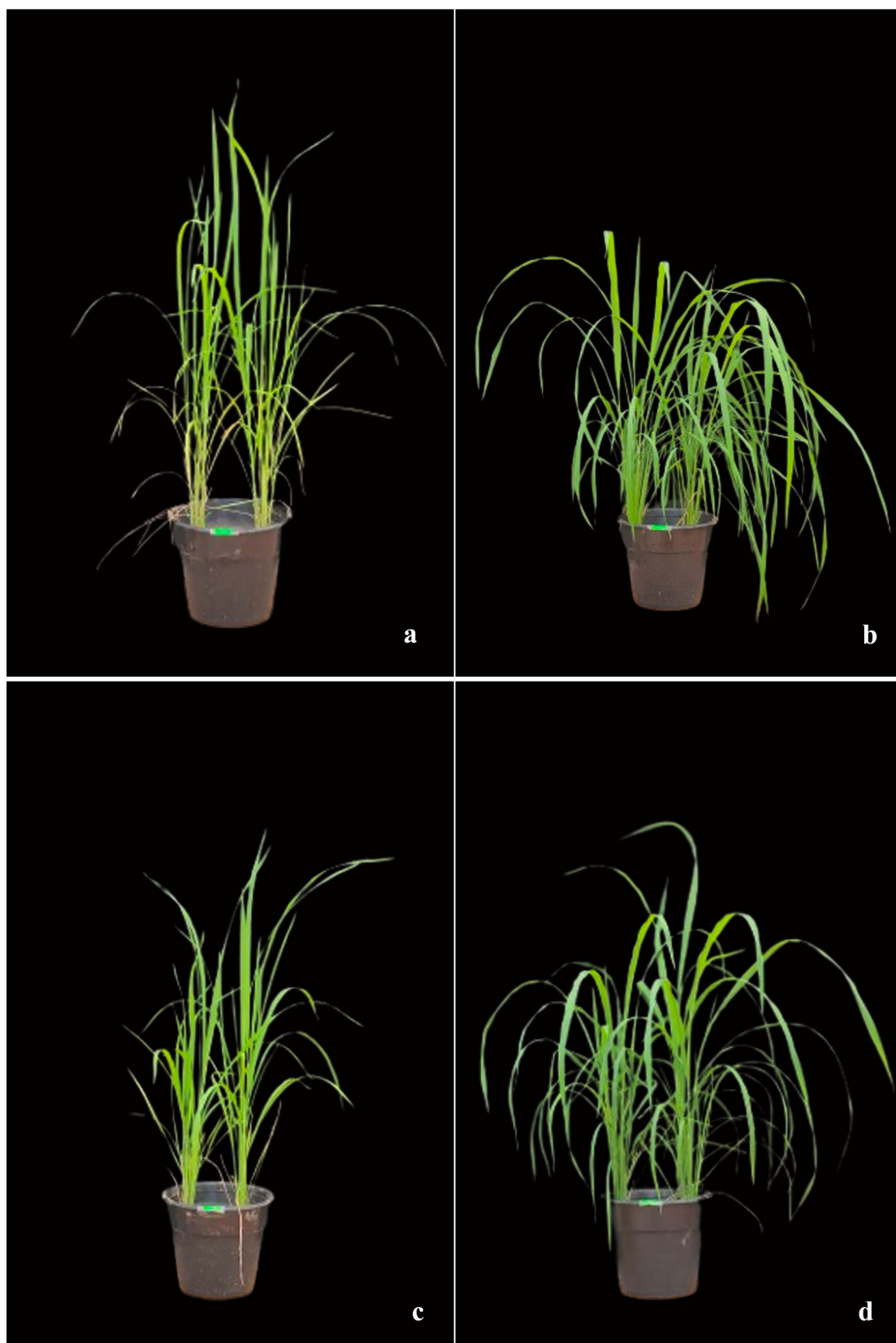


Fig. 14: Effect of biostimulant application on the booting stage of var. Manu ratna
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

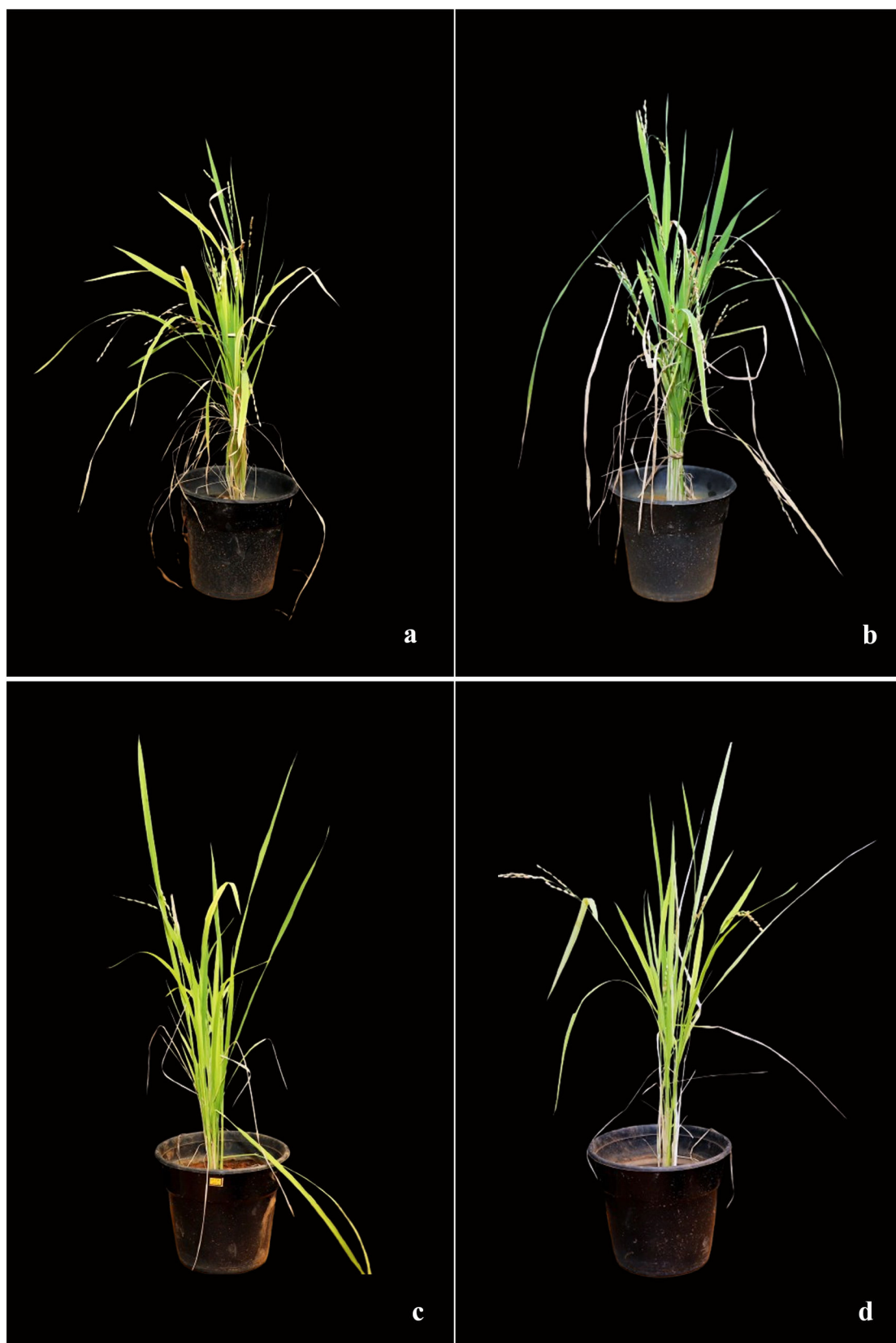


Fig. 15: Effect of biostimulant application on the reproductive stage of var. Hraswa
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).



Fig. 16: Effect of biostimulant application on the reproductive stage of var. Manu ratna **a.** C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).



Fig. 17: Effect of biostimulant application on the ripening stage of var. Hraswa
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S+B (Biostimulant + Nutrient deficient condition) **d.** S (Stress-nutrient deficient condition).

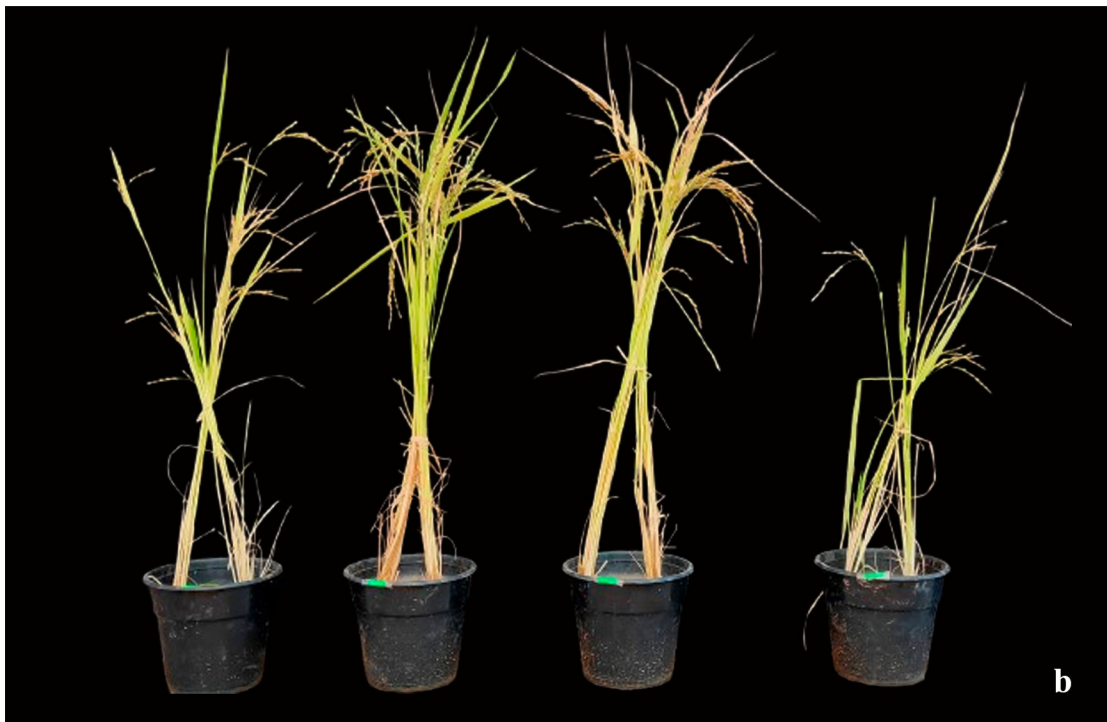


Fig. 18: Effect of biostimulant application on the ripening stage of var. Manu ratna
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S+B (Biostimulant + Nutrient deficient condition) **d.** S (Stress-nutrient deficient condition).

4. RESULTS

4.1 Screening of biostimulants

4.1.1 pH of different biostimulant formulations

The pH of 10 different biostimulant formulations are summarised in Table 1. The pH of the formulations ranged between 4.32 to 8.10. The pH of Yl (7.2), Ys (8.1), Y (7.7) showed more basic nature whereas the combined formulations were acidic in nature i.e., Bl (6.2), Bs (6.0), B (5.9), PH (5.6), HA (5.8) and also for individual formulation of A (4.38), Cys (5.90).

4.1.2 EC of different biostimulant formulations

The EC of different biostimulants ranged within 268 μ s-5.55ms. Yeast derived (Yl, Ys) and combined formulations (B) showed lower conductivity <0.6ms i.e., 560 μ s for Yl, 1710 μ s for B, 1465 μ s for Ys and 268 μ s for HA. Y (4.22ms), Bl (3.48ms), A (3.58ms), showed moderate conductivity. However, Bs (5.12ms), Cys (5.15ms) and PH (5.88ms) recorded excessively high conductivity > 0.6ms (Table. 1) .

4.1.3 Total N%, C% and C:N ratio

N%, C% and C:N ratio of different biostimulant formulations are represented in Table.1. The highest N% available in the biostimulant formulations were seen in Bl (8.2), B (7.8), Bs (6.8), HA (6.0), PH (5.8) and Yl (5.2), while lowest were observed in A (1.7), Cys (2.3), Y (4.0) and Ys (4.8).

C% in a formulation represents the percentage of carbon by weight in the product. It indicates how much of the formulation's composition is made up of carbon-containing compounds. The highest C% were seen in Bl (22.5), PH (18.71), Yl (15.2) and HA (11.2), while lowest were observed in Y (6.8), B (7.0) and moderate amount were observed in Cys (8.4), Ys (8.8), Bs (9.0) and A (9.2).

The C:N ratio in biostimulant formulations depends on the composition and intended function of the product. The highest C:N ratio were observed in A (5.41), Cys (3.65) and PH (3.22) whereas all other formulations showed lowest C:N ratio ranging from 0.89 to 2.92.

4.1.4 Structural analysis using XRD

The crystallite size of the 10 different biostimulant formulations was measured by XRD (Fig. 19). The size range were found to be 1.0 μm to 3.03 μm . The different formulations had varied size; PH (3.03 μm), HA (2.87 μm), Bs (2.54 μm), B (2.1 μm), Yl; Ys; Y (1.9 μm), Cys (1.8 μm), A (1.65 μm) and Bl (1.0 μm). The lowest crystallite size was observed for Bl and largest for PH. The X-ray diffraction plots the intensity of the signal for various angles of diffraction at their respective two theta positions. The two theta positions correspond to a certain spacing between the crystals or atoms in the samples, determined by the angle of diffraction from the x-ray beam incident on the sample. The intensity of the peaks is related to the number of molecules in that phase or with that spacing. The greater intensity of the peaks was observed in Bl, HA and PH and this indicates that majority of crystals or molecules are with distinct spacing. The width of the peaks is inversely proportional to the crystal size. Thinner peaks were observed in PH, Bl and HA, corresponding to a bigger size and crystalline nature. A broader peak means a smaller crystal, with a defect in the crystalline structure, or even the sample might be of amorphous in nature, a solid lacking perfect crystallinity.

4.1.5 Functional characterisation using FTIR

The FTIR graph displays spectra for 10 different samples (Fig. 20 A, B, C and D). The number of peaks are 3, and when it is less than 5, it indicates that the formulations include simple organic compounds. Here the main regions of interest are the broad peaks around 3200–3600 cm^{-1} which is likely due to O–H or N–H stretching (alcohols, phenols, or amines). A broad absorption band in the range of 2850– 3850 indicates hydrogen bond and confirms the presence of hydroxyl, ammonium and amino band. There is no sharp bond at about 3500 cm^{-1} , signifying the absence of oxygen-related bonding. The peaks between 3000 and 3200 cm^{-1}

Table 1. Characterization studies of different biostimulant formulations. Yl, Bl, Ys, Bs, Y, B, A, C, PH and HA represent different biostimulant formulations. The error represents statistical data with 1 sigma confidence interval.

Formulations										
	Yl	Bl	Ys	Bs	Y	B	A	C	PH	HA
PH	8.89±0.9	6.2±0.03	9.70±0.01	6.0±1.1	7.70±0.07	5.90±0.5	4.38±0.8	5.90±0.1	5.67±0.4	5.80±0.4
Conductivity	560µs ±0.2	3.48ms ±0.31	1465µs ±0.20	5.12ms ±0.088	4.22ms ±0.11	1710µs ±0.35	1358ms ±0.53	5.15ms ±0.28	5.88ms ±0.15	268µs ±0.30
Total N%	5.2±0.1	8.2±0.1	4.5±0.1	6.8±0.1	4.0±0.1	7.8±0.1	1.7±0.1	2.3±0.1	5.8±0.1	6.0±0.1
Total C%	15.2±0.1	22.5±0.1	8.8±0.2	9.0±0.1	6.8±0.4	7.0±0.1	9.2±0.4	8.4±0.1	18.7±0.2	11.2±0.1
C:N ratio	2.92	2.74	1.95	1.32	1.7	0.89	5.41	3.65	3.22	1.86

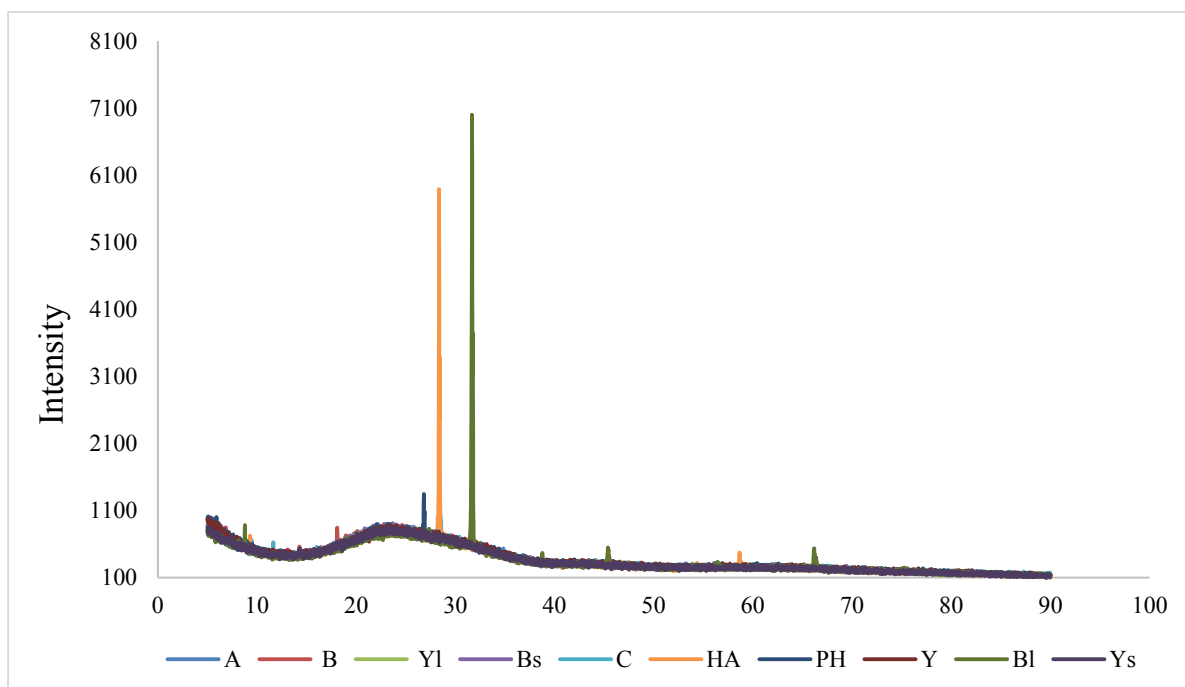


Fig. 19: XRD analysis of 10 different biostimulant formulations

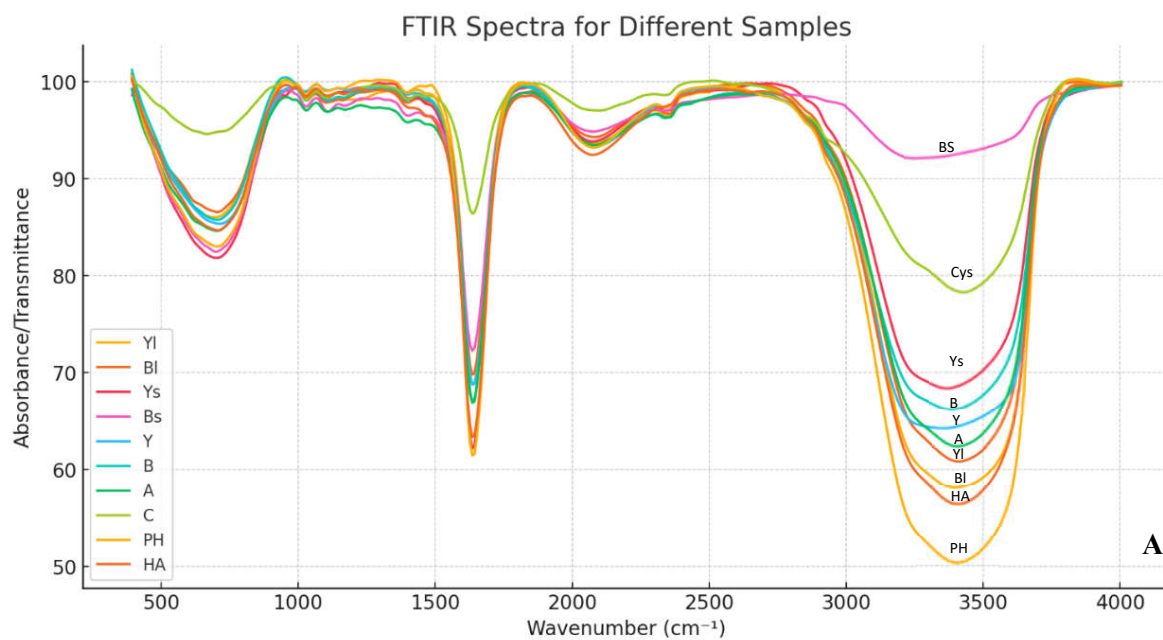


Fig. 20: (A) FTIR analysis of 10 different biostimulant formulations

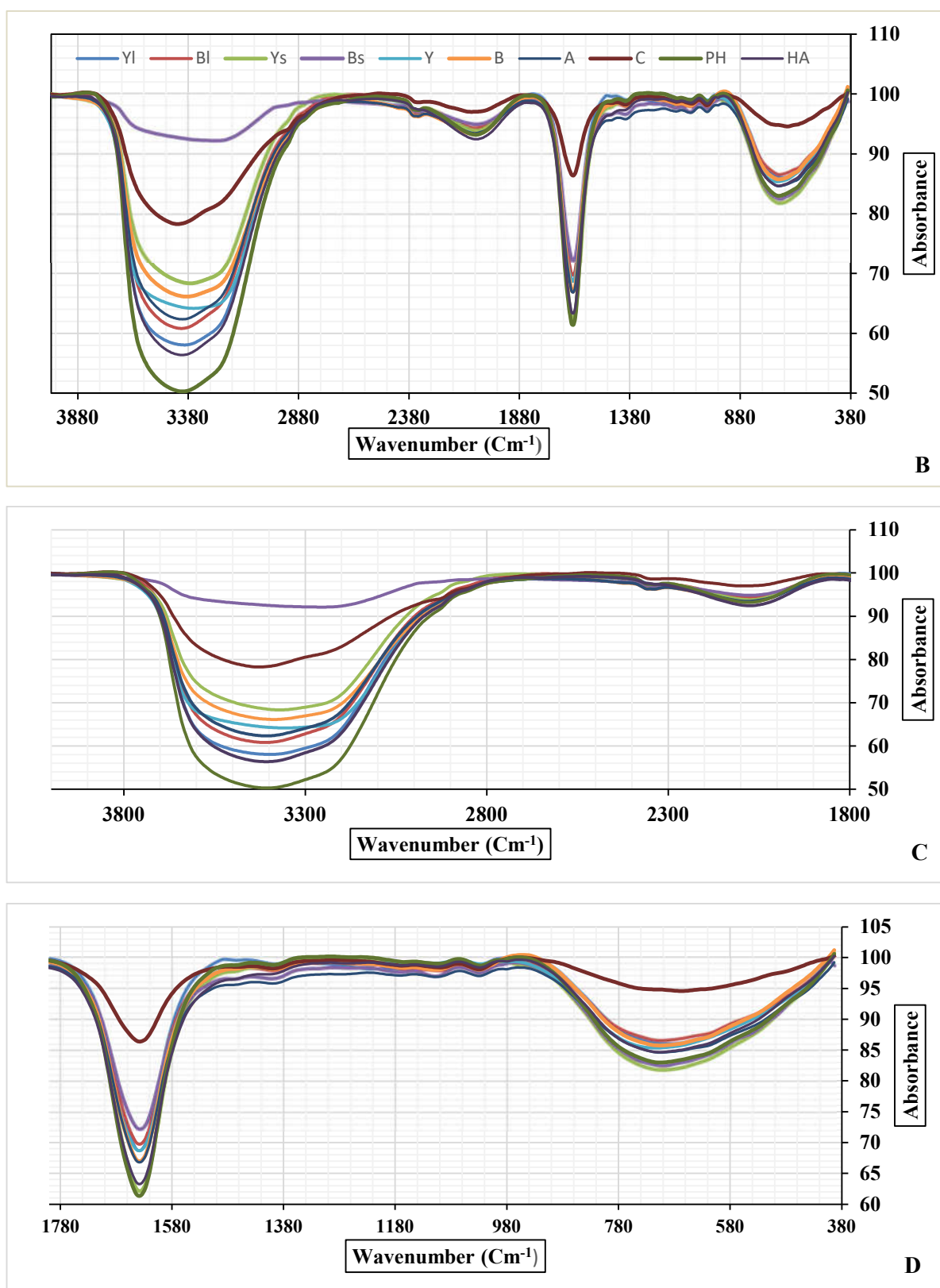


Fig. 20: (B) General overview of peaks in the range of 380-4000 cm⁻¹ band (C) peaks in the range of 1800 - 4000 cm⁻¹ band (D) peaks in the range of 380 - 1790 cm⁻¹ band

indicates the presence of aromatic structure and narrow bond at less than 3000 cm^{-1} corresponds to the C-C bond. No specific peak for aldehyde has been found between 2700 and 2800 cm^{-1} . Peaks around $2800\text{--}3000\text{ cm}^{-1}$ indicate C-H stretching (alkyl groups). Strong peaks near $1650\text{--}1750\text{ cm}^{-1}$ suggest the presence of C=O groups (ketones, aldehydes, or carboxylic acids). Peaks in the $1000\text{--}1300\text{ cm}^{-1}$ region corresponds to C-O stretching or C-H bending. Variations between spectra suggest differences in the chemical composition of the samples. Shifting of peaks or differing in intensity reflect variations in number of functional groups.

4.1.6 XRF

The elemental analysis of 10 different formulations were analysed using XRF (Table 2). Each formulation had specific elements of higher or lower accumulations. Among the different biostimulants, Bl and Yl accumulated most of the elements in higher amount followed by Ys, Bs, PH, Cys, Y and B as compared to other formulations. Bl had higher accumulation of Mg (215ppm), Zn (8.0ppm), S (58.8ppm), Si (108.3ppm), Al (82.3ppm), P (124.3ppm), K (23.3ppm) and Fe (2.7ppm). Yl shows accumulation of P (251.5ppm), K (107.7ppm), Ca (9.3ppm), Mn (1.9ppm) and Fe (3.1ppm). Ys showed higher accumulation of Zn (8.4ppm), Mn (2.5ppm), Ca (11.4ppm) and Al (62.2ppm) whereas, Bs recorded Zn (7.0ppm), S (65.6ppm), P (28.9ppm) and Al (47.1ppm). PH had higher accumulation of Si (34.5ppm), Al (98.2ppm) and Ca (24.8ppm). Cys showed higher accumulation of S (120ppm) and Si (37.7ppm) while, A recorded Zn (7.4ppm), Ca (4.4ppm) and Al (37.2ppm).

4.2 Selection of biostimulants based on seed growth parameters

4.2.1 Germination and seedling growth

Among the 10 different formulations, seed germination % recorded an increase in Bl, Yl, Bs, Ys, B, Y, A and HA (Table 3). The maximum increase in germination % was observed in Bl (28%) and Bs (26%) and followed by Yl (12%) and Ys (10%) with respect to control. While rest of the formulations showed least significant difference as compared to control. Root length (RL) and shoot length

Table 2. XRF analysis of different biostimulant formulations. Yl, Bl, Ys, Bs, Y, B, A, Cys, PH and HA represent different biostimulant formulations. Data represent Mean $\pm$ SE (n=3).

Treatments										
Elements (ppm)	Yl	Bl	Ys	Bs	Y	B	A	Cys	PH	HA
Mg	<0.1	215 $\pm$ 62	<0.1	<0.1	<0.1	<22	<0.1	<0.1	<0.1	<0.1
Si	58 $\pm$ 1.3	108.3 $\pm$ 1.9	7.7 $\pm$ 0.5	17.8 $\pm$ 1.0	19.3 $\pm$ 0.8	19.1 $\pm$ 0.9	4.7 $\pm$ 0.3	37.7 $\pm$ 1.9	34.5 $\pm$ 1.2	9.3 $\pm$ 0.5
Al	58.8 $\pm$ 1.7	82.3 $\pm$ 2.2	62.2 $\pm$ 1.8	47.1 $\pm$ 1.3	52.7 $\pm$ 1.5	<8.8	37.2 $\pm$ 1.1	37.8 $\pm$ 0.7	98.2 $\pm$ 2.6	31.3 $\pm$ 0.9
P	251.5 $\pm$ 2.0	124.3 $\pm$ 1.5	89.8 $\pm$ 1.2	28.9 $\pm$ 1.1	2.8 $\pm$ 0.4	2.0 $\pm$ 0.6	<0.5	2.4 $\pm$ 1.0	4.6 $\pm$ 0.7	0.9 $\pm$ 0.3
S	45.1 $\pm$ 0.7	58.8 $\pm$ 0.8	52.6 $\pm$ 0.9	65.6 $\pm$ 1.0	8.4 $\pm$ 0.3	49.4 $\pm$ 0.8	2.5 $\pm$ 0.2	120.1 $\pm$ 1.4	14.9 $\pm$ 0.6	8.2 $\pm$ 0.3
K	107.7 $\pm$ 1.0	23.3 $\pm$ 0.5	49.6 $\pm$ 0.7	15.9 $\pm$ 0.5	4.3 $\pm$ 0.3	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2	1.4 $\pm$ 0.2	15.1 $\pm$ 0.4	37.2 $\pm$ 0.6
Ca	9.3 $\pm$ 0.4	4.8 $\pm$ 0.2	11.4 $\pm$ 0.4	3.4 $\pm$ 0.2	3.6 $\pm$ 0.2	4.3 $\pm$ 0.2	4.4 $\pm$ 0.2	2.4 $\pm$ 0.2	24.8 $\pm$ 0.5	8.4 $\pm$ 0.3
Mn	1.9 $\pm$ 0.2	1 $\pm$ 0.1	2.5 $\pm$ 0.2	1.4 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	1.8 $\pm$ 0.2	<0.5	1.9 $\pm$ 0.2	1.9 $\pm$ 0.2
Fe	3.1 $\pm$ 0.2	2.7 $\pm$ 0.2	1.7 $\pm$ 0.1	0.8 $\pm$ 0.1	1.9 $\pm$ 0.21	1.4 $\pm$ 0.1	0.8 $\pm$ 0.1	<0.8	1.3 $\pm$ 0.1	1.4 $\pm$ 0.2
Ni	<0.6	<0.7	<0.6	<0.7	<0.6	<0.6	<0.6	<0.7	<0.6	<0.6
Zn	7.9 $\pm$ 0.2	8 $\pm$ 0.2	8.4 $\pm$ 0.2	7 $\pm$ 0.2	6.2 $\pm$ 0.2	9.9 $\pm$ 0.2	7.4 $\pm$ 0.2	6.6 $\pm$ 0.2	7.5 $\pm$ 0.2	7.7 $\pm$ 0.2

Table 3. Germination parameters of rice using different biostimulant formulations. C represents control, Yl, Bl, Ys, Bs, Y, B, A, Cys, PH and HA represent different biostimulant formulations. Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Treatments											
Morphological Parameters	C	Yl	Bl	Ys	Bs	Y	B	A	Cys	PH	HA
Germination %	72.22 $\pm$ 1.1 ^c	93.66 $\pm$ 1.9 ^a	97.33 $\pm$ 1.9 ^a	86.11 $\pm$ 1.1 ^b	87.74 $\pm$ 1.1 ^b	92.66 $\pm$ 0.9 ^a	94.22 $\pm$ 1.5 ^a	88.88 $\pm$ 1.8 ^b	66.66 $\pm$ 1.1 ^d	82.22 $\pm$ 0.4 ^b	90 $\pm$ 1.4 ^{ab}
Seed water uptake (%)	64 $\pm$ 0.17 ^d	88 $\pm$ 0.45 ^b	92 $\pm$ 0.16 ^a	76 $\pm$ 0.23 ^c	85 $\pm$ 0.38 ^b	82 $\pm$ 0.21 ^b	90 $\pm$ 0.34 ^a	78 $\pm$ 0.41 ^c	73 $\pm$ 0.29 ^c	91 $\pm$ 0.44 ^a	85 $\pm$ 0.56 ^b
Root length (Cm)	5.43 $\pm$ 0.23 ^d	6.23 $\pm$ 0.14 ^c	7.03 $\pm$ 0.31 ^a	6.4 $\pm$ 0.20 ^b	5.96 $\pm$ 0.088 ^d	6.8 $\pm$ 0.11 ^b	7.53 $\pm$ 0.35 ^a	6.23 $\pm$ 0.53 ^b	6.5 $\pm$ 0.28 ^b	7.3 $\pm$ 0.15 ^a	6.6 $\pm$ 0.30 ^b
Shoot length (Cm)	3.03 $\pm$ 0.03 ^c	3.63 $\pm$ 0.08 ^a	2.7 $\pm$ 0.057 ^d	3.5 $\pm$ 0.17 ^a	3.8 $\pm$ 0.11 ^a	3.2 $\pm$ 0.11 ^b	3.6 $\pm$ 0.2 ^a	3.6 $\pm$ 0.20 ^a	3.26 $\pm$ 0.12 ^b	3.67 $\pm$ 0.23 ^a	3.23 $\pm$ 0.14 ^b
SVI	695.7 $\pm$ 15.0 ^d	902.1 $\pm$ 19.7 ^b	931.8 $\pm$ 19.7 ^a	874.4 $\pm$ 34.5 ^c	906.2 $\pm$ 39.2 ^b	862 $\pm$ 15.3 ^c	951.7 $\pm$ 20.9 ^a	873.5 $\pm$ 22.8 ^c	649.5 $\pm$ 8.21 ^d	901.5 $\pm$ 26.7 ^b	884.4 $\pm$ 37.8 ^c

Table 4. Seed hydrolytic enzyme activity and soluble sugars of rice using different biostimulant formulations. C represents control, Yl, Bl, Ys, Bs, Y, B, A, Cys, PH and HA represent different biostimulant formulations. Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Formulations											
	C	Yl	Bl	Ys	Bs	Y	B	A	Cys	PH	HA
α-Amylase (mg starch hydrolysed mg protein ⁻¹ h ⁻¹)	13.67 $\pm$ 0.03 ^k	18.8 $\pm$ 0.5 ^f	26.76 $\pm$ 0.43 ^a	15.0 $\pm$ 0.21 ^h	24.2 $\pm$ 0.30 ^c	16.2 $\pm$ 0.54 ^g	22.9 $\pm$ 0.8 ^d	13.8 $\pm$ 0.04 ^j	14.2 $\pm$ 0.21 ⁱ	25.43 $\pm$ 0.89 ^b	19.4 $\pm$ 0.4 ^e
Protease (μ g protein hydrolysed g ⁻¹ FW)	21.4 $\pm$ 0.15 ⁱ	27.3 $\pm$ 0.28 ^e	35.8 $\pm$ 0.54 ^b	22.5 $\pm$ 0.10 ^h	32.3 $\pm$ 0.12 ^c	25.6 $\pm$ 0.19 ^g	26.2 $\pm$ 0.35 ^f	22.4 $\pm$ 0.87 ^h	21.9 $\pm$ 0.23 ⁱ	36.2 $\pm$ 0.19 ^a	30.1 $\pm$ 0.3 ^d
Soluble Sugars (mg/g FW)	1.4 $\pm$ 0.4 ⁱ	2.2 $\pm$ 0.7 ^h	3.5 $\pm$ 0.1 ^a	2.8 $\pm$ 0.5 ^c	3.0 $\pm$ 0.2 ^b	2.5 $\pm$ 0.5 ^e	2.7 $\pm$ 0.9 ^d	1.7 $\pm$ 0.3 ⁱ	2.3 $\pm$ 0.11 ^g	3.0 $\pm$ 0.23 ^b	2.4 $\pm$ 0.2 ^f

(SL) were also higher in Bl, BS and B i.e., combined formulations followed by PH (Table 3).

4.2.2 Seed water uptake (SWU)

Seeds primed with different biostimulant formulations were observed to have higher level of water uptake. Seed water uptake % recorded significant increase in Bl (43%), PH (42%), B (40%), Bs (38%) Yl (32%), Y (28%) and HA (25%), while rest of the biostimulant primed seeds were observed with least significant increase as compared to control (Table 3).

4.2.3 Seedling vigor index (SVI)

SVI was significantly increased in all the seeds primed with different biostimulant formulations while decreased in Cys as compared to control. SVI significantly increased in Bl (35%), B (33%), Bs (30%), Yl (30%), Ys (26%), Y (24%), A (25%), PH (29%) and HA (27%) with respect to control (Table 3).

4.2.4 Seed hydrolytic enzyme activity (α -amylase, protease) and soluble sugar content

The activity of seed hydrolytic enzymes increased in all the biostimulant primed seeds. α -amylase activity recorded significant increase in seeds on priming with Bl (89%), Bs (75%), B (64%), followed by PH, Yl, Ys and Y as compared to control (Table 4). While the protease activity showed more pronounced effect in seeds primed with PH (70%), Bl (67%), Bs (50%) and B (22%) followed by other biostimulants with respect to control. The total soluble sugars were significantly increased in seeds primed with Bl (86%), PH (74%), Bs (72%), Ys (66%), followed by rest of the formulations (Table 4).

It was determined that among the different biostimulants, Bl performed better than the other treatments after a thorough analysis of the applied biostimulants and an assessment of their impact on several seed growth metrics. Bl was recognized and chosen as a strong and successful biostimulant to be used in further phases of

the study because of its constant and noteworthy impact on improving seed vigor, germination, and promoting faster growth characteristics.

4.3 Soil analysis for determination of nutrient deficiency in the soil

The comparative analysis of soil physicochemical properties among the four different soil samples revealed statistically significant differences across nearly all measured parameters (Table 5). The soil pH exhibited a decreasing trend from sample 1 (6.9) to sample 4 (4.8), indicating progressive acidification. Similarly, electrical conductivity (EC) declined markedly from 2.21 ms/cm in sample 1 to 0.046 ms/cm in sample 4, reflecting reduced ionic concentration in the soil solution.

Organic carbon content was highest in sample 1 (2.51 %) and decreased significantly across the samples, reaching 0.3 % in sample 4. Nitrogen content followed a similar trend, with values ranging from 358.3 kg/ha in sample 1 to 123.2 kg/ha in sample 4. Significant differences were also observed in the levels of phosphorus (27 to 8 kg/ha) and potassium (280 to 90 kg/ha), suggesting a progressive depletion of essential macronutrients.

Among the secondary and micronutrients, sulphur content dropped from 50.31 ppm in sample 1 to 4.9 ppm in sample 4. The levels of boron, iron, manganese, zinc, and copper all followed similar statistically significant decreasing trends. Boron decreased from 1.22 to 0.3 ppm; iron from 5.8 to 1.6 ppm; manganese from 3.8 to 1.4 ppm; zinc from 1.9 to 0.3 ppm; and copper from 1.3 to 0.5 ppm.

These observations collectively suggest a declining soil fertility gradient from sample 1 to sample 4, likely resulting from nutrient deprivation (Table 5).

4.4 Selection of tolerant and sensitive varieties based on tolerance index

Two different *O. sativa* varieties i.e., Hraswa and Manu ratna were selected for the present study and their stress tolerance potential was analyzed. Both varieties were grown in nutrient deficient soil, the growth parameters were analysed and compared with that of respective controls. Based on the cumulative stress response index (CSRI) tolerant and sensitive varieties were analysed further. The seedlings of

Table 5. Soil nutrient content as well as physical and chemical properties. Sample1 - Control, sample 2 - 75:25 (soil: sand), sample 3 - 50:50 (soil: sand) and sample 4 - 25:75 (soil:sand). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$)

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
pH	6.9 $\pm$ 0.34 ^a	6.4 $\pm$ 0.32 ^b	5.9 $\pm$ 0.29 ^c	4.8 $\pm$ 0.24 ^d
Electrical conductivity (mS/cm)	2.21 $\pm$ 0.11 ^a	2.0 $\pm$ 0.1 ^b	1.4 $\pm$ 0.07 ^c	0.046 $\pm$ 0.0023 ^d
Organic carbon (%)	2.51 $\pm$ 0.12 ^a	2.33 $\pm$ 0.11 ^b	1.53 $\pm$ 0.07 ^c	0.3 $\pm$ 0.015 ^d
Nitrogen (kg/ha)	358.3 $\pm$ 19.2 ^a	324.8 $\pm$ 16.2 ^b	212.8 $\pm$ 10 ^c	123.2 $\pm$ 6.16 ^d
Phosphorus (Kg/ha)	27 $\pm$ 1.35 ^a	19.17 $\pm$ 0.9 ^b	10 $\pm$ 0.5 ^c	8 $\pm$ 0.4 ^d
Potassium (Kg/ha)	280 $\pm$ 14 ^a	235 $\pm$ 11 ^b	145 $\pm$ 7.25 ^c	90 $\pm$ 4.5 ^d
Sulphur (ppm)	50.31 $\pm$ 2.5 ^a	42 $\pm$ 2.1 ^b	27 $\pm$ 1.35 ^c	4.9 $\pm$ 0.22 ^d
Boron (ppm)	1.22 $\pm$ 0.061 ^a	1.10 $\pm$ 0.05 ^b	0.7 $\pm$ 0.035 ^c	0.3 $\pm$ 0.01 ^d
Iron (ppm)	5.8 $\pm$ 0.29 ^a	4.2 $\pm$ 0.21 ^b	2.9 $\pm$ 0.14 ^c	1.6 $\pm$ 0.08 ^d
Manganese (ppm)	3.8 $\pm$ 0.19 ^a	3.1 $\pm$ 0.15 ^b	2.7 $\pm$ 0.13 ^c	1.4 $\pm$ 0.07 ^d
Zinc (ppm)	1.9 $\pm$ 0.095 ^a	1.2 $\pm$ 0.06 ^b	0.9 $\pm$ 0.04 ^c	0.3 $\pm$ 0.015 ^d
Copper (ppm)	1.3 $\pm$ 0.065 ^a	1.0 $\pm$ 0.05 ^b	0.8 $\pm$ 0.04 ^c	0.5 $\pm$ 0.025 ^d

Table 6. Cumulative stress responsive index (CSRI) of both var. Hraswa and Manu ratna

Varieties	Treatments	
	C	S
Hraswa	92271	76860
Manu ratna	102885	64499

Table 7. Effect of BI priming on fresh weight (mg), dry weight (mg), shoot length (cm), root length (cm), pigment composition and MDA content in the seedlings of rice varieties Hraswa and Manu Ratna. Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Variety	Concentration (ppm)	Shoot Length (cm)	Root Length (cm)	FW (g)	DW (g)	Chlorophyll a ($\mu\text{g/g DW}$)	Chlorophyll b ($\mu\text{g/g DW}$)	Total chlorophyll ($\mu\text{g/g DW}$)	Carotenoid ($\mu\text{g/g DW}$)	MDA ($\mu\text{mol/g DW}$)
Hraswa	Control (0)	13.5 $\pm$ 0.6 ^d	20 $\pm$ 0.34 ^{bc}	0.48 $\pm$ 0.1 ^c	0.05 $\pm$ 0.43 ^c	1205.318 $\pm$ 0.5 ^d	466.856 $\pm$ 0.46 ^e	8453 $\pm$ 0.1 ^e	384 $\pm$ 0.46 ^e	2.5 $\pm$ 0.24 ^d
	25	14 $\pm$ 0.7 ^c	28 $\pm$ 0.65 ^a	0.84 $\pm$ 0.2 ^a	0.07 $\pm$ 0.06 ^a	1467.645 $\pm$ 0.3 ^a	714.066 $\pm$ 0.58 ^a	11631 $\pm$ 0.32 ^a	799 $\pm$ 0.23 ^a	2.2 $\pm$ 0.87 ^e
	30	16 $\pm$ 0.8 ^b	22.5 $\pm$ 0.7 ^{bc}	0.75 $\pm$ 0.1 ^b	0.064 $\pm$ 0.4 ^b	1211.283 $\pm$ 0.2 ^c	519.426 $\pm$ 0.73 ^d	8956 $\pm$ 0.42 ^d	514 $\pm$ 0.54 ^d	2.8 $\pm$ 0.64 ^c
	35	19.6 $\pm$ 0.9 ^a	20.5 $\pm$ 0.31 ^b	0.64 $\pm$ 0.3 ^c	0.063 $\pm$ 0.3 ^b	1236.704 $\pm$ 0.6 ^b	540.722 $\pm$ 0.24 ^c	9240 $\pm$ 0.2 ^c	533. $\pm$ 0.28 ^c	3.2 $\pm$ 0.92 ^b
	40	14.5 $\pm$ 0.7 ^c	16.75 $\pm$ 0.1 ^c	0.57 $\pm$ 0.7 ^d	0.058 $\pm$ 0.1 ^b	1338.23 $\pm$ 0.6 ^b	575.576 $\pm$ 0.53 ^b	9911 $\pm$ 0.3 ^b	545 $\pm$ 0.32 ^b	3.4 $\pm$ 0.43 ^a
Manu Ratna	Control (0)	13.5 $\pm$ 0.6 ^c	20 $\pm$ 0.16 ^{bc}	0.51 $\pm$ 0.6 ^c	0.04 $\pm$ 0.26 ^d	1055.447 $\pm$ 0.8 ^b	357.818 $\pm$ 0.26 ^b	6933 $\pm$ 0.46 ^b	398 $\pm$ 0.76 ^b	3.1 $\pm$ 0.24 ^d
	25	18 $\pm$ 0.9 ^a	27 $\pm$ 0.25 ^a	0.92 $\pm$ 0.2 ^a	0.083 $\pm$ 0.2 ^a	1560.615 $\pm$ 0.5 ^a	522.042 $\pm$ 0.78 ^a	10187 $\pm$ 0.76 ^a	488 $\pm$ 0.14 ^a	2.5 $\pm$ 0.32 ^c
	30	14.5 $\pm$ 0.7 ^b	26 $\pm$ 0.65 ^a	0.84 $\pm$ 0.1 ^b	0.08 $\pm$ 0.3 ^b	762.822 $\pm$ 0.86 ^d	172.17 $\pm$ 0.56 ^d	4216 $\pm$ 0.75 ^d	229 $\pm$ 0.25 ^d	3.3 $\pm$ 0.14 ^c
	35	14 $\pm$ 0.7 ^b	23.3 $\pm$ 0.28 ^b	0.7 $\pm$ 0.32 ^c	0.06 $\pm$ 0.29 ^c	830.208 $\pm$ 0.94 ^c	311.628 $\pm$ 0.47 ^c	5731 $\pm$ 0.45 ^c	319 $\pm$ 0.34 ^c	3.8 $\pm$ 0.16 ^b
	40	13.8 $\pm$ 0.6 ^c	21 $\pm$ 0.24 ^b	0.65 $\pm$ 0.7 ^d	0.04 $\pm$ 0.16 ^d	586.998 $\pm$ 0.12 ^e	118.71 $\pm$ 0.86 ^e	3118 $\pm$ 0.63 ^e	178 $\pm$ 0.76 ^e	4.4 $\pm$ 0.43 ^a

the variety which showed ~50% decrease in various growth attributes (shoot length, fresh and dry weight) were selected as sensitive variety and those which maintained the same growth features without drastic reduction was identified as tolerant variety. In comparison to their respective controls, both rice varieties under stress (S) exhibited a significant decrease in the CSRI. A significant drop in total growth and physiological performance was indicated by the CSRI calculated for Manu ratna, which dropped to 64,499 under stress. While, Hraswa recorded a CSRI of 76,860 under stress, which demonstrated that Hraswa showed a comparatively higher CSRI under stress indicating greater adaptability and tolerance than Manu ratna. Here var. Hraswa maintained the growth features, whereas variety Manu ratna showed 60% growth retardation at nutrient deficient condition and accordingly Hraswa was considered as tolerant and Manu ratna as sensitive variety. In contrast to Manu ratna, which is relatively more sensitive to stress, Hraswa is more resilient/tolerant to nutrient stress (Table 6).

4.5 Standardization of biostimulant for priming and soil application

4.5.1 Selection of optimal concentration of BI for priming

The *O. sativa* seeds were treated with various concentration of BI solutions (0, 25, 30, 35 and 40 ppm) and after these treatments the seeds were germinated as described in materials and methods. The growth parameters like shoot length, root length, fresh and dry weight, pigment composition and MDA accumulation in seedlings raised from BI primed seeds were recorded. The above growth parameters and biochemical features were maximum in the seedlings of two varieties when the seeds were primed with 25 ppm.

Priming at 25 ppm resulted in a significant improvement in the majority of characteristics in Hraswa var. when compared to the control. Improved below-ground growth was indicated by a significant 40% increase in root length and a slight (4%) increase in shoot length. Fresh weight and dry weight increased by 75% and 40%, respectively, over the control, indicating a positive impact on biomass accumulation. The results of the pigment study showed a significant improvement in photosynthetic pigments, with total chlorophyll rising by 38%, chlorophyll *a* by

22% and chlorophyll *b* by 53%. Furthermore, the MDA content dropped by 12% in comparison to the control, indicating less lipid peroxidation.

While in Manu ratna, biomass accumulation was more pronounced than pigment enhancement in response to 25 ppm priming. There was a 33% rise in shoot length and a 35% increase in root length followed by fresh weight and dry weight. Chlorophyll *a* and *b* levels increased by 28% and 24%, respectively, while carotenoids increased by 41%. MDA levels in Manu ratna remained almost constant, in contrast to Hraswa, indicating very modest progress in mitigating oxidative stress. Therefore, 25 ppm concentration of BI, was selected for priming the two varieties of *O. sativa* seeds subjected to stress and without any stress treatments (Table 7).

4.5.2 Standardization of priming duration

The time period for seed priming (the hours for which seeds were incubated in the respective priming solution) which brought about maximum increase in the growth attributes (shoot length, fresh and dry weight), pigment composition and MDA accumulation in the seedlings emerging from primed seeds was fixed for the entire experiments. Among the different priming period such as 6, 12 and 18 hour, it was found that 12 hour priming period showed maximum enhancement in growth attributes when compared to other time intervals in the case of both varieties of *O. sativa* subjected to stress and without any stress treatments (Table 8).

In Hraswa (25 ppm), chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids increased by 16, 38, 27 and 64% respectively at 12 hour compared to the control, while MDA content decreased by 8% at 12 hour indicating reduced lipid peroxidation. However, at 18-hour, pigment levels declined and MDA increased, reflecting reduced efficiency of prolonged priming.

In Manu ratna (25 ppm), the optimum priming duration was 12 hour. Chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids increased by 19, 15, 25 and 42% at 6 hour. Moreover, MDA levels decreased significantly by 29% at 6 hour indicating better stability with respect to oxidative stress. With extended priming (18 hour), pigments declined (chlorophyll *a* reduced by 32% and total

Table 8. Pigment composition and MDA content in seedling of rice varieties Hraswa and Manu ratna raised from seeds subjected to different priming periods 0 (control), 6, 12 and 18h priming. Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Variety	Hour	Chlorophyll <i>a</i> ($\mu\text{g/g DW}$)	Chlorophyll <i>b</i> ($\mu\text{g/g DW}$)	Total Cholorophyll ($\mu\text{g/g DW}$)	Carotenoid ($\mu\text{g/g DW}$)	MDA ($\mu\text{mol/g DW}$)
Hraswa (25 ppm)	Control (0)	1539.2 $\pm$ 0.25 ^c	641.9 $\pm$ 0.54 ^b	2179.2 $\pm$ 065 ^b	442.02 $\pm$ 0.56 ^b	1.4 $\pm$ 0.67 ^c
	6	1362.8 $\pm$ 0.65 ^d	556.8 $\pm$ 0.43 ^d	1920.4 $\pm$ 0.87 ^d	105.62 $\pm$ 0.54 ^d	2.0 $\pm$ 0.87 ^a
	12	1467.6$\pm$0.35^a	714.0$\pm$0.97^a	2461.7$\pm$0.85^a	721.49$\pm$0.48^a	1.2$\pm$0.82^d
	18	1459.2 $\pm$ 0.12 ^b	606.1 $\pm$ 0.67 ^c	2092.3 $\pm$ 0.56 ^c	191.68 $\pm$ 0.34 ^c	2.2 $\pm$ 0.36 ^b
Manu Ratna (25 ppm)	Control (0)	1358.7 $\pm$ 0.97 ^a	517.3 $\pm$ 0.56 ^b	1946.4 $\pm$ 0.45 ^b	442.02 $\pm$ 0.43 ^b	1.3 $\pm$ 0.65 ^c
	6	1310.8 $\pm$ 0.83 ^b	511.8 $\pm$ 0.43 ^b	1824.8 $\pm$ 0.76 ^c	405.35 $\pm$ 0.14 ^c	1.7 $\pm$ 0.37 ^a
	12	1560.6$\pm$0.79^a	522.0$\pm$0.45^a	2086.7$\pm$0.56^a	489.06$\pm$0.37^a	1.2$\pm$0.85^d
	18	883.6 $\pm$ 0.80 ^d	347.0 $\pm$ 0.67 ^c	1236.0 $\pm$ 0.45 ^d	279.02 $\pm$ 0.26 ^d	1.4 $\pm$ 0.57 ^b

Table 9: Standardization of different concentrations of biostimulant on soil application C (Control), ND (Nutrient deficient condition), 200 ppm 250 ppm and 300 ppm. Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Parameters	C	ND	200 (ppm)	250 (ppm)	300 (ppm)
Sucrase (mg.24h ⁻¹ g ⁻¹)	0.3 $\pm$ 0.011 ^c	0.15 $\pm$ 0.021 ^d	0.35 $\pm$ 0.007 ^b	0.46 $\pm$ 0.004 ^a	0.37 $\pm$ 0.001 ^b
Protease (mg.24h ⁻¹ g ⁻¹)	0.02 $\pm$ 0.01 ^d	0.015 $\pm$ 0.009 ^{cd}	0.028 $\pm$ 0.003 ^b	0.039 $\pm$ 0.005 ^a	0.023 $\pm$ 0.007 ^c
Dehydrogenase (mg.24h ⁻¹ g ⁻¹)	0.35 $\pm$ 0.02b ^c	0.20 $\pm$ 0.05 ^d	0.42 $\pm$ 0.013 ^b	0.49 $\pm$ 0.023 ^a	0.43 $\pm$ 0.003 ^b
Urease (mg.24h ⁻¹ g ⁻¹)	2.2 $\pm$ 0.06 ^c	1.4 $\pm$ 0.08 ^d	2.9 $\pm$ 0.03 ^b	3.8 $\pm$ 0.01a	3.3 $\pm$ 0.06 ^{ab}
SVI	634 $\pm$ 13.2 ^b	258 $\pm$ 4.9 ^e	622 $\pm$ 12.5 ^c	663 $\pm$ 15.4 ^a	614 $\pm$ 0.11.3 ^d

Table 10: Shoot length, root length, dry weight % and photosynthetic pigment composition in the seedlings of two rice varieties raised from BI primed and non-primed seedlings subjected to nutrient deficient stress. C (Control), C+B (Biostimulant treated unstressed condition), S (Stress-nutrient deficient condition) and S+B (Biostimulant treated seeds subjected to stress-nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Variety	Treatments	Shoot length (cm)	Root length (cm)	DW%	Chl <i>a</i> (mg/g FW)	Chl <i>b</i> (mg/g FW)	Chl <i>a+b</i> (mg/g FW)	Carotenoid (mg/g FW)
Hraswa	C	14 $\pm$ 0.43 ^c	20 $\pm$ 0.16 ^{bc}	9.54 $\pm$ 0.21 ^c	1.25 $\pm$ 0.003 ^c	0.465 $\pm$ 0.03 ^c	89.56 $\pm$ 0.003 ^c	0.398 $\pm$ 0.14 ^c
	C+B	20 $\pm$ 0.54 ^b	27 $\pm$ 0.12 ^a	9.84 $\pm$ 0.15 ^b	1.39 $\pm$ 0.32 ^b	0.575 $\pm$ 0.14 ^b	99.11 $\pm$ 0.16 ^b	0.533 $\pm$ 0.06 ^b
	S	11 $\pm$ 0.65 ^d	18 $\pm$ 0.16 ^c	8.33 $\pm$ 0.12 ^d	0.84 $\pm$ 0.16 ^d	0.466 $\pm$ 0.055 ^c	84.53 $\pm$ 0.29 ^d	0.214 $\pm$ 0.13 ^d
	S+B	28 $\pm$ 0.47 ^a	32 $\pm$ 0.05 ^a	10.41 $\pm$ 0.34 ^a	1.49 $\pm$ 0.01 ^a	0.714 $\pm$ 0.03 ^a	116.31 $\pm$ 0.13 ^a	0.799 $\pm$ 0.42 ^a
Manu ratna	C	12 $\pm$ 0.21 ^c	18 $\pm$ 0.012 ^c	8.8 $\pm$ 0.006 ^b	1.05 $\pm$ 0.24 ^c	0.352 $\pm$ 0.14 ^c	59.3 $\pm$ 0.05 ^c	0.38 $\pm$ 0.007 ^c
	C+B	16 $\pm$ 0.86 ^b	23 $\pm$ 0.03 ^b	9.46 $\pm$ 0.04 ^a	1.56 $\pm$ 0.06 ^a	0.522 $\pm$ 0.32 ^a	101.8 $\pm$ 0.01 ^a	0.488 $\pm$ 0.04 ^a
	S	10 $\pm$ 0.43 ^d	15 $\pm$ 0.17 ^d	6.11 $\pm$ 0.07 ^c	0.76 $\pm$ 0.15 ^d	0.172 $\pm$ 0.05 ^d	31.18 $\pm$ 0.45 ^d	0.229 $\pm$ 0.12 ^d
	S+B	18 $\pm$ 0.32 ^a	26 $\pm$ 0.18 ^a	8.99 $\pm$ 0.01 ^b	1.16 $\pm$ 0.04 ^b	0.398 $\pm$ 0.16 ^b	69.33 $\pm$ 0.27 ^b	0.419 $\pm$ 0.11 ^b

chlorophyll by 32% at 18 hour), while MDA increased by 23%, suggesting negative effects of longer exposure.

4.5.3 Standardization of effective concentration of biostimulants for soil application

The influence of different concentrations (200, 250, and 300 ppm) of biostimulant formulation on soil enzymatic activities—sucrase, protease, dehydrogenase, and urease—as well as on the seedling vigor index (SVI), was compared with the control (C) and nutrient deficient (ND) soil samples (Table 9).

Sucrase activity in the control was $0.30 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$, which decreased by 50% in the ND sample ($0.15 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$). Upon treatment, sucrase activity increased by 16.7%, 53.3%, and 23.3% at 200, 250, and 300 ppm, respectively, compared to the control. The highest activity ($0.46 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$) was observed at 250 ppm, indicating the optimal dose for sucrase stimulation.

Protease activity in the control ($0.020 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$) decreased by 25% under the ND condition ($0.015 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$). The activity increased by 40%, 95%, and 15% at 200, 250, and 300 ppm, respectively. Similar to sucrase, the highest protease activity was recorded at 250 ppm ($0.039 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$), suggesting enhanced protein degradation potential at this concentration.

Dehydrogenase activity followed a comparable trend. Compared to the control ($0.35 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$), ND sample reduced activity by 42.9%. The enzymatic activity increased by 20%, 40%, and 22.9% at 200, 250, and 300 ppm, respectively. The maximum dehydrogenase activity ($0.49 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$) occurred at 250 ppm, representing a 40% enhancement over the control.

Urease activity in the control ($2.2 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$) decreased by 36.4% in the ND sample ($1.4 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$). With treatment, the activity increased by 31.8%, 72.7%, and 50.0% at 200, 250, and 300 ppm, respectively. The 250 ppm treatment again exhibited the highest urease activity ($3.8 \text{ mg } 24 \text{ h}^{-1} \text{ g}^{-1}$).

In the SVI, ND treatment reduced drastically by 59.3%. Subsequent treatments at 200, 250, and 300 ppm resulted in -1.9%, +4.6%, and -3.2% changes relative to the control. The maximum SVI value at 250 ppm indicates the most favorable seedling vigor characteristics.

Overall, the 250 ppm treatment consistently produced the greatest increases in enzymatic activities and SVI, demonstrating that this concentration enhances microbial and biochemical performance most effectively, while higher concentrations (300 ppm) showed mild inhibitory effects.

4.6 Growth parameters

For each experiment, there were four treatments with three replicates in both varieties of *O. sativa*. The four treatments include C (control: non-treated seeds not subjected to any stress treatment), C+B (BI primed seeds not subjected to any stress treatment), S (non-primed seeds subjected to nutrient deficient stress condition), S+B (BI primed seeds subjected to nutrient deficient stress condition).

C+B treatment significantly increased root length (45 and 38%), shoot length (52 and 46%) and DW% (38 and 23%) in both tolerant and sensitive varieties in comparison to the control. Reduced root length (12 and 18%), shoot length (16 and 22%) and DW % (15 and 20%) were observed in seedlings subjected to S treatment of both varieties when compared to that of control. On the other hand, S+B treatment showed an enhancement of root length (24 and 30%), shoot length (20 and 44%) and dry weight % (12 and 28%) in sensitive and tolerant varieties respectively when compared to that of control. C+B and S+B elicited an enhancement of various growth-related parameters under both stress and non-stress conditions (Table 10).

4.7 Physio-chemical parameters and carbohydrate composition

Various physiological and biochemical parameters was recorded in leaves of both rice varieties emerging from treated and non-treated seedlings at 10th day of seedling stage, 21th day of tillering stage, 40th day of booting stage and 75th day of ripening stage. At the end of tillering stage, the soil was amended with the application of 250 ppm of BI biostimulant, three times on alternative days in C+B

and S+B treatments. The control and S treatments were amended with water, instead of the biostimulant.

4.7.1 Photosynthetic pigments

Total chlorophyll and carotenoid content in leaves of both varieties showed a decreasing trend from tillering to ripening stage in nutrient deficient condition but only negligible change was observed in seedling stage as compared to control (Fig. 3). In var. Hraswa, C+B and S+B treatment showed higher accumulation of total chlorophyll (35-55%) and carotenoid (30-48%) content in tillering and booting stages followed by a decrease of the same in ripening stage. However, these pigment contents were increased significantly only in the booting stage in var. Manu ratna. In booting stage of var. Manu ratna, S treatment recorded a decrease in total chlorophyll (47 and 5%) and carotenoid content (40 and 46%) relative to control plants. Whereas S+B and C+B treatments, showed increased levels of total chlorophyll (46% and 31%) and carotenoid content (51% and 37%) respectively with respect to the control. The increase in carotenoid content was much more than that observed in the case of chlorophyll content under treated condition (Fig. 21).

4.7.2 Sucrose

Sucrose concentration in leaves showed significant variation ($p < 0.05$) across developmental stages, treatments, and between the two-rice var. Hraswa and Manu ratna. In tolerant var. Hraswa, sucrose level steadily increased from the seedling to the booting stage. The C+B treatment recorded the highest sucrose accumulation at the booting stage (48%), significantly higher than the untreated control. S+B treatment also markedly enhanced sucrose content (49%) compared to control. During the ripening stage, sucrose levels declined across all treatments, but C+B (21%) and S+B (26%) treatments maintained significantly higher sucrose concentrations as compared to control (Fig. 21).

A similar pattern was observed in sensitive var. Manu ratna. However, the magnitude of sucrose accumulation was generally lower than in var. Hraswa. At the booting stage, C+B treated plants exhibited the highest sucrose levels (35%),

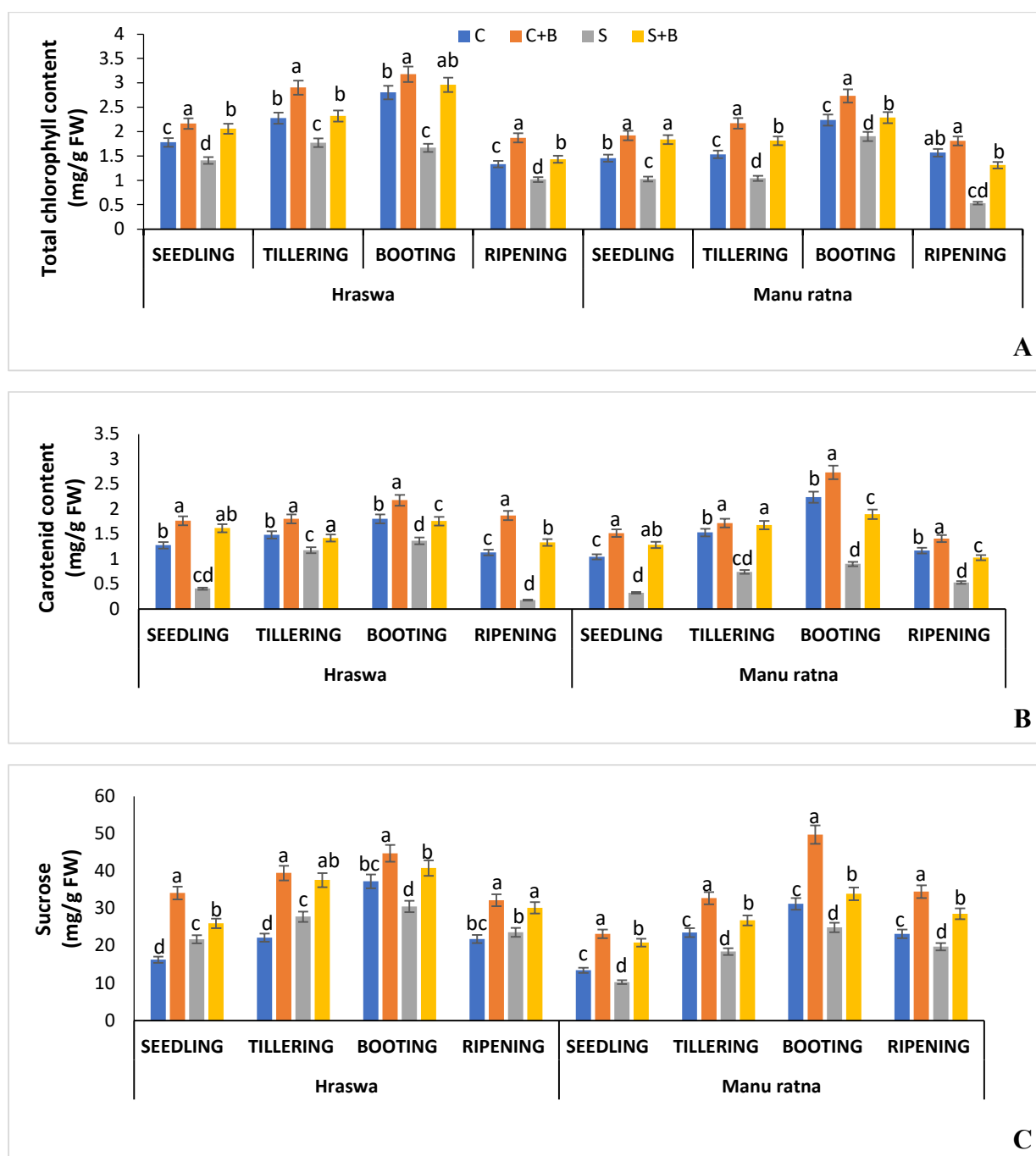


Fig. 21: Total chlorophyll (A), Carotenoid (B) and Sucrose (C) contents in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

significantly outperforming other treatments ($p < 0.05$). Sucrose content was notably lower in the S treatment at all stages, indicating the adverse impact of stress. However, S+B treatment improved sucrose levels, especially during tillering (23%) and ripening stages (33%) with respect to control. Across both varieties, sucrose accumulation was highest at the booting stage under C+B treatment, while stress alone (S) consistently resulted in the lowest sucrose levels. Both C+B and S+B treatments effectively mitigated the negative effects of stress, maintaining higher sucrose content throughout various developmental stages (Fig. 21).

4.7.3 Total soluble sugars

In the tolerant var. Hraswa, total soluble sugar levels progressively increased from seedling to booting stages followed by a slight decrease in ripening stage (Fig. 22). Plants subjected to C+B treatment exhibited the highest accumulation of total sugars at the booting stage, showing an increase of 71% followed by S+B treatment which recorded a notable rise of 38% relative to control. In S treatment, a significant decline in total soluble sugar content was observed at different growth stages specifically at booting and ripening stages as compared to control. While biostimulant treatments i.e., C+B (53%) and S+B (28%) maintained significantly higher total soluble sugar content in booting stage than the untreated control.

Similarly, in var. Manu ratna, a comparable trend was observed, although with lower magnitudes. Under S treatment, total soluble sugar content decreased across different stages with significant reduction recorded in ripening stage (42%) as compared to control. The C+B treatment enhanced total soluble sugar accumulation in seedling (42%), tillering (37%), booting (41%) and ripening stage (48%) relative to the control, while S+B treatment resulted in a 28% increase during booting and a 42% increase during ripening. Overall, both treatments C+B and S+B consistently promoted higher total soluble sugar accumulation across different developmental stages compared to control plants, with the booting and ripening stages identified as the critical periods of maximum sugar accumulation (Fig. 22).

4.7.4 Reducing sugars

In tolerant var. Hraswa, the S treatment resulted in a significant decrease of reducing sugars throughout the growth stages but a negligible increase in reducing

sugars was observed during the booting (13%) stage as compared to untreated control. While an increase in reducing sugars content was observed under the S+B treatment, with the highest increase recorded at the seedling (104%), ripening stage (58%) followed by the booting (33%) and tillering stages (10%) with respect to control. In the C+B treatment, reducing sugars accumulation was significantly improved across all growth stages, with substantial increases noted at the seedling (66%), tillering (12%), booting (18%) and ripening (49%) stages compared to control (Fig. 22).

While in sensitive var. *Manu ratna*, the S+B treatment recorded the highest increase in reducing sugars content at the seedling stage (57%), followed by tillering (32%), booting (28%) and ripening stages (21%) as compared to control. The C+B treatment showed significantly enhanced reducing sugars level during seedling (71%) and tillering (40%) followed by booting and ripening stages compared to control. The S treatment resulted in moderate increases during earlier growth stages of tillering and booting but a substantial reduction occurred during ripening stage (21%) (Fig. 22).

4.7.5 Non-reducing sugars

In tolerant var. *Hraswa*, non-reducing sugars levels increased progressively from the seedling to the booting stage, with the C+B treatment recording the highest accumulation at the booting stage (120%), followed closely by the S+B treatment (77%). The S treatment showed reduction across the different growth stages with significant decrease at booting stage (33%) as compared to control. During the ripening stage, non-reducing sugars declined across all treatments; however, the C+B (54%) and S+B (37%) treatments maintained significantly higher non-reducing sugars content compared to the control (Fig. 22).

In sensitive var. *Manu ratna*, the maximum non-reducing sugars accumulation was observed at the booting stage as compared across all stages. The C+B treatment showed the highest increase in seedling (41%), followed by booting (57%) and ripening stage (45%). The S+B treatment resulted in highest increase of

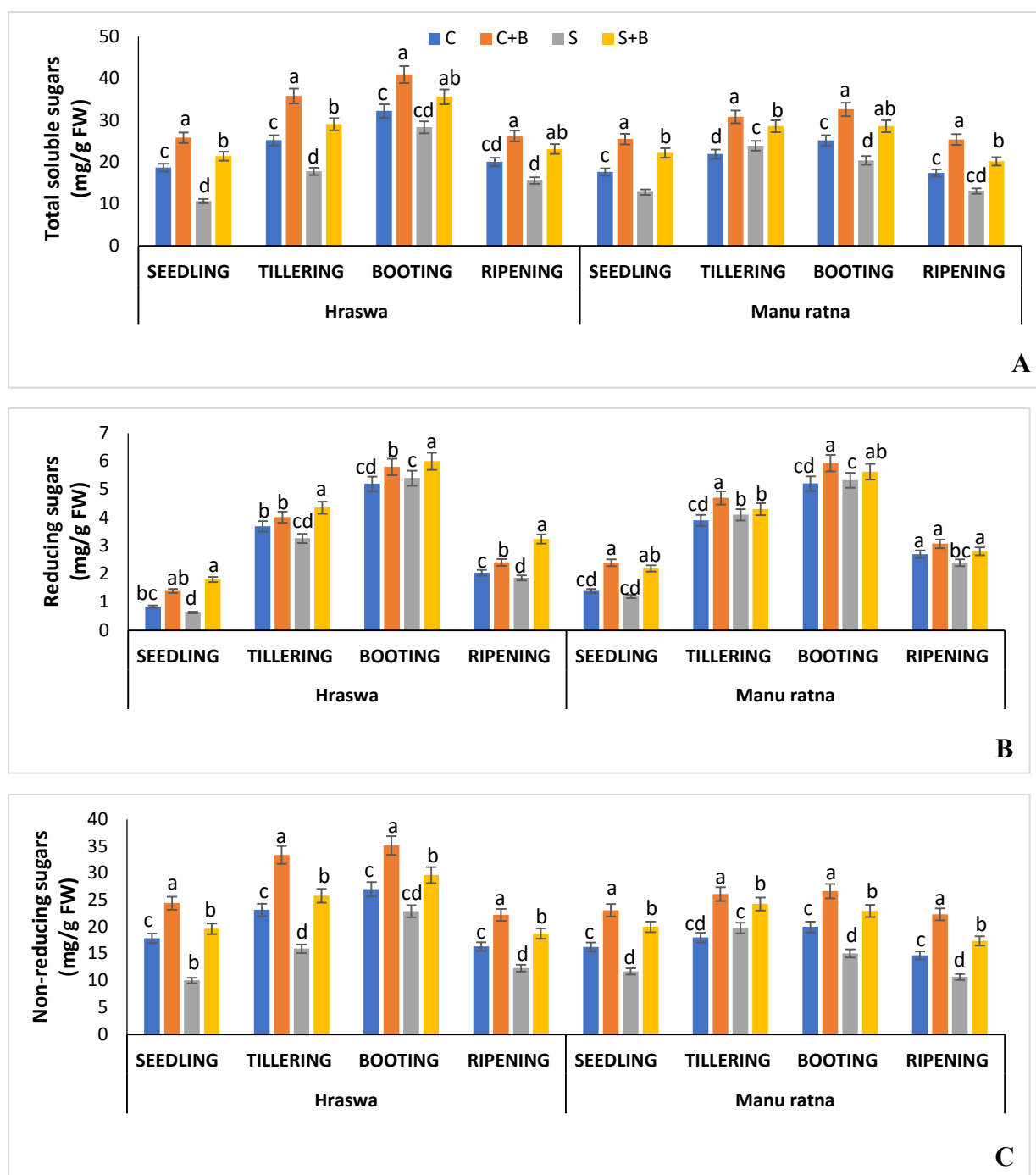


Fig. 22: Total soluble sugars (A), Reducing sugars (B) and Non-reducing sugars (C) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant +Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

non-reducing sugars in ripening (35%) and booting stage (21%) while negligible decrease in seedling stage was observed compared to control (Fig. 22).

4.7.6 Sucrose synthase

In tolerant var. Hraswa, sucrose synthase activity steadily increased from the seedling to the ripening stage (Fig. 23). The C+B treatment consistently recorded the highest enzyme activity at each developmental stage, peaking during the seedling (32%), booting (54%) and ripening stages (34%), which was significantly higher than the untreated control. The S+B treatment also markedly enhanced sucrose synthase activity across all stages, particularly at booting (30%) and ripening stages (48%) compared to control. Although S treatment reduced enzyme activity, its negative effect was mitigated when combined with biostimulant treatment, as seen in the S+B treatment. Overall, the C+B and S+B treatments effectively enhanced sucrose synthase activity in Hraswa, with the highest activity was recorded at the ripening stage.

A similar trend was observed in the sensitive var. Manu ratna, and the magnitude of sucrose synthase activity was generally higher than in Hraswa (Fig. 23). The C+B treatment led to the highest activity at the booting stage (48%), significantly outperforming other treatments ($p < 0.05$). The S+B treatment also improved enzyme activity, particularly during the booting (25%) and ripening stages (18%) compared to the control. The S treatment consistently resulted in the lowest activity across all stages, highlighting the adverse effect of stress. However, both C+B and S+B treatments effectively alleviated stress-induced reduction in sucrose synthase activity throughout different stages of plant development.

Across both varieties, sucrose synthase activity was highest under C+B treatment, especially during the booting and ripening stages. The S treatment consistently showed the lowest levels, while combination treatments (C+B, S+B) successfully mitigated stress impacts, sustaining higher enzymatic activity across different developmental stages (Fig. 23).

4.7.7 Sucrose phosphate synthase

In the tolerant variety Hraswa, sucrose phosphate synthase activity showed a steady increase from the seedling stage, reaching a peak at the booting stage before declining at ripening (Fig. 23). The C+B treatment consistently exhibited the highest activity, with maximum at seedling (33%) and ripening stages (39%) significantly higher than the untreated control. The S+B treatment also led to enhanced activity, particularly at tillering (23%) and ripening (18%), demonstrating its effectiveness in mitigating stress effects. In contrast, the S treatment resulted in the lowest enzyme activity at all stages, especially during the seedling (33%) and ripening stages (40%) as compared to the control.

In the sensitive variety Manu ratna, a similar trend was observed, although the levels of sucrose phosphate synthase activity were generally lower than those in Hraswa (Fig. 23). The highest activity was recorded under the C+B treatment during the seedling (71%) and tillering stages (45%), followed by the S+B treatment in seedling (38%) with respect to control. The S treatment consistently resulted in reduced activity of the enzyme across all stages, with the lowest values recorded during seedling and ripening stages. Overall, both varieties exhibited the highest sucrose phosphate synthase activity at the booting stage under C+B treatment, while the S treatment alone led to the most pronounced decline. The S+B treatment showed a clear protective effect, improving activity of SPS under stress and maintaining higher levels throughout the different stages of development.

4.7.8 Starch content

Starch concentrations showed significant variation ($p < 0.05$) across developmental stages, treatments, and between the two rice varieties, Hraswa and Manu ratna (Fig. 23). In tolerant var. Hraswa, starch levels steadily increased from the seedling to the ripening stage. The S+B treatment recorded the highest starch accumulation at the ripening stage (42%), significantly higher than the untreated control. C+B treatment also markedly enhanced starch content (68%) compared to control. During the early stages, starch levels were moderately higher under C+B and S+B treatments, while the S treatment consistently showed the lowest values. At

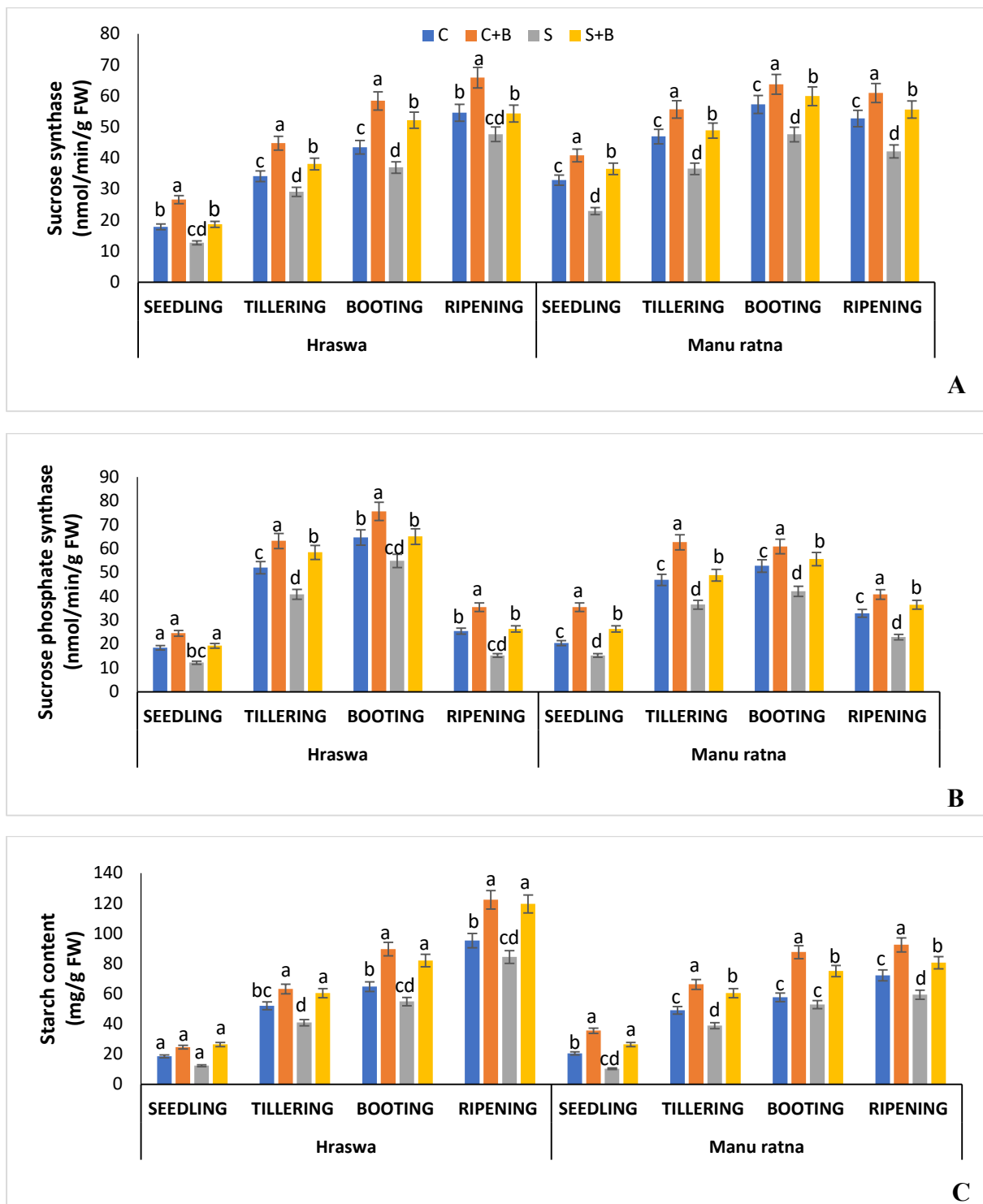


Fig. 23: Sucrose synthase activity (A), Sucrose phosphate synthase (B) and Starch contents (C) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

ripening, starch content declined under S, but C+B and S+B treatments maintained significantly higher starch levels as compared to control.

A similar pattern was observed in sensitive var. Manu ratna, although the magnitude of starch accumulation was generally lower than in var. Hraswa (Fig. 23). At the ripening stage, C+B treated plants exhibited the highest starch levels (43%), significantly outperforming other treatments ($p < 0.05$). Starch content was notably lower in the S treatment at all stages, indicating the adverse impact of stress. However, S+B treatment improved starch levels, especially during the booting (32%) and ripening stages (41%) with respect to control. Across both varieties, starch accumulation was highest at the ripening stage under C+B or S+B treatments, while S treatment consistently resulted in the lowest starch levels. Both C+B and S+B treatments effectively mitigated the negative effects of stress, maintaining higher starch content throughout the different stages of development.

4.8 Biochemical parameters

4.8.1 Leaf osmolality

Leaf osmolality becomes more negative (reduced) during S treatment in both varieties while tolerant variety Hraswa exhibited remarkable reduction in seedling stage (73%) and ripening stage (45%) followed by an increase in tillering (53%) and booting (62%) stages as compared to control. Similarly, S+B treatment recorded negligible increase in leaf osmolality in seedling stage (34%) followed by significant increment in tillering (54%), booting stages (62%) and a decrease was recorded in ripening stage. In C+B treatment significant increase in osmolality was recorded throughout the growth stages except ripening stage where 22% decrease was observed (Fig. 24).

In sensitive var. Manu ratna, considerable reductions in osmolality was observed in S condition with maximum recorded at seedling stage (43%). An increment in osmolality was recorded in tillering (41%), booting (62%) and ripening stages (24%) as compared to control. Nutrient deficiency driven reduction in leaf osmolality was recovered with the biostimulant treatment. In S+B treatment a

significant increase was recorded in tillering (48%) and booting stages (62%) while seedling stage (18%) and ripening stage (15%) recorded a least significant increase with respect to control. Seedlings subjected to C+B treatment also showed increased osmolality with significant increase in booting (43%) and tillering stages (32%) followed by seedling (23%) and ripening stages (18%) as compared to control (Fig. 24).

4.8.2 Proline content

Generally, proline content was higher in var. Manu ratna as compared to var. Hraswa and a similar trend of increase was observed in various developmental stages of both varieties subjected to different treatments. When the different stages of rice were analysed in both varieties in booting stage a steep increase in proline content was observed when compared to seedling and tillering stages whereas in ripening stage a constant value was recorded same as that in booting stage (Fig. 24).

In var. Hraswa under S treatment, proline accumulation was significantly higher in seedling stage (65%) followed by tillering (47%), booting (69%) and ripening stages (31%) as compared to control. Similar to that, S+B treatment recorded increased proline accumulation across different stages and highest accumulation was recorded in seedling (34%) and ripening (21%) stages but negligible increase in tillering (16%) and booting stages (15%) were observed. Whereas in C+B treatment, proline accumulation showed least significant increase and negligible accumulation were seen in seedling and tillering stages as compared to the control (Fig. 24).

In var. Manu ratna under S treatment, decreased proline accumulation was observed in seedling (25%) followed by negligible increase in tillering, booting and ripening stages with respect to control. Under S+B treatment, significant increase of proline content was recorded in seedling (65%) and booting stages (54%) followed by tillering (41%) and ripening stages (20%) as compared to control. In C+B treatment, least significant increase of proline accumulation was recorded across all stages while only in seedling stage an increase of 24% was observed as compared to the control (Fig. 24).

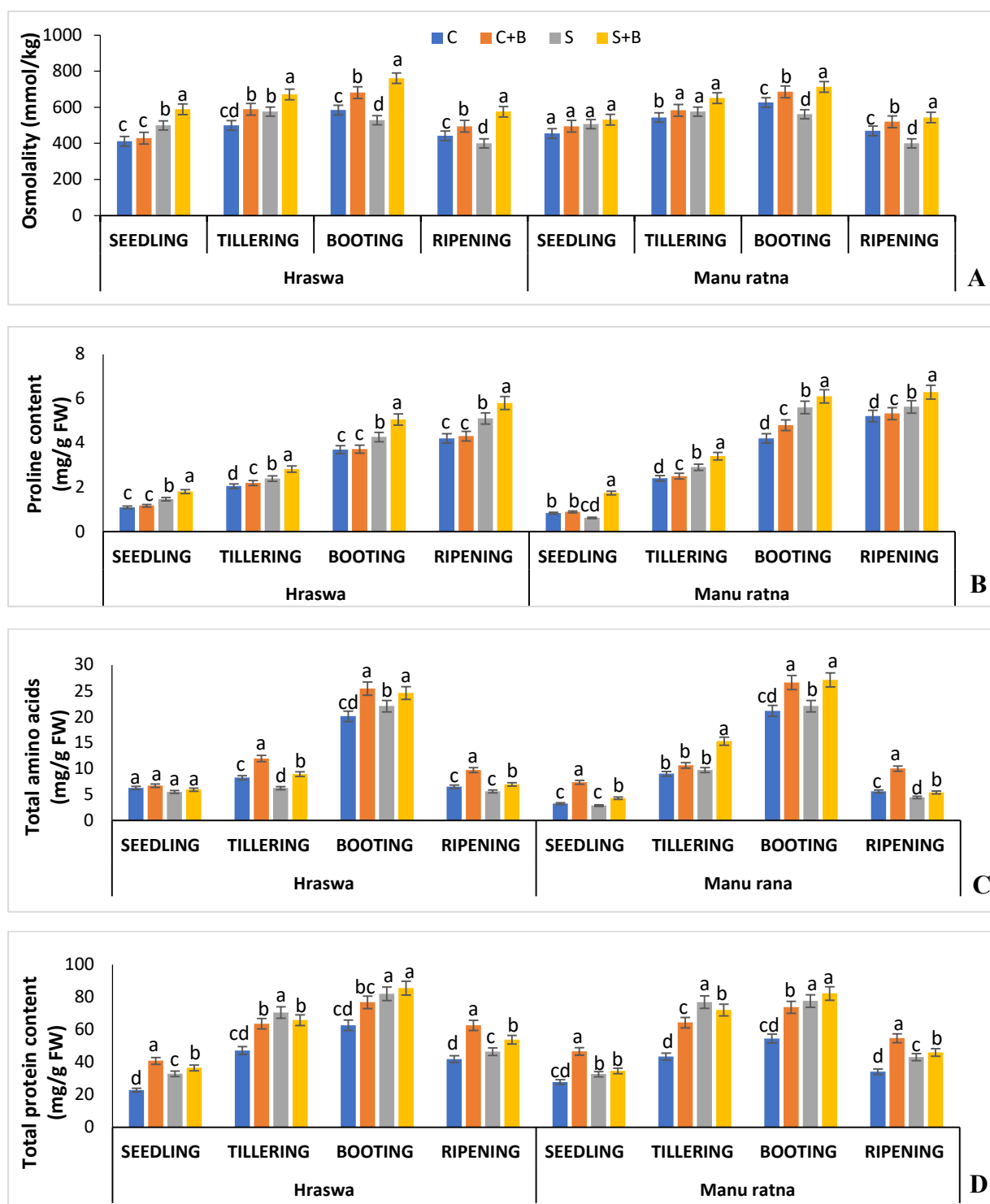


Fig. 24: Osmolality (A), Proline (B), Total amino acids (C) and Total protein content (D) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

4.8.3 Total free amino acids content

In tolerant var. Hraswa total free amino acids content was enhanced under S treatment in seedling stage (54%) and further negligible increase was observed in tillering, booting and ripening stages over that of control plants. Whereas under S+B treatment, a steady increase was recorded, starting with a minimum increase in seedling stage but maximum accumulation of free amino acids was recorded in booting stage (69%) as compared to control. In C+B treatment, tillering (59%) and ripening stages (39%) recorded an increase in total free amino acids content with respect to control (Fig. 24).

Under S treatment, var. Manu ratna showed a decrease in total free amino acids content by 21% and 34% in seedling and tillering stages respectively but recorded negligible increase in booting and ripening stages with respect to control. Similarly, in S+B treatment maximum enhancement in free amino acids was recorded in booting stage (69%) followed by ripening (45%) as compared to control. Under C+B treatment, higher accumulation of free amino acids content was observed in seedling stage (157%) and tillering stage (89%) but increase in booting and ripening stage was below 25% as compared to control plants (Fig. 24).

4.8.4 Total protein content

In tolerant var. Hraswa, under S treatment the accumulation of total protein was higher in all the growth stages of rice but maximum increase was observed in booting (62%), ripening (57%) and seedling stages (48%) whereas least enhancement was recorded in tillering stage (30%) as compared to control. However, in S+B treatment maximum increase was observed in seedling (64%) and booting stages (48%) followed by negligible increase in tillering and ripening stages as compared to control. In C+B treatment significant increase in total protein content was observed in seedling stage (84%) whereas a decreasing trend was observed throughout other growth stages (Fig. 24)

In sensitive variety Manu ratna, under S treatment maximum enhancement in total protein content was recorded in booting (87%) followed by ripening stages

(66%) as compared to control. Similarly, in S+B treatment the maximum accumulation was observed in booting (67%) followed by ripening stage (50%) as compared to control. However, a varied and enhanced response was shown in C+B treatment, where seedling (77%) and tillering stages (70%) recorded significant increase as compared to control condition (Fig. 24).

4.8.5 Malondialdehyde (MDA) content

Lipid peroxidation induced biological damage to membrane was measured in terms of MDA content. In Var. Hraswa, under S treatment higher MDA accumulation was observed across growth stages among which significant increase was seen in ripening (48%) and booting stages (45%) with respect to control. However, as a result of S+B treatment, a reduction in MDA was recorded and it was significant in seedling (40%) and booting stages (38%) but negligible increase in MDA content was observed in tillering and ripening stages as compared to control. But C+B treatment led to significant reduction in MDA content both in ripening and seedling stages with respect to control (Fig. 25).

Under S treatment in var. Manu ratna an increasing trend in MDA content was observed throughout the different growth stages of rice. The maximum accumulation was recorded in booting stage (38%) followed by seedling and ripening stages as compared to control. However, S+B treatment resulted in negligible decrease of MDA content in all stages of rice with respect to control. Under C+B treatment, significant decline in MDA content was observed in booting (53%), ripening (48%) and seedling (37%) stages as compared to control (Fig. 25).

4.8.6 Superoxide (O_2^-) content

Rate of ROS production in rice plants was varied, depending on the different growth stages. In var. Hraswa, under S treatment maximum superoxide production was recorded in ripening (45%) followed by booting stages (37%) as compared to control. Whereas S+B treatment initially reduced the superoxide content in tillering (29%), booting (17%) and seedling stages but an increase was observed in ripening stage (20%) of rice plants as compared to control. In C+B treatment significant

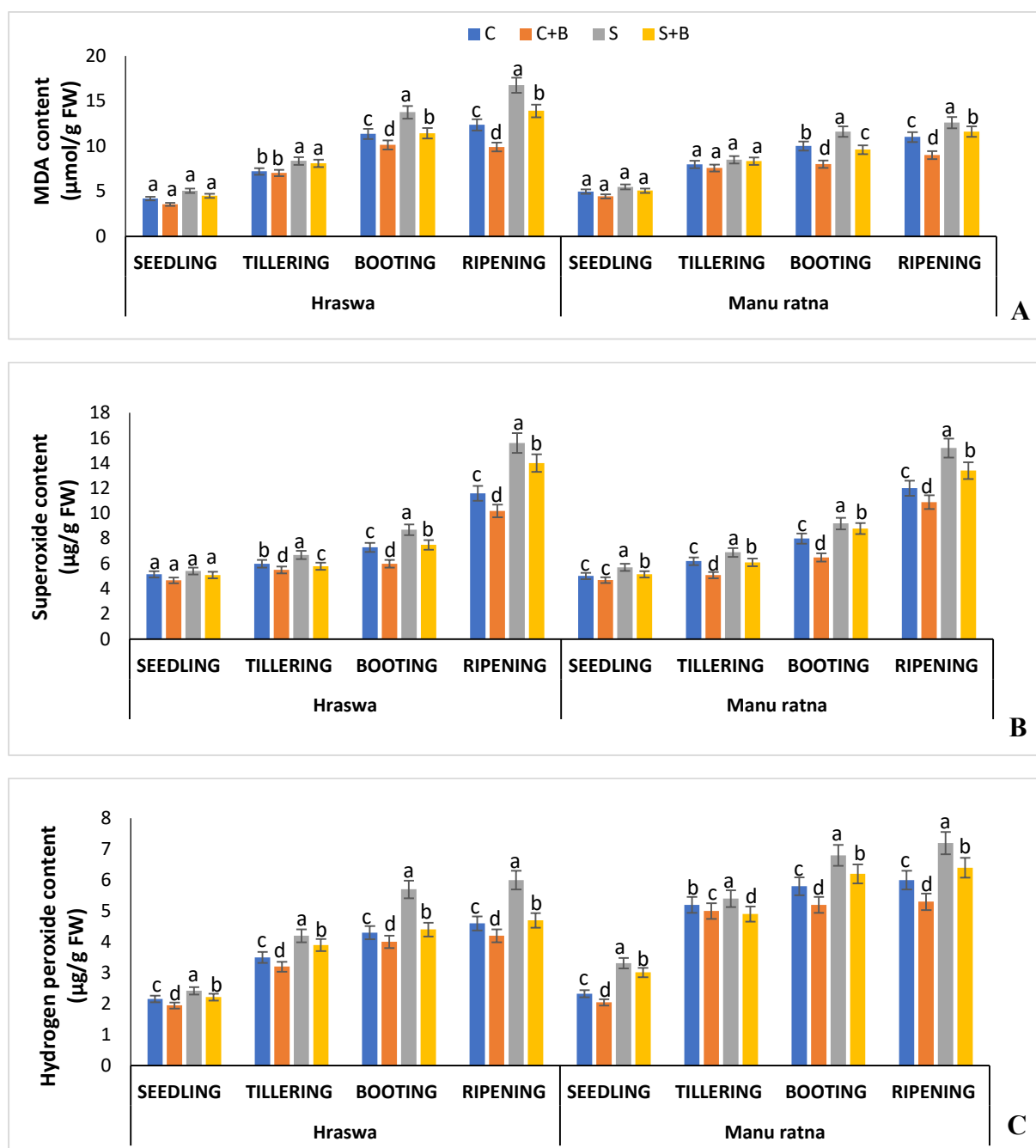


Fig. 25: MDA (A), Superoxide (B) and Hydrogen peroxide (C) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

decrease in superoxide content was observed and maximum decrease was shown in booting stage (54%) followed by ripening stage as compared to control (Fig. 25).

In var. Manu ratna under S treatment, significant increase of superoxide's was recorded in ripening stage followed by booting and seedling stages with respect to control plants. Whereas negligible difference was exhibited in S+B treatment with respect to control. In C+B treatment significant decrease in superoxide content was recorded and maximum decline was observed in booting (37%) and tillering stages (25%) as compared to control plants (Fig. 25).

4.8.7 Hydrogen peroxide (H₂O₂) content

In Var. Hraswa, higher rate of hydrogen peroxide accumulation was observed in ripening (58%) and booting stages (55%) under S treatment with respect to control. However, in S+B treatment, a reduction in hydrogen peroxide was recorded and it was significant in seedling (30%) and booting stages (48%) but negligible increase was observed in tillering and ripening stages as compared to control. But C+B treatment led to significant reduction of H₂O₂ in both ripening and seedling stages with respect to control (Fig. 25).

Under S treatment in var. Manu ratna an increasing trend in hydrogen peroxide production was observed throughout the different growth stages of rice. The maximum accumulation was recorded in booting stage (58%) followed by seedling and ripening stages as compared to control. However, S+B treatment resulted in negligible decrease in all stages of rice with respect to control. Under C+B treatment, significant decline in hydrogen peroxide production was observed in booting (53%), ripening (48%) and seedling (37%) stages as compared to control (Fig. 25).

4.8.8 Non-Enzymatic antioxidants

4.8.8.1 Total phenolics content

In the tolerant var. Hraswa, a minimal rise of total phenolics content was noticed under S treatment in seedling, tillering and booting stages but further in

ripening stage a severe decline was recorded as compared to control plants. Whereas under S+B treatment an increase in total phenolic content was recorded with maximum increase in seedling (80%) and tillering stages (74%) but in later stages such as booting and ripening only a negligible increase was seen as compared to control. In C+B treatment, total phenolic accumulation was enhanced and significant enhancement was recorded in seedling stage (74%), followed by tillering stage (65%) with respect to control (Fig. 26).

Under S treatment, decrease in total phenolics content was recorded in var. Manu ratna and the decrease was 41% and 34% in seedling and tillering stages respectively but further decrease was negligible in booting and ripening stages with respect to control. But in the case of S+B treatment, negligible enhancement was recorded in different growth stages as compared to control. Under C+B treatment, higher accumulation of total phenolics content was observed in seedling stage (68%) and tillering stages (46%) but further increase was lesser and it was 28% in booting and 21% in ripening stages over that of control plants (Fig. 26).

4.8.8.2 Ascorbate (AsA) content

Under S treatment, a negligible increase in AsA content was recorded in var. Hraswa and maximum increase was recorded in seedling stage (25%) as compared to control. In other stages decrease in AsA content was recorded and maximum decrease was observed in ripening stage (34%). In S+B treatment, increased AsA content was observed in seedling (44%) and tillering stages (23%) but further a negligible decrease was observed in booting and ripening stages compared to control plants. However, in the case of C+B treatment a general trend of increasing AsA content was seen, and maximum content was observed in seedling stage (51%) followed by booting and ripening stages as compared to control (Fig. 26).

Var. Manu ratna under S treatment also exhibited a decreasing trend of AsA content and maximum decline was shown in booting (40%) and ripening stages (36%) whereas a negligible decrease was observed in seedling and tillering stages as compared to control. But in S+B treatment, initially a negligible increase was observed in seedling stage but further stages recorded a decreasing trend and

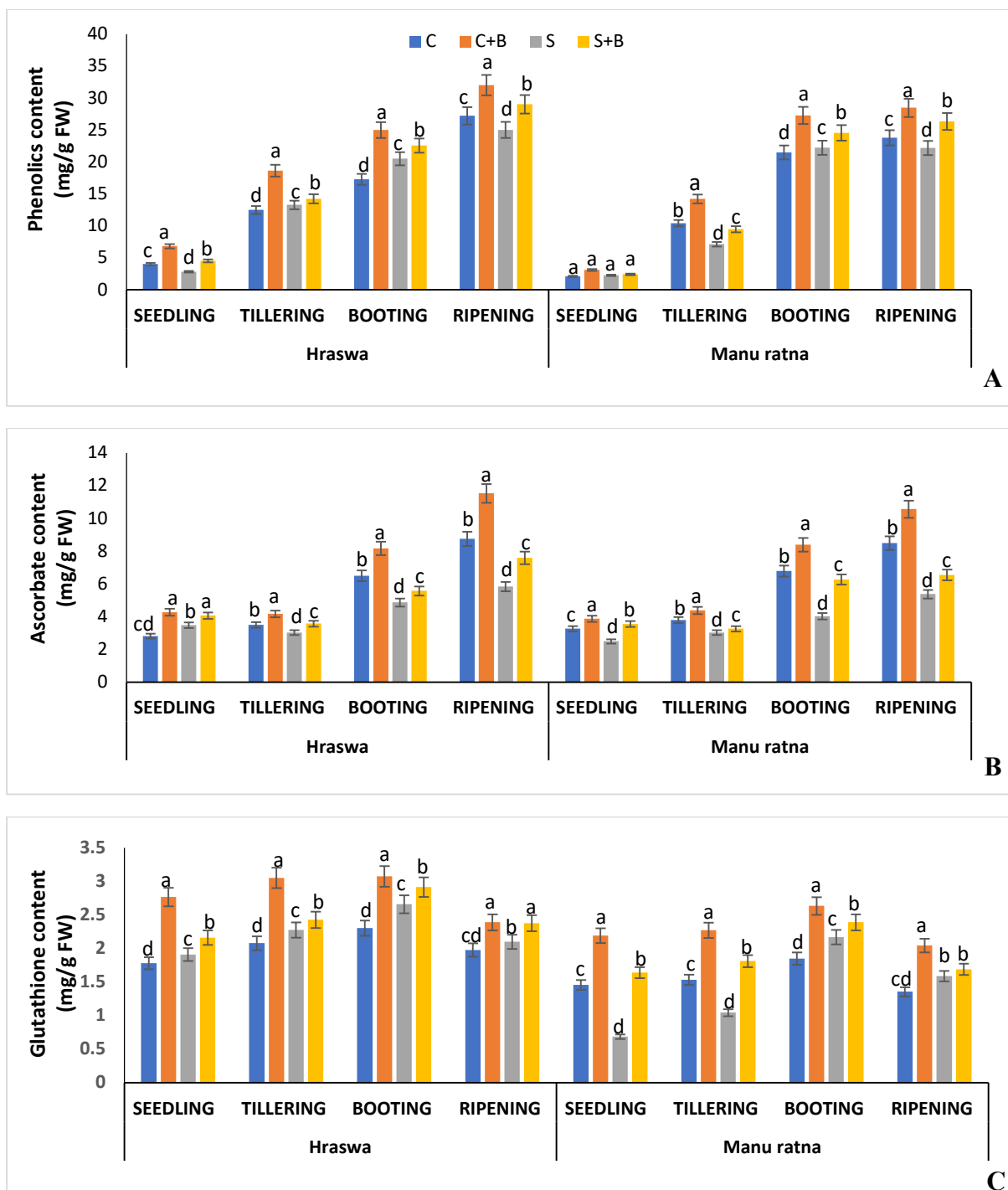


Fig. 26: Phenolics (A), Ascorbate (B) and Glutathione (C) content in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant +Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

profound decrease was recorded in ripening stage (38%) with respect to control. However, in the case of C+B treatment, the AsA content showed a steady increase throughout the different growth stages and maximum accumulation was recorded in ripening stage (46%) over that of control (Fig. 26).

4.8.8.3 Glutathione (GSH) content

In tolerant var. Hraswa under S treatment, GSH accumulation was significantly increased and maximum accumulation was observed in booting stage (77%) followed by ripening stage whereas a negligible increase was observed in seedling, and tillering stages. In the case of S+B treatment, the GSH content was maximally increased in booting (100%), seedling stages (77%) and followed by ripening stage but only a negligible increase was achieved in tillering stage. In C+B treatment, maximum accumulation of GSH content was observed in booting (91%), seedling (60%) and tillering stages but further a decline was recorded in ripening stage as compared to control (Fig. 26).

Similar trend was observed in var. Manu ratna where treated plants accumulated higher GSH content as compared to non-treated ones. When subjected to S treatment, significant decrease in GSH accumulation was recorded in seedling (42%) and tillering stages (41%) whereas further increase was observed in booting (55%) and a lower level of increase in ripening stage (26%) as compared to control. Under S+B treatment, maximum accumulation of GSH content was observed in seedling (176%), followed by tillering and booting stages but further enhancement of GSH content in ripening stage was negligible with respect to control. In C+B treatment, GSH accumulation was far more enhanced in seedling (42%), tillering (56%), booting (66%) and ripening stages (39%) as compared to control (Fig. 26).

4.8.9 Enzymatic antioxidants

4.8.9.1 Superoxide dismutase (SOD)

In tolerant variety Hraswa, under S treatment the decrease of SOD activity was in the range of 25%-45% in different growth stages. Whereas the SOD activity in S+B treatment was enhanced upto an extent of 35-67% with maximum activity

being recorded in seedling (67%) followed by booting stages (54%). In the case of C+B treatment, an increasing trend in SOD activity was observed and highest activity was found in seedling (128%) followed by ripening (83%) and booting (76%) stages (Fig. 27).

Under S treatment a decreasing trend in SOD activity was observed in var. Manu ratna and it was to an extent of 26- 44% in different growth stages and the highest decrease was found in ripening stage (44%). Whereas in S+B treatment decrease in SOD activity was initially observed in seedling and tillering stages but further there was an increase in activity to an extent of 35-48% in booting and ripening stages. In the case of C+B treatment, a steady increase in SOD activity was observed with maximum accumulation in booting (72%) followed by ripening stages (60%) (Fig. 27).

4.8.9.2 Catalase (CAT)

In var. Hraswa, the decrease of CAT activity was in the range of 32-78% in different growth stages under S treatment and the decrease was highest in seedling and booting stages as compared to control. Whereas in S+B treatment, CAT activity enhancement was in the range of 27%-44% in seedling-ripening stages. Maximum increase in CAT activity was observed in booting and ripening stages, over control plants on imparting nutrient deficiency stress. Under C+B treatment, the increase of CAT activity was higher in seedling followed by booting stages over control plants (Fig. 27).

In var. Manu ratna under S treatment, the decrease of CAT activity in seedling, tillering, booting and ripening stages was 25, 32, 48 and 54% respectively as compared to control, but in S+B treatment, the decrease was very negligible initially but further CAT activity showed an increase over that of control plants. However, in C+B treatment the CAT activity showed an increasing trend from seedling to ripening stages and maximum activity was observed in seedling (70%) followed by ripening stage with respect to control (Fig. 27).

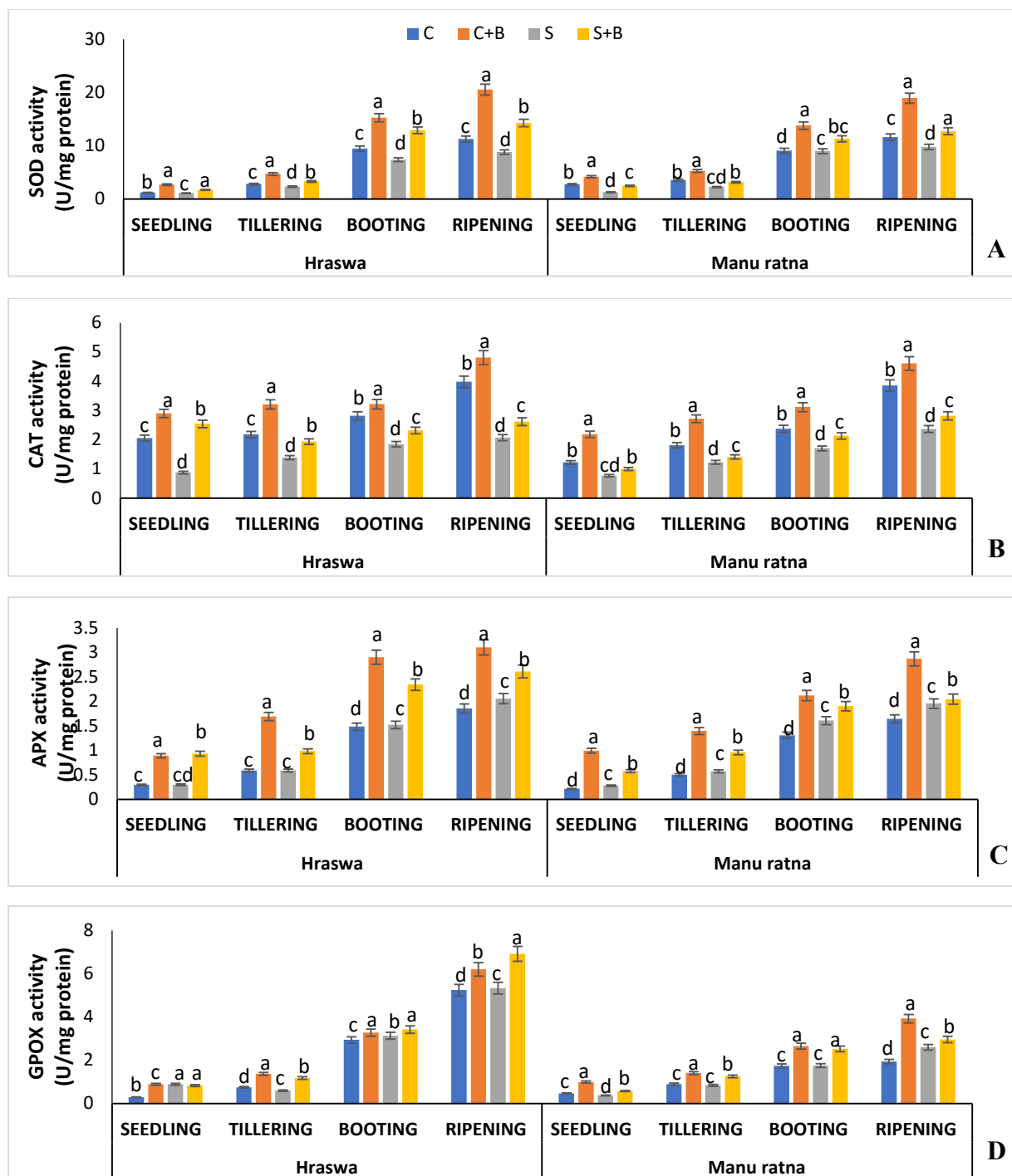


Fig. 27: SOD (A), CAT (B), APX (C) and GPOX (D) content in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at different stages of growth. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant +Nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$)

CAT activity was decreased in both varieties under nutrient deficiency as compared to their controls. But maximum activity was recorded in biostimulant treated plants as compared to control. When both varieties were compared, maximum CAT activity was observed in Hraswa under S+B treatment. However, CAT activity was more prominently observed in seedling and booting stages in both varieties under S+B and C+B treatments.

4.8.9.3 Ascorbate peroxidase (APX)

In tolerant var. Hraswa under S treatment, the APX activity showed negligible increase with highest increase in ripening stage (24%) followed by booting and tillering stages as compared to control. Whereas in the case of S+B treatment, significant increase in APX activity was recorded to an extent of 64-110% in seedling-ripening stages and maximum activity was shown in seedling stage (110%) with respect to control. In C+B treatment, the APX activity was enhanced and highest increase was found in seedling (200%) followed by tillering (112%), booting (97%) and ripening stages (88%) as compared to control (Fig. 27).

The S treatment induced enhancement in APX activity was least in var. Manu ratna (12-32%) in seedling to ripening stages and the increase was highest in seedling (32%) and booting stages (22%) as compared to control plants. In S+B treatment, significant increase in APX activity was observed to the extent of 35%-145% in seedling to ripening stages. The maximum increase of APX activity was comparatively higher in seedling (145%) and booting stages (98%) over that of the control. The C+B treatment also led to an increase in APX activity and it was to the extent of 28-37% in different growth stages (Fig. 27).

4.8.9.4 Guaiacol peroxidase (GPOX)

In var. Hraswa the decrease of GPOX activity was in the range of 32-78% in different growth stages under S treatment and the decrease was highest in seedling and booting stages as compared to control. Whereas in S+B treatment the increase in GPOX activity was in the range of 27%-94% in seedling-ripening stages with maximum increase was observed in seedling and booting stages, over control plants.

Under C+B treatment, the increase of GPOX activity was higher in seedlings followed by booting stage over control plants (Fig. 27).

In var. Manu ratna under S treatment the decrease of GPOX activity in seedling, tillering, booting and ripening stages was 25, 32, 48 and 54% respectively as compared to control, but in S+B treatment the increase in GPOX activity was very negligible initially but further increased upto 68% in ripening stage over that of control plants. However, in C+B treatment the GPOX activity showed an increasing trend from seedling to ripening stages with respect to control (Fig. 27).

4.9 Chlorophyll *a* fluorescence parameters

Various Chl *a* fluorescence parameters were analyzed in the leaves of both tolerant and sensitive varieties to study the effect of BI on the photochemistry at different growth stages (seedling, tillering, booting and ripening) of rice. Pronounced changes were observed in the Chl *a* fluorescence parameters of both varieties subjected to S+B condition as compared to control seedlings.

4.9.1 Electron donation to PSII (Fv/Fo)

In tolerant var. Hraswa, the electron donation to PSII (Fv/Fo) was increased significantly in C+B treatment when compared to the control plants. The increase in electron donation was maximum in booting stage (72%) followed by tillering (56%), seedling (34%) and ripening stages (21%) with respect to control. Under S treatment, Fv/Fo was decreased significantly throughout the different growth stages of rice but the highest decrease was observed in booting stage (83%). S+B significantly improved the rate of Fv/Fo even under nutrient deficient soil condition, and maximum increase was shown in booting stage (64%) followed by ripening and tillering stages as compared to control. However, a notable decrease in the rate of Fv/Fo was observed in seedling stage exposed to S+B condition with respect to control (Table 11).

Var. Manu ratna under S treatment also exhibited a decrease in Fv/Fo in seedling (24%), tillering (39%), booting (57%) and ripening (62%) stages as compared to control. However, S+B treatment elicited an increase of Fv/Fo in

Table 11: Area, Fo, Fm, Fo/Fm, Fv/Fm, Fv/Fo, Vj and PI_(abs) measured in rice var. Hraswa raised from BI treated and non-treated seedlings subjected to nutrient deficient stress at different stages of growth. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). Data represented are average mean of n=3.

Stages		Tfm	Area	Fv/Fo	Fo/Fm	Fv/Fm	SFI	Φ(Eo)	PI _(abs)
Seedling	C	190	17519	4.8	0.17	0.82	1.21	0.47	2.92
	C+P	220	23501	4.8	0.17	0.82	1.92	0.33	1.35
	S	180	18842	4.9	0.16	0.83	1.04	0.42	2.59
	S+P	175	24651	4.5	0.17	0.82	1.45	0.39	1.81
Tillering	C	185	24706	3.9	0.20	0.79	1.25	0.29	1.05
	C+P	210	29989	4.2	0.18	0.80	1.67	0.36	1.50
	S	190	22184	4.2	0.19	0.80	1.34	0.38	1.37
	S+P	210	34922	4.5	0.17	0.82	1.70	0.41	2.18
Booting	C	600	9041	2.3	0.32	0.67	7.2	0.46	28.0
	C+P	450	8020	2.5	0.29	0.70	12.6	0.47	29.3
	S	700	5324	1.3	0.48	0.51	4.3	0.22	3.93
	S+P	410	9740	3.1	0.24	0.75	12	0.59	18.7
Ripening	C	132	7034	2.9	0.25	0.57	6.6	0.58	18.2
	C+P	149	8956	3.7	0.37	0.64	7.8	0.64	20.2
	S	108	4859	1.8	0.19	0.38	4.9	0.49	13.6
	S+P	138	8153	3.5	0.36	0.60	6.0	0.63	15.9

Table 12: Area, Fo, Fm, Fo/Fm, Fv/Fm, Fv/Fo, Vj and PI_(abs) measured in rice var. Manu ratna raised from BI treated and non-treated seedlings subjected to nutrient deficient stress at different stages of growth. C (Control), C+B (Control + Biostimulant), S (stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). Data represented are average mean of n=3.

Stages		Tfm	Area	Fv/Fo	Fo/Fm	Fv/Fm	SFI	Φ(Eo)	PI _(abs)
Seedling	C	195	22995	4.6	0.17	0.82	1.2	0.35	1.52
	C+B	260	36531	4.4	0.18	0.85	1.9	0.42	2.52
	S	240	25540	4.4	0.18	0.71	0.9	0.38	1.75
	S+B	215	27077	4.4	0.18	0.81	1.2	0.36	1.66
Tillering	C	550	23197	4.0	0.20	0.79	1.8	0.26	2.26
	C+B	950	29671	4.4	0.17	0.81	2.3	0.35	2.89
	S	190	18763	3.8	0.29	0.80	1.3	0.26	1.48
	S+B	535	22542	4.0	0.19	0.80	1.7	0.42	2.19
Booting	C	350	9248	3.0	0.24	0.75	6.8	0.56	22.4
	C+B	425	10600	3.5	0.21	0.78	10.2	0.63	23.7
	S	575	6734	2.1	0.33	0.66	2.2	0.38	4.6
	S+B	347.5	10293	3.3	0.23	0.76	8.6	0.61	17.4
Ripening	C	125	6542	2.5	0.22	0.52	6.1	0.51	15.2
	C+B	132	8456	3.0	0.28	0.54	6.3	0.54	18.2
	S	100	4359	1.2	0.15	0.32	4.6	0.41	10.6
	S+B	108	6453	1.8	0.19	0.41	5.0	0.43	13.9

ripening (58%) followed by booting (39%) and tillering stages (10%) whereas a significant reduction was observed in seedling stage (25%) with respect to control. In C+B condition, a linear increasing trend in Fv/Fo was observed throughout different growth stages starting with seedling (39%) and continued in tillering (50%), booting (57%) and ripening (68%) over that of control plants (Table 12).

4.9.2 Performance index (PI_{abs})

In tolerant Var. Hraswa, the performance index/plant vitality index (PI_{abs}) exhibited a decreasing trend in S treatment, and maximum decline was observed in booting stage (85%) as compared to control. However, S+B treatment improved the plants performance index throughout the growth stages with maximum effect on seedling (78%) and booting (54%) stages. Whereas, C+B seedlings elicited a maximum enhancement of PI_{abs} in tillering stage (58%) and negligible increase was further observed in other stages with respect to control (Table 11).

Under S treatment, an increase in PI_{abs} was observed in seedling stage (28%) in the var. Manu ratna but thereafter a decreasing trend was observed in tillering, booting followed by ripening stages. However, S+B enhanced the PI_{abs} with highest increase in booting (72%) and ripening stages (66%) and negligible increase was observed in seedling and tillering stages. In C+B treatment, maximum increment in PI_{abs} occurred during booting (72%), seedling (66%) and ripening stages (45%) followed by a least increase in tillering stage as compared to control (Table 12).

4.9.3 Structure function index (SFI)

Structure function index (SFI) of PSII provides information about the structural and functional changes in PSII reaction center. In tolerant Var. Hraswa, significant reduction in SFI was observed throughout the growth stages of rice under S treatment but S+B treatment induced a significant increase of SFI in booting stage (66%) followed by negligible increase in tillering, ripening and seedling stages. However, in C+B treatment an increasing trend was recorded for this parameter of which maximum effect was observed in booting (75%), followed by seedling (58%) and ripening stages (52%) with respect to control (Table 11).

In sensitive var. Manu ratna under S treatment, a decreasing trend was observed in SFI throughout the different growth stages, among which drastic reduction was observed in ripening (72%) followed by booting stages (67%) but least decrease was seen in seedling and tillering stages. However, S+B treatment elicited an increase in this parameter with maximum effect in booting (43%), followed by ripening and seedling stages and a negligible increase was seen in tillering stage. On the other hand, C+ B treatment improved the SFI in seedling (53%), tillering (43%), booting (50%) and ripening stages (34%) as compared to the control (Table 12).

4.9.4 Electron transport quantum yield (ϕ_{Eo})

In tolerant var. Hraswa, a decreasing trend in ϕ_{Eo} was observed under S treatment with significant reduction in booting (44%) and ripening stages (42%) but negligible increase was observed in seedling and tillering stages as compared to control. However significant improvement in ϕ_{Eo} was observed in S+B treatment, with substantial increase recorded in seedling (44%) and tillering stages (39%) followed by least increase in ripening and booting stages (Table 11).

Var. Manu ratna exhibited a considerable reduction of ϕ_{Eo} at all growth stages of rice whereas S+B treatment significantly improved the quantum yield with maximum effect in seedling and tillering stages nevertheless a negligible increase was observed in booting and ripening stages. A significant increase in ϕ_{Eo} was observed in C+B treatment, with maximum effect noted in seedling (64%) and booting stages (48%) followed by a decrease in tillering and ripening stages (Table 12).

4.9.5 Energy flux model of PSII

Specific energy flux model of PSII was studied in Bl treated and untreated plants in control and stress. Using specific energy flux model of PSII per cross section, the parameters investigated were absorption per cross section (ABS/CSm), trapping energy per cross section (TR/CSm), rate of electron transport per cross section (ET/CSm) and dissipation energy per cross section (DI/CSm). Specific energy flux model of PSII per reaction center was also used to analyze absorption

(ABS/RC), trapping energy (TRO/RC) rate of electron transport (ETO/RC) and dissipation energy (DIO/RC) per active reaction center of PSII investigated.

4.9.5.1 Absorption per cross section (ABS/CSm) and reaction center (ABS/RC)

In tolerant var. Hraswa, significant reduction was recorded under S treatment in ABS/CSm and ABS/RC throughout different growth stages of rice whereas an increase was observed in S+B treatment with maximum absorption in booting (57-65%) and seedling stages (41-48%) followed by tillering and ripening stages. In C+B treatment, an increasing trend was recorded in absorption of energy with highest effect in seedling (38-42%) and booting stages (42-51%) while negligible enhancement in tillering and ripening stages as compared to control (Fig. 28, 30, 32 and 34).

Similarly, in var. Manu ratna under S treatment a decreasing trend of energy absorption was observed with maximum reduction in booting (60-65%) followed by tillering stages (43-52%) but a negligible decrease was observed in seedling and ripening stages. In S+B treatment, a linear increase was observed throughout the different growth stages of rice. C+B treatment recorded highest energy absorption rate in seedling (48-54%) and booting stages (52-58%) and a negligible increase was recorded in tillering and ripening stages with respect to control plants (Fig. 29, 31, 33 and 35).

4.9.5.2 Dissipation per cross section (DIO/CSm) and reaction center (DIO/RC)

In tolerant var. Hraswa, S treatment resulted in an increase of 37- 47% in DIO/CSm and DIO/RC whereas under S+B treatment resulted in significant decrease of DIO/CSm and DIO/RC in seedling (47-54%), tillering stages (52-62%) thereafter recorded a negligible increase in ripening and booting stages as compared to control. In C+B treatment, a decreasing trend was seen throughout the different growth stages of rice with maximum effect in booting (78-86%) followed by seedling and ripening stages with respect to control (Fig. 28, 30, 32 and 34).

In sensitive var. Manu ratna, under S treatment significant increase in DIO/RC (41-45%) and DIO/CSm (32-38%) was observed in booting and seedling stages whereas in ripening and tillering stages a negligible increase was observed as

compared to control plants. However, S+B treatment recorded a decrease in Dlo/RC with maximum reduction in seedling (45-52%), booting (61-67%) and ripening stages (68-72%) but negligible increase was recorded in tillering stage (10-12%). Significant reduction of these parameter was recorded in C+B treatment throughout the different growth stages of rice with highest decrease recorded in booting (40-45%) and seedling stages (51-57%) as compared to control (Fig. 29, 31, 33 and 35).

4.9.5.3 Trapping energy per cross section (TRo/CSm) and reaction center (TRo/RC)

In tolerant var. Hraswa under S treatment a slight increase in TRo/CSm and TRo/RC was observed in tillering (23-28%) and booting stages (36-25%) and a negligible decrease was observed in seedling (15-11%) and ripening stages (8-10%). Significant increase in TRo/CSm and TRo/RC was recorded in S+B treatment during booting and seedling stages (45-52%) and least increase was observed in tillering and ripening stages. In C+B treatment, TRo/CSm and TRo/RC linearly increased throughout the growth stages with maximum effect in seedling (62-68%) and booting stages (57-60%) (Fig. 28, 30, 32 and 34).

Under S treatment, var. Manu ratna showed a higher reduction in TRo/CSm and TRo/RC with maximum decrease in seedling (55-61%) and ripening stages (34-45%) followed by an increase in tillering (12-24%) and booting stages (36-47%) as compared to control. S+B treatment improved the trapping efficiency with significant increase in tillering (54-67%), booting (50-52%) and seedling stages (43-48%), however a significant decline was recorded in ripening stage (30-35%) as compared to control. In C+B treatment significant increase of both parameters was observed throughout the growth stages with maximum trapping recorded in booting (52-62%) and seedling stages (51-58%) (Fig. 29, 31, 33 and 35).

4.9.5.4 Electron transport per cross section (ETo/CSm) and reaction center (ETo/RC)

In tolerant var. Hraswa a significant decrease in ETo/CSm and ETo/RC was observed in S treatment with highest decrease recorded in booting stage (63-72%) followed by ripening stage (35-48%). The ETo/CSm and ETo/RC in the S+B treatment recorded a significant increase in booting (55-63%), followed by tillering

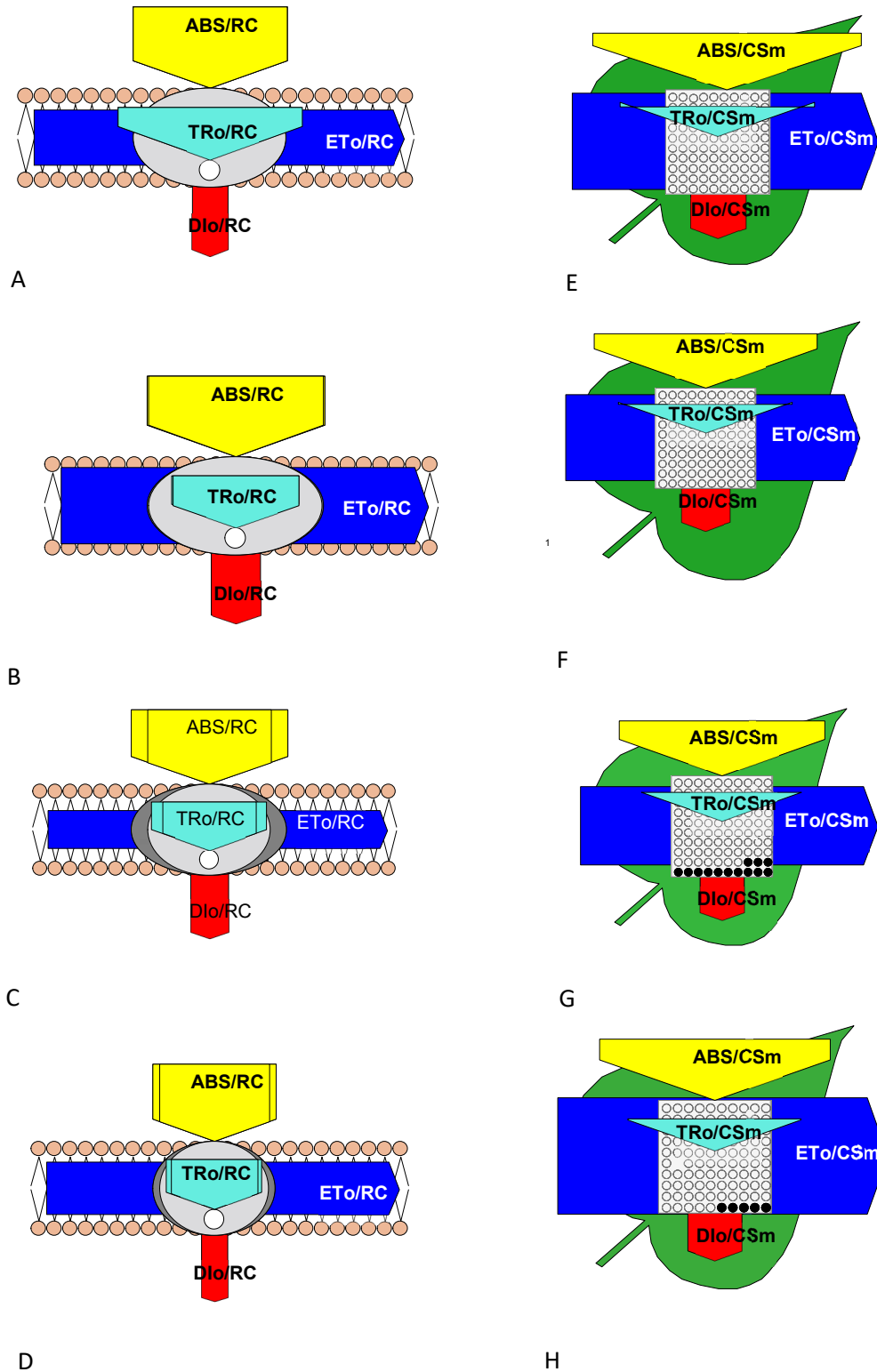


Fig. 28: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Hraswa at seedling stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C; B&F-C+B; C&G-S; D&H-S+B.

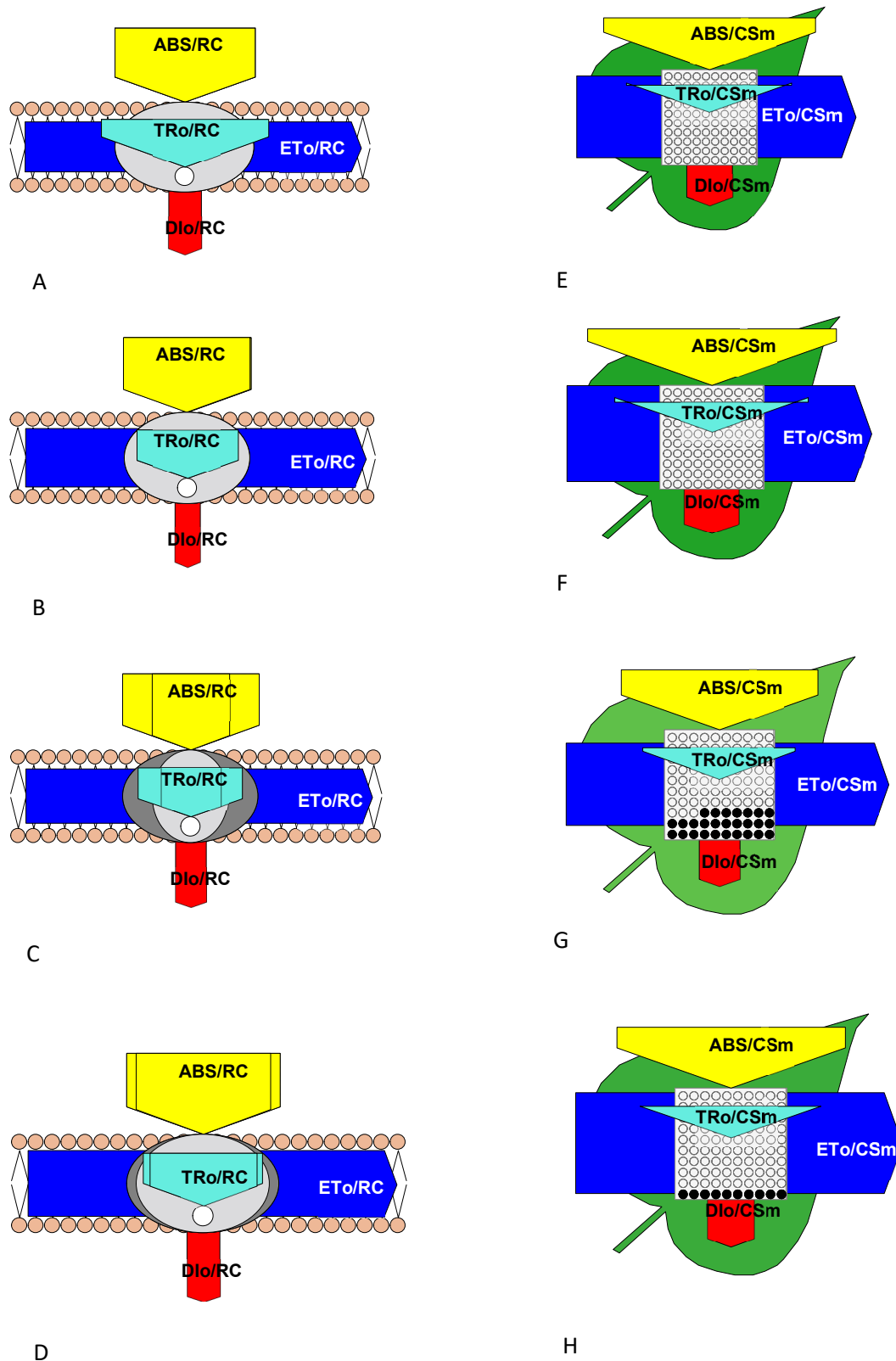


Fig. 29: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Manu ratna at seedling stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

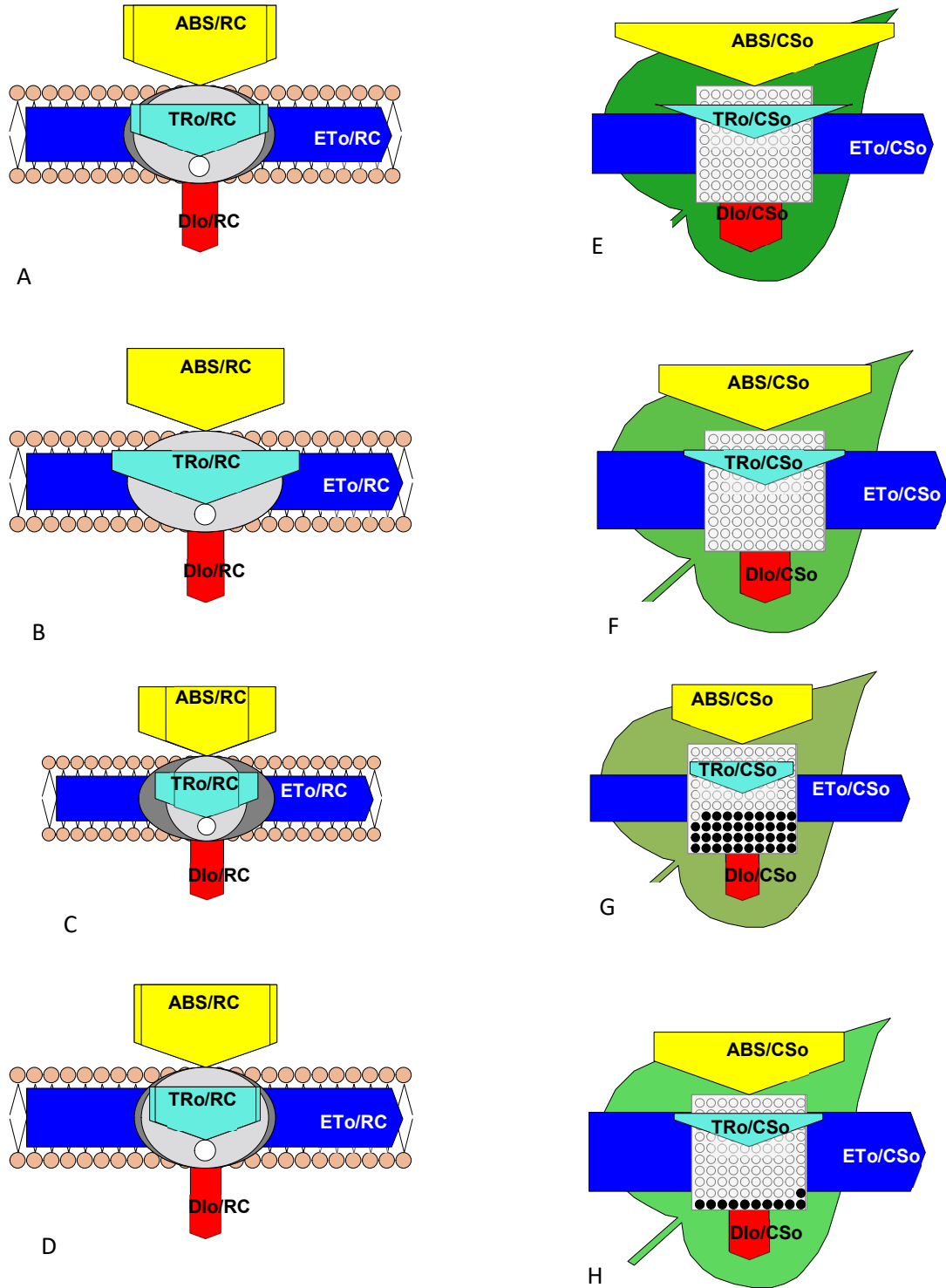


Fig. 30: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Hraswa at tillering stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

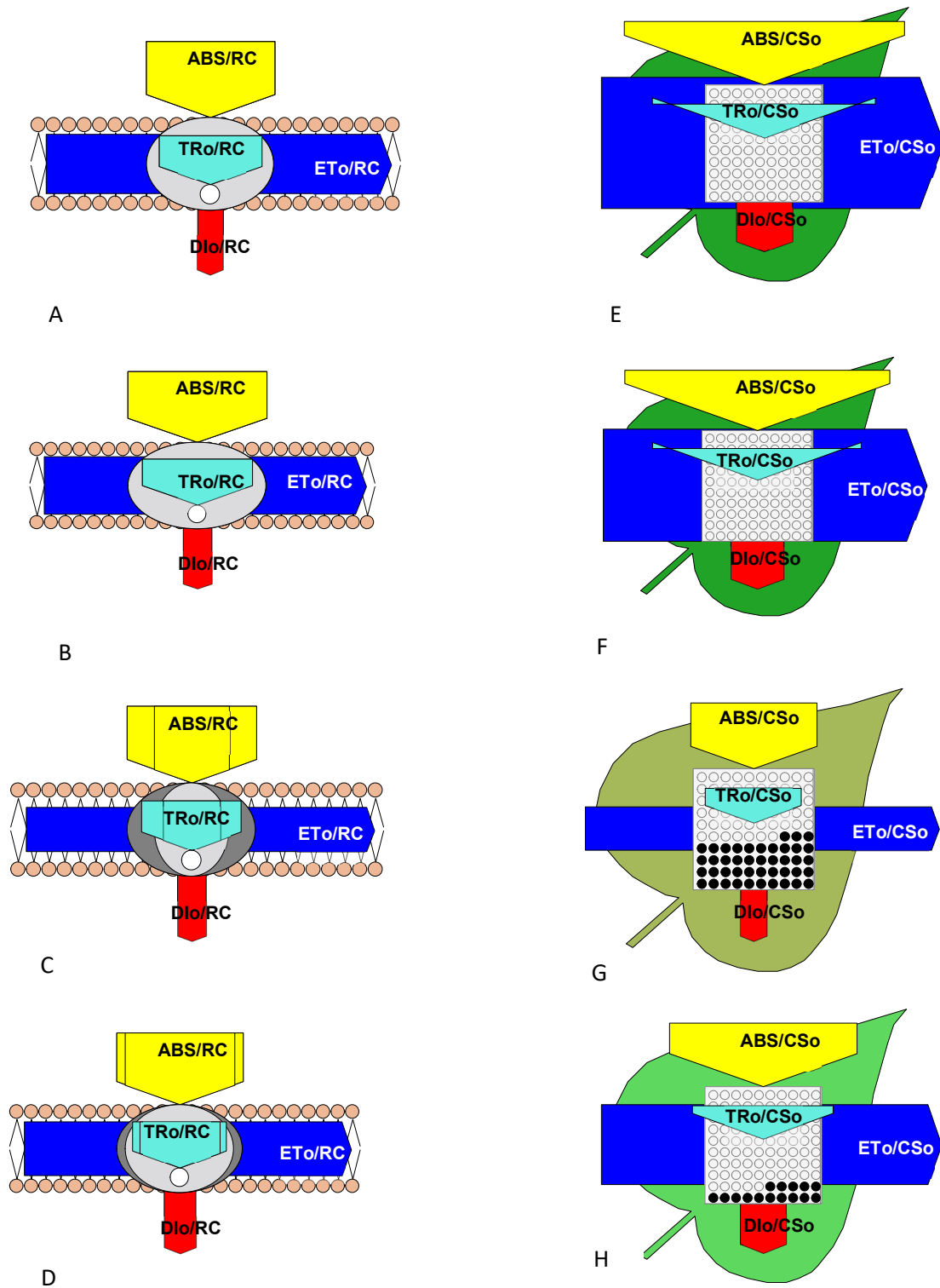
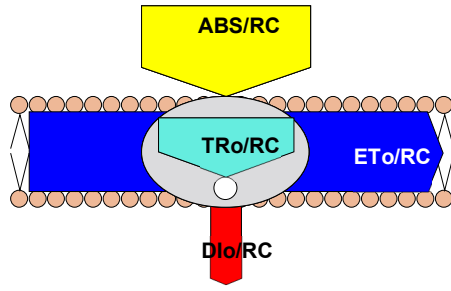
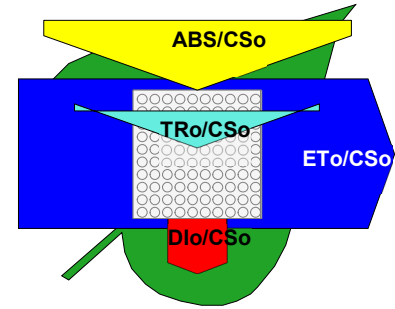


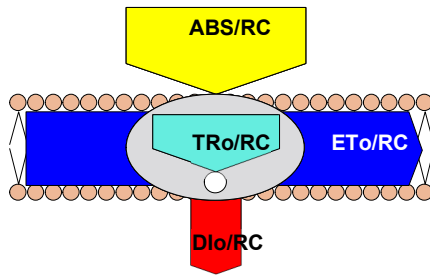
Fig. 31: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Manu ratna at tillering stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.



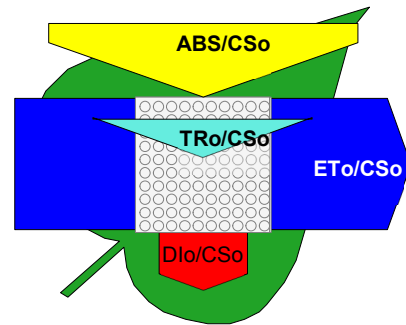
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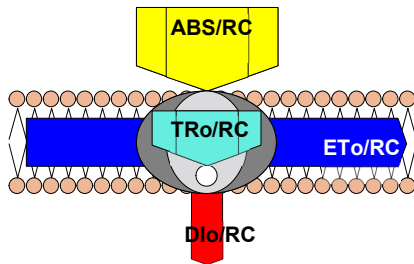
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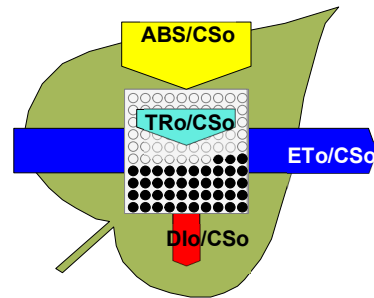
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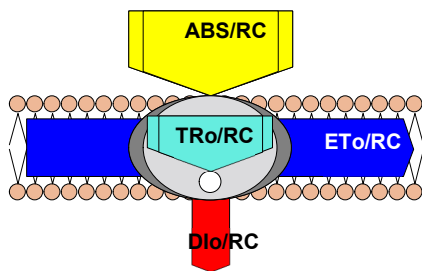
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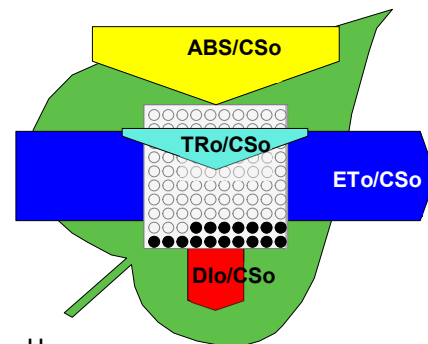
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Fig. 32: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Hraswa at booting stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

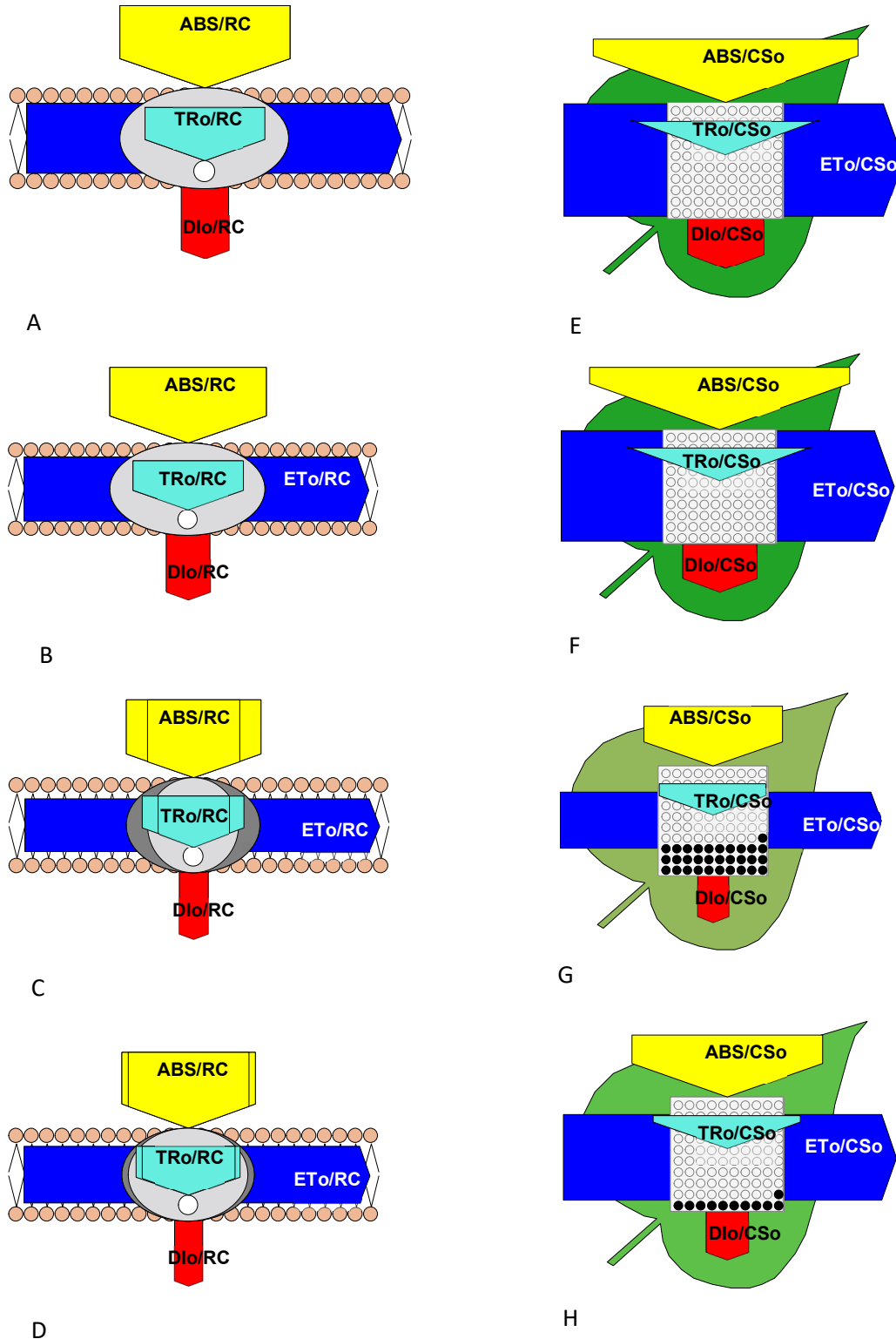


Fig. 33: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Manu ratna at booting stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

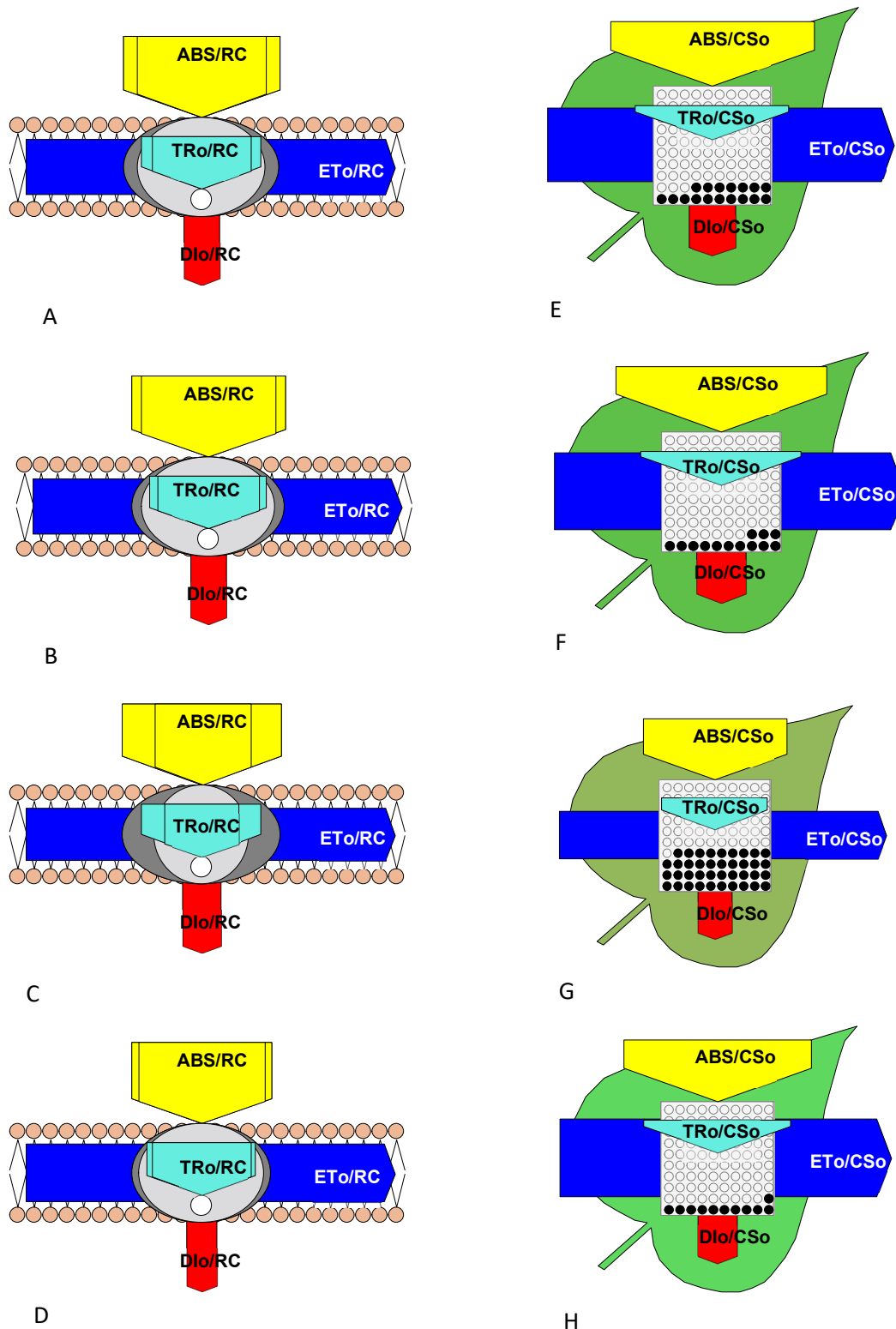


Fig. 34: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Hraswa at ripening stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

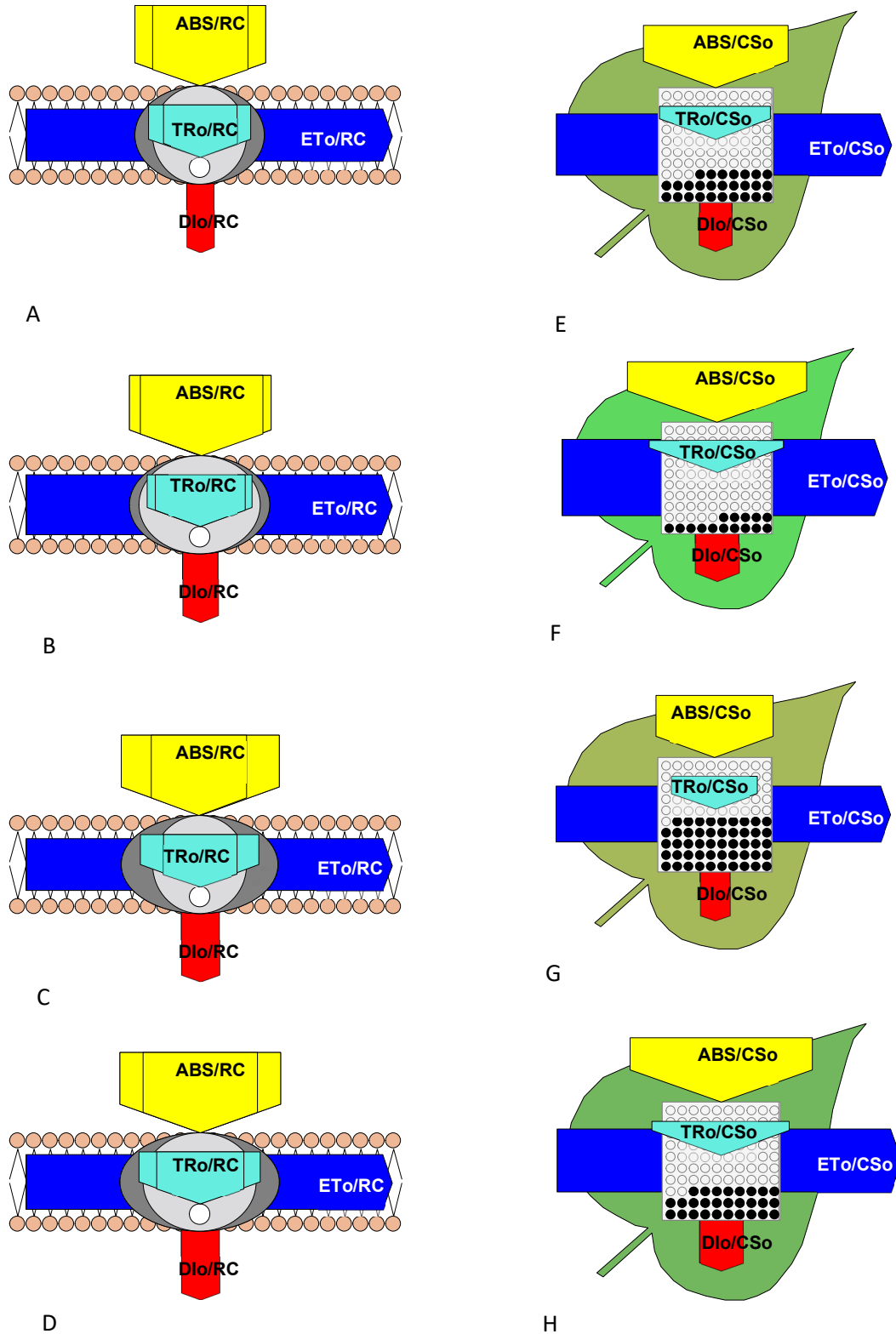


Fig. 35: Specific membrane model and Energy leaf model of phenomenological fluxes per cross-section (CSm) and per reaction centre (RC) in leaves of rice var. Manu ratna at ripening stage. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). The value of each parameter can be seen in relative changes in width of each arrow. Active RCs are shown as open circles and inactive RCs are shown as closed circles. A&E-C: B&F-C+B; C&G-S; D&H-S+B.

(48-57%) and seedling (30-46%) stages whereas a reduction was observed in ripening stage (28-34%). In C+B treatment, significant improvement of electron transport was recorded in all the growth stages and substantial increase was noted in booting (58-61%) and seedling stages (42-58%) as compared to control (Fig. 28, 30, 32 and 34).

Under S treatment in var. Manu ratna, drastic reductions were observed in electron transport throughout the growth stages with maximum decline in ripening (47-56%) and booting stages (34-41%), however initial seedling and tillering stages showed negligible decrease as compared to control. In S+B treatment, significant increase was recorded in seedling (41-48%) and booting stages (53-57%) followed by a transient decline in ripening stage. Under C+B treatment, the efficiency of electron transport improved throughout the growth stages with maximum effect in seedling (65-72%) and booting (58-61%) followed by negligible increment in tillering stage (Fig. 29, 31, 33 and 35).

4.10 Gas exchange parameters

4.10.1 Net photosynthetic rate (P_n)

In var. Hraswa, S treatment led to a 46% reduction in P_n compared to the control. However, S+B treatment improved P_n by 41% and C+B by 15% as compared to control (Fig. 36).

However, var. Manu ratna when exposed to S treatment caused a 44% reduction in P_n while S+B led to a 19% improvement, indicating a moderate recovery effect. Under C+B treatment, a 10% increase in P_n was observed compared to the control, showing a mild enhancement in photosynthetic performance (Fig. 36).

4.10.2 Stomatal conductance (g_s)

In the tolerant var. Hraswa, g_s was markedly reduced in the case of S treated plants, showing a 59% decline compared to the control, indicating restricted stomatal opening likely due to water conservation mechanisms or impaired gas exchange. Interestingly, in the S+B treated plants, there was a 35% increase in g_s relative to the S, suggesting partial restoration of stomatal opening and improved

stomatal function. While C+B treatment led to a negligible (16%) enhancement in g_s over the untreated control, reflecting a subtle positive influence of the treatment even under optimal growing conditions (Fig. 36).

In the sensitive var. Manu ratna, S treated plants led to a pronounced (67%) reduction in g_s compared to the control, indicating a significant restriction in stomatal aperture. However, under S+B treatment, an impressive 88% improvement in g_s relative to the S condition occurred, nearly doubling the stomatal conductance and suggesting a strong stimulatory effect on gas exchange mechanisms. Interestingly, C+B treatment led to a 33% increase in g_s compared to the control, a response more pronounced than in the tolerant variety, indicating greater physiological responsiveness of the sensitive genotype to biostimulant treatment even in the absence of stress (Fig. 36).

4.10.3 Transpiration rate (Tr)

In the tolerant var. Hraswa, the S treatment led to a notable 42% decrease in Tr compared to the control, highlighting the suppressive effect of stress on water loss through stomata. Under S+B treatment, Tr increased by 22% relative to the S treatment, indicating a partial recovery of water regulation mechanisms. The C+B treatment resulted in a 13% increase as compared to the control, suggesting that the biostimulant positively influenced transpiration even in the absence of stress (Fig. 36).

In contrast, the sensitive var. Manu ratna experienced a more severe (56%) reduction in Tr under S treatment compared to control, reflecting greater vulnerability to stress. However, the S+B treatment improved Tr by 39% over S, indicating a strong restorative effect of the biostimulant. Interestingly, the C+B treatment led to a 4% decrease in Tr compared to control, suggesting a slightly inhibitory effect under non-stress conditions (Fig. 36).

4.10.4 Intercellular CO₂ concentration (Ci)

In the var. Hraswa, Ci declined markedly under S treatment, showing a 48% reduction compared to the control, reflecting potential stomatal closure or photosynthetic inhibition under stress. While S+B treatment led to a 47% increase in Ci compared to the S treatment, indicating that internal CO₂ availability can lead to

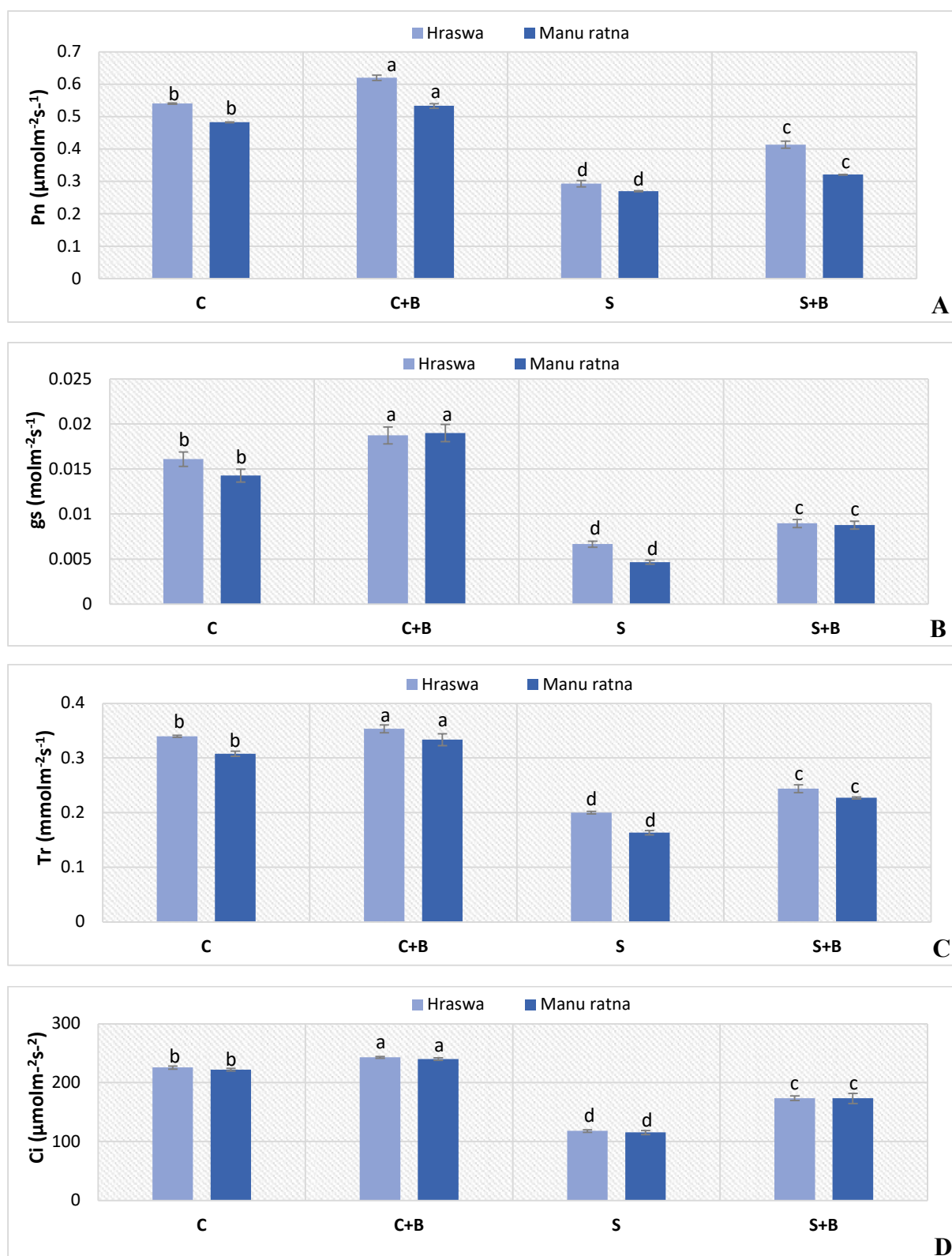


Fig. 36: Pn (A), g_s (B) Tr (C) and Ci (D) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

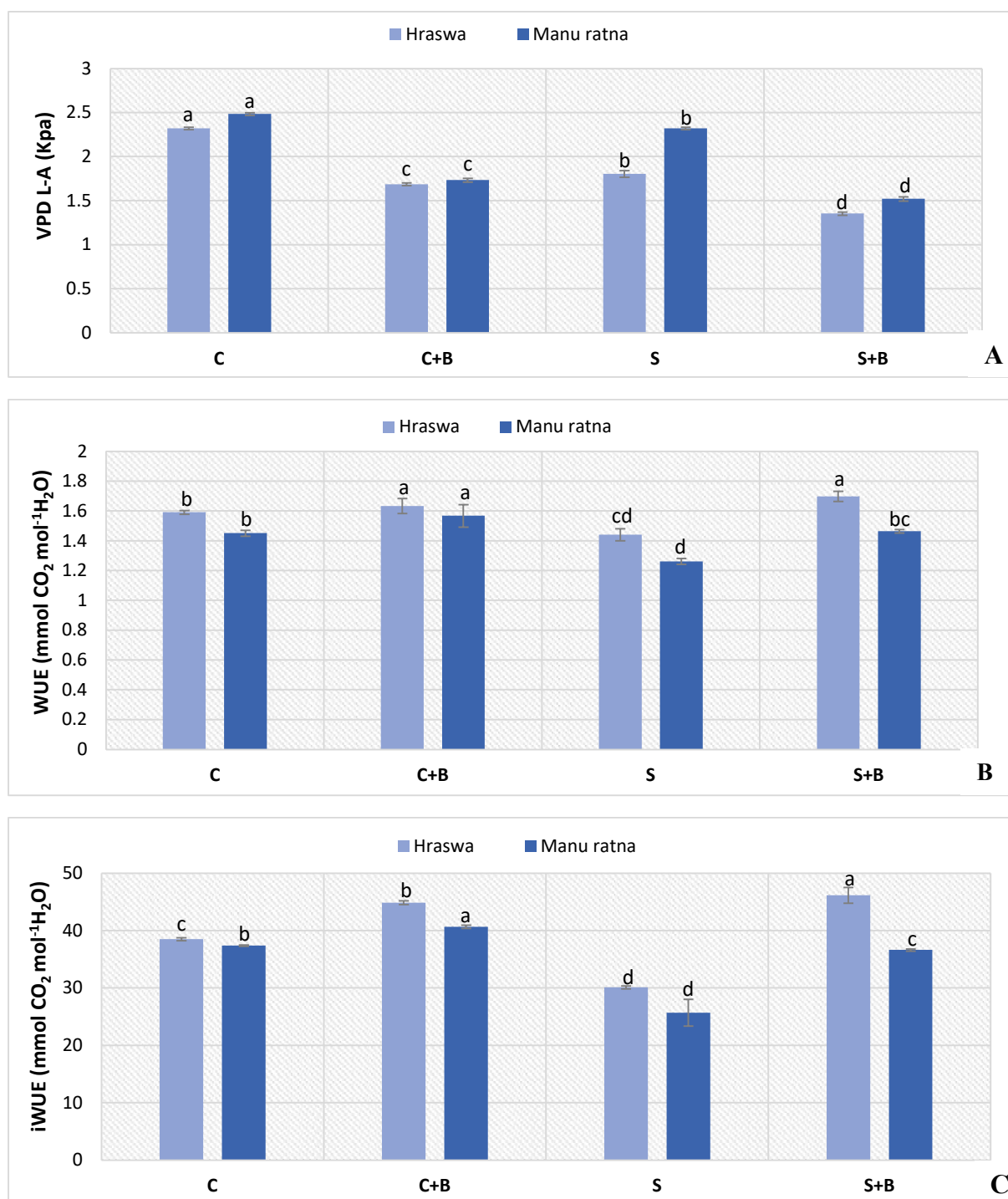


Fig. 37: VPD (A), WUE (B) and iWUE (C) in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

Table. 13: Concentration of different elements in soil subjected to different treatments (C, ND and B). C (Control), ND (Stress-nutrient deficient condition) and B (Biostimulant subjected to stress-nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences between means ($p < 0.05$).

Elements	C	ND	B
K (g/kg Dw)	0.781 $\pm$ 0.05 ^b	0.577 $\pm$ 0.02 ^a	0.950 $\pm$ 0.07 ^c
Ca (mg/kg Dw)	8.54 $\pm$ 0.54 ^b	6.787 $\pm$ 0.31 ^a	10.150 $\pm$ 0.62 ^c
Mg (g/kg Dw)	0.804 $\pm$ 0.08 ^c	0.734 $\pm$ 0.01 ^a	0.848 $\pm$ 0.06 ^b
Mn (mg/kg Dw)	60.18 $\pm$ 0.16 ^a	48.59 $\pm$ 0.32 ^c	61.28 $\pm$ 0.24 ^b
Zn (mg/kg Dw)	2.55 $\pm$ 0.13 ^b	1.73 $\pm$ 0.15 ^c	3.20 $\pm$ 0.11 ^a
B (mg/kg Dw)	0.74 $\pm$ 0.01 ^a	0.41 $\pm$ 0.04 ^b	1.14 $\pm$ 0.05 ^c
Ni (mg/kg Dw)	37.41 $\pm$ 0.11 ^c	13.88 $\pm$ 0.09 ^b	45.31 $\pm$ 0.03 ^a
Se (mg/kg Dw)	0.068 $\pm$ 0.003 ^b	0.040 $\pm$ 0.004 ^c	0.078 $\pm$ 0.001 ^a
Mo (mg/kg Dw)	0.237 $\pm$ 0.03 ^b	0.182 $\pm$ 0.01 ^a	0.324 $\pm$ 0.05 ^c

enhanced carbon assimilation. Interestingly, C+B treatment enhanced C_i by 18% relative to the control, suggesting improved mesophyll conductance or stomatal function (Fig. 36).

In var. Manu ratna, S treatment led to a 48% reduction in C_i compared to the control. However, S+B treatment significantly enhanced C_i by 50% over the S treatment, suggesting an ameliorative effect on stomatal or mesophyll conductance. The negligible increase of 12% was observed under C+B treatment as compared to control, indicating a mild positive influence on internal CO_2 dynamics even in the absence of stress (Fig. 36).

4.10.5 Water use efficiency (WUE)

In var. Hraswa, WUE declined by approximately 25% under S treatment relative to the control. However, under S+B, WUE increased substantially by 38% compared to S treatment. Interestingly, under optimal conditions, C+B treated plants recorded a 13% increase in WUE over the control, demonstrating a marginal but consistent improvement in water use even in control condition (Fig. 37).

In var. Manu ratna, S treatment led to a 33% decrease in WUE compared to the control, which can affect the physiological performance of the plant under stress. However, S+B significantly improved WUE by 46% relative to the S treatment, suggesting an ameliorative effect. And C+B treatment, enhanced the WUE by 18% over the control plants, reflecting improved water absorption efficiency even in the absence of stress (Fig. 37).

4.11 Elemental analysis of biostimulant application

4.11.1 In soil

The elemental composition of the soil under different treatments—Control (C), Nutrient Deficient (ND), and Biostimulant-treated (B)—was analyzed to assess the influence of biostimulant application on soil nutrient status (Table 13).

The results revealed that the biostimulant-treated soil exhibited higher concentrations of most essential elements compared to both the control and nutrient-

deficient soils. Potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), zinc (Zn), boron (B), nickel (Ni), selenium (Se), and molybdenum (Mo) levels were all positively influenced by biostimulant application.

Among the macronutrients, K increased by 22% in the biostimulant treatment, while Mg and Ca also increased by 6 and 19%, respectively, compared to the control. Similarly, Mn showed a modest rise of 2%, whereas Zn and B increased by 26% and 54%, respectively.

Micronutrients such as Ni, Se, and Mo also demonstrated notable increases in the biostimulant-treated soil. Ni content increased by 12%, Se by 15%, and Mo by 37% increase.

In contrast, the ND soil consistently exhibited the lowest elemental concentrations, with substantial reductions in K (27%), Mg (9%), Ca (21%), and trace elements such as Zn (33%) and Ni (63%) relative to the control.

4.11.2 In flag leaves

The elemental composition of var. Hraswa and Manu ratna was analyzed using ICP-MS under four treatments: C, C+B, S and S+B during booting stage. The concentrations of macro and micronutrients showed distinct patterns across treatments and between varieties, demonstrating the impact of stress and the ameliorative role of biostimulants.

In Var. Hraswa, K content increased by 25% under C+B compared to control, while S caused a 20% decrease. The S+B treatment led to a 25% increase in K compared to S. Mg followed a similar trend: C+B induced a 30% increase, S treatment led to a 5% reduction, while S+B showed a 30% improvement over S. Ca exhibited a 40% increase under C+B, a 15% decrease under S, and a remarkable 60% increase under S+B relative to S (Fig. 38).

Micronutrients such as Mn, Zn, and Fe showed notable shifts. In Hraswa, Mn increased by 30% with C+B treatment and by 35% under S+B relative to S. Zn showed a 10% rise under C+B treatment, a 30% decline under S treatment, and a

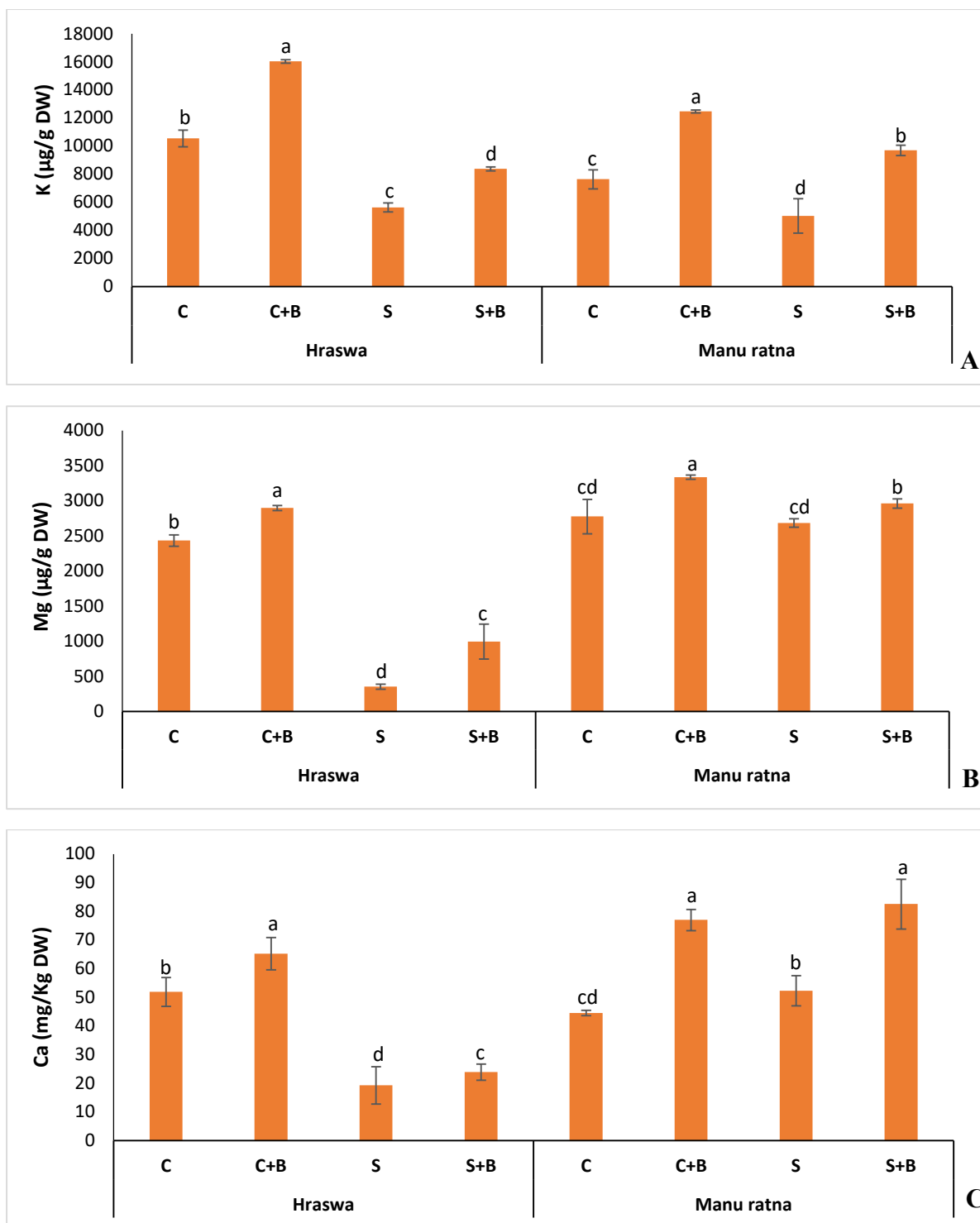


Fig. 38: K (A), Mg (B) and Ca (C) ions in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

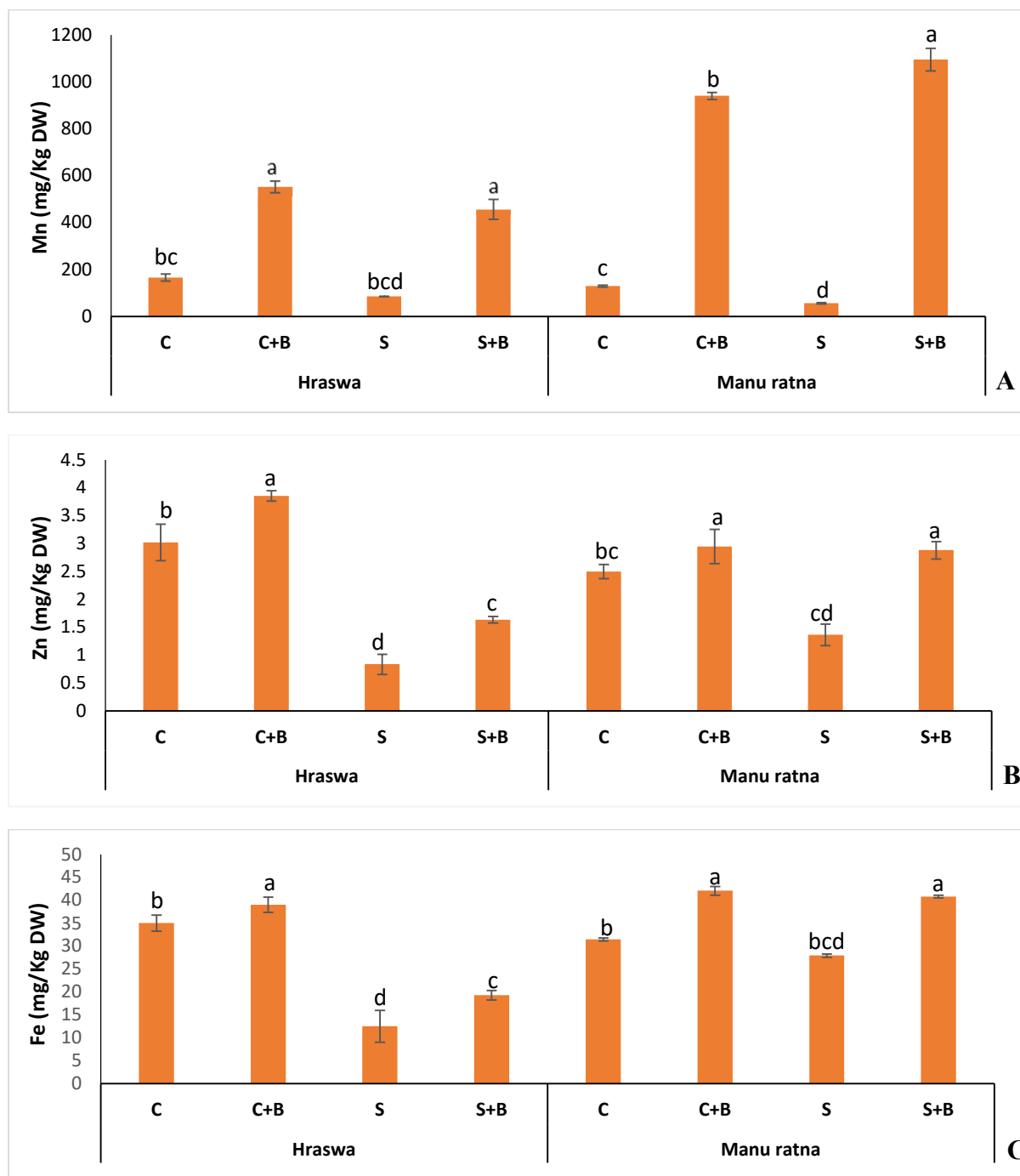


Fig. 39: Mn (A), Zn (B) and Fe (C) ions in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

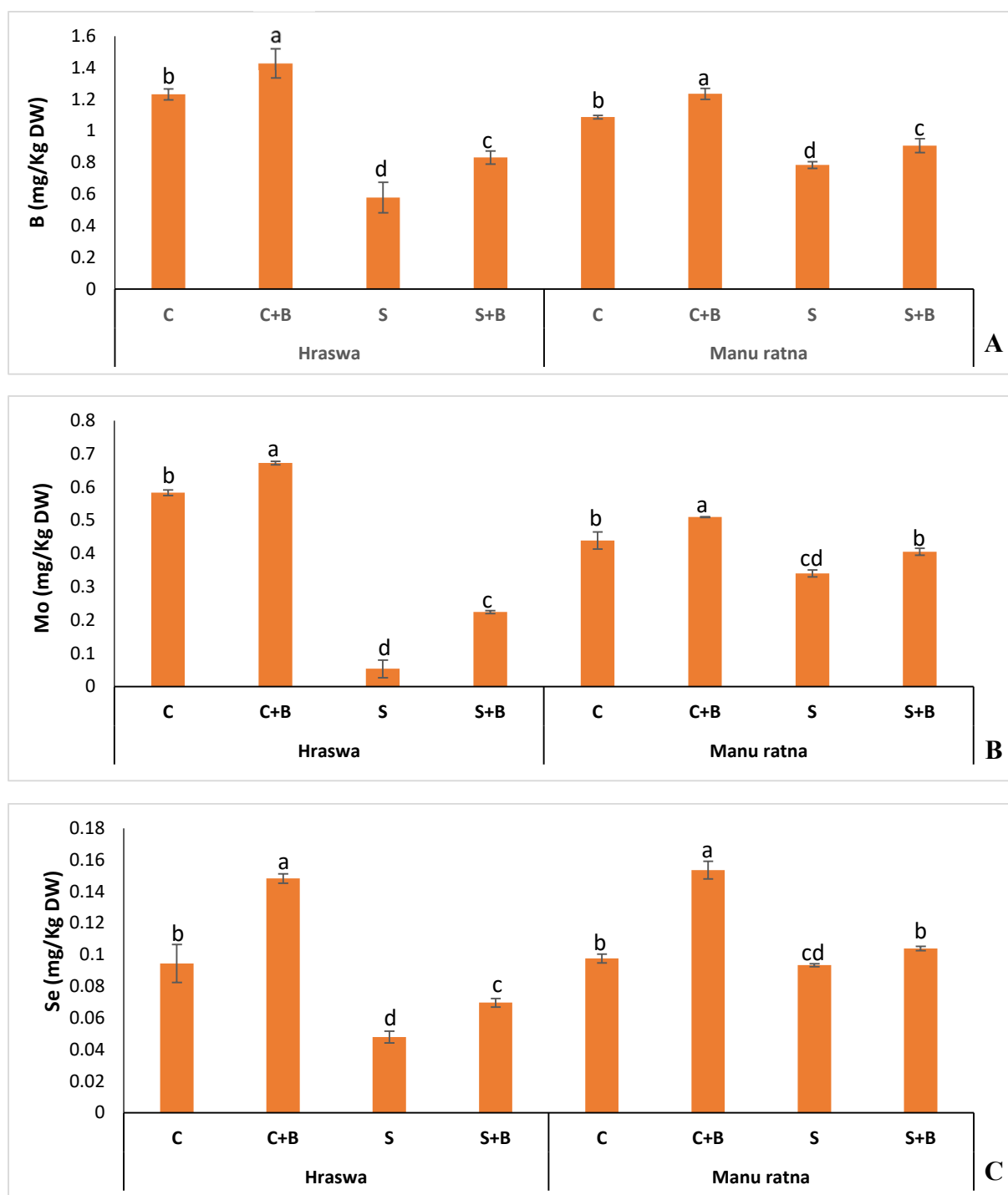


Fig. 40: B (A), Mo (B) and Se (C) ions in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

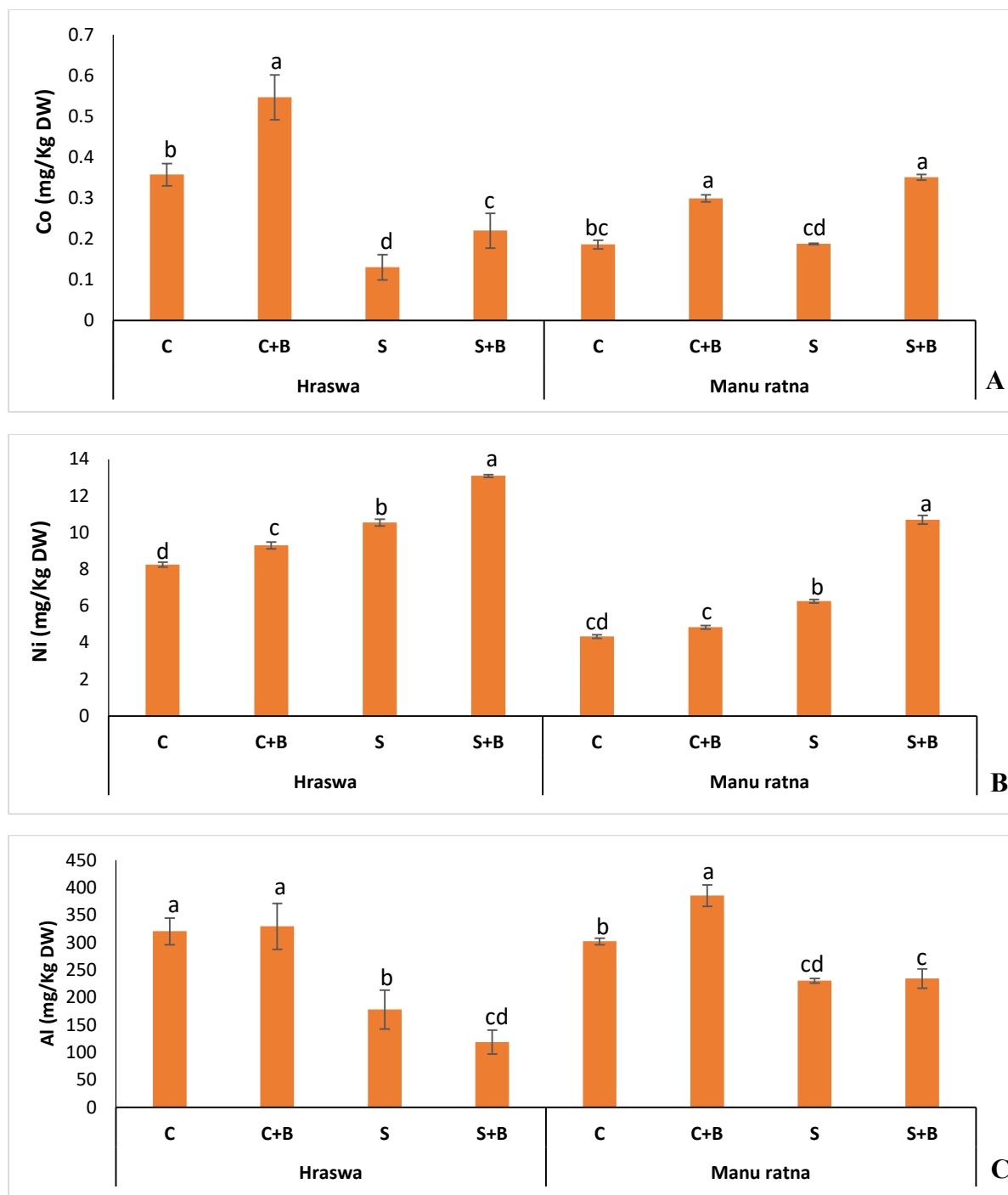


Fig. 41: Co (A), Ni (B) and Al (C) ions in leaves of biostimulant treated rice varieties (Hraswa & Manu ratna) at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Different letters indicate significant differences ($P < 0.05$).

substantial 25% increase under S+B treatment. Fe content increased by 20% under C+B, dropped by 25% under S, and improved by 45% with S+B treatment. In var. Manu ratna, the trends were directionally similar but less pronounced: K, Mg, and Ca increased by 10%, 15%, and 30%, respectively, under C+B; S caused 15–20% reductions; and S+B treatments restored and enhanced these levels by 20–50% (Fig. 39)

Trace element uptake was also positively influenced by biostimulants. In var. Hraswa Co, Ni, B, Mo, and Se increased by 25%, 20%, 10%, 15%, and 25%, respectively, under C+B. S treatment alone led to moderate decline (5–20%) of these elements, but S+B treatments significantly improved their uptake, with increases of Co (40%), Ni (30%), B (35%), Mo (30%), and Se (40%) over S. In var. Manu ratna, S+B treatment increased accumulation of Co (35%), Ni (25%), B (30%), Mo (25%), and Se (35%), but the absolute values remained lower than in var. Hraswa (Fig. 40 and 41).

Interestingly, Al, showed a slight reduction of 15–20% under S and S+B in var. Hraswa, while var. Manu ratna exhibited a 20–35% reduction in S+B, suggesting a potential role of biostimulants in limiting Al accumulation (Fig. 41).

4.12 Agronomic traits and yield components

Agronomic traits studied include plant height, number of filled and unfilled grains, number of panicles bearing tillers and yield components included grain weight, spikelet fertility, seed setting, biological yield and HI. These parameters were studied in plants emerging from treated and non-treated seeds subjected to nutrient deficiency (Fig. 42 and Table 14).

4.12.1 Plant height

Under S treatment, plant height was considerably reduced in Manu ratna (56%) and Hraswa (45%) as compared to control. S+B treatment exhibited significant increase of height in both varieties with highest increase in var. Hraswa (53%) followed by Manu ratna (34%) over that of control. Similarly, in C+B treatment, maximum increase was recorded in both varieties with 58% and 52%

increase in var. Hraswa and Manu ratna respectively as compared to control (Fig. 42A and Table 14).

4.12.2 Panicle density

Panicle density was significantly influenced by S treatment with higher reduction in Manu ratna (66%) followed by Hraswa (45%) as compared to control. However, both varieties exhibited an increase in panicle density with significant enhancement in S+B treatment. In S+B, maximum increment in panicle density was observed in tolerant var. Hraswa (45%) followed by Manu ratna (38%). Similarly, C+B treatments recorded a significant increase of panicle density in both varieties and it was upto 45-58% as compared to control (Fig. 42A and Table 14).

4.12.3 Grain yield (filled vs unfilled grains)

The number of filled grains significantly decreased in both varieties under S treatment as compared to control. But treated ones (S+B) recorded a different response, with maximum filled grains recorded in tolerant var. Hraswa (48%) whereas slight decrease was seen in var. Manu ratna (12%) respectively. C+B treatment recorded an increase in filled grains with maximum effect in Hraswa (64%) followed by Manu ratan (46%) as compared to control (Fig. 42A and Table 14).

Unfilled grains increased drastically under S treatment and a decrease of the same was seen in plants emerged from treated seeds. Significant reduction in unfilled grains was recorded in C+B followed by S+B treatments as compared to control. However considerable variations was observed in both varieties with maximum increase in unfilled grains noticed in var. Manu ratna and negligible increase in var. Hraswa as compared to control (Fig. 42A and Table 14).

4.12.4 Grain weight

Under S treatment, grain weight was considerably reduced in Manu ratna (48%) and Hraswa (32%) as compared to control. S+B treatment exhibited significant increase in grain weight in the case of both varieties with highest in var.

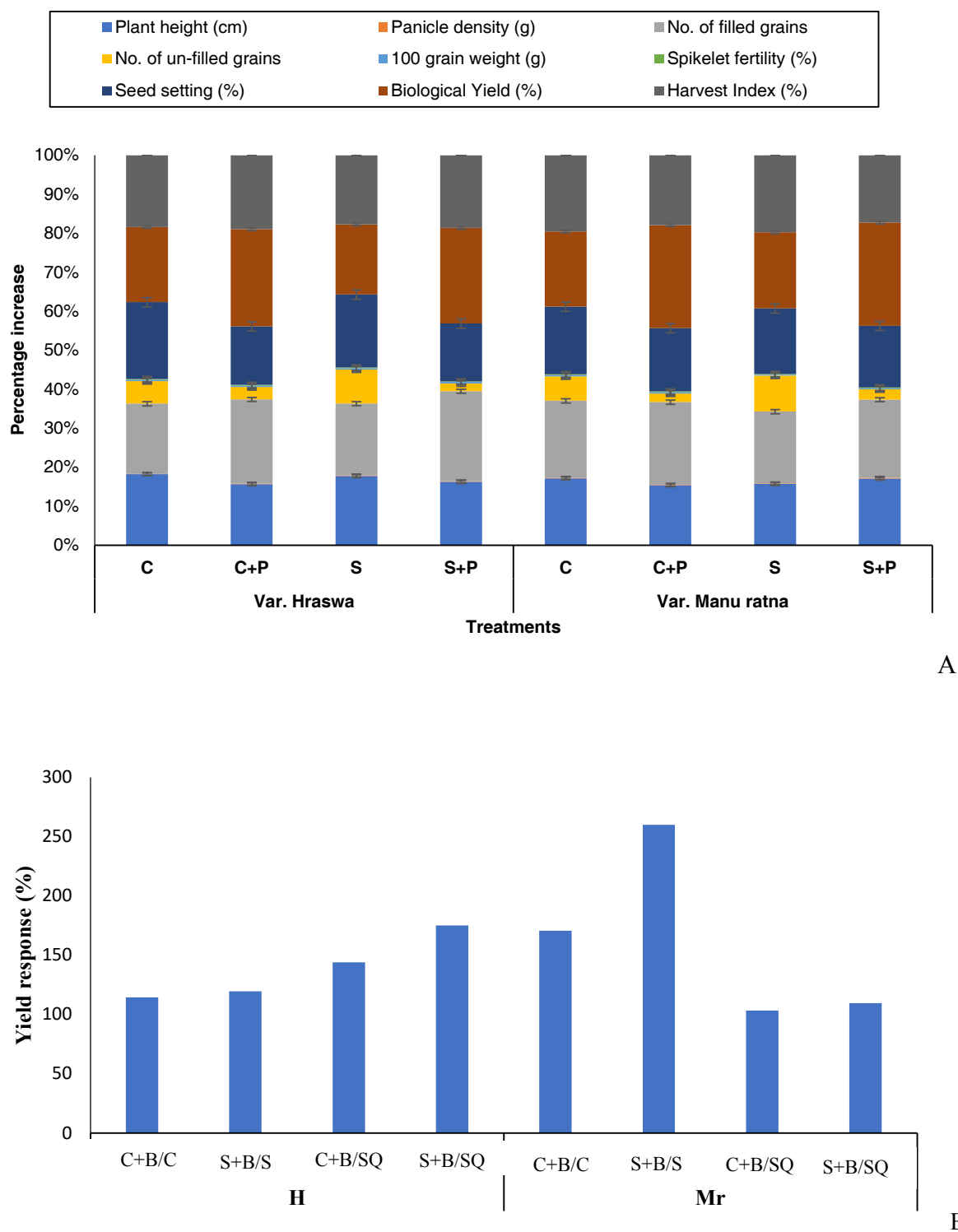


Fig. 42: Effect of biostimulant treatment on (A) yield parameters (B) yield response (%) in the rice seedlings (var. Hraswa and var. Manu ratna). C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+nutrient deficient condition), SQ (Syngenta quantis: biostimulant from market). H (Hraswa) and Mr (Manu ratna). Data represent Mean $\pm$ SE (n=3).

Table. 14: Effect of biostimulant on yield parameters in the rice seedlings (var. Hraswa and var. Manu ratna). C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Means within the same row followed by the different small letters show that there are significant differences between means ($p < 0.05$).

Parameters	Var. Hraswa				Var. Manu ratna			
Parameters	C	C+P	S	S+P	C	C+P	S	S+P
Plant height (cm)	85 $\pm$ 0.24 ^c	99 $\pm$ 0.43 ^b	71 $\pm$ 0.22 ^d	108 $\pm$ 0.20 ^a	71 $\pm$ 0.16 ^c	90 $\pm$ 0.36 ^b	58 $\pm$ 0.14 ^d	97 $\pm$ 0.32 ^a
Panicle density (g)	0.54 $\pm$ 0.36 ^c	0.83 $\pm$ 0.16 ^b	0.48 $\pm$ 0.37 ^d	0.85 $\pm$ 0.14 ^a	0.63 $\pm$ 0.25 ^c	1.04 $\pm$ 0.53 ^a	0.45 $\pm$ 0.11 ^d	0.87 $\pm$ 0.36 ^b
No. of filled grains	84 $\pm$ 0.51 ^c	137 $\pm$ 0.26 ^b	74 $\pm$ 0.39 ^d	154 $\pm$ 0.34 ^a	82 $\pm$ 0.29 ^c	124 $\pm$ 0.26 ^a	68 $\pm$ 0.17 ^d	114 $\pm$ 0.26 ^b
No. of un-filled grains	27 $\pm$ 0.37 ^b	20 $\pm$ 0.29 ^c	35 $\pm$ 0.42 ^a	13 $\pm$ 0.29 ^d	26 $\pm$ 0.46 ^b	13 $\pm$ 0.5 ^d	34 $\pm$ 0.21 ^a	15 $\pm$ 0.16 ^c
100 grain weight (g)	1.51 $\pm$ 0.12 ^c	2.47 $\pm$ 0.34 ^b	1.25 $\pm$ 0.21 ^d	2.50 $\pm$ 0.24 ^a	1.3 $\pm$ 0.32 ^c	2.0 $\pm$ 0.25 ^a	0.8 $\pm$ 0.38 ^d	1.5 $\pm$ 0.19 ^b
Spikelet fertility (%)	0.95 $\pm$ 0.63 ^{ab}	0.97 $\pm$ 0.42 ^a	0.77 $\pm$ 0.32 ^b	0.98 $\pm$ 0.41 ^a	0.89 $\pm$ 0.14 ^b	0.93 $\pm$ 0.29 ^a	0.79 $\pm$ 0.32 ^c	0.90 $\pm$ 0.34 ^a
Seed setting (%)	92 $\pm$ 0.34 ^{ab}	95 $\pm$ 072 ^{ab}	75 $\pm$ 0.76 ^b	99 $\pm$ 0.36 ^a	72 $\pm$ 0.54 ^c	95 $\pm$ 0.89 ^a	62 $\pm$ 0.64 ^d	90 $\pm$ 0.12 ^b
Biological Yield (%)	90 $\pm$ 0.26 ^c	158 $\pm$ 0.45 ^b	72 $\pm$ 0.47 ^d	163 $\pm$ 0.30 ^a	80 $\pm$ 0.21 ^c	155 $\pm$ 0.43 ^a	72 $\pm$ 0.54 ^d	150 $\pm$ 0.24 ^b
Harvest Index (%)	86 $\pm$ 0.21 ^c	120 $\pm$ 0.26 ^b	71 $\pm$ 0.32 ^d	124 $\pm$ 0.28 ^a	81 $\pm$ 0.34 ^b	105 $\pm$ 0.62 ^a	73 $\pm$ 0.32 ^c	98 $\pm$ 0.36 ^{ab}

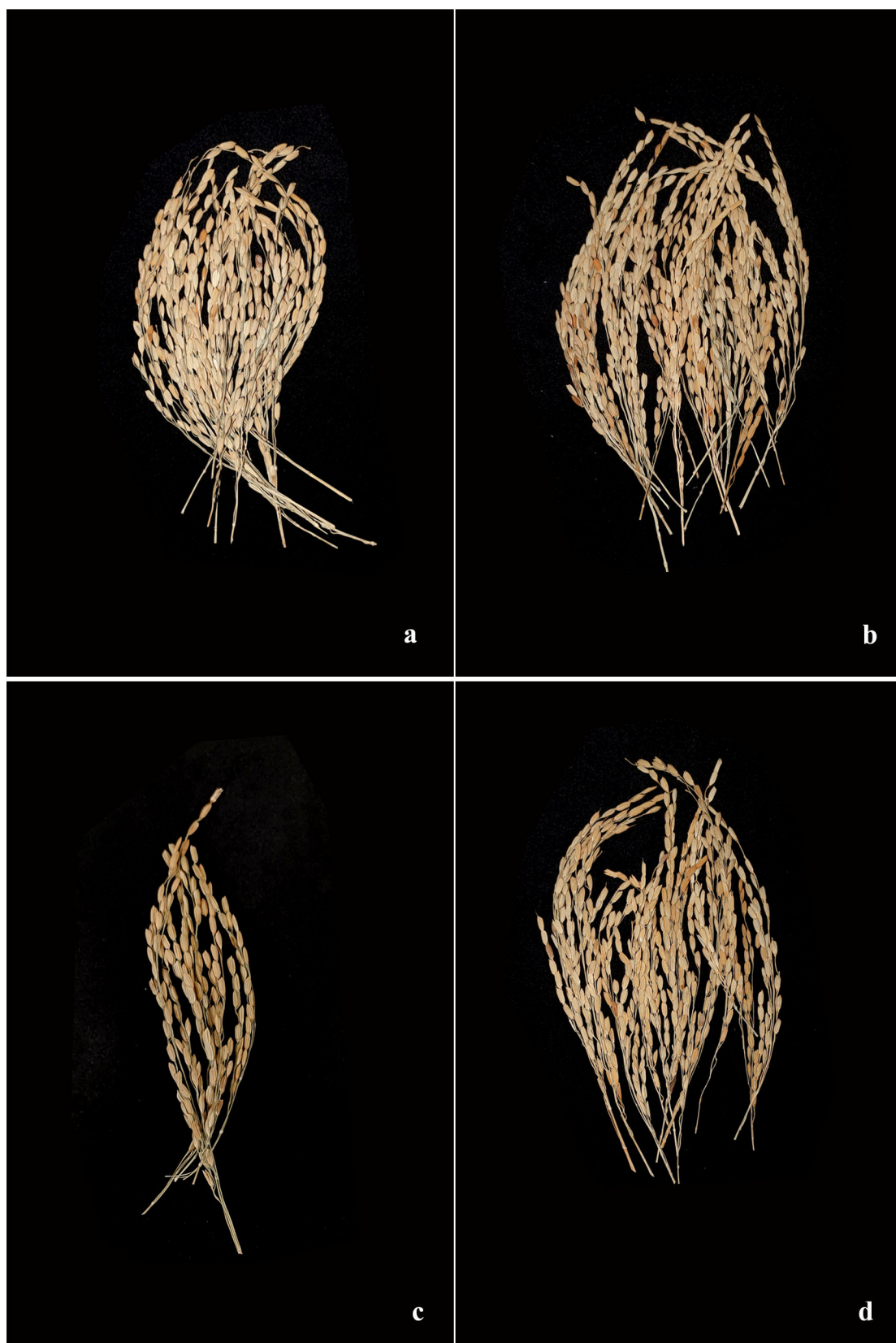


Fig. 43: Panicle morphology of rice var. Hraswa under different treatments.
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

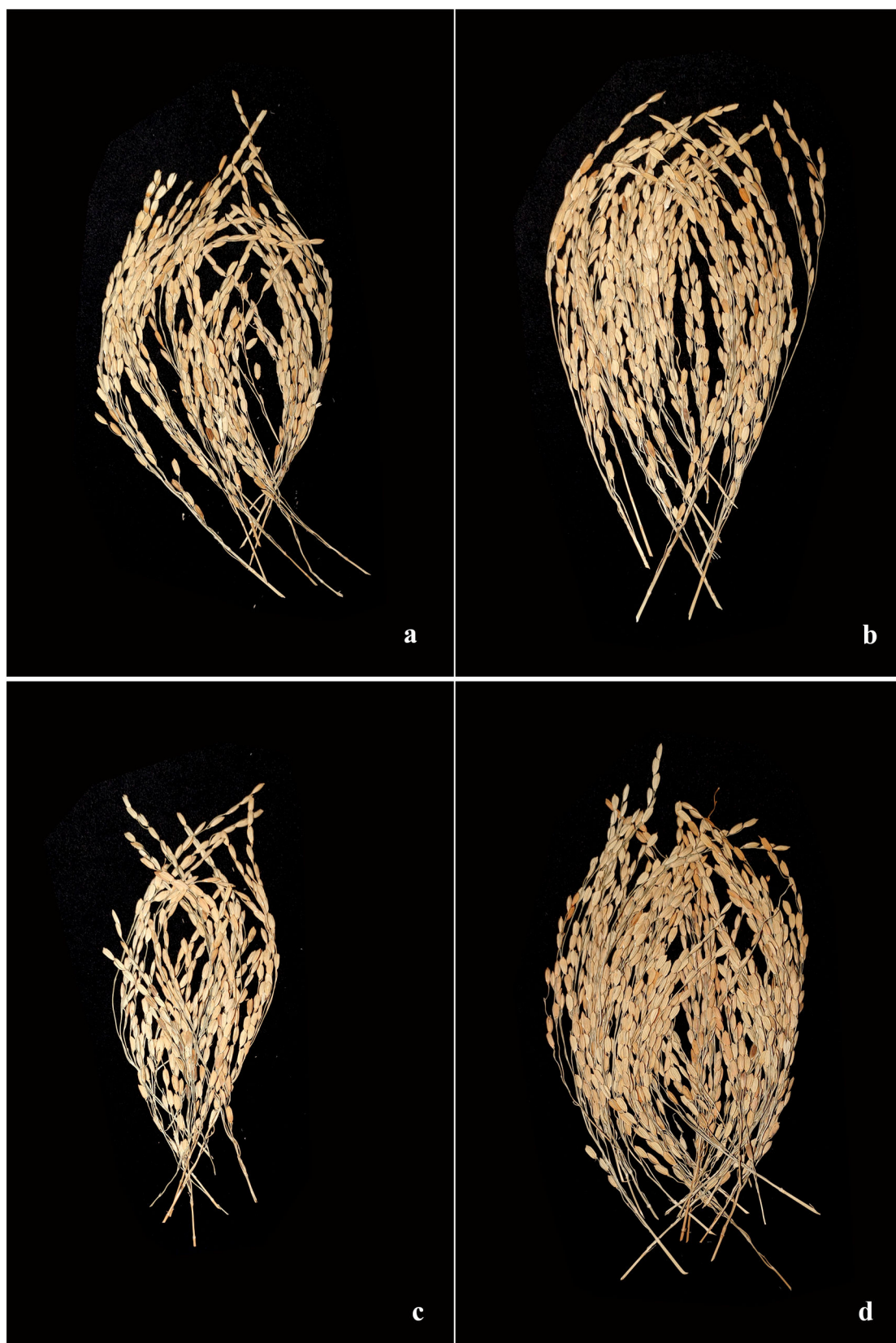


Fig. 44: Panicle morphology of rice var. Manu ratna under different treatments.
a. C (Control) **b.** C+B (Control + Biostimulant) **c.** S (Stress-nutrient deficient condition) **d.** S+B (Biostimulant + Nutrient deficient condition).

Hraswa (58%) followed by Manu ratna (44%) over that of control. Similarly, in C+B treatment, maximum increase was recorded in both varieties with 68% and 62% increase in Hraswa and Manu ratna respectively as compared to control (Fig. 42A and Table 14).

4.12.5 Spikelet fertility

Spikelet fertility was significantly influenced by S treatment with higher reduction in Manu ratna (62%) followed by Hraswa (41%) as compared to control. However, under S+B treatment both varieties exhibited an increase in spikelet fertility with significant enhancement observed in tolerant var. Hraswa (48%) and followed by Manu ratna (39%). Similarly, C+B treatment elicited a significant increase in spikelet fertility of both varieties and it was upto 52-58% as compared to control (Fig. 42A and Table 14).

4.12.6 Seed setting

Under S treatment, seed setting was considerably reduced in Manu ratna (49%) and Hraswa (30%) as compared to control. S+B treatment exhibited significant increase of seed setting in both varieties with highest recorded in var. Hraswa (52%) followed by Manu ratna (40%) over that of control. Similarly, in C+B treatment maximum increase in seed setting was recorded in both varieties with 48% and 22% increase in Hraswa and Manu ratna respectively as compared to control (Fig. 42A and Table 14).

4.12.7 Biological yield

Biological yield was significantly influenced by S treatment with higher reduction in Manu ratna (63%) followed by Hraswa (42%) as compared to control. However, under S+B treatment both varieties exhibited an increase in biological yield with significant enhancement recorded in tolerant var. Hraswa (49%) and followed by Manu ratna (34%). Similarly, C+B treatment showed a significant increase in biological yield in both varieties and it was upto 53-62% as compared to control (Fig. 42A and Table 14).

4.12.8 Harvest index (HI)

Under S treatment, HI was considerably reduced in Manu ratna (52%) and Hraswa (40%) as compared to control. S+B treatment exhibited significant increase in HI of both varieties, with highest HI recorded in var. Hraswa (56%) followed by Manu ratna (43%) over that of control. Similarly, in C+B treatment maximum increase in HI was recorded in both varieties and the increase was 46% and 27% in Hraswa and Manu ratna respectively as compared to control (Fig. 42A and Table 14).

4.12.9 Yield response (%)

The yield response (%) indicated significant variation among the different treatment combinations under both varieties (Fig. 42B). Here SQ represents Syngenta quantis (biostimulant purchased from market). In var. Hraswa, the yield response ranged from approximately 110% to 170%, with the highest value observed in the S+B/SQ treatment (170%), followed by C+B/SQ (140%). Comparatively, in var. Manu ratna, yield responses were generally higher, varying from about 100% to 260%. The maximum yield response was recorded in the S+B/S treatment (260%), followed by C+B/C (160%), whereas C+B/SQ and S+B/SQ exhibited relatively lower responses (100% and 110%, respectively). These results suggest that in var. Manu ratna the yield responses were more effectively enhanced than the Hraswa, particularly in the S+B/S treatment, indicating a possible synergistic effect of the treatment combination under these conditions (Fig. 43 and 44).

4.13 Heat map with hierarchial cluster

Each component's concentration level is shown on the heatmap using a color gradient. Typically, this gradient runs from blue (low concentration) to red (high concentration). The precise values, which show the relative levels or changes of the biological components, fall between -1 and 2.

From the analysis of heat map, it was revealed that there were 4 distinct groups in Var. Hraswa and 5 groups in Var. Manu ratna based on the observation of

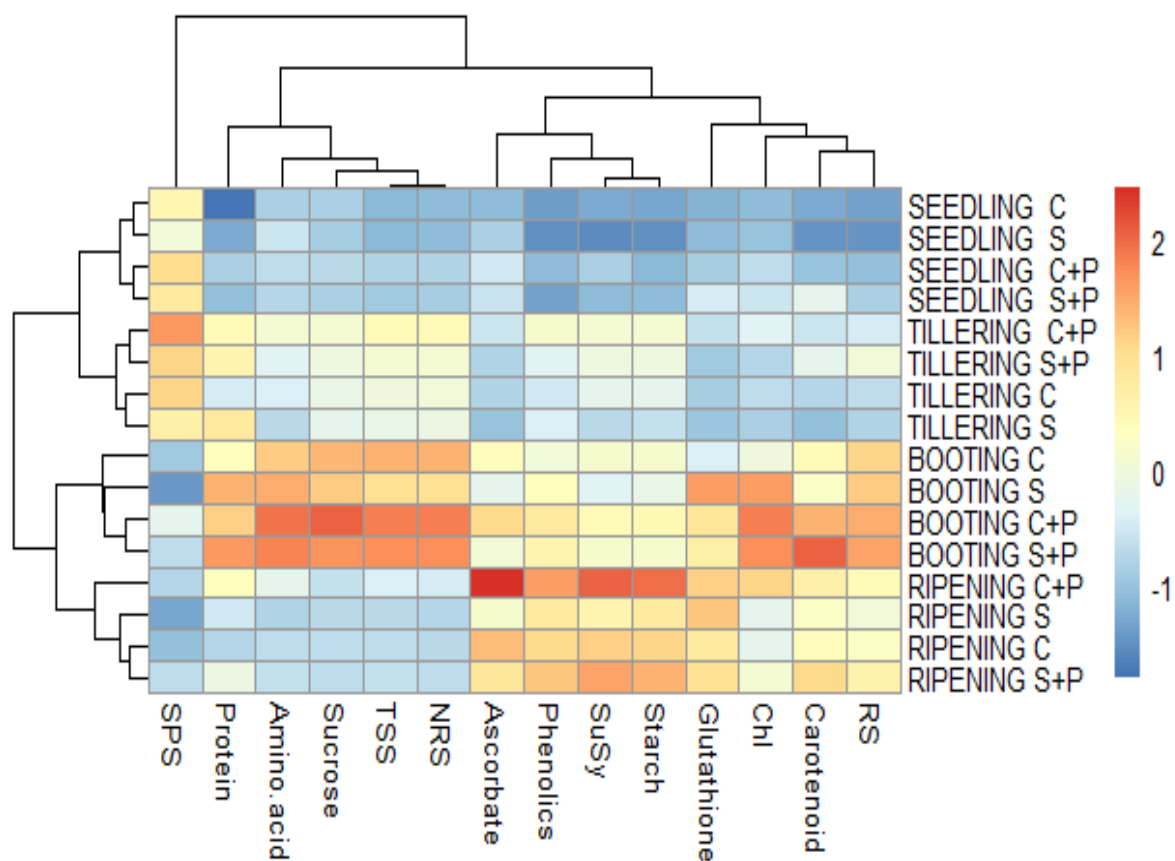


Fig. 45: Heat map with hierarchical cluster based on the effect of biostimulant treatment on carbohydrate and physiological traits in the rice seedlings var. Hraswa at different stages representing S (Seedling), T (Tillering), B (Booting) and R (Ripening). The scale bar represents Z score values of data of each parameter.

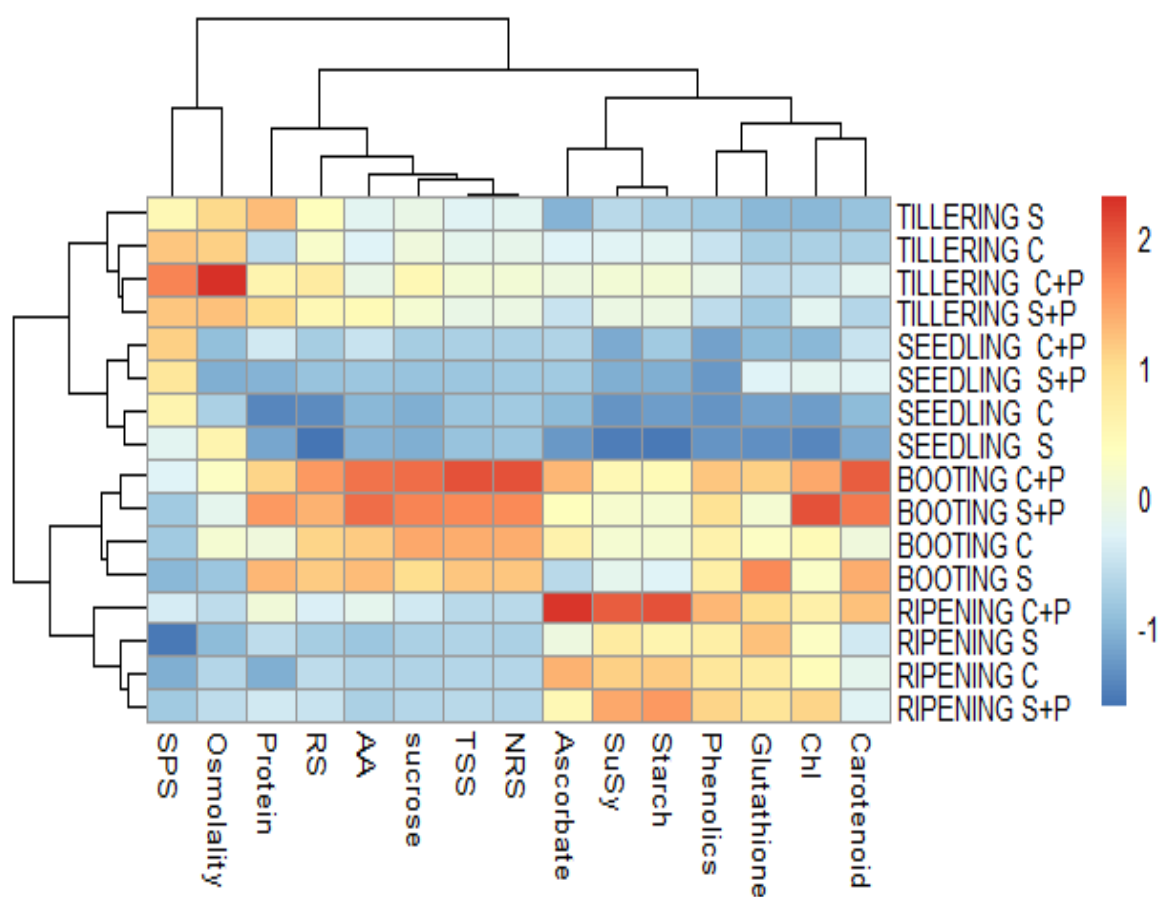


Fig. 46: Heat map with hierarchical cluster based on the effect of biostimulant treatment on carbohydrate and physiological traits in the rice seedlings B) Var. Manu ratna at different stages representing S (Seedling), T (Tillering), B (Booting) and R (Ripening). The scale bar represents Z score values of data of each parameter.



Fig. 47: FTIR analysis of biostimulant treated rice leaves var. Hraswa at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition).



Fig. 48: FTIR analysis of biostimulant treated rice leaves var. Manu ratna at booting stage. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant+Nutrient deficient condition).

cluster linkage (Fig. 45 and 46). In var. Hraswa, the four distinct groups were observed among the parameters during the different growth stages of rice. The 1st group includes RS, carotenoid, Chl, glutathione. The 2nd group include starch, SuSy, phenolics, ascorbate. The 3rd group include NRS, TSS, sucrose, AA and protein while the 4th group includes only SPS (Fig. 45).

In var. Manu ratna, the 1st group include chlorophyll and carotenoid, the 2nd group include glutathione and phenolics, 3rd group include starch, SuSy and ascorbate, 4th group include NRS, TSS, sucrose, AA, RS and protein, 5th group include osmolality and SPS (Fig.46).

Among the four growth stages (seedling, tillering, booting, and ripening), carbohydrate composition and key metabolites were most prominent in the booting stage. However, their accumulation decreased during ripening. Among the four treatments (C, C+B, S, S+B), C+B shows higher levels of most biochemical components across all stages compared to control. And in S+B, the levels of biochemical components (TSS, sucrose, AA, RS and protein) are consistently moderate to high with respect to S (Fig. 45 and 46).

4.14 Functional group characterisation using FTIR

The FTIR spectral analysis of Var. Hraswa and Manu ratna was conducted over the wavenumber range of 4300–300 cm^{-1} (as peaks started and observed from these regions) to identify functional groups present in the samples under various treatments. The corresponding transmittance spectra are shown in Fig. 47 and 48.

In var. Hraswa, broad absorption bands was observed around 3300–3200 cm^{-1} in all treatments, corresponding to O–H stretching vibrations, indicative of hydroxyl groups and the intensity of this band increased notably in C+B and S+B treatments, suggesting enhanced hydrogen bonding or moisture retention. Sharp bands near 2918 and 2850 cm^{-1} are attributed to C–H stretching vibrations from aliphatic $-\text{CH}_2$ and $-\text{CH}_3$ groups. These bands were consistently present across all treatments with marginal intensity variations. A distinct peak at approximately 1720–1700 cm^{-1} , most prominent in the C+B treatment, corresponds to C=O stretching vibrations. This indicates the presence of carbonyl groups, possibly from esters, aldehydes, or acids and the significant enhancement of this peak is seen in

C+B treatment. In the 1600–1500 cm^{-1} region, bands were observed which are characteristic of amide II (N–H bending) or aromatic C=C stretching. These were more intense in C+B and S treatments, indicating potential changes in aromatic compound content. The fingerprint region (1300–1000 cm^{-1}) displayed multiple strong bands across all treatments, attributed to C–O–C and C–O stretching vibrations, typically associated with esters, ethers, or carbohydrate structures. These bands were significantly more pronounced in the C+B and S+B treatments, suggesting structural modification of polysaccharides or formation of new ester linkages. The fingerprint region below 900 cm^{-1} showed complex and distinct patterns unique to each sample and the most prominent features were observed in the C+B treatment (Fig. 47).

Whereas in var. Manu ratna, a broad absorption band between 3300–3200 cm^{-1} was observed across all treatments, indicative of O–H stretching vibrations. This band was most intense in the S+B and C+B treatments. C–H stretching bands appeared near 2920 cm^{-1} and 2850 cm^{-1} , consistent in all treatments, confirming the presence of aliphatic hydrocarbons. A sharp peak around 1730–1700 cm^{-1} was detected in all treatments, with significantly higher intensity in C+B and S+B, corresponding to C=O stretching vibrations, likely from esters or acids. The 1600–1500 cm^{-1} region exhibited prominent peaks, especially in S and S+B, indicating the presence of amide II or aromatic C=C vibrations, which may be associated with protein or phenolic compounds. In the 1300–1000 cm^{-1} range, strong and multiple peaks were observed in all treatments, particularly in S+B, suggesting C–O stretching typical of polysaccharides or ester linkages. The fingerprint region (900–600 cm^{-1}) showed complex patterns and differences among treatments. The S+B treatment exhibited more defined and deeper absorption bands (Fig. 48).

4.15 Metabolomic approach using GC-MS/MS

4.15.1 Metabolome Profiling

Differential accumulation of metabolites was observed in each treatment of C, C+B, S and S+B in leaves by application of biostimulant under control and nutrient deficient conditions. A total of 305 metabolites were detected across all samples. Among these, amino acids, amines, organic acids, sugars, sugar acids,

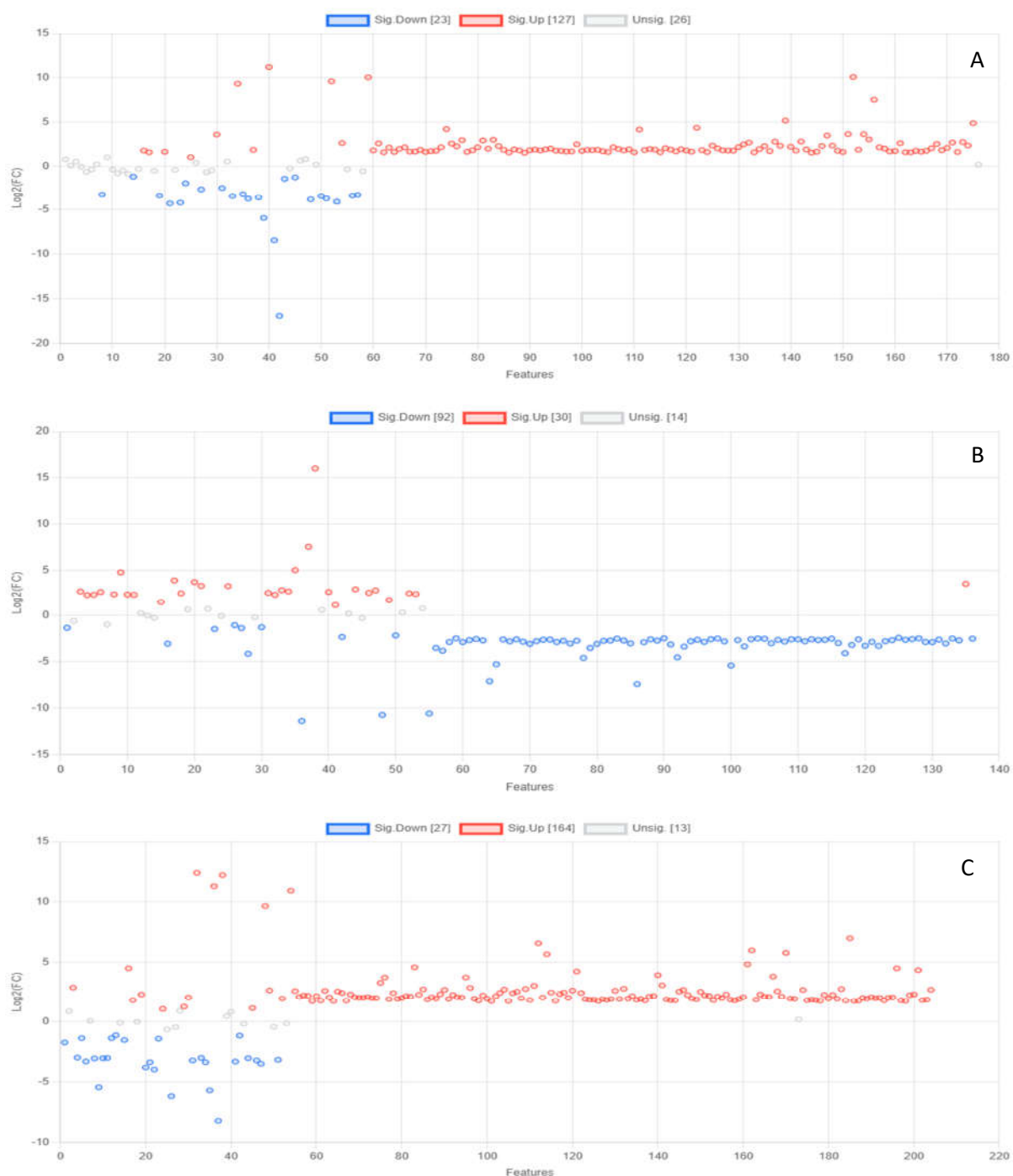


Fig. 49: Fold change analysis depicting significantly upregulated and downregulated metabolites accumulated in leaves of var. Manu ratna in response to biostimulant application under different treatments in rice. A) Biostimulant application under control condition in rice (C+B vs C); B) nutrient deficient condition in rice (S vs C); C) biostimulant application under nutrient deficient condition in rice (S+B vs C). Metabolite data were derived from GC-MS. Blue colour represents downregulated metabolites; Red colour represents upregulated metabolites and Grey colour represents insignificant metabolites.

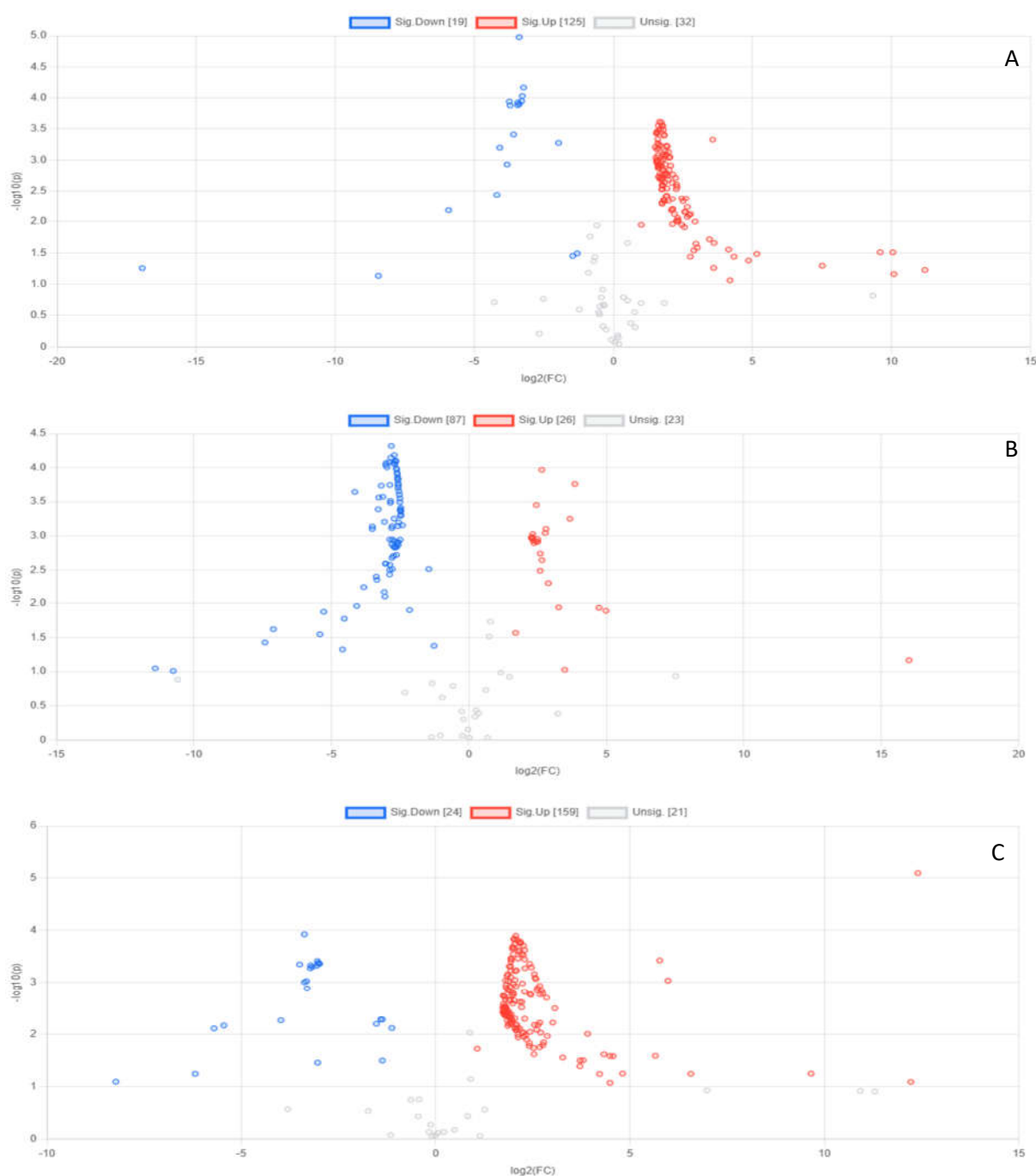


Fig. 50: Volcano plot depicting significantly upregulated and downregulated metabolites accumulated in leaves of var. Manu ratna in response to biostimulant application under different treatments in rice. A) Biostimulant application under control condition in rice (C+B vs C); B) nutrient deficient condition in rice (S vs C); C) biostimulant application under nutrient deficient condition in rice (S+B vs C). Metabolite data were derived from GC-MS. Blue colour represents downregulated metabolites; Red colour represents upregulated metabolites and Grey colour represents insignificant metabolites.

sugar alcohols, and fatty acids were categorized as major groups based on their relative abundance and well-established roles in different treatments of control and stress response. Only the metabolites having a fold change of more than 2 across different treatment groups and a p value less than 0.05 were considered differentially abundant. Fold change analysis and volcano plot revealed the number of upregulated and downregulated metabolites in different treatments. In C+B treatment, 127 metabolites were significantly upregulated, 23 metabolites were significantly downregulated and 26 were found as insignificant metabolites as compared to control. While S treatment recorded 92 metabolites as significantly downregulated, 32 metabolites as significantly upregulated and 14 insignificant metabolites with respect to control. In S+B treatment, 164 metabolites were significantly upregulated, 27 metabolites were significantly downregulated and 13 were insignificant metabolites as compared to control (Fig. 49 and 50).

4.15.2 Partial least square discriminant analysis (PLS-DA) and score plot

Principal Component Analysis (PCA) was performed to explore the overall variance and clustering patterns in the metabolic profiles of the four treatment groups: C+B, S+B, C, and S. The resulting pairwise PCA presents the first five principal components (PCs), cumulatively explaining a substantial 94% of the total variance in the dataset. Specifically, PC1, PC2, and PC3 account for 40%, 21%, and 10% of the variance, respectively, indicating that the majority of the biologically meaningful variation among samples is well captured by these three components (Fig. 51). While, the PLS-DA score plot represents 69% of the total variance with PC1 individually contributing variance of 44% and PC2 25% (Fig. 52).

In the PC1 vs PC2 projection, clear group-level patterns were observed. Samples from the C+B (red circles) and S+B (green triangles) treatments tend to cluster towards the right-hand side along PC1, while the S (blue crosses) and C (blue plus signs) treatments were more dispersed along the left of PC1. This spatial separation suggests that PC1 predominantly captures variation associated with the metabolic changes induced by C+B and S+B treatments, possibly related to lipid metabolism or antioxidant responses, as suggested by the VIP score analysis. Along

PC2, which explains 21% of the variance, the S+B group shows partial separation from C+B, further indicating that S+B induces a distinct metabolic profile. Meanwhile, C and S treatments show more overlap in the PC2 dimension, however some individual variation is evident, suggesting subtle metabolic differences between these two conditions.

The PC3 axis (10%) further refines group differentiation. In the PC1 vs PC3 and PC2 vs PC3 projections, the treatments exhibit additional clustering patterns, particularly with S+B samples consistently occupying a distinct region in the multivariate space, reinforcing the hypothesis of a treatment-specific metabolic shift. PC4 and PC5, which contribute 19% and 2% of the variance respectively, offer minimal additional separation among the groups, indicating that most of the relevant biological information is captured by the first three components. Overall, the PCA analysis demonstrates a clear separation of metabolic profiles among the four treatments, with C+B and S+B forming relatively tight clusters and showing strong divergence from S and C. This separation reflects the systematic biochemical changes triggered by each treatment and underscores the utility of PCA in capturing global patterns in complex metabolomics data.

4.15.3 Principal component analysis (PCA) biplot

The principal component analysis (PCA) biplot constructed using the metabolomic profiles of the four treatment groups: C+B, S+B, C, and S is represented in Fig 53. The biplot visualizes both the scores (samples) and loadings (metabolites) in the plane defined by PC1 and PC2, which together explain a total of 67% of the overall variance (PC1: 41%, PC2: 26%). This high cumulative variance indicates that the primary sources of variation in the dataset are well represented in this two-dimensional space (Fig. 53).

Distinct clustering of treatment groups is observed. The C+B group (pink circles) is clearly separated in the upper left quadrant, the S+B group (green circles) clusters in the lower right quadrant, while the C (blue crosses) and S (light blue circles) groups localize predominantly in the lower left quadrant. This spatial

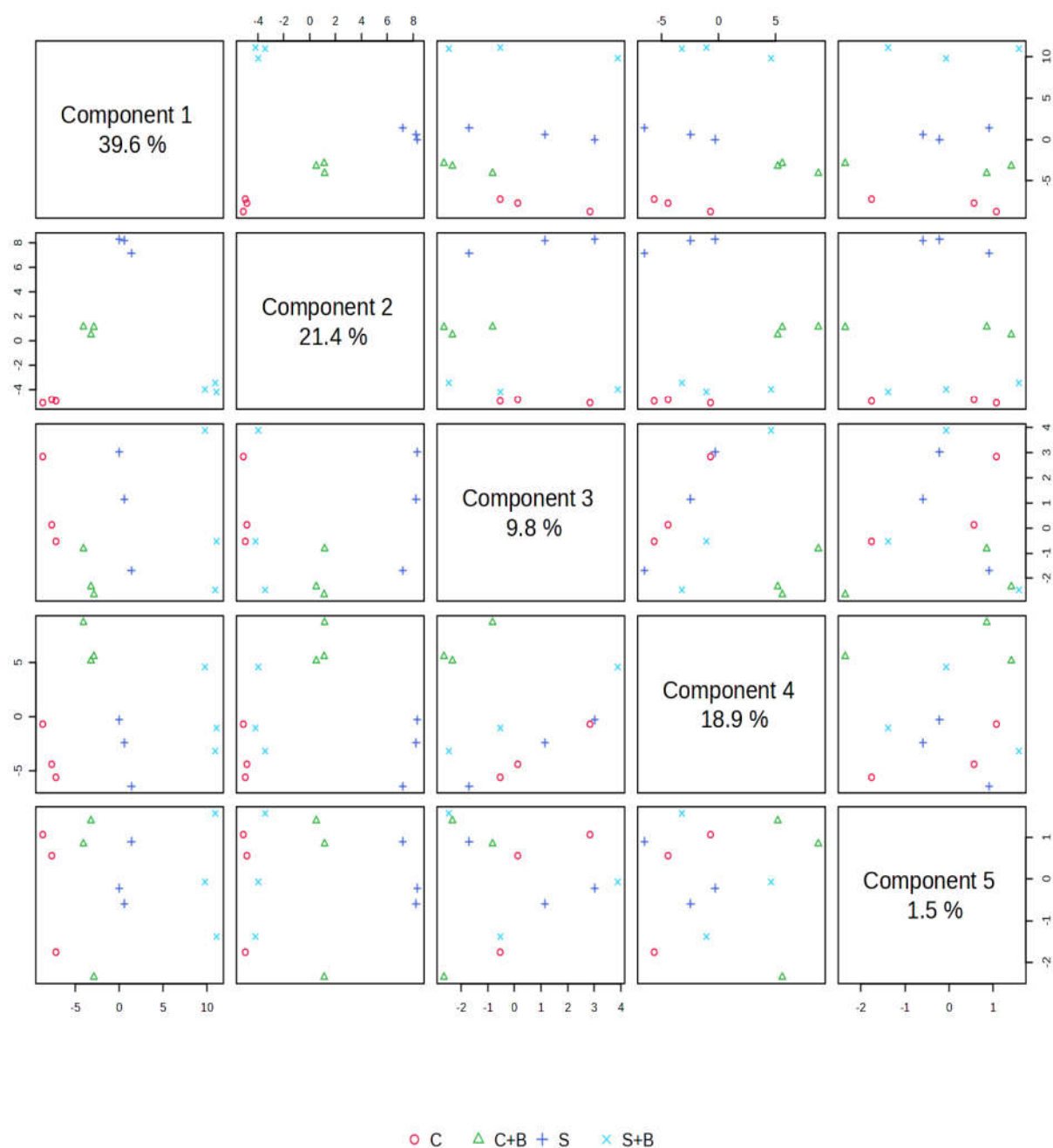


Fig. 51: Partial least squares discriminant analysis (PLS-DA) plots depicting variation in metabolites accumulated in leaves of var. Manu ratna in response to biostimulant application under different treatments in rice. Metabolite data were derived from GC-MS. PLS components represent the major axes of variation, highlighting treatment-induced metabolic shift.

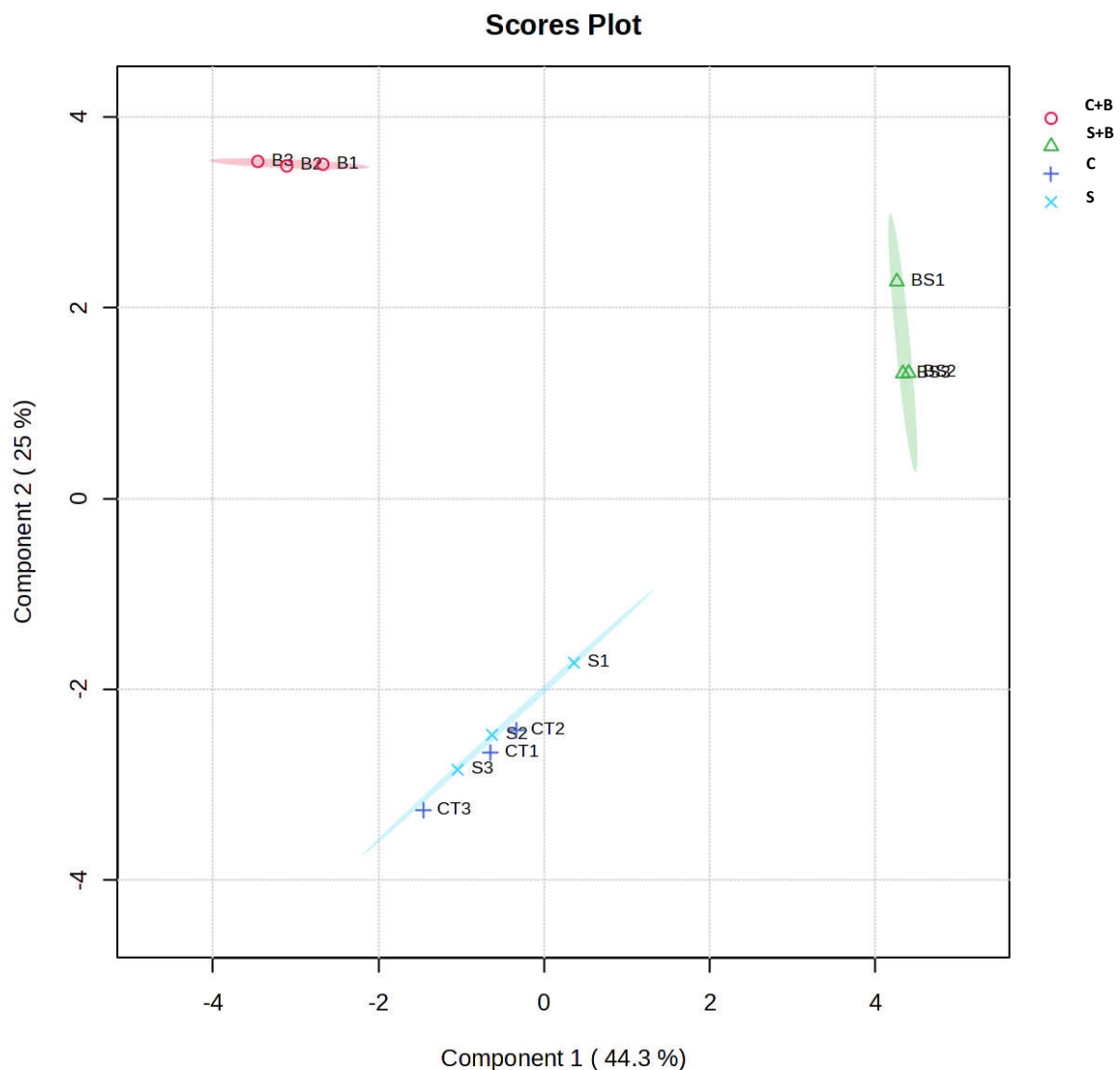


Fig. 52: PLS-DA scores plot illustrating the separation of metabolite profiles in leaves of var. Manu ratna under different treatments in rice: C+B-Biostimulant application alone (red circles), S+B- Biostimulant under nutrient deficiency (green triangles), C-Control (blue plus symbols), and S-Nutrient deficiency alone (blue crosses). The distinct clustering of each treatment group highlights the variation in metabolite composition, with clear discrimination particularly between BS and other conditions. This suggests a strong metabolic response to biostimulant application under nutrient-deficient conditions.

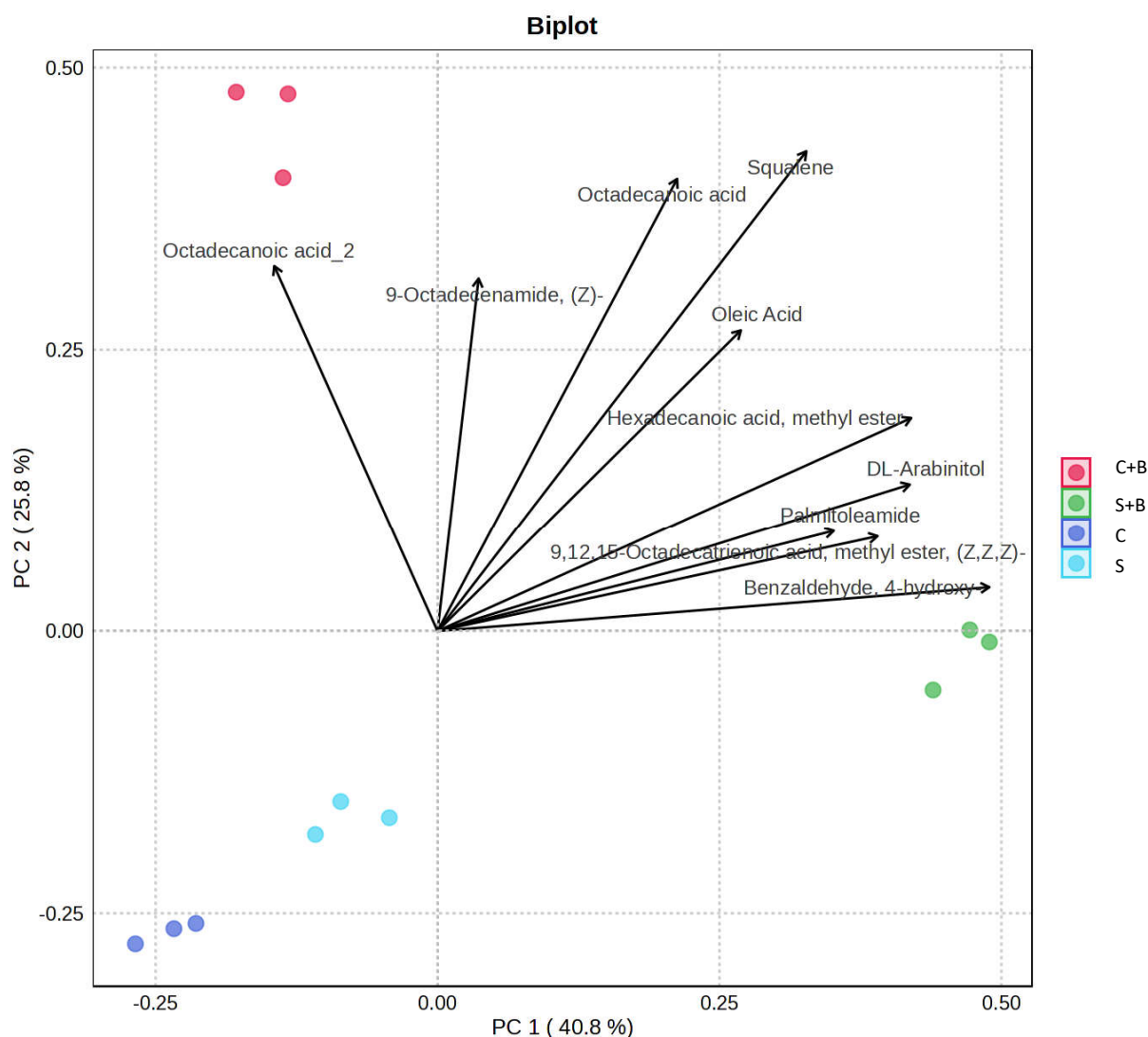


Fig. 53: Biplot representing the principal component analysis (PCA) of metabolites in leaves of var. Manu ratna under different treatments in rice. The treatments include: C+B-Biostimulant alone (red); S+B-Biostimulant application under nutrient deficiency (green); C-Control plants (blue); and S-Nutrient-deficient condition without biostimulant (cyan). The analysis demonstrates distinct clustering of treatments, highlighting differential accumulation of metabolites. Metabolites such as octadecanoic acid, squalene, and oleic acid are associated with BS treatment, indicating their potential role in biostimulant-mediated stress mitigation. Data were obtained using GC-MS.

separation signifies unique metabolic signatures associated with each treatment condition. The loading vectors (arrows) represent the influence of specific metabolites on sample distribution and indicate the direction and magnitude of each variable's contribution to the PCs. Longer arrows denote stronger influence. Several key metabolites are highly influential along PC1, driving the separation between the C+B/S+B treatments and the C/S treatments.

Some of the specifically important metabolite features are:

- Squalene, octadecanoic acid, oleic acid, hexadecanoic acid (methyl ester), and DL-arabinitol are positively correlated with PC1 and strongly associated with the S+B group, which appears in the same direction of the vectors.
- Conversely, octadecanoic acid and 9-octadecenamide are aligned towards the C+B group, indicating their elevated levels under this treatment.
- The C and S treatments, found in the lower left quadrant, are located opposite to the main lipid-related metabolite vectors, implying a comparatively lower abundance of these compounds.
- Metabolites such as 4-hydroxy benzaldehyde and 9,12,15-octadecatrienoic acid methyl ester are also positioned in the direction of the S+B cluster, suggesting a possible link to oxidative signaling or aromatic compound metabolism in this treatment.
- The orthogonal orientation of certain vectors indicates distinct metabolic roles or regulation patterns.

In conclusion, the biplot effectively demonstrates treatment-specific metabolic reprogramming, with S+B treatment showing a pronounced shift in lipid-related metabolites, C+B treatment linked to specific fatty acid amides, and C/S treatments displaying a distinct, less lipid-intensive profile. This visualization corroborates the VIP score findings and provides mechanistic insights into how each treatment modulates the rice metabolome differently.

4.15.4 Variable important projectile (VIP) score

The Variable Importance in Projection (VIP) scores derived from the Partial Least Squares Discriminant Analysis (PLS-DA), highlighting the top metabolites contributing most significantly to the separation among the four treatment groups: C+B, S+B, C, and S (Fig 34). VIP scores provide a quantitative measure of each metabolite's influence in class discrimination within the PLS-DA model, with values greater than 1 generally considered as indicative of high importance. In this analysis, the top-ranking metabolites include squalene, octadecanoic acid, hexadecanoic acid, and oleic acid, all of which exhibit VIP scores above 2.5, suggesting a strong role in distinguishing between treatments (Fig. 54).

The accompanying heatmap offers a visual representation of the relative abundance of each metabolite across treatments, with a color gradient ranging from red (high abundance) to blue (low abundance). Squalene, a triterpenoid involved in sterol biosynthesis and stress response, was notably elevated in treatment S+B, indicating a possible enhancement of isoprenoid or antioxidant pathways. Similarly, octadecanoic acid (stearic acid) and hexadecanoic acid (palmitic acid), both long-chain saturated fatty acids, were significantly more abundant in C+B and S+B treatments, pointing towards a lipid accumulation or remodeling response under these conditions. Oleic acid, a monounsaturated fatty acid, also was positioned at a higher level in these groups, which may reflect shifts in membrane fluidity or stress-related lipid metabolism.

Other key metabolites such as 9-octadecenamide, L-serine, catechin, L-cysteine, and DL-arabinitol also contributed to class separation in S+B. For instance, L-serine and L-cysteine, both amino acids linked to stress signaling and redox balance, showed higher relative abundance in C and S treatments, suggesting altered nitrogen metabolism or oxidative stress response. Catechin, a polyphenolic compound with known antioxidant activity, was also elevated in these groups, reinforcing the possibility of an enhanced defense mechanism. Notably, trehalose-6-phosphate, a sugar signaling molecule, and malic acid, a TCA cycle intermediate, showed differential abundance across treatments, hinting at metabolic shifts in

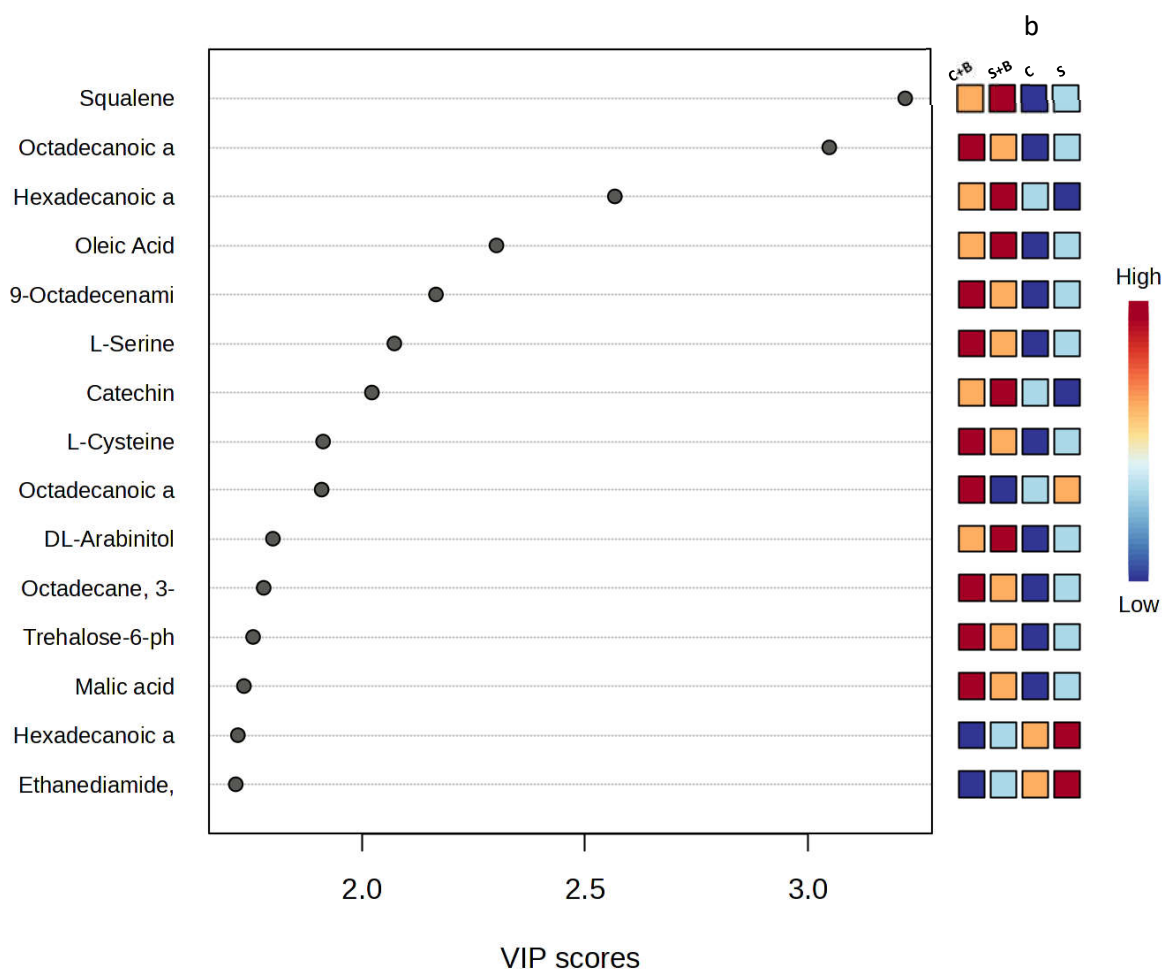


Fig. 54: Variable Importance in Projection (VIP) score plot showing the top discriminating metabolites identified in leaves of var. Manu ratna under different treatments in rice. Treatments include: C+B-Biostimulant application alone, S+B-Biostimulant under nutrient deficiency, C-Control, and S-Nutrient deficiency alone. The heatmap alongside each metabolite indicates relative abundance across treatments, with red representing high abundance and blue indicating low abundance. Squalene, octadecanoic acid, hexadecanoic acid, and oleic acid were among the most influential metabolites contributing to treatment discrimination, as derived from GC-MS analysis.

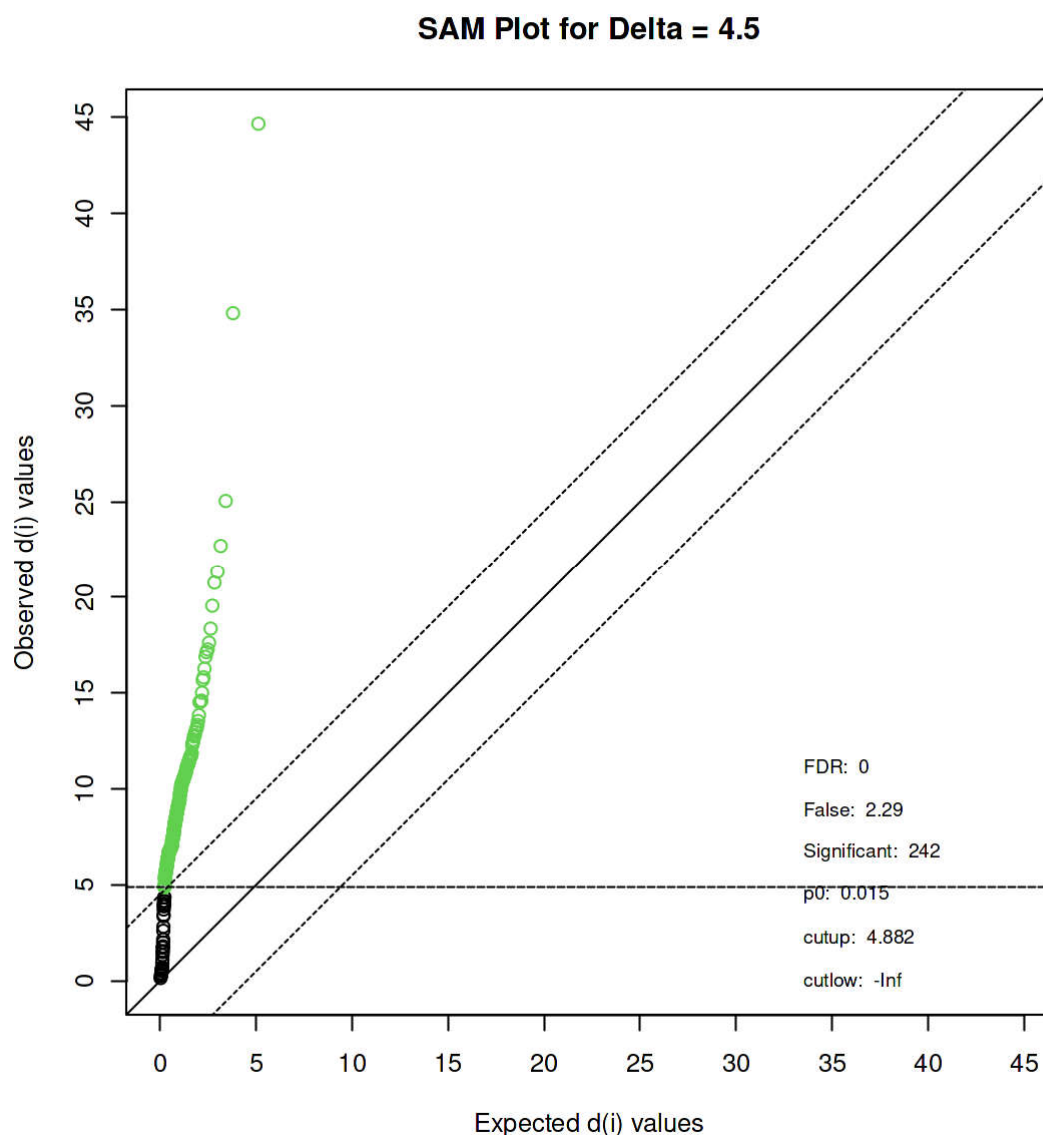


Fig. 55: Significance Analysis of Microarrays (SAM) plot for Delta = 4.5 showing differential metabolite expression in leaves of var. Manu ratna under different treatments in rice. The plot displays the observed d(i) values versus the expected d(i) values under the null hypothesis. Metabolites outside the upper and lower dotted lines are considered significantly upregulated or downregulated. A total of 242 metabolites were identified as significant (FDR = 0), indicating a strong metabolic response to treatment with minimal false positives. The analysis highlights the robust discriminative power of the experimental conditions on metabolite profiles.

carbohydrate and energy pathways. Overall, the VIP analysis effectively highlights treatment-specific metabolic reprogramming, particularly emphasizing the differential regulation of lipids, amino acids, and antioxidant-related pathways. These findings provide insight into the underlying biochemical mechanisms influenced by each treatment and support the hypothesis that the metabolic response is distinctly tailored under different experimental conditions.

4.15.5 Significance Analysis of Microarrays (SAM) plot

The SAM (Significance Analysis of Microarrays) plot is used to identify significant and differentially expressed metabolites across the treatment groups (Fig 53). In this plot, the expected $d(i)$ values (under the null hypothesis of no difference) are plotted on the x-axis, while the observed $d(i)$ values (actual relative difference between groups) are plotted on the y-axis. The black diagonal line represents the null hypothesis (i.e., no differential expression), while the dashed lines indicate the thresholds for significance at a chosen delta value of 4.5.

A total of 242 metabolites exceeded the significance threshold, as indicated by green dots falling outside the dashed boundaries. These represent C+B and S+B treatments whose observed expression are greater than expected, marking them as significantly altered metabolites. The plot shows a clear deviation from the diagonal line, highlighting a strong treatment effect. The estimated number of false positives is only 2.29, which is exceptionally low relative to the total number of significant features, supporting the robustness of the findings (Fig. 55).

The cutup value is 4.882, meaning any metabolite with a $d(i)$ score greater than this threshold is considered significant. The p_0 value (0.015) indicates the estimated proportion of truly null features, further supporting the reliability of the significance in treatments. Overall, the SAM analysis identified a large number of metabolites with statistically significant changes across treatments, reinforcing the presence of widespread metabolic reprogramming. These significantly altered metabolites can now be prioritized for further biological interpretation, pathway analysis, and biomarker discovery.

4.15.6 Pathway enrichment analysis

MetaboAnalyst-based pathway analysis identified key metabolic pathways influenced by biostimulant treatment under control and nutrient deficient condition. Key pathway statistics include impact score (which reflects the pathway topological score) and match status (indicating coverage as observed hits over total pathway compounds). The differential coloring and size of the nodes in the plot correspond to the statistical significance and pathway impact, respectively, with red-colored and larger-sized nodes indicating the most significantly perturbed metabolic routes.

The metabolic pathway analysis revealed several significantly impacted pathways in response to the C+B, S and S+B treatment as compared to control. In C+B treatment, *starch and sucrose metabolism* was the most enriched and impactful pathway, exhibiting the highest $-\log_{10}(p)$ value and a substantial pathway impact score. This suggests a prominent alteration in carbohydrate metabolism. Other significantly affected pathways included *alpha-linolenic acid metabolism*, *glycine, serine and threonine metabolism*, and *phenylalanine, tyrosine and tryptophan biosynthesis*, indicating notable changes in lipid and amino acid metabolism. Additionally, pathways such as *terpenoid backbone biosynthesis*, *glycerolipid metabolism*, and *valine, leucine and isoleucine biosynthesis* were moderately impacted, reflecting a broader reprogramming of primary and secondary metabolism. Lesser, but still notable, impacts were observed in energy-related pathways like the *TCA cycle* and *pyruvate metabolism* (Fig. 56).

In S treatment, the most significantly enriched pathway was *alanine, aspartate and glutamate metabolism*, with the highest $-\log_{10}(p)$ value, indicating robust alterations in amino acid metabolism. *Flavone and flavonol biosynthesis* and *linoleic acid metabolism* were also highly impacted, both exhibiting strong pathway impact scores nearing 1.0, suggesting critical changes in secondary metabolite and lipid pathways, respectively. Additional affected pathways include *starch and sucrose biosynthesis*, *pyruvate metabolism*, and *alpha-linolenic acid metabolism*, reflecting broader shifts in carbohydrate and lipid metabolism under nutrient stress. Amino acid-related pathways such as *phenylalanine, tyrosine and tryptophan*

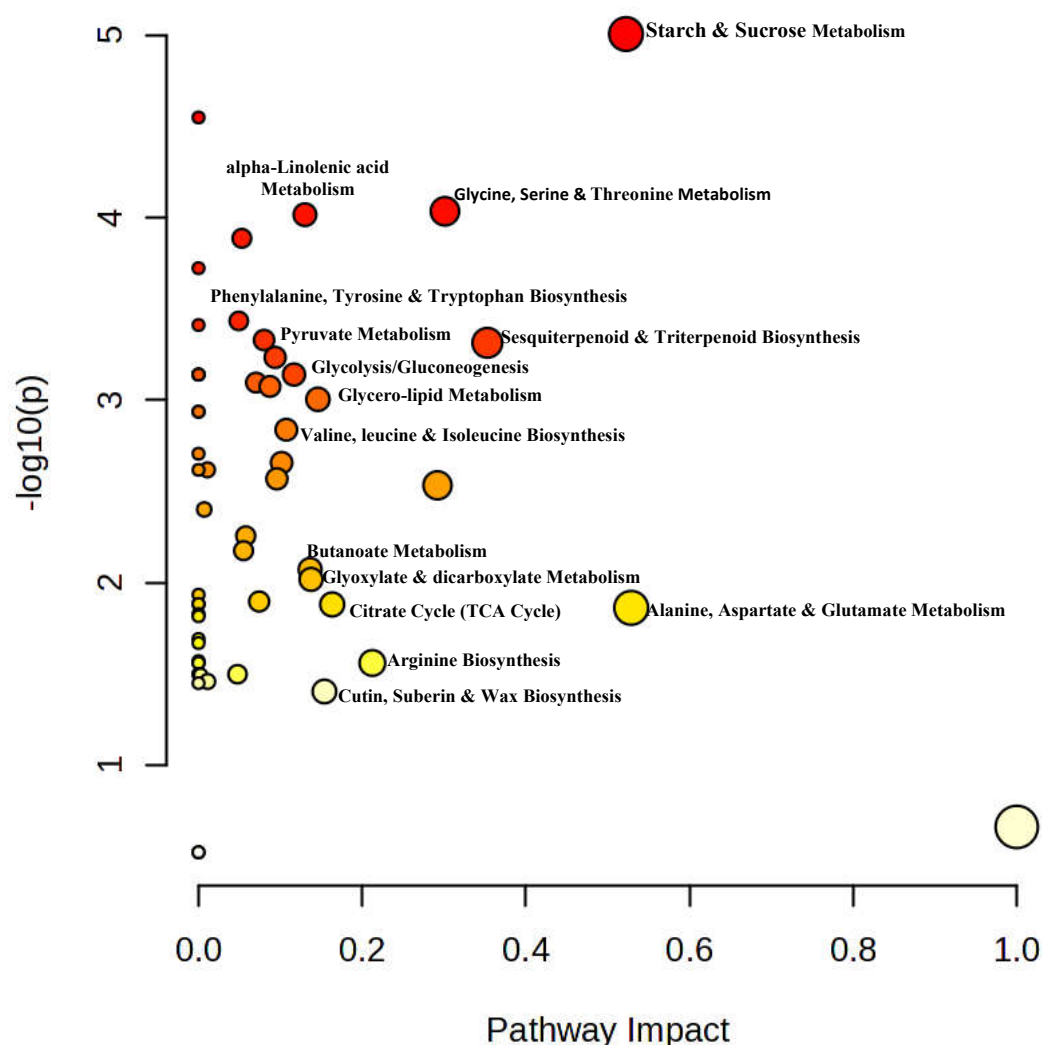


Fig. 56: MetaboAnalyst-generated pathway enrichment plots ("metabolome view") illustrating significantly affected metabolic pathways in response to biostimulant application under control condition in rice (C+B vs C). Arranged by the statistical significance ($-\log_{10}$ of p-value) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

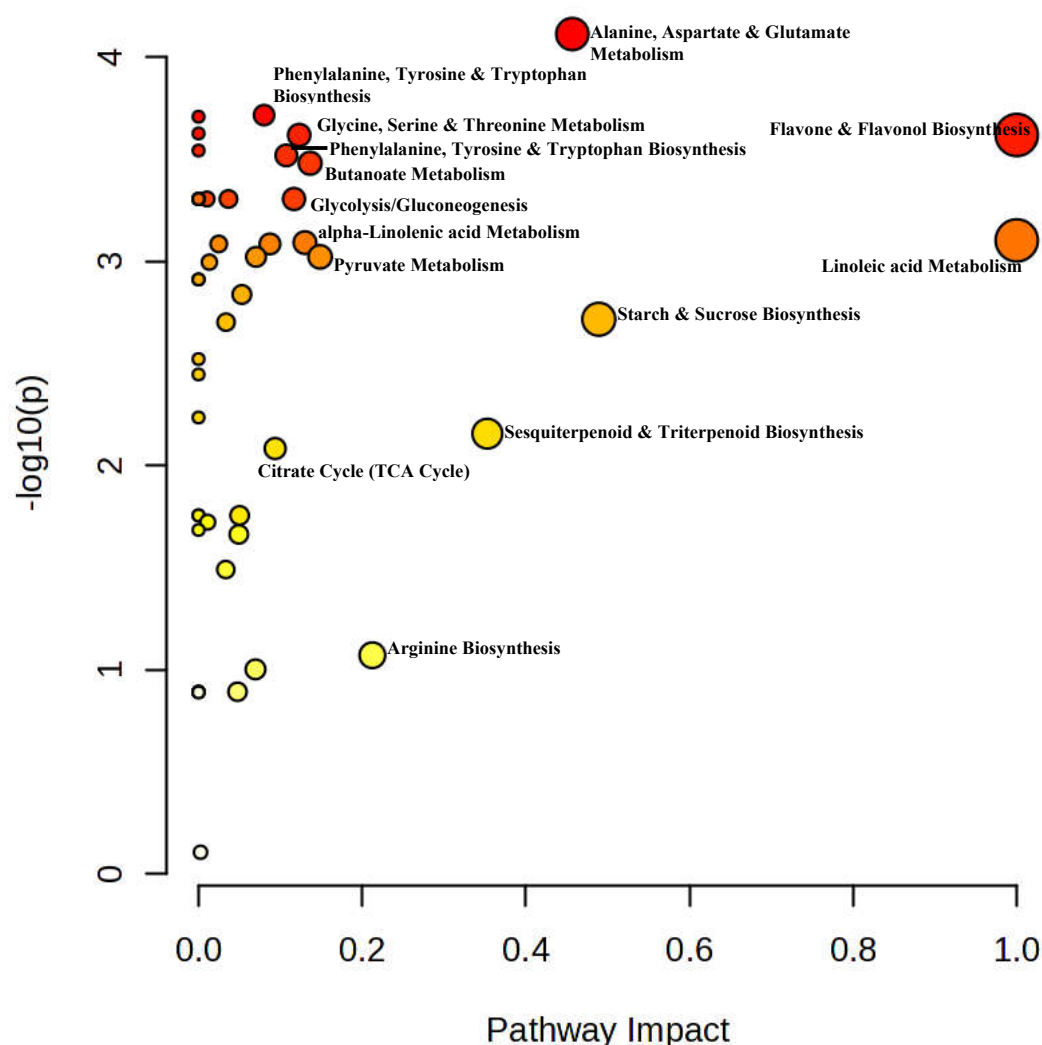


Fig. 57: MetaboAnalyst-generated pathway enrichment plots (“metabolome view”) illustrating significantly affected metabolic pathways in response to nutrient deficient condition in rice (S vs C). Arranged by the statistical significance ($-\log_{10}$ of p-value) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

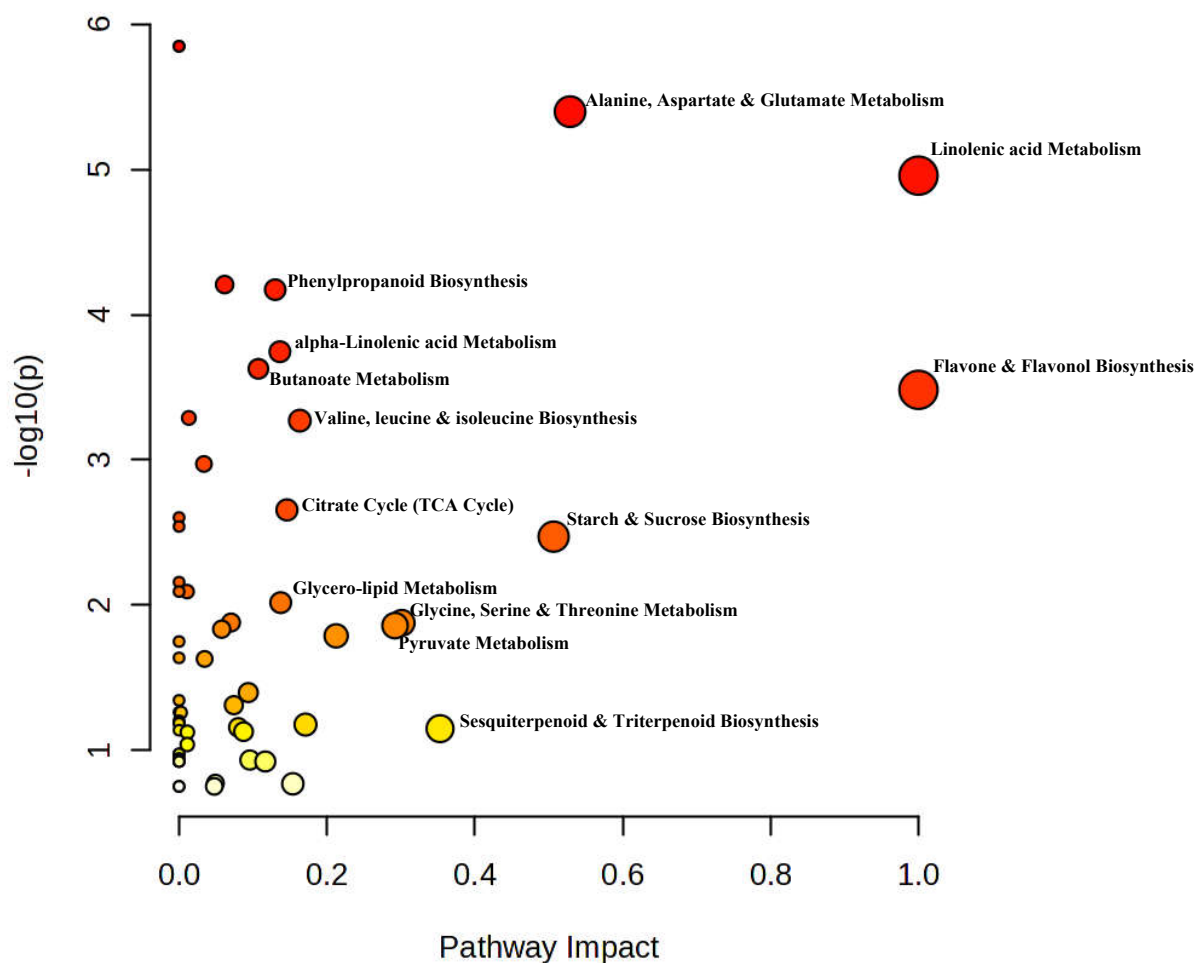


Fig. 58: MetaboAnalyst-generated pathway enrichment plots (“metabolome view”) illustrating significantly affected metabolic pathways in response to biostimulant application under nutrient deficient condition in rice (S+B vs C). Arranged by the statistical significance ($-\log_{10}$ of p-value) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

biosynthesis and *glycine, serine and threonine metabolism* also showed moderate impact and significance. The size of the nodes represents the degree of metabolite coverage within each pathway, with larger circles indicating more metabolites involved. The color gradient from yellow to red further emphasizes statistical significance, with red indicating highly significant pathways. These findings highlight extensive reprogramming of metabolic networks in response to nutrient deprivation in rice, particularly involving amino acid biosynthesis, energy metabolism, and defense-related secondary metabolism (Fig. 57).

In contrast, under biostimulant application during stress (S+B), there was a marked enhancement in flavone and flavonol biosynthesis, starch and sucrose biosynthesis, and glucose-lipid metabolism, suggesting improved energy status and activation of antioxidant and protective secondary metabolite pathways. Notably, linoleic acid metabolism remained significantly impacted across both conditions, but with higher pathway impact and significance in the biostimulant-treated plants, implying enhanced modulation of lipid signaling or membrane dynamics. The observed increase in pathway coverage and statistical significance (larger, redder nodes) for energy and antioxidant-related pathways under biostimulant treatment further supports the idea that the biostimulant mitigates stress by activating specific protective metabolic circuits (Fig. 58).

4.16 Metabolomic approach using HRLC-MS

4.16.1 Metabolome Profiling

High-resolution liquid chromatography-mass spectrometry (HR-LCMS) analysis was conducted to identify metabolomic alterations across the different treatment groups, including control (C), control with biostimulant (C+B), stress (S), and stress with biostimulant (S+B). The data were subjected to univariate statistical analysis to identify significant metabolic features that distinguish between treatment conditions.

A total of 1,410 metabolic features were detected and processed. Among these, 561 features were identified as statistically significant based on a p-value

threshold of < 0.05 (represented as $-\log_{10}(p) > 1.3$), and are depicted in purple in the scatter plot. The remaining features, shown in grey, did not exhibit statistically significant changes. The significant features were distributed across the entire feature set, suggesting that the observed metabolic variations were not limited to specific retention times or mass ranges, but rather reflect a widespread metabolic response to treatment. The $-\log_{10}(p)$ values of significant features reached as high as ~ 28 , indicating highly robust statistical significance for several metabolites.

In C+B treatment, 342 metabolites were significantly upregulated, 205 metabolites were significantly downregulated and 80 were found as insignificant metabolites as compared to control. While S treatment recorded 232 metabolites as significantly upregulated, 307 metabolites as significantly downregulated and 95 as insignificant metabolites with respect to control. In S+B treatment, 293 metabolites were significantly upregulated, 186 metabolites were significantly downregulated and 112 insignificant metabolites were observed as compared to control (Fig. 59 and 60).

These results highlight a strong influence of the experimental conditions—particularly stress exposure and biostimulant application—on the plant's metabolome. The large number of significant features suggests extensive metabolic reprogramming, likely associated with activation of stress response pathways and/or biostimulant-induced regulatory effects. Such changes may include alterations in primary metabolism (e.g., sugars, amino acids, organic acids) as well as secondary metabolism (e.g., phenolics, flavonoids, alkaloids). Further multivariate analysis (e.g., PCA and PLS-DA), were performed to functionally interpret these significant features impacted by the treatments.

4.16.2 Principal component analysis (PCA)

Principal Component Analysis (PCA) was performed to explore the variance structure and clustering patterns among the treatment groups C, C+B, S, and S+B—based on the metabolic features obtained from HR-LCMS profiling. The first five principal components (PCs) were used to visualize group separation, assess inter-

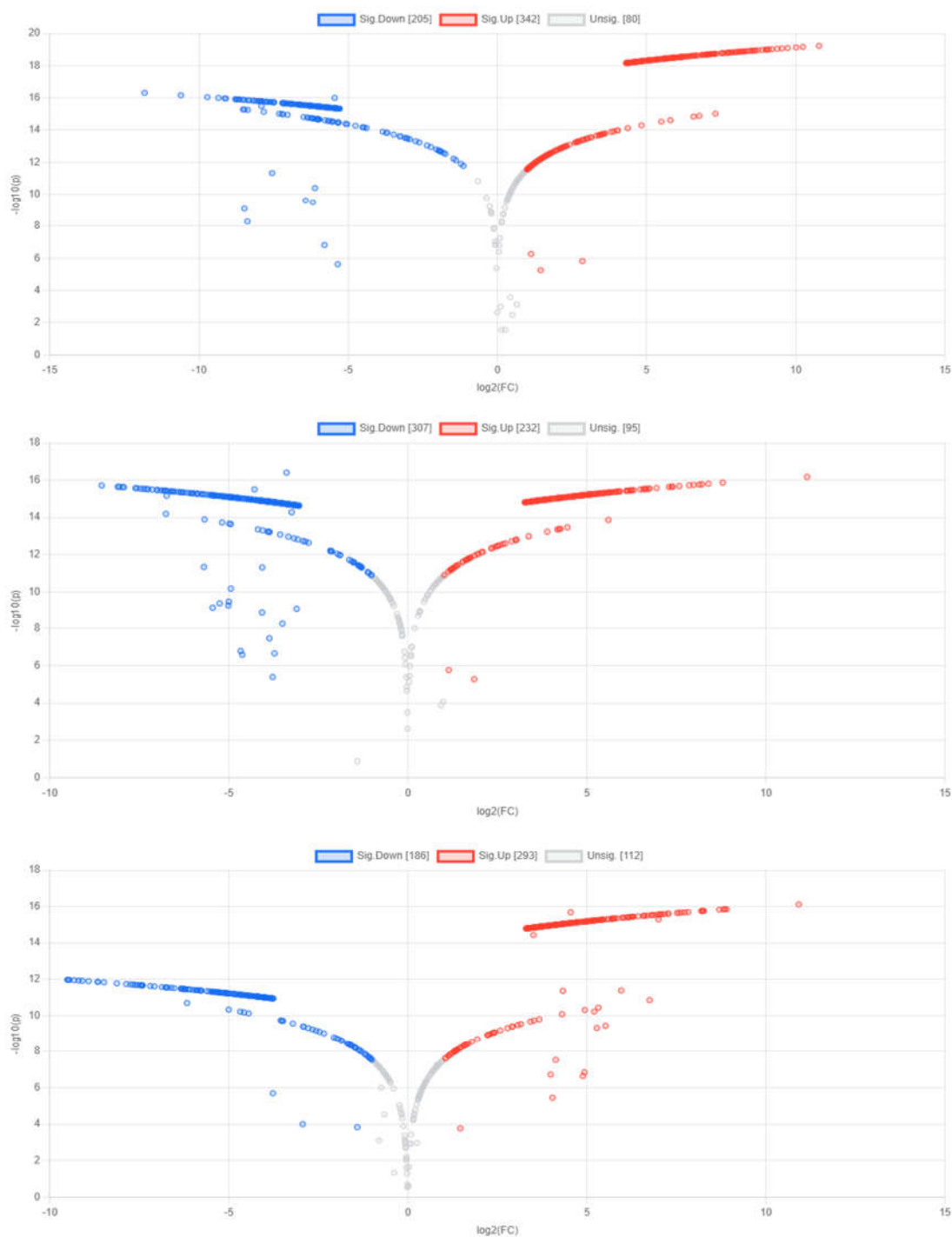


Fig. 59: Volcano plot depicting significantly upregulated, downregulated and insignificant metabolites accumulated in leaves of var. Manu ratna in response to biostimulant application under different treatments in rice. A) Biostimulant application under control condition in rice (C+B vs C); B) Nutrient deficient condition in rice (S vs C); C) Biostimulant application under nutrient deficient condition in rice (S+B vs C). Metabolite data were derived from HRLC-MS. Blue colour represents downregulated metabolites; Red colour represents upregulated metabolites and grey colour represents insignificant metabolites.

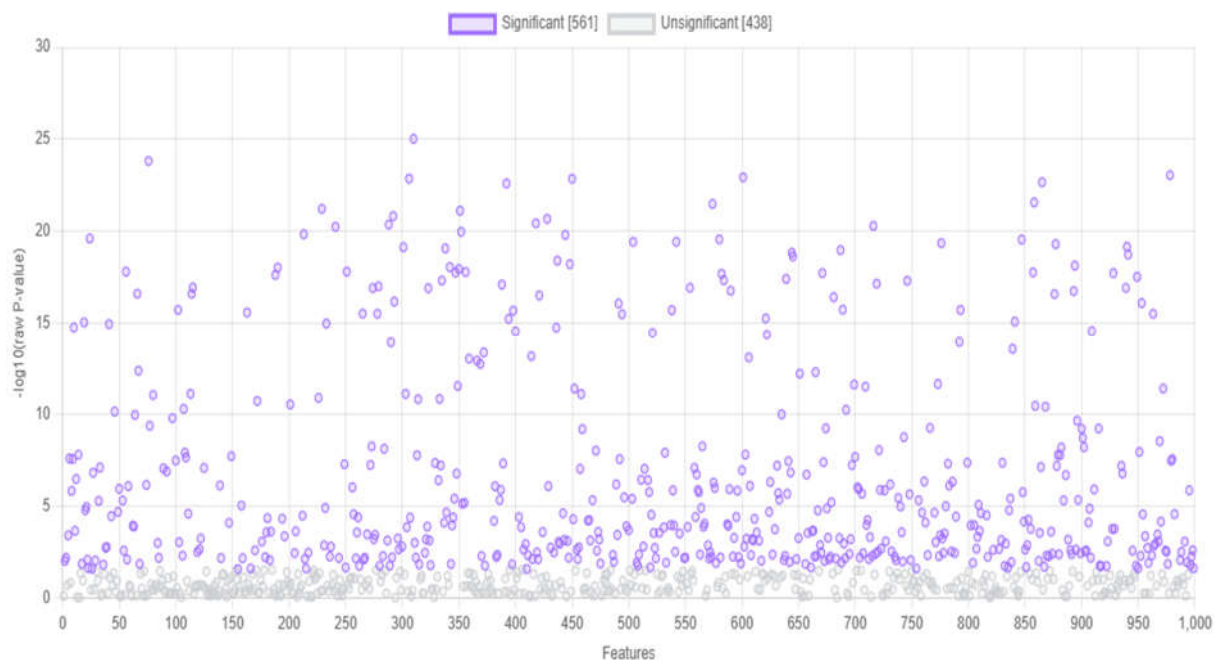


Fig. 60: Fold change analysis depicting significantly upregulated and downregulated metabolites accumulated in different treatment (C+B, S, S+B vs C) of leaves of var. Manu ratna in response to biostimulant application under different treatments in rice. Biostimulant application under control condition in rice (C+B); Nutrient deficient condition in rice (S); Biostimulant application under nutrient deficient condition in rice (S+B). Metabolite data were derived from HRLC-MS. Blue colour represents downregulated metabolites; and grey colour represents insignificant metabolites.

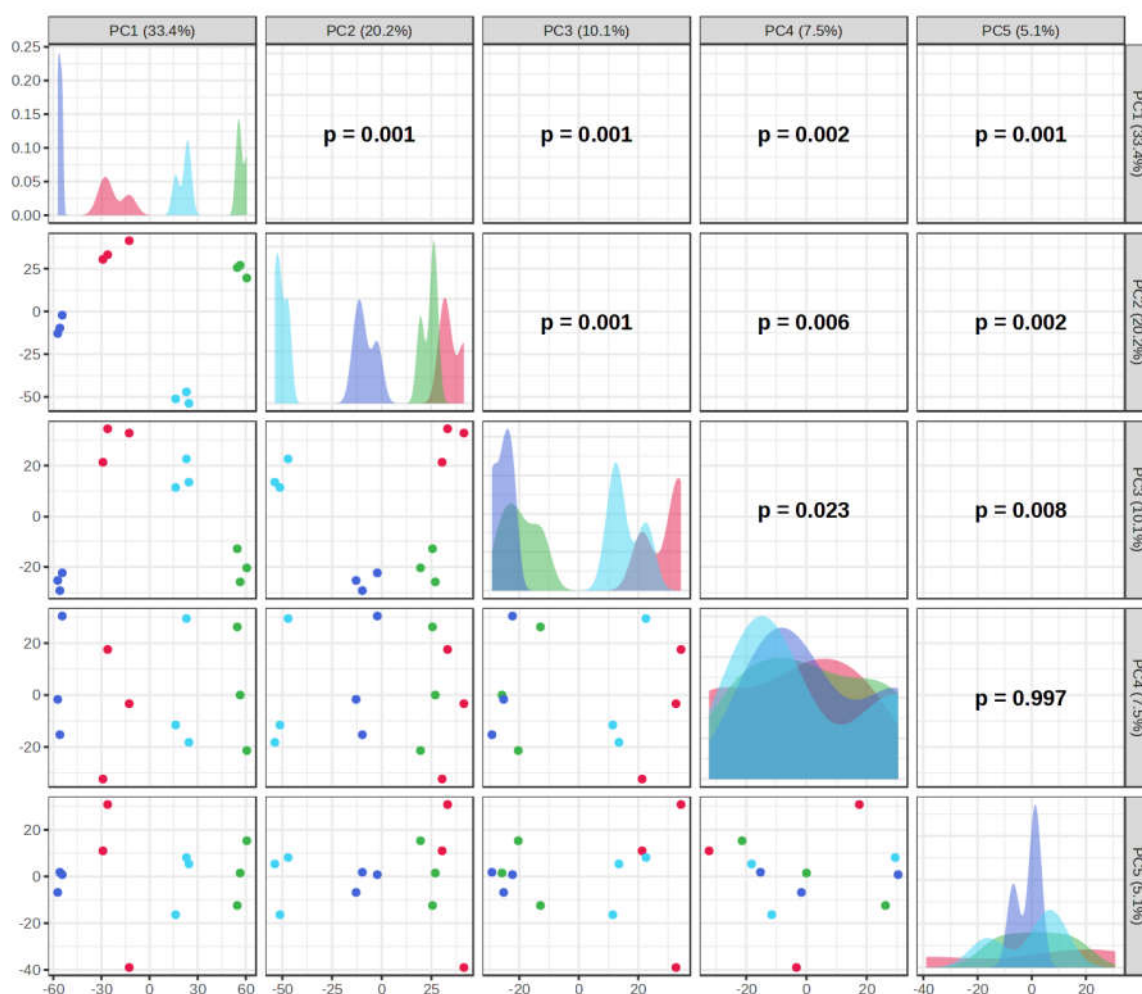


Fig. 61: Multivariate pairwise comparison of principal components (PCs) depicting separation among treatments. The scatterplot matrix displays the first five principal components (PC1–PC5) derived from PCA of metabolite data, cumulatively explaining 76.2% of the total variance. Diagonal panels show the distribution density of individual PCs across treatments (red, green, blue, and cyan), while off-diagonal scatterplots visualize pairwise relationships among PCs. The p-values represent statistical significance from pairwise multivariate analysis between groups for each component combination, indicating significant separation especially in PC1–PC3 ($p \leq 0.001$) and moderate significance in PC4 and PC5.

sample variability, and identify dominant sources of variation across the metabolomic dataset.

The PCA model explained a cumulative variance of 76%, distributed as follows: PC1 (33%), PC2 (20%), PC3 (10%), PC4 (8%), and PC5 (5%) (Fig. 61). This distribution reflects a moderately complex data structure, with the first two components capturing the majority of the biologically relevant variance. Clear separation among treatment groups was observed along the primary components:

PC1 and PC2 collectively contributed to strong discrimination between the C and S treated groups, indicating that S and S+B exhibited significantly different metabolic profiles compared to C and C+B treatments. The associated p-value for PC2 ($p = 0.001$) confirms the statistical significance of this separation.

Along PC3 and PC4, additional patterns of variation were evident, especially between C+B and S+B, suggesting that the biostimulant elicited a differential metabolic response depending on the presence or absence of stress. These components captured subtler changes, which may correspond to specific biochemical pathways modulated under combined treatments.

While PC5 explained only 5% of the variance, it still exhibited a statistically significant group difference ($p = 0.001$), indicating that even low-variance components may harbor biologically meaningful signals.

The p-value matrix accompanying the PCA pairwise plots revealed strong statistical separation between most principal components, especially for PC1–PC3 (all with $p < 0.01$). A few comparisons (e.g., PC4 vs PC5, $p = 0.997$) indicated no significant separation, highlighting that not all components contribute equally to group discrimination. The observed clustering patterns and statistically significant separations strongly suggest that both stress conditions and biostimulant treatments result in marked metabolic alterations. The pronounced shifts in metabolic profiles under stress, as captured by PC1 and PC2, point toward substantial reprogramming of primary and secondary metabolic pathways. Furthermore, the separation between

biostimulant-treated and untreated samples, especially under stress, indicates that the biostimulant plays a modulatory role in shaping the metabolic response.

The PCA biplot and score plot incorporates both sample distribution and loading vectors, allowing for the simultaneous interpretation of group clustering and metabolite influence on the principal components. The first two principal components, PC1 and PC2, accounted for 57% and 24% of the total variance, respectively, thereby capturing 81% of the overall variability in the dataset. This high percentage suggests a strong discriminatory power of the included variables (Fig. 62).

The distribution of samples across the biplot revealed distinct clustering patterns corresponding to the four treatment groups: C samples clustered primarily along the negative side of PC1. C+B samples were positioned near the control group but with a slight shift towards positive PC2, suggesting metabolic difference due to biostimulant application under optimal conditions. S samples were located on the far-right of PC1, reflecting substantial metabolic alterations in response to stress conditions. S+B samples also appeared in the right quadrant, overlapping partially with the stress group but shifted along PC2, indicating biostimulant-mediated modulation of stress-responsive metabolites. This spatial distribution underscores the biochemical divergence between treatments, particularly highlighting the mitigating influence of the biostimulant under stress (Fig. 63).

The loading vectors superimposed on the PCA space represent the direction of metabolite contribution towards group separation. The orientation of each vector indicates its correlation with the principal components, and its length signifies the strength of influence i.e., longer arrows signifies stronger metabolite influence. Metabolites aligned with S and S+B groups, located in the positive PC1 region, included phenolics, flavonoids and anthocyanin. These metabolites appear to be upregulated in response to stress and remain significantly elevated even upon biostimulant treatment under stress, indicating their role in stress adaptation pathways. Biostimulant-responsive metabolites, particularly under non-stress and stress conditions (C+B and S+B), include: Lysolecithin, Hexyl 2-[N-(diethylamino)-

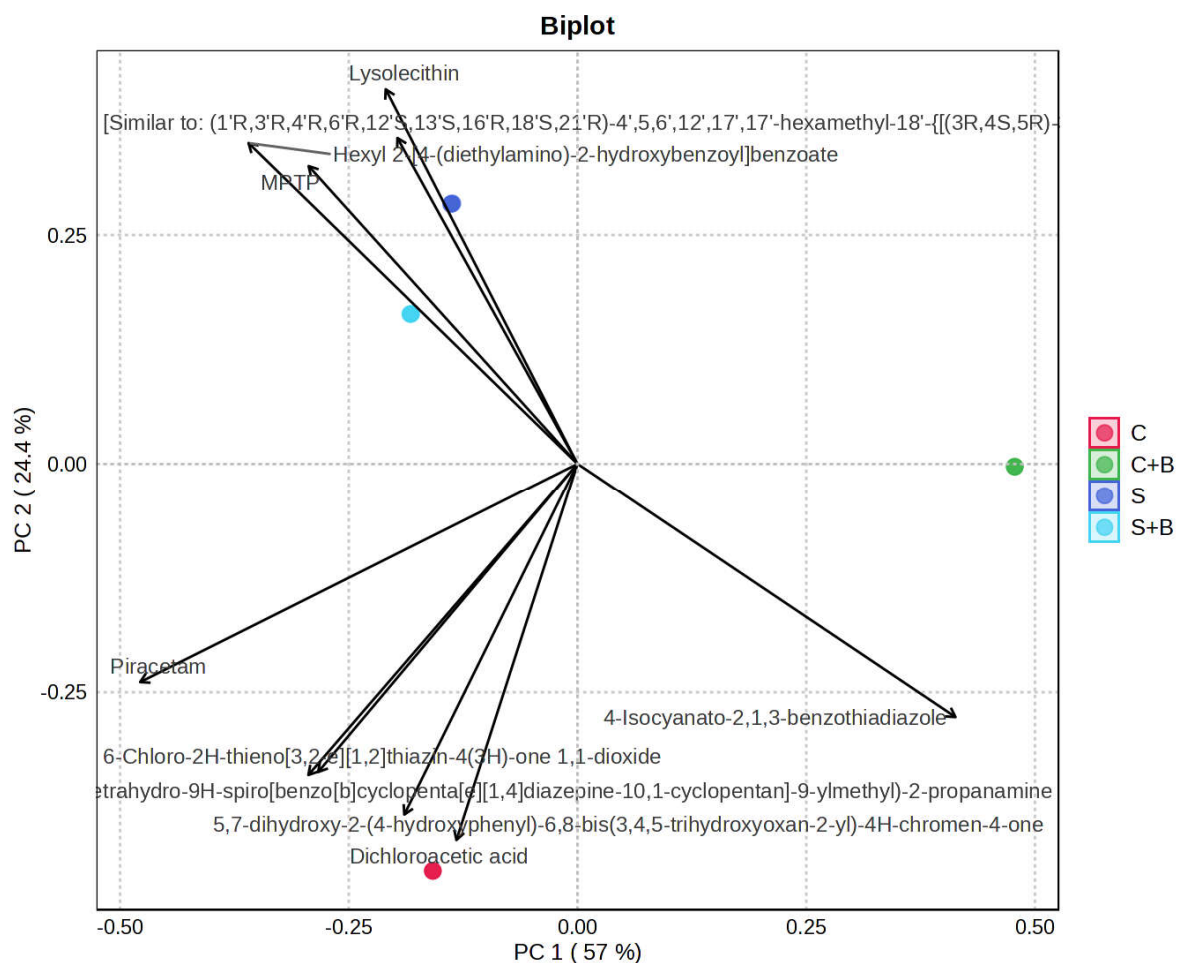


Fig. 62: Biplot representing the principal component analysis (PCA) of metabolites in leaves of var. Manu Ratna under different treatments in rice. The treatments include: C-Control plants (pink); C+B-Biostimulant alone (green); S-Nutrient-deficient condition without biostimulant (blue); and S+B-Biostimulant application under nutrient deficiency (cyan). The analysis demonstrates distinct clustering of treatments, highlighting differential accumulation of metabolites.

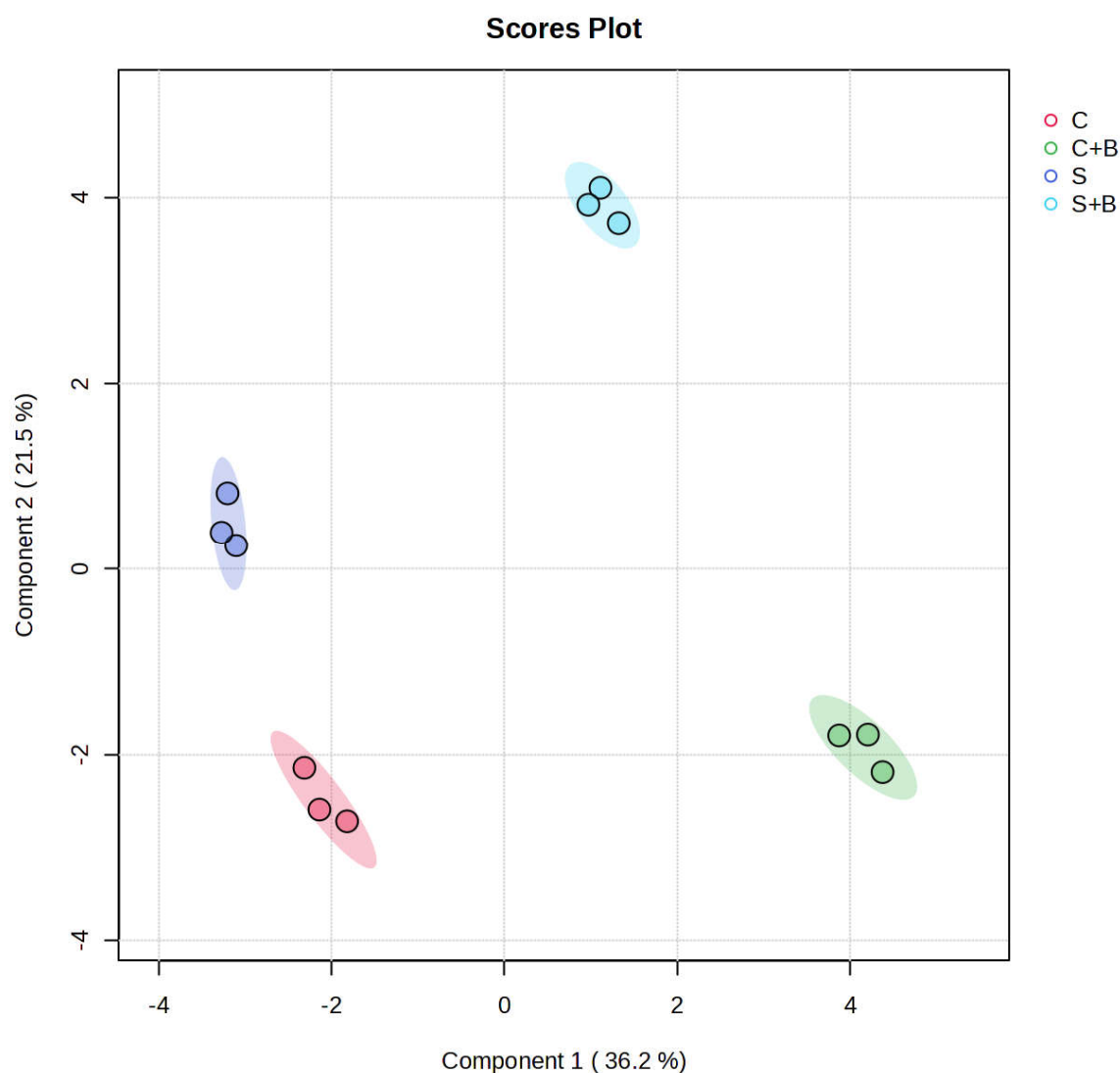


Fig. 63: PLS-DA scores plot illustrating the separation of metabolite profiles in leaves of var. Manu ratna under different treatments in rice: C-Control plants (pink); C+B-Biostimulant alone (green); S-Nutrient-deficient condition without biostimulant (blue); and S+B-Biostimulant application under nutrient deficiency (cyan). The distinct clustering of each treatment group highlights the variation in metabolite composition.

2-hydroxybenzoyl] benzoate. These vectors (arrows) are positively loaded on PC2, indicating distinct modulation due to biostimulant application across both stress and non-stress conditions. The PCA biplot analysis provided a visual summary of how individual metabolites contribute to the observed separation among treatment groups. The clear demarcation between control and stressed groups, along with the intermediate positioning of biostimulant-treated samples, demonstrates the metabolic plasticity induced by biostimulant application. The strong directional vectors associated with certain metabolites emphasize their potential as biomarkers for distinguishing stress responses and evaluating biostimulant efficacy.

4.16.3 Pathway enrichment analysis

The perturbed metabolic pathway analysis revealed several significantly impacted pathways in response to the C+B, S and S+B treatment as compared to control. In C+B treatment, sphingolipid metabolism exhibited the highest statistical significance and impact, indicating a prominent role of membrane lipid remodeling and signaling under biostimulant influence. Similarly, alanine, aspartate, and glutamate metabolism showed substantial pathway impact and significance, suggesting enhanced nitrogen assimilation and amino acid interconversion. Purine metabolism, glycine, serine and threonine metabolism, and cysteine and methionine metabolism were also significantly enriched, reflecting activation of nucleotide biosynthesis and sulfur-containing amino acid pathways. These are often associated with cellular growth and redox homeostasis. Notably, nitrogen metabolism and phenylalanine metabolism displayed moderate to high pathway impacts, further supporting the role of biostimulant in boosting primary nitrogen metabolism and aromatic amino acid biosynthesis, which are precursors to various plant growth regulators and phenylpropanoids. Other enriched pathways included alpha-linolenic acid metabolism, pantothenate and CoA biosynthesis, and glyoxylate and dicarboxylate metabolism, pointing towards enhanced energy metabolism, fatty acid signaling, and stress-preparedness mechanisms (Fig. 64).

In S treatment, phenylalanine metabolism showed the highest statistical significance and pathway impact, indicating elevated biosynthesis of

phenylpropanoids, which are critical for stress defense, lignin formation, and secondary metabolite production. Sphingolipid metabolism was also highly enriched, suggesting possible remodeling of membrane lipids and signaling in response to stress. Notably, arginine biosynthesis and purine metabolism were activated, highlighting a possible upregulation of nitrogen remobilization and nucleotide turnover under nutrient-deprived conditions. Enrichment in pantothenate and CoA biosynthesis, butanoate metabolism, and glycine, serine and threonine metabolism further support a shift towards energy conservation and osmoprotection mechanisms. Lower but significant impacts were also observed in tyrosine metabolism, cysteine and methionine metabolism, and glycerophospholipid metabolism, reflecting broader metabolic stress adaptation. Collectively, these metabolic alterations indicate an extensive reprogramming under stress, with priority given to stress tolerance, redox balance, and maintenance of key biosynthetic functions (Fig. 65).

In S+B treatment, phenylalanine metabolism continued to be highly enriched with increased pathway impact, indicating further upregulation of phenylpropanoid biosynthesis, potentially amplifying structural and antioxidant defense responses. Nitrogen metabolism and alanine, aspartate and glutamate metabolism displayed markedly increased pathway impact, suggesting improved nitrogen assimilation and amino acid interconversion, possibly aiding in stress recovery and biomass maintenance. Interestingly, butanoate metabolism showed the highest statistical significance among all pathways, indicating enhanced production of GABA and related compounds associated with stress mitigation. The involvement of one-carbon metabolism by folate, glutathione metabolism, and sphingolipid metabolism points towards a stronger activation of redox regulation, folate cycling, and membrane stabilization. Additional enrichment of tyrosine, cysteine and methionine, and isoquinoline alkaloid biosynthesis pathways reflect broader metabolic remodeling towards resilience. These results suggest that biostimulant application under nutrient stress conditions not only offsets stress-induced damage but also primes the plant for improved defense and metabolic recovery (Fig. 66).

4.17 Amino acids profiling

4.17.1 PCA Biplot

The PCA biplot clearly distinguishes the treatment groups based on their amino acid composition in leaves (Fig. 67). The S+B treatment was distinctly separated along the positive axis of PC1 and PC2, indicating a marked alteration in amino acid metabolism under stress and biostimulant treatment. Amino acids such as L-Citrulline, L-Cysteine, L-Phenylalanine, 4-Hydroxyproline, and L-Arginine were positively correlated with the treatment.

In contrast, the C+B treatments were located on the negative side of PC1, with L-Glutamine and L-Aspartic acid showing stronger association. The S treatment appeared segregated along the negative axis of PC2 and was primarily associated with L-Arginine and 4-Hydroxyproline. Overall, the PCA highlights that biostimulant application under stress conditions (S+B) significantly influenced the amino acid profile, contributing to distinct clustering patterns compared to other treatments.

4.17.2 Heat map with hierarchial clustering

A hierarchical clustering heatmap was generated to evaluate the differential accumulation of amino acids in response to biostimulant application under control and stress conditions (Fig. 68). The heatmap distinctly grouped the treatment samples based on their amino acid profiles, demonstrating consistent biological replicates and treatment-specific clustering.

The S treatment exhibited a marked accumulation of several amino acids, including L-Arginine, L-Phenylalanine, L-Citrulline, L-Cysteine, L-Tryptophan, L-Methionine, and L-Tyrosine, indicating stress-induced activation of nitrogen metabolism and secondary metabolite precursors. These metabolites were significantly upregulated in the stress-alone condition compared to the control and C+B groups. Interestingly, S+B treatment altered the amino acid profile distinctly as compared to control with some amino acids such as L-Cysteine, L-Glutamic acid, and L-Arginine remained elevated, however other stress-induced amino acids

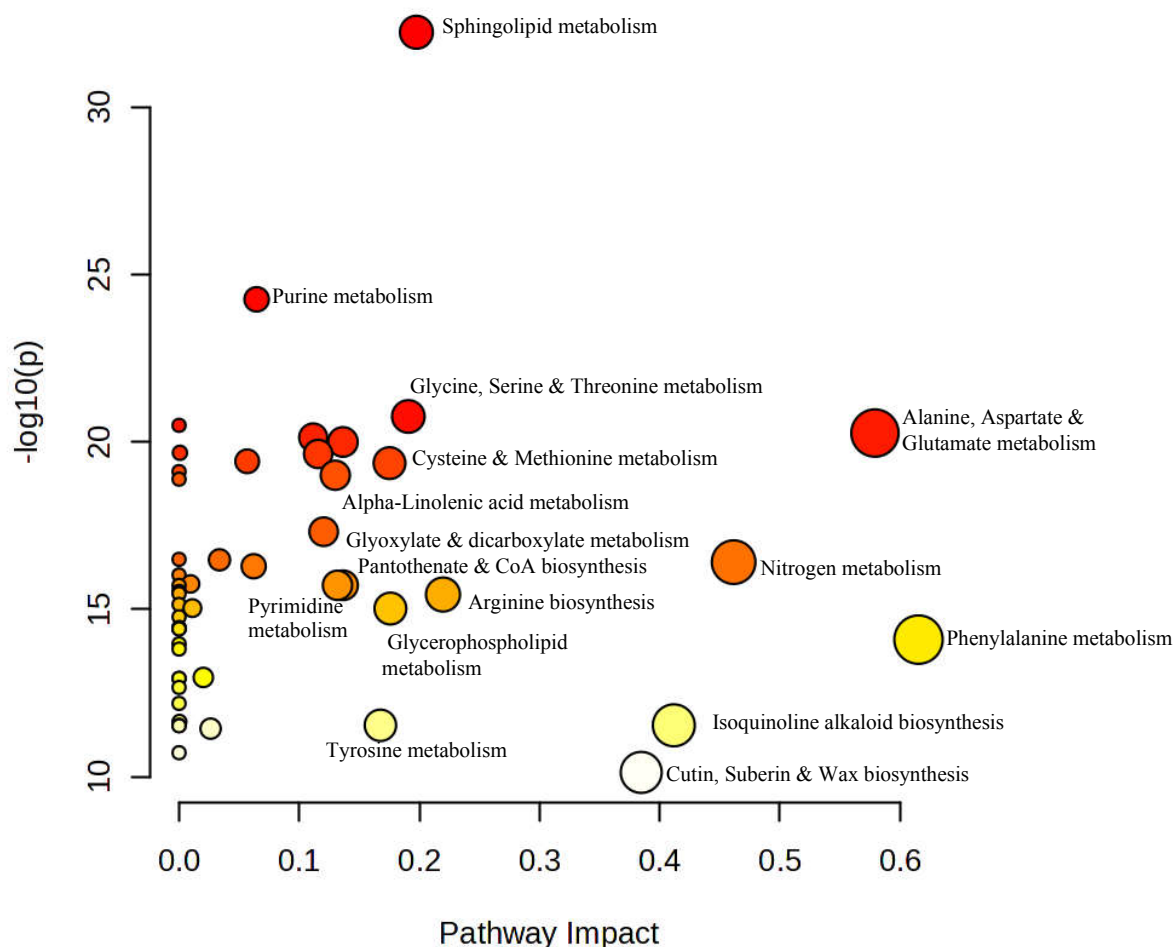


Fig. 64: MetaboAnalyst-generated pathway enrichment plots (“metabolome view”) illustrating significantly affected metabolic pathways in response to biostimulant application under control condition in rice (C+B vs C). Arranged by the statistical significance ($-\log_{10} > 1$ and $p < 0.05$) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

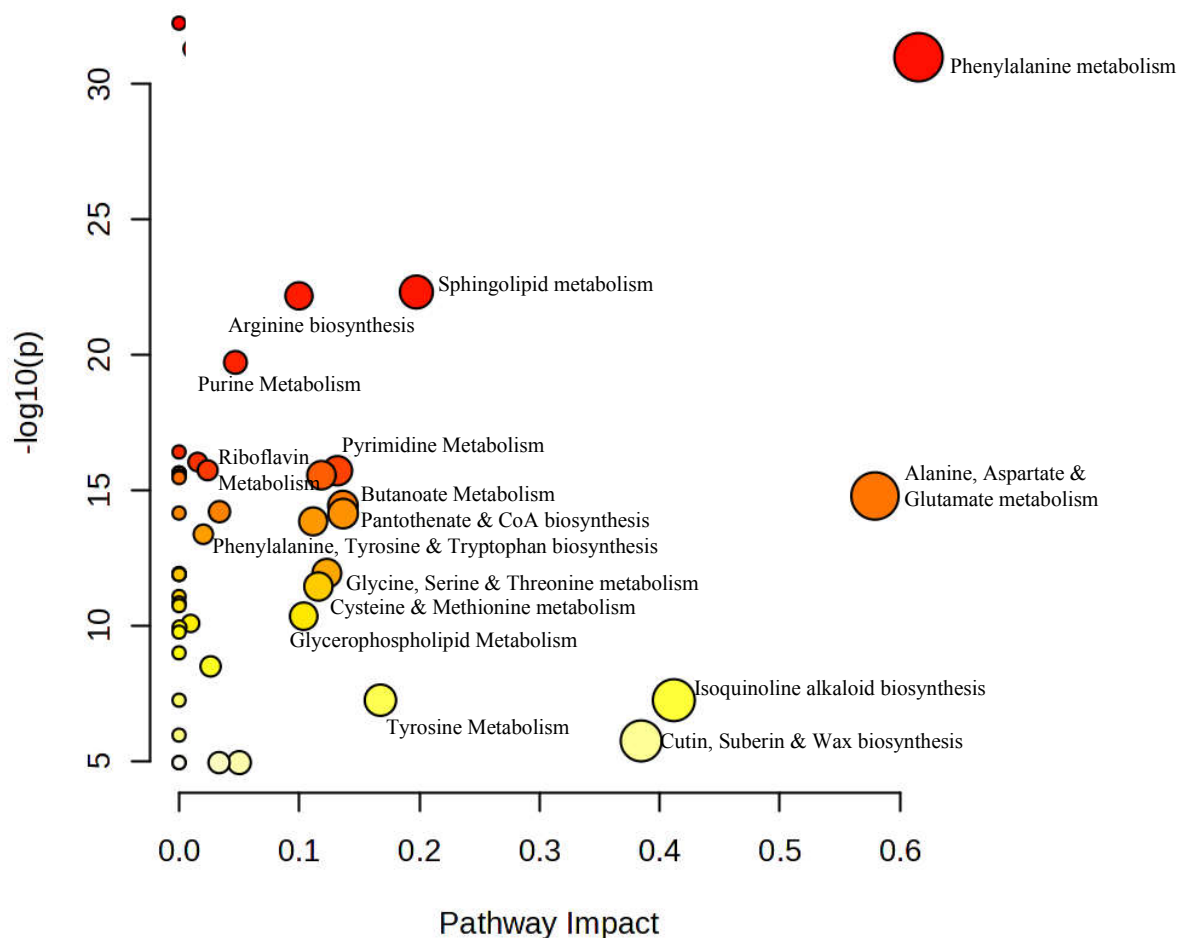


Fig. 65: MetaboAnalyst-generated pathway enrichment plots ("metabolome view") illustrating significantly affected metabolic pathways in response to nutrient deficient condition in rice (S vs C). Arranged by the statistical significance ($-\log_{10} p > 1$ and $p < 0.05$) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

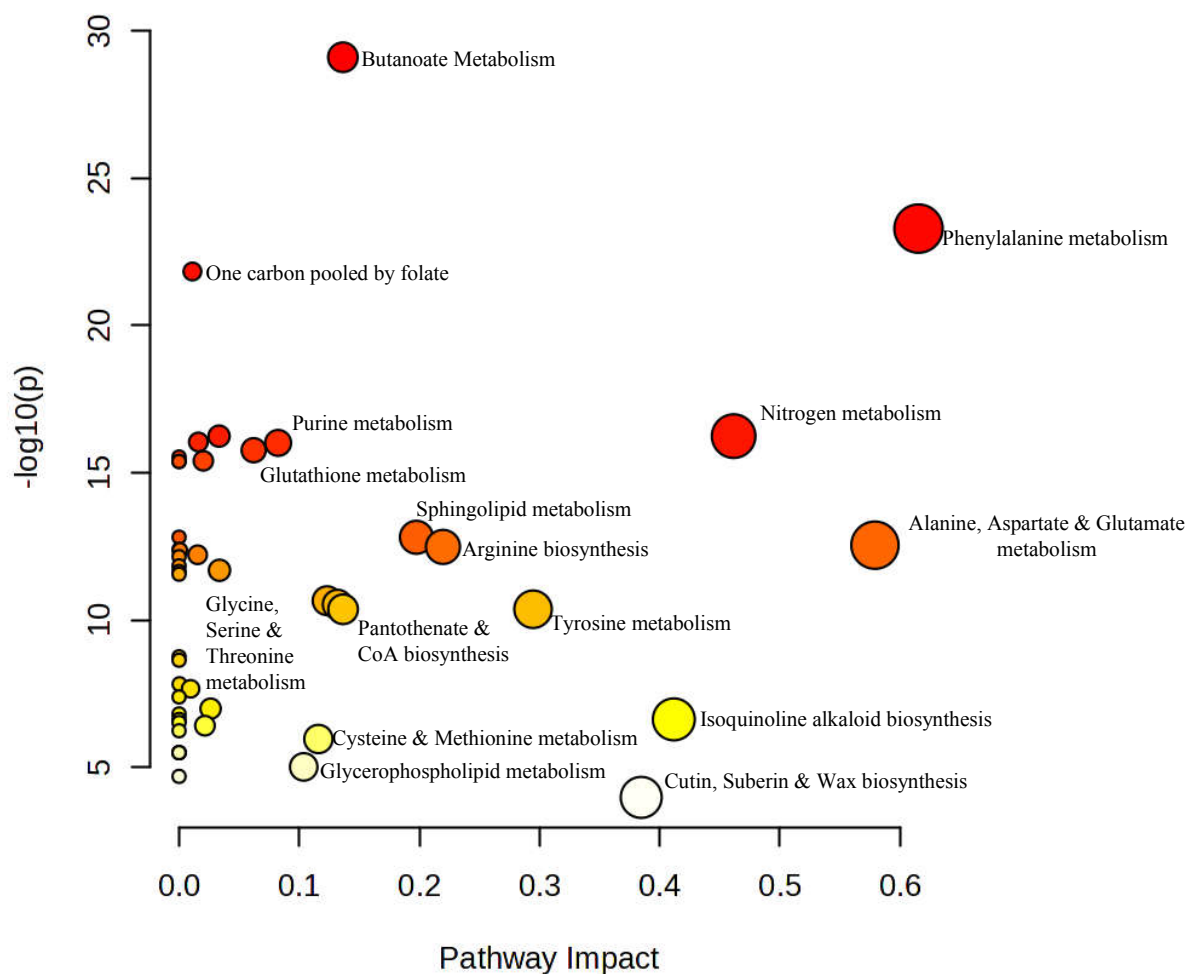


Fig. 66: MetaboAnalyst-generated pathway enrichment plots (“metabolome view”) illustrating significantly affected metabolic pathways in response to biostimulant application under nutrient deficient condition in rice (S+B vs C). Arranged by the statistical significance ($-\log_{10} > 1$ and $p < 0.05$) on the y-axis and the pathway impact scores derived from pathway topology analysis on the x-axis. Pathway impact values refer to the cumulative percentage from the matched metabolite nodes, and the maximum importance of each pathway is 1. Circle size reflects the degree of pathway coverage (number of matched metabolites). The color of the circles represents the p-value, with a gradient from yellow (higher p-values) to red (lower p-values), indicating increasing statistical significance.

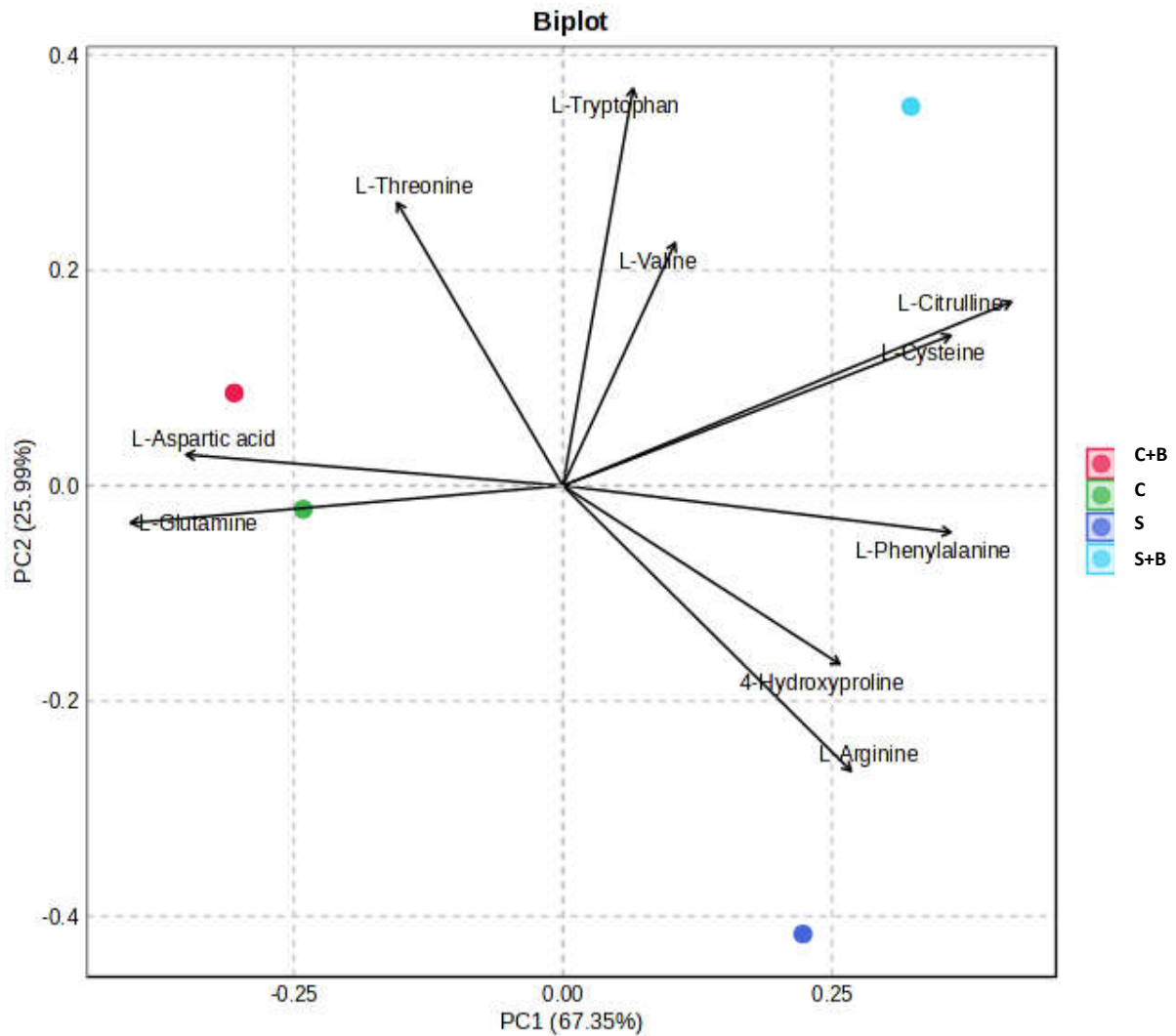


Fig. 67: PCA biplot of amino acid profiling under different treatment conditions in leaves of rice. The plot shows principal component 1 (PC1) and principal component 2 (PC2), which explain 67.35% and 25.99% of the variance, respectively. Colored dots represent different treatments: C-Control plants (pink); C+B-Biostimulant alone (green); S-Nutrient-deficient condition without biostimulant (blue); and S+B-Biostimulant application under nutrient deficiency (cyan). Arrows indicate the contribution and directionality of individual amino acids to the overall variance.

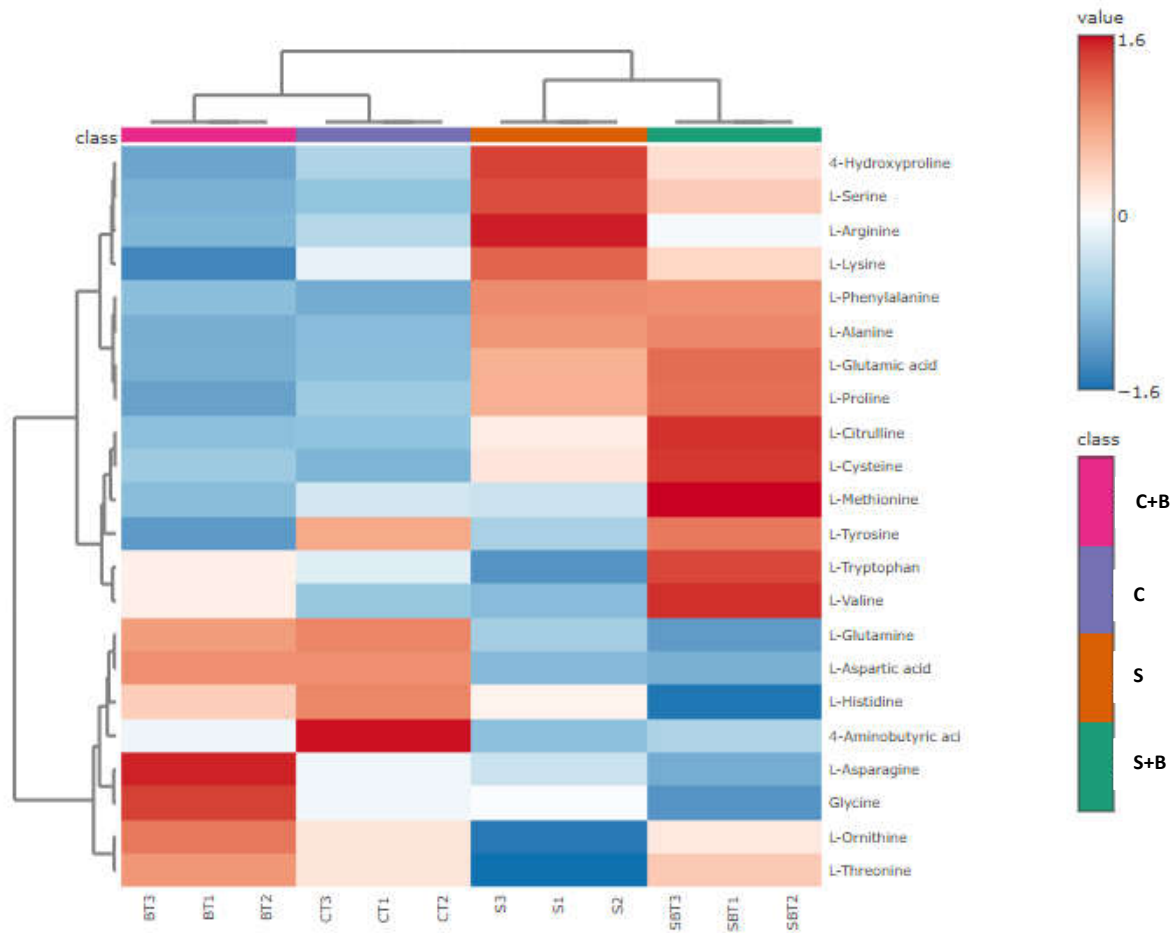


Fig. 68: Heatmap representing the differential abundance of amino acids in leaves of rice under four treatment conditions: C+B (Biostimulant), C (Control), S (Stress), and S+B (Stress + Biostimulant). Rows indicate individual amino acids and columns represent biological replicates from each treatment. The color scale denotes relative expression values ranging from low (blue) to high (red). Clustering of rows and columns reveals patterns of similarity among treatments and amino acid profiles.

showed reduced accumulation. In contrast, C+B treatment groups showed relatively lower abundance of most amino acids, forming a separate cluster characterized by downregulation of key stress-responsive amino acids with some amino acids such as L-Aspartic acid, L-Threonine, and Glycine were relatively more abundant in this treatment as compared to control.

4.17.3 Pathway enrichment analysis

In C+B treatment, a total of 15 metabolic pathways were found to be enriched, with varying degrees of statistical significance and biological impact (Fig. 69). The most significantly enriched pathway was arginine biosynthesis ($p = 2.215E-6$, $FDR = 2.02E-5$), indicating a prominent modulation of arginine metabolism, followed by glyoxylate and dicarboxylate metabolism ($p = 1.666E-5$, $FDR = 7.58E-4$) which was also significantly enriched. Other notable enriched pathways included cyanoamino acid metabolism, alanine, aspartate and glutamate metabolism, and arginine and proline metabolism, all of which are central to amino acids interconversion and nitrogen assimilation. The alanine, aspartate and glutamate metabolism pathway showed the highest impact value (0.64388), indicating its central role in the overall metabolic perturbation observed. Additional pathways such as glycine, serine and threonine metabolism, lysine biosynthesis, and nitrogen metabolism were also observed. Further, it was observed that statistically less significant pathways like glutathione metabolism, cysteine and methionine metabolism, are also associated with maintaining redox balance and stress tolerance mechanisms.

In S treatment, pathways like alanine, aspartate and glutamate metabolism (Impact: 0.77338) displayed the highest pathway impact score, indicating a central role in metabolic reprogramming (Fig. 70). Similarly, glycine, serine and threonine metabolism (Impact: 0.5249), arginine biosynthesis (Impact: 0.39613), and arginine as well as proline metabolism (Impact: 0.33639) also demonstrated relatively high impact values, emphasizing the modulation of nitrogen-rich amino acids that are vital for stress defense and protein synthesis. Glutathione metabolism (Impact: 0.12837) also emerged with a moderate impact, suggesting the involvement of redox buffering systems and antioxidant responses. Other pathways like glyoxylate and dicarboxylate metabolism (Impact: 0.17255) and sulfur metabolism (Impact:

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact	Details
Lipoic acid metabolism	1/24	2.3694E-8	7.6254	1.0188E-6	1.0188E-6	0.0016	KEGG
Lysine degradation	1/20	1.8594E-7	6.7306	7.8094E-6	2.8378E-6	0.0	KEGG
Glyoxylate and dicarboxylate metabolism	4/29	2.4299E-7	6.6144	9.9626E-6	2.8378E-6	0.10876	KEGG
Lysine biosynthesis	2/9	2.6398E-7	6.5784	1.0559E-5	2.8378E-6	0.0	KEGG
Alanine, aspartate and glutamate metabolism	6/22	4.255E-7	6.3711	1.6595E-5	2.9436E-6	0.77338	KEGG
Phenylpropanoid biosynthesis	2/33	6.0568E-7	6.2178	2.3016E-5	2.9436E-6	0.0	KEGG
Isoquinoline alkaloid biosynthesis	1/6	7.1815E-7	6.1438	2.6571E-5	2.9436E-6	0.41176	KEGG
Tyrosine metabolism	1/18	7.1815E-7	6.1438	2.6571E-5	2.9436E-6	0.16757	KEGG
Ubiquinone and other terpenoid-quinone biosynthesis	1/48	7.1815E-7	6.1438	2.6571E-5	2.9436E-6	0.0	KEGG
Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	7.2443E-7	6.14	2.6571E-5	2.9436E-6	0.02152	KEGG
Valine, leucine and isoleucine biosynthesis	2/22	7.5301E-7	6.1232	2.6571E-5	2.9436E-6	0.0	KEGG
Valine, leucine and isoleucine degradation	1/37	9.621E-7	6.0168	3.0787E-5	3.1823E-6	0.0	KEGG
Glucosinolate biosynthesis	1/22	9.621E-7	6.0168	3.0787E-5	3.1823E-6	0.0	KEGG
Cyanoamino acid metabolism	5/22	1.1252E-6	5.9488	3.3756E-5	3.456E-6	0.0	KEGG
D-Amino acid metabolism	2/7	1.4753E-6	5.8311	4.2785E-5	4.2293E-6	0.0	KEGG
One carbon pool by folate	3/21	1.9575E-6	5.7083	5.4809E-5	5.2607E-6	0.02815	KEGG
Tryptophan metabolism	1/23	2.239E-6	5.65	6.0452E-5	5.3487E-6	0.1938	KEGG
Indole alkaloid biosynthesis	1/5	2.239E-6	5.65	6.0452E-5	5.3487E-6	0.0	KEGG
Glycine, serine and threonine metabolism	5/33	2.4378E-6	5.613	6.0946E-5	5.5172E-6	0.5249	KEGG
Thiamine metabolism	2/22	2.9417E-6	5.5314	7.0601E-5	6.3247E-6	0.0	KEGG

Fig. 69: Pathway enrichment analysis of amino acid metabolism in response to biostimulant application under control condition in rice (C+B vs C) based on KEGG database using MetaboAnalyst. The table lists significantly enriched metabolic pathways in response to treatment, sorted by statistical significance. Key columns include the number of matched metabolites to the total in the pathway (Match Status), p-value, corrected p-values (Holm and FDR), and pathway impact scores derived from topological analysis.

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact	Details
Lysine biosynthesis	2/9	2.5784E-7	6.5886	1.1087E-5	5.0945E-6	0.0	KEGG
Purine metabolism	1/72	4.7391E-7	6.3243	1.9904E-5	5.0945E-6	0.0	KEGG
Pyrimidine metabolism	1/40	4.7391E-7	6.3243	1.9904E-5	5.0945E-6	0.0	KEGG
Vitamin B6 metabolism	1/12	4.7391E-7	6.3243	1.9904E-5	5.0945E-6	0.0	KEGG
Pantothenate and CoA biosynthesis	2/25	1.29E-6	5.8894	5.0312E-5	8.4015E-6	0.0	KEGG
Monobactam biosynthesis	1/8	1.5631E-6	5.806	5.9396E-5	8.4015E-6	0.0	KEGG
beta-Alanine metabolism	1/18	1.5631E-6	5.806	5.9396E-5	8.4015E-6	0.0	KEGG
Nicotinate and nicotinamide metabolism	1/13	1.5631E-6	5.806	5.9396E-5	8.4015E-6	0.0	KEGG
Phenylpropanoid biosynthesis	2/33	3.3101E-6	5.4802	1.1585E-4	1.5815E-5	0.0	KEGG
Alanine, aspartate and glutamate metabolism	6/22	4.3137E-6	5.3652	1.4666E-4	1.8549E-5	0.77338	KEGG
Carbon fixation by Calvin cycle	2/21	5.0218E-6	5.2991	1.6572E-4	1.9631E-5	0.0	KEGG
Nitrogen metabolism	2/11	6.7664E-6	5.1696	2.1652E-4	2.4246E-5	0.0	KEGG
Glycine, serine and threonine metabolism	5/33	1.0589E-5	4.9751	3.2826E-4	3.2816E-5	0.5249	KEGG
Cysteine and methionine metabolism	4/46	1.2076E-5	4.9181	3.6227E-4	3.2816E-5	0.16061	KEGG
Phenylalanine metabolism	1/12	1.2211E-5	4.9133	3.6227E-4	3.2816E-5	0.42308	KEGG
Tropane, piperidine and pyridine alkaloid biosynthesis	1/9	1.2211E-5	4.9133	3.6227E-4	3.2816E-5	0.0	KEGG
Lysine degradation	1/20	1.8672E-5	4.7288	5.0415E-4	4.4767E-5	0.0	KEGG
Thiamine metabolism	2/22	1.874E-5	4.7272	5.0415E-4	4.4767E-5	0.0	KEGG
Arginine biosynthesis	6/18	2.8958E-5	4.5382	7.2394E-4	6.5428E-5	0.45781	KEGG
Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	3.0432E-5	4.5167	7.3036E-4	6.5428E-5	0.02152	KEGG

Fig. 70: Pathway enrichment analysis of amino acid metabolism in response to nutrient deficient condition in rice (S vs C) based on KEGG database using MetaboAnalyst. The table lists significantly enriched metabolic pathways in response to treatment, sorted by statistical significance. Key columns include the number of matched metabolites to the total in the pathway (Match Status), p-value, corrected p-values (Holm and FDR), and pathway impact scores derived from topological analysis.

Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact	Details
Alanine, aspartate and glutamate metabolism	6/22	6.7394E-10	9.1714	2.8979E-8	2.8979E-8	0.77338	KEGG
Glycine, serine and threonine metabolism	5/33	2.2832E-7	6.6415	9.5893E-6	4.9088E-6	0.5249	KEGG
Arginine biosynthesis	6/18	3.6477E-7	6.438	1.4955E-5	5.2283E-6	0.45781	KEGG
Carbon fixation by Calvin cycle	2/21	6.8208E-7	6.1662	2.7283E-5	7.3324E-6	0.0	KEGG
Valine, leucine and isoleucine biosynthesis	2/22	1.0636E-6	5.9732	4.1479E-5	8.9222E-6	0.0	KEGG
Monobactam biosynthesis	1/8	1.6599E-6	5.7799	6.3078E-5	8.9222E-6	0.0	KEGG
beta-Alanine metabolism	1/18	1.6599E-6	5.7799	6.3078E-5	8.9222E-6	0.0	KEGG
Nicotinate and nicotinamide metabolism	1/13	1.6599E-6	5.7799	6.3078E-5	8.9222E-6	0.0	KEGG
Lysine biosynthesis	2/9	2.1448E-6	5.6686	7.5068E-5	9.1274E-6	0.0	KEGG
Valine, leucine and isoleucine degradation	1/37	2.3349E-6	5.6317	7.9387E-5	9.1274E-6	0.0	KEGG
Glucosinolate biosynthesis	1/22	2.3349E-6	5.6317	7.9387E-5	9.1274E-6	0.0	KEGG
Glyoxylate and dicarboxylate metabolism	4/29	3.3262E-6	5.4781	1.0644E-4	9.8969E-6	0.10876	KEGG
Purine metabolism	1/72	3.5152E-6	5.454	1.0897E-4	9.8969E-6	0.0	KEGG
Pyrimidine metabolism	1/40	3.5152E-6	5.454	1.0897E-4	9.8969E-6	0.0	KEGG
Vitamin B6 metabolism	1/12	3.5152E-6	5.454	1.0897E-4	9.8969E-6	0.0	KEGG
Nitrogen metabolism	2/11	3.7605E-6	5.4248	1.0897E-4	9.8969E-6	0.0	KEGG
Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	3.9127E-6	5.4075	1.0897E-4	9.8969E-6	0.02152	KEGG
Selenocompound metabolism	1/13	5.244E-6	5.2803	1.3634E-4	1.2527E-5	0.0	KEGG
One carbon pool by folate	3/21	1.451E-5	4.8383	3.6275E-4	3.251E-5	0.02815	KEGG
Lipoic acid metabolism	1/24	1.5121E-5	4.8204	3.629E-4	3.251E-5	0.0016	KEGG

Fig. 71: Pathway enrichment analysis of amino acid metabolism in response to biostimulant application under nutrient deficient condition in rice (S+B vs C) based on KEGG database using MetaboAnalyst. The table lists significantly enriched metabolic pathways in response to treatment, sorted by statistical significance. Key columns include the number of matched metabolites to the total in the pathway (Match Status), p-value, corrected p-values (Holm and FDR), and pathway impact scores derived from topological analysis.

0.06077) point to possible adjustments in energy and sulfur-containing compound synthesis under the treatment conditions. . In contrast, there was no major impact on the pathways such as purine metabolism, pyrimidine metabolism, lysine degradation, and carbon fixation. These low impact/no impact on the above mentioned enrichment pathways suggest that only minor metabolites are changed while the core function of the pathway likely remained unchanged and thus they are not driving the key stress responses or growth enhancement mechanisms.

The treatment (S+B), brought about significant alterations in multiple metabolic pathways, particularly those related to amino acid biosynthesis and nitrogen metabolism (Fig. 71). All listed pathways demonstrated statistical significance ($p = 0.025347$, $FDR = 0.025347$), but the biological relevance was better interpreted through pathway impact scores. Among the enriched pathways, alanine, aspartate and glutamate metabolism (Impact: 0.77338) exhibited the highest impact, highlighting the central involvement of these amino acids in stress mitigation and energy redistribution. Similarly, arginine biosynthesis (Impact: 0.45781) and glycine, serine and threonine metabolism (Impact: 0.5249) were substantially impacted, indicating a pronounced shift in nitrogen metabolism and osmoprotectant production under S+B treatment. Moderate enrichment was also observed in glutathione metabolism (Impact: 0.12837) including glyoxylate and dicarboxylate metabolism (Impact: 0.10876), reflecting the activation of redox balancing mechanisms and alternative carbon flow pathways, which are known to play protective roles during stress adaptation. Other pathways such as sulfur metabolism (Impact: 0.07047) and vitamin B6 metabolism may contribute to the synthesis of cofactors and sulfur-containing amino acids, further supporting biochemical adjustments in response to stress.

4.18 Nutritional quality analysis of 1st generation seeds

4.18.1 Amino acid profiling

The amino acid composition of first-generation seeds exhibited distinct variations across the four treatments (C, C+B, S, and S+B), demonstrating the impact of stress and the ameliorative effect of biostimulant application. In the control, amino acid concentrations were maintained at basal levels and served as a benchmark for comparative analysis (Fig. 72).

Seeds obtained from C+B treatment had enhanced accumulation of several amino acids. Notably, essential amino acids such as leucine, isoleucine, valine, and phenylalanine increased by approximately 15–25% compared to the control, indicating improved proteinogenic potential. Similarly, glutamic acid, aspartic acid, and alanine showed a 20–30% enhancement, suggesting that biostimulant supplementation promoted nitrogen assimilation and amino acid biosynthesis in developing seeds.

While seeds obtained from S treatment, a substantial decline in amino acid content was observed relative to the control. Glutamic acid and aspartic acid, which are central to nitrogen metabolism, were reduced by nearly 30–40%, while branched-chain amino acids (valine, leucine, isoleucine) showed a 25–35% decrease. Stress also negatively impacted aromatic amino acids (phenylalanine, tyrosine, tryptophan), with reductions of around 20–30%, reflecting impaired carbon–nitrogen flux under stress.

Interestingly, the seeds obtained from combined treatment of S+B treatment, alleviated the negative effects of stress and substantially improved amino acid levels. Glutamic acid and aspartic acid showed a 20–25% recovery compared to stress alone, while branched-chain amino acids increased by 30–35%, nearly reaching control levels. Aromatic amino acids also displayed partial recovery (15–20% increase over stress), highlighting the protective effect of biostimulant supplementation in maintaining amino acid homeostasis under adverse conditions.

4.18.2 Elemental analysis of 1st Generation Seeds

The elemental composition of first-generation seeds demonstrated considerable variation under different treatments (C, C+B, S, and S+B), reflecting the influence of both stress and biostimulant application on nutrient accumulation (Table 15).

Under C+B treatment, a clear enhancement in nutrient accumulation was observed compared to control. K and Mg increased by 12% and 25%, respectively, while Ca content by 12%. Among micronutrients, Mn improved by 8%, Zn by 33%,

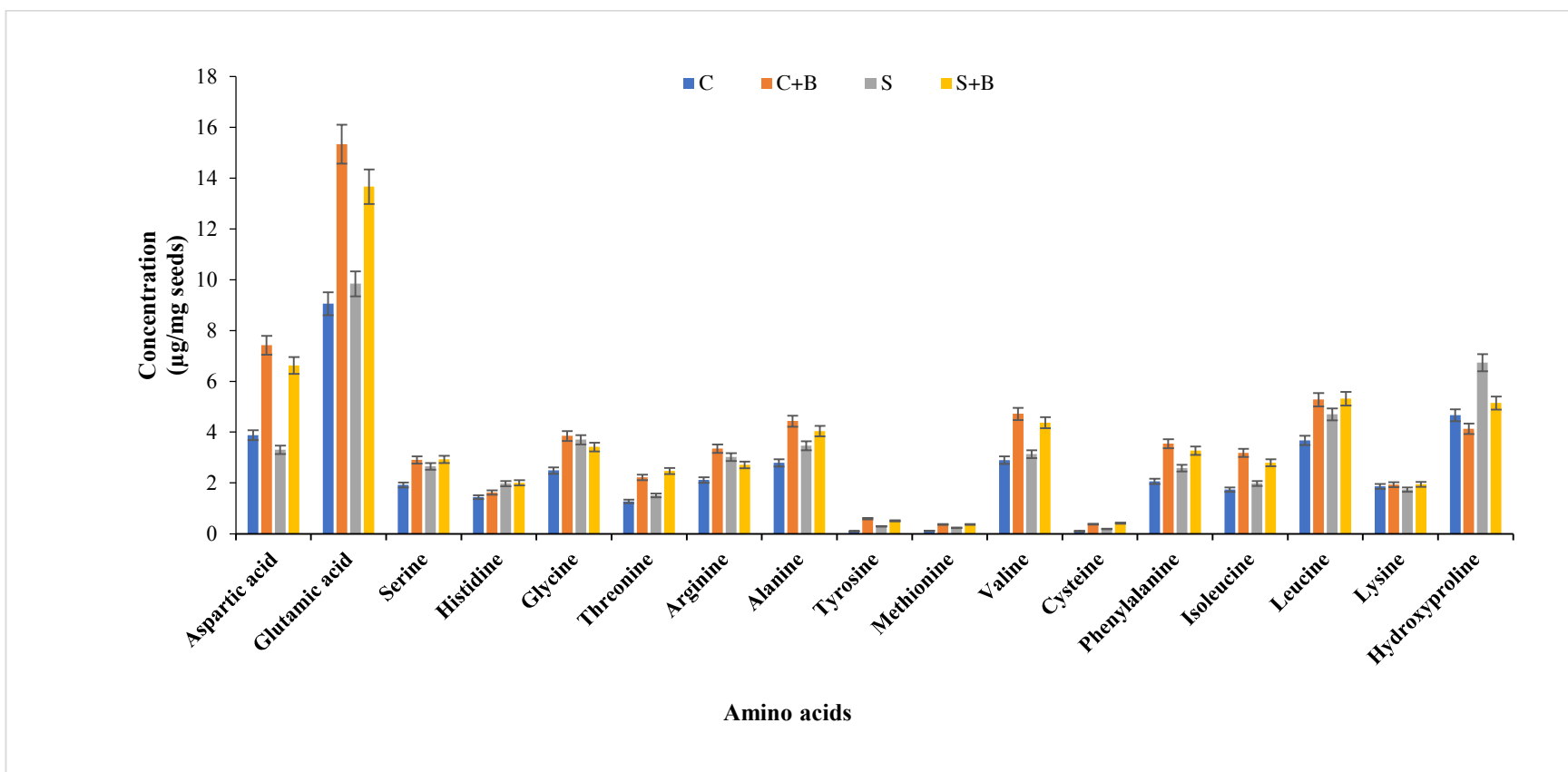


Fig. 72: Amino acid profiling of 1st generation seeds obtained from different treatments in rice. C (Control), C+B (Control+Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant +nutrient deficient condition). Data represent Mean $\pm$ SE (n=3)

Table. 15: Elemental analysis of 1st generation seeds obtained from different treatments in rice of var. Manu ratna. C (Control), C+B (Control + Biostimulant), S (Stress-nutrient deficient condition) and S+B (Biostimulant subjected to stress-nutrient deficient condition). Data represent Mean $\pm$ SE (n=3). Means within the same row followed by the different small letters show that there are significant differences between means ($p < 0.05$).

Elements	C	C+B	S	S+B
K (g/Kg DW)	1.97 $\pm$ 0.16 ^c	2.21 $\pm$ 0.13 ^d	1.04 $\pm$ 0.20 ^a	1.34 $\pm$ 0.28 ^b
Mg (g/Kg DW)	1.23 $\pm$ 0.21 ^c	1.54 $\pm$ 0.37 ^d	0.84 $\pm$ 0.11 ^a	1.12 $\pm$ 0.07 ^b
Mn (mg/Kg DW)	27.1 $\pm$ 0.32 ^c	29.23 $\pm$ 0.27 ^d	18.03 $\pm$ 0.45 ^a	22.28 $\pm$ 0.12 ^b
Zn (mg/Kg DW)	21.23 $\pm$ 0.03 ^a	28.39 $\pm$ 0.13 ^c	14.8 $\pm$ 0.42 ^d	19.1 $\pm$ 0.11 ^b
Ca (mg/Kg DW)	57.72 $\pm$ 0.54 ^d	64.83 $\pm$ 0.21 ^b	23.12 $\pm$ 0.3 ^a	43.84 $\pm$ 0.27 ^c
Fe (mg/Kg DW)	1.84 $\pm$ 0.11 ^b	2.14 $\pm$ 0.02 ^d	1.08 $\pm$ 0.06 ^a	1.36 $\pm$ 0.03 ^c
B (mg/Kg DW)	0.62 $\pm$ 0.24 ^c	0.73 $\pm$ 0.28 ^d	0.21 $\pm$ 0.11 ^a	0.35 $\pm$ 0.10 ^b
Ni (mg/Kg DW)	0.15 $\pm$ 0.09 ^c	0.18 $\pm$ 0.03 ^d	0.09 $\pm$ 0.04 ^a	0.12 $\pm$ 0.06 ^b
Mo (mg/Kg DW)	0.84 $\pm$ 0.1 ^c	0.92 $\pm$ 0.14 ^d	0.35 $\pm$ 0.21 ^a	0.43 $\pm$ 0.07 ^b

and Fe by 16%. Trace elements exhibited similar gains, with B, Ni, and Mo showing increases of 18%, 20%, and 9%, respectively. These improvements indicate the positive role of biostimulant in promoting nutrient enrichment under non-stress conditions.

In contrast, S treatment caused a pronounced decline in elemental concentrations relative to control. K decreased by 47%, Mg by 32%, and Ca by 60%. Micronutrients were severely affected, with Mn declining by 33%, Zn by 30%, and Fe by 41%. Reductions in trace elements were more drastic, with B showing a 66% decrease, Ni 40% and Mo 58%, reflecting the adverse effect of stress on nutrient uptake and deposition in seeds.

The S+B treatment partially reversed the negative effects of stress. Compared to stressed seeds, K and Mg content increased by 29% and 33%, respectively, while Ca exhibited a strong recovery of 36%. Micronutrients also improved, with Mn, Zn, and Fe showing recoveries of 24%, 29%, and 26%, respectively. Trace elements such as B, Ni, and Mo were enhanced by 47%, 33%, and 23%, respectively, relative to stress. The recovery trend clearly highlights the ameliorative influence of biostimulant supplementation in restoring nutrient balance under stress.

5. DISCUSSION

Two rice varieties (Hraswa and Manu ratna) were evaluated for their performance in nutrient-deficient conditions in response to the application of different biostimulants. The experimental design was structured across four developmental stages—seedling, tillering, booting, and ripening—to capture dynamic changes in plant physiology and metabolism. At each stage, parameters such as carbohydrate translocation, photosynthate allocation, metabolite accumulation, reactive oxygen species (ROS) generation, antioxidant enzyme activity, and chlorophyll *a* fluorescence based physiological aspects were systematically assessed.

This stage-wise evaluation provided a comprehensive understanding of how biostimulant treatment modulated plant responses under stress at different stages of its lifecycle. Among the four stages, the booting phase emerged as particularly critical, showing pronounced changes in physiological performance and metabolic regulation. The booting stage marks a crucial shift from vegetative to reproductive growth in cereals like rice. The mechanism that ultimately determines spikelet fertility and yield potential occurs at this period, when the panicle primordia rapidly differentiate and elongate within the flag leaf sheath. Numerous studies have emphasized the physiological importance of this stage because at this stage of reproductive development, plants are more vulnerable to abiotic stressors such as temperature extremes, water shortages, and nutritional deficiencies (Yang et al. 2024; Adzigbe et al. 2025). Stress exposure during booting can cause irreversible yield loss by interfering with pollen viability, assimilate partitioning, and floral organ development (Mehmood et al. 2025). Furthermore, the booting period marks the peak demand for mineral elements, especially potassium and nitrogen, which are essential for the transfer of carbohydrates and the synthesis of proteins. During this phase, lack of nutrients results in sterility and inadequate grain filling (Haider et al. 2023). Booting stage analysis may also be used as a diagnostic window to evaluate source-sink dynamics, translocation patterns, and nutrient absorption efficiency in both ideal and stressful conditions. Therefore, detailed analysis such as gas

exchange measurements and ICP-MS-based nutrient profiling were carried out at this stage. Subsequently, yield traits and FTIR spectroscopy were employed to examine grain development and functional group modifications of metabolites in leaves of plants subjected to biostimulant application. The study revealed that among the both varieties studied, the var. Manu ratna appeared more sensitive to nutrient deficiency, however, biostimulant treatment induced more structural and biochemical changes that improved its tolerance.

5.1 Screening of different biostimulants

Biostimulants are certain substances and/or microorganisms that can improve plant growth, stress tolerance, and nutrient uptake. Identifying new biostimulants, understanding how biostimulants work, and developing efficient methods for characterizing biostimulants is important. Biostimulant characterization is the process of identifying the composition and mode of action of biostimulants. Different biostimulant formulations including microbial and non-microbial were characterized and it is important to note that different formulations show the range and importance of their constituent parts, emphasizing their usefulness individually and in combination for field and agricultural applications. The microbial biostimulant formulations were developed utilizing various derivatives of *Saccharomyces cerevisiae*, including live yeast extract (YI), yeast supernatant (Ys), and powdered yeast (Y). Conversely, the non-microbial biostimulants encompassed a sulfur-based amino acid, L-cysteine (Cys); an antioxidant- ascorbic acid (A); protein hydrolysate (PH); and humic acid (HA) that is available commercially.

From the results, it was observed that pH of these different formulations was in the range of 4.3 to 8.1. YI recorded a pH of 6.7 which is better for enhancing seed germination whereas Ys and Y had pH above 7. According to Hellequin et al. (2020), if the biostimulant had a higher pH (6.4) than the soil (5.5), it will induce a neutralizing effect on biostimulant-treated. The optimal pH range for most plants is 5.5–7.0, and this is also considered the best pH for plant growth. This range of pH would induce changes in the bacterial and fungal communities of the soil (Wang and

Kuzyakov 2024). pH affects plant nutrient availability by effecting changes to the chemical forms of the nutrients and influencing the chemical reactions they undergo. Biostimulant formulations with 5.5-7.0 pH is considered fertile because most nutrients and minerals are more soluble in slightly acidic condition. If the pH is too high, plants may not be able to absorb nutrients properly. The pH of combined formulations i.e., Bl, Bs, B and PH showed a range of 5.6 -6.2 which indicates the optimal range for better growth and development.

Electrical conductivity of biostimulants is an important parameter as it indicates the concentration of dissolved ions (i.e., nutrients, salts, other active components). A moderate conductivity (<5ms) was observed for Bl, A and Y, which can balance the ions in the solution and make stronger electrolytes. While lower conductivity (<0.5ms) as observed in B, Yl, Ys and HA indicate insufficient available nutrient content, which can reduce the efficacy of seed growth. And excessively high conductivity (>5ms) as recorded in Bs, PH and Cys can lead to osmotic stress. EC is generally maintained in the range of 1.5 to 3.5 ms/cm to obtain the optimal growth and yield (Lu et al. 2022). It has been hypothesized that moderate EC level may be beneficial to plants as it can improve the nutritional availability for the growth.

N% and C% in a formulation represents the percentage of nitrogen (N)/carbon (C) by weight present in the product. It is a crucial parameter in agricultural formulations, such as fertilizers and biostimulants. Higher N% as seen in Bl, Yl and PH indicates a product rich in nitrogen, suitable for promoting vegetative growth. While some biostimulants contain nitrogen in lesser quantity (i.e., Ys, Y, A, Cys) and such a situation can promote nutrient uptake or stress tolerance rather than directly supply nutrients. And higher C% in PH, Bl, Yl, HA signifies a product rich in organic matter, as reported for humic acids, fulvic acids, composts, or biochar. Organic carbon plays a vital role in improving soil health by enhancing microbial activity, water retention, and nutrient exchange (Pandao et al. 2024). The C:N ratio represents the ratio of carbon (C) to nitrogen (N) in a formulation. The carbon-to-nitrogen (C:N) ratio is critical for understanding how organic matter will decompose in soil. Products with a very high C% relative to N%

such as A, Cys and PH may decompose slowly and immobilize nitrogen temporarily. While a favorable C:N ratio as observed in Bl, Bs, B, Yl, Ys, Y supports active microbial populations, which are essential for soil health and nutrient cycling. Similar results were observed by Sharma et al. (2016) where two biostimulant formulations “Manek” and “Algavyt” recorded highest and lowest C:N ratio respectively which affects the stability of the formulations and its long-term effects. Thus, optimizing the C:N ratio in biostimulants is essential to achieve both immediate growth promotion and sustained soil health benefits.

To study the structure, composition, and physical properties of biostimulant formulation XRD is considered to be a powerful analytical technique. It is primarily employed to analyze crystalline substances. The intensity of the diffraction peaks is related to the amount of molecules in that crystal phase or having specific interplanar spacing. Greater intensity of the peaks were observed in Bl, HA and PH which indicates higher amount of crystals or molecules with distinct spacing. The width of the peaks is inversely proportional to the crystal size and thinner peaks were observed in PH, Bl and HA that corresponds to a bigger crystal indicating that the samples are crystalline in nature. And rest of the formulations recorded broader peak which means that these may be constituted of defects in the crystalline structure, or that the sample might be amorphous in nature (Tritean et al. 2024). The lowest crystallite size were observed for Bl and larger for PH.

From the number of peaks on the FTIR spectra, it was confirmed that the ten different biostimulant formulations have 3 peaks and this indicates that the formulations include simple organic compounds. While if the peaks are more than 5 then it is considered as complex molecule (Nandiyanato et al. 2019). The infrared absorption bands of Bl, Yl, PH, HA, BS, YS, A, B and Y showed distinct characteristics at ~2936, 2921, 2854, 2207, 1741, 1731, 1630, 1591, 1562, 1582, 1464, 1397, 1375, 1247, 1103, 1077, 1038, 952, 856, 775 and 722 cm^{-1} , displaying composite characteristics of major and minor components. Peaks at 3373, 2926, 1731, 1591, 1375, and 1038 cm^{-1} detected for Bl, PH and HA are linked to functional groups of amino groups, aromatic compounds and polysaccharides. The combination of spectral bands at 3386, 2921, 2854, 1741, 1562, 1464, 1102, 952,

856 and 722 cm^{-1} represent functional groups of fatty acids and polysaccharides, are unique to Bl and can be used to distinguish this product from the other formulations. The FTIR spectra of PH are dominated by bands at 3355, 1582, 1397, 1077, 980, 934, 898 and 719 cm^{-1} representing functional groups of peptides used in the formulation. Each of the six formulations can be identified by their unique combination of absorption peaks/bands, which can help to identify major components of the formulation. The absorption bands in 3300–3500 cm^{-1} are due to stretching vibrations of -OH or -NH groups with varying degrees of hydrogen bonding. The aliphatic groups were confirmed by the presence of two peaks at 2900 and 2840 cm^{-1} , due to the asymmetrical and symmetrical stretching of methylene groups, respectively (Conselvan et al. 2018). From the results it was clear that Bl, Yl, HA and PH shows presence of more functional groups such as amino groups, polysaccharides, fatty acids as compared to other formulations and this can be beneficial for growth and survival of plants under stress condition.

To determine the elemental composition of a material, the elements present in a biostimulant were analysed using XRF. The elements in each formulations depend on balanced mix of essential nutrients, bioactive molecules, and, in some cases, beneficial microbes. These components work synergistically to enhance plant growth, stress tolerance, and nutrient uptake. Among the different formulations, Bl and Yl accumulated most of the elements in highest ratio i.e., Mg, Mn, Zn, S, Si, Al, P, K and Fe. Mg and Mn is directly involved in photosynthesis and nitrogen metabolism, Zn is essential for enzyme activation and hormonal regulation, Si strengthens cell walls, P is important for energy transfer (ATP), root development, and flowering, K for water regulation, stress tolerance, and enzyme activation, Fe is crucial for chlorophyll synthesis and electron transport.

Based on the characterization study of different formulations, it is revealed that Bl, PH and B showed optimal pH and electrical conductivity, ensuring better nutrient availability and stability of active compounds. The higher content of beneficial bioactive molecules, such as amino acids, organic acids, and micronutrients, contributes to improved plant metabolism and growth. In addition, the presence of both microbial and non-microbial components in Bl enhances

nutrient assimilation and stress tolerance. These combined features make BI a more effective and balanced formulation compared to the others.

5.2 Effect of selected biostimulants on seeds

Seed water uptake (SWU) is the first step in the process of seed germination. A prominent increase in SWU capacity due to the treatment with BI, PH and B indicates a higher water inflow into the seed and the accompanied movement of these biostimulants into the seed through the seed coat facilitated by the upregulation of genes involved in water uptake (Qian et al. 2013). The increase in germination % can be attributed to the presence of elements in biostimulant formulations i.e., BI has higher level of Zn (as discussed in XRF elemental characterization of biostimulants section 5.1) and it can induce breaking of seed dormancy, hydrolysis, metabolization of inhibitors, imbibition and enzyme activation. The elements present in biostimulant formulation (BI) also play a role in biosynthesis of endogenous auxins and gibberellins that lead to increase in the radicle and plumule length and thus increase the SVI. An increase in germination % and SVI of seeds primed with BI, PH and B in this study could be explained as a consequence of increased activity of amylase allowing faster rate of starch hydrolysis in germinating primed seeds. This resulted in more availability of soluble sugars necessary for seedling growth. Similarly, the activity of proteases involved in the hydrolysis of proteins in germinating seeds as was enhanced in PH and BI. The breakdown of storage proteins facilitated by proteases ensures a rapid supply of nutrients, leading to faster and more uniform germination in primed seeds.

A significant increase in FAA was observed in seeds primed with PH and HA while BI and Bs showed higher accumulation of sugars. These biostimulant formulations maintain the osmoticum of the cell by the accumulation of osmolytes such as TSS and FAA. The accumulated sugars play an important role in enhancing osmoticum of cell, causing increased uptake of water and thereby expansion of cells, which altogether results in the increased germination and further growth of seedlings. During seed germination, osmotic adjustment of cells is mainly carried out by soluble sugars besides its detoxification role (Afzal et al. 2021).

5.3 Soil analysis

The study involves the analysis of biostimulant (BI) application in two different varieties of rice to tolerate nutrient deprived condition in the soil. Therefore, the soil nutrient properties of nutrient optimum (NO)-control and nutrient deficient (ND) soil were studied. Nutrient deficient soil was prepared by mixing soil and sand in different ratios such as 75:25, 50:50, 25:75 respectively. The result of the soil analysis showed that the NO (control) soil have OC (organic carbon) 2.51% while N (nitrogen), P (phosphorous) and K (Potassium) to the extent of 358, 27 and 280 kg/ha respectively. The ND (25:75) soil have OC, N, P and K content to the extent of 0.3%, 123, 8 and 90 kg/ha, respectively. If the values of OC, N, P and K are higher than 0.75%, 135, 24.6, 280 kg/ha respectively, the soil is generally considered as highly fertile. When the soil parameters such as OC, N, P and K is lower than 0.5%, 127, 10 and 108 kg/ha, respectively it is generally considered as less fertile (Upadhyay et al. 2022). In this study, OC of ND soil has a value 0.3% and if the OC value is below the range of 0.5%, it is generally regarded as soil with low fertility (Upadhyay et al. 2022) and N, P and K values of the ND soil was in the range that was generally assigned to low fertility soil. Similarly, other macro and micro-nutrients of soil was lower as compared to control soil and therefore 25:75 soil was considered as nutrient deficient soil.

5.4 Preliminary studies

The duration of priming is an important factor in any seed priming technique adopted for any crop. Earlier works showed that the success of seed priming was influenced by a combination of factors, among which the duration of seed priming is an important one (Rehman et al. 2024). The seed priming duration significantly affects the germination percentage and germination rate in many plants. If the priming duration exceeds the optimum level, the seeds may undergo over-imbibition, leading to premature metabolic activation and even lead to radicle protrusion before sowing. This prolonged hydration can also disturb membrane integrity leading to solute leakage, and create oxygen-deficient conditions that weaken enzyme activity (Adhikari et al. 2022). While if the priming duration is of

lesser duration then seeds may not attain sufficient water uptake to activate key metabolic processes. As a result, enzymatic activities like amylase and protease synthesis remain incomplete, restricting reserve mobilization and delaying germination (Kakar et al. 2023). In the present study, it was found that 12 h priming period was optimal for both *O. sativa* varieties, because this particular seed priming duration brought about maximum enhancement in the growth attributes (measured in terms of shoot length, fresh and dry weight) of the seedlings. Similar results were observed in *Oryza sativa* while biopriming with *Bacillus megaterium* for 12 h led to increased seed germination, seedling vigor and root traits (Vaishnavi et al. 2024).

The two varieties of *O. sativa* i.e., Manu ratna and Hraswa used in the present study differed in their abiotic stress tolerance nature based on the study of cumulative stress response index (CSRI). Therefore, the nutrient deficient stress tolerance potential of both varieties was tested to identify a soil condition which imparted ~ 50% growth retardation. In *O. sativa*, among the two different genotypes studied, Hraswa was tolerant and Manu ratna as sensitive under nutrient deficient soil condition. Seedling growth difference under stressed conditions depends on the tolerance nature of varieties. In barley, among the different genotypes studied, the NaCl tolerant one showed more seedling growth under NaCl stress when compared to that of the sensitive genotype subjected to NaCl stress (Javed et al. 2022). It was also necessary to check the optimal concentration of BI extract required for bringing about effective seed priming effects in these varieties, since both varieties had different levels of nutrient deficiency tolerance potential. It was found that among the *O. sativa* varieties, Hraswa (tolerant) and Manu ratna (sensitive) was effectively primed with BI extract (in terms of better root length, shoot length, fresh and dry weight) at 25 ppm concentration. The results recorded in this study are in correspondence with the reports of Amooaghaie and Rajaie (2025), wherein they found that foliar application of yeast extract at the rate of 6 g/L overcome the deleterious effect of water stress and consequently resulted in improved productivity of *Hypericum perforatum* and its grain quality.

It was interesting to note that a single concentration of BI (25 ppm) was most effective in bringing about priming effects in both the varieties of *O. sativa* under

nutrient deficient stress condition. From the results, it was also clear that priming of seeds with a concentration higher than 25 ppm of BI could not bring about any enhancement in the growth of seedlings in both the varieties of *O. sativa*. This implies that a particular concentration of BI might bring about some saturating effects in promoting the growth of seedlings. Similar results were reported by Jisha and Puthur (2014) in rice and Zhong et al. (2014) in soya bean were treatment with high concentration (>50 mM) of beta amino butyric acid (BABA) showed growth inhibitory effects, resulting in significant reduction of shoot length, fresh and dry weight and root vitality in the treated plants and optimal concentrations (<50 mM) brought about an enhancement in the above parameters.

5.5 Effect of biostimulant on soil application

The results clearly demonstrate that the application of biostimulants significantly influenced soil enzymatic activities and seedling vigor index (SVI), with 250 ppm emerging as the most effective concentration. The marked improvement in sucrase, protease, dehydrogenase, and urease activities at this concentration indicates enhanced microbial activity and metabolic functioning within the soil microenvironment. This enhancement is likely due to improved nutrient availability and stimulation of soil microorganisms by the optimal biostimulant dose.

Sucrase is responsible for catalyzing the hydrolysis of sucrose into glucose and fructose, which serve as immediate energy sources for soil microorganisms while protease is attributed to the stimulation of proteolytic microbial communities and increased substrate availability (Rajasaheb et al. 2025). The increase in sucrase and protease activity at 250 ppm over the control implies improved microbial energy availability, carbon turnover, protein degradation and nitrogen cycling. Dehydrogenase activity act as an indicator of overall microbial oxidative activity and soil respiration while urease activity facilitates the hydrolysis of urea into ammonium (Daunoras et al. 2024). The observed 40% increase at 250 ppm demonstrates an enhancement in microbial metabolic potential, reflecting an active and balanced microbial community. Also, the marked improvement in SVI

corresponds well with the observed increase in enzymatic activities, suggesting that enhanced microbial functions and nutrient transformations directly translate into better seedling establishment and vigor.

At lower (200 ppm) and higher (300 ppm) concentrations, enzymatic activities and SVI were comparatively lower, suggesting that the beneficial effects of biostimulants are dose-dependent. Excessive application (300 ppm) may have led to substrate saturation or mild inhibitory effects on microbial metabolism, resulting in reduced enzymatic efficiency. The significant reduction in all measured parameters under nutrient-deficient conditions further underscores the importance of nutrient and biostimulant supplementation in maintaining soil biological activity.

Here, 250 ppm represents the standardized effective concentration for soil application of the biostimulant formulation. At this level, the formulation optimizes microbial enzyme activity and seedling vigor, promoting improved soil health and plant growth potential without inducing inhibitory effects, generally observed at higher concentrations.

5.6 Effect of biostimulant on growth parameters

The results indicated a severe decline in growth attributes of rice under nutrient deficient stress condition, while BI priming elicited positive effects and enhanced the growth parameters such as root length, shoot length and DW%. Particularly the root length development was much stronger with formation of finer and longer roots as stimulated by BI extract priming. The reduction in the seedling growth attributes under nutrient deficient condition is invariably due to lack of nutrient availability in the soil. The presence of nutrients ensures proper functioning of cells, which will reflect in enhanced growth. Plants under stress conditions certainly make changes in some of their physiological and biochemical features which will finally enable them to cope up with the stressed situations but with reduced growth. The reduction in plant growth in a stressful environment is also ascribed to the altered level of plant hormones (Adhikari et al. 2022; Cardarelli et al. 2022).

Both the *O. sativa* varieties responded in a similar way to the priming method. Generally, the seedlings raised from primed seeds of tolerant *O. sativa* varieties showed more enhancements in the seedling growth parameters when the seedlings were grown under nutrient deficient condition rather than the seedlings without any stress conditions. This clearly indicates that the seed priming effects in the tolerant varieties are more prominent when they are encountered with stress conditions. It was already reported in rice that the growth advantage as a result of seed priming (with yeast/ salicylic acid / zinc) was more prominent in the seedlings which were grown in stress conditions when compared to seedlings grown without stress (Johnson and Puthur 2022; Khan et al. 2025; Veena et al. 2025). Interestingly, when both varieties were compared, tolerant var. Hraswa showed maximum enhancement in shoot length, fresh and dry weight of seedlings under BI priming which indicates its greater response towards BI priming by exhibiting enhanced tolerance towards nutrient deficient condition.

The increase in growth parameters of the seedlings raised from primed seeds is due to the increased cell division or cell elongation due to increased water uptake of seeds during the seed priming treatments, which finally resulted in an increase of the seedling vigour. Seed priming is known to decrease the resistance of the endosperm envelope, which is a hindrance for the expansive growth of the embryo. This situation allows the turgor threshold for germination to be reached at a faster rate in primed seeds than in non-primed seeds, thereby helping the seedlings from primed seeds to attain greater root and shoot length. The increased shoot and root length in plants raised from primed seeds can also be due to changes in germination events such as reducing the lag time between imbibition and radical emergence (Campobenedetto et al. 2020).

The seed priming treatments are reported to reduce the time taken to initiate the germination process, improves the rate of germination and synchronization, moreover enhances the lengths of shoots and roots and thus increases the fresh and dry mass of the *O. sativa* seedlings (Yadav et al. 2023). Devi et al. (2023) reported that primed wheat seedlings had significantly better results for height, root length and the root dry weight than the non-primed control. According to Khalequzzaman

(2023), in cotton, the increase in the seedling growth can be attributed to the higher water uptake by primed seeds as compared to non-primed ones, which further supports the faster and enhanced growth of the seedlings. Likewise, in soyabean (Sheteiwy et al. 2021) and *O. sativa* (Basit et al. 2022), the enhanced growth of seedlings indicates the stimulative role of priming solution (Jasmonic acid/Spermine), which finally helps in acclimation process of the seedlings. In earlier studies, it was observed that seedlings from NaCl primed canola seeds maintained greater mean seedling dry weights and cell membrane stability than untreated seedlings (Bibi et al. 2024). Seed priming with biostimulant caused increase in seedling vigour and resulted in enhanced shoot weight of maize seedlings (Abdel Lateef et al. 2019).

The improvement of growth parameters such as shoot and root length is due to the presence of cytokinins and other macro and micro nutrients, growth regulators, sugars, vitamins (especially vitamin B) (Sarhan et al. 2011) which is mainly present in yeast (Sarhan et al. 2011). According to Farhadi et al. (2023) yeast extract is a natural source of cytokinins that stimulates cell proliferation and differentiation, controlling shoot and root morphogenesis and chloroplast maturation. Also, the increased amino acids accumulation act as a precursor for the synthesis of these hormones, which have an influential role in enhancing growth responses. Ascorbate and cysteine maintains cellular redox balance and protect from oxidative stress, thereby promoting better root and shoot elongation. Moreover, ascorbate acts as a cofactor for several enzymatic reactions involved in cell wall expansion and hormone regulation, while cysteine serves as a sulfur donor for the synthesis of essential metabolites and antioxidants like glutathione. Similar to the observations of this study, Pathak et al. (2024) have reported that the integrating microbial and non-microbial biostimulants will enhance the level of macro and micro nutrients, together with large proportion of free amino acids and short peptides of two or three amino acids as well as long chain protein hydrolysates.

5.7 Effect of biostimulant on carbohydrate translocation and photosynthate allocation

Biostimulants commonly regulate some pathways which are connected with abiotic stress tolerance, carbohydrate synthesis, cell wall metabolism, growth and development, lipid metabolism and signalization (Chambard et al. 2023). The use of biostimulants enhances the efficient translocation of growth regulators and other metabolites from the leaves to reproductive organs, resulting in improved and higher yields (Bartucca et al. 2022). In field investigations, it was observed that stress from nutrient deficiency significantly impedes the growth of rice seedlings, disrupting their associated physiological and metabolic functions. These deficient conditions ultimately lead to reduced plant productivity and crop yield losses. The visible signs of stress resulting from the lack of specific macro- and micronutrients are well documented (Jiaying et al. 2022). Moreover, to withstand particular stress conditions, plants require energy and adequate resources. However, nutrient limitations or deprivation hinder the plant's ability to tolerate major stressors. Here the study, examined the impact of biostimulant application on various growth stages of rice under nutrient-deficient soil conditions, with notable results observed during the booting stage. The booting stage is associated with substantial alterations in photosynthetic activity, carbohydrate metabolism, and antioxidant enzyme regulation. Enhanced photosynthate translocation from source leaves to developing reproductive tissues is essential to support panicle growth and grain initiation. The booting phase is characterized by the swelling of the flag leaf sheath due to the enlargement of the panicle as it emerges from the sheath. Critical physiological processes like microsporogenesis occur during this stage, making it a crucial phase for rice grain yield and quality (Mohapatra et al. 2025). Throughout this process, the developing gametes act as robust photosynthetic sinks, accumulating carbon assimilates transported from the leaves (Ferguson et al. 2021). Under stress conditions, however, the reduction in chlorophyll content, photosystem efficiency, and rubisco activity adversely affects assimilate production (Gautam et al. 2022). Consequently, studies conducted at the booting stage are instrumental in elucidating how biostimulant or nutrient interventions mitigate these stress-induced impairments

by maintaining photosynthetic integrity, osmotic adjustment, and redox homeostasis (Rouphael and Colla 2020b).

Under nutrient deficient condition, the chlorophyll content was reduced from tillering to ripening stage because of chlorophyll degradation or hindrance in the synthesis of chlorophyll as compared to control. Earlier reports of Razi and Muneer (2021) reported that chlorophyll content seems to get reduced under osmotic as well as other stresses as a result of chloroplast damage, pigment-protein complex instability and due to the suppression of the enzymes involved in the chlorophyll synthesising pathway. Whereas biostimulant treated seeds i.e., C+B and S+B were observed with increased chlorophyll content in tillering and booting stages than non-primed control. According to Sarhan et al. (2011), the main constituent of biostimulant extract are cytokinins and other macro and micro nutrients, growth regulators, sugars, vitamins (especially vitamin B) which improve various physiological activities indicating chlorophyll synthesis. Among these, cytokinins play a crucial role in chlorophyll biosynthesis and delayed leaf senescence. It imparts a positive influence on the chlorophyll synthesis which reflects in the increase of total chlorophyll content in the leaves of rice seedlings (Bulgari et al. 2019). Similarly, ascorbate and cysteine, which are included in the biostimulant formulation, help to preserve redox equilibrium and shield chlorophyll molecules from oxidative deterioration. Also, ascorbate maintain chlorophyll stability and improve photosynthetic efficiency by acting as a potent antioxidant and cofactor in chloroplast enzymatic processes and cysteine as a precursor for glutathione formation. This will positively reflect on the activity of photosynthesis and the synthesized photosynthates such as total sugar and starch content. Biostimulant treatment significantly improved the activation of carotenogenesis, increasing carotenoids, which have the capacity to neutralise ROS and thus protect the photosynthetic apparatus and the pigments associated with it from oxidative reactions and similar effect have been reported in biostimulant treatment in rice (Johnson and Puthur 2022).

Carbohydrates which represent one of the major components of dry matter was affected under nutrient deficient condition, with decrease recorded in the case of

total sugars, starch, reducing and non-reducing sugars at different stages of rice. This drastic reduction is a clear indication of decline in chlorophyll content, photosynthetic rate, which are responsible factors for reduced net synthesis of sugars in leaves and that ultimately decreased starch content in leaves. Whereas, biostimulant treated rice seedlings (i.e., C+B and S+B) accumulated more total sugars, starch, reducing and non-reducing sugars than non-treated seedlings in booting stage. The sugar accumulation in booting stage has got significance as booting stage is directly related to panicle initiation, which majorly accounts for the yield (Parida et al. 2022). The increase in sugar content in rice seedlings subjected to S+B suggests that it could be due to significant accumulation of carbohydrates which can be correlated with higher chlorophyll content, which influences energy harvest and accompanied water oxidation and the associated photosynthetic process, reflecting in higher crop yield.

Similar results were observed by Chambard et al. (2023) where biostimulants induce carbohydrate synthesis and abiotic stress resistance pathway to protect plant against various stresses during plant development and maintain a higher level of sugars. Whereas the decrease in non-reducing sugars observed under the S+B condition during the ripening stage suggests a dynamic turnover of these sugars into starch and other metabolic products as the leaves and grains approach maturity. This pattern can be attributed to the enhanced activity of sucrose synthase (SuSy), which catalyzes the reversible conversion of sucrose into UDP-glucose and fructose, thereby promoting starch biosynthesis and energy storage in developing grains (Jaiswal et al. 2025). These hexoses serve not only as vital substrates for respiration but also act as signaling molecules facilitating sugar transport from source (leaves) to sink (grains) tissues. The mobilization and utilization of these sugars enhance grain filling and carbohydrate allocation, ensuring grain development and helping the plant to cope with stress while sustaining yield.

The commencement of grain-filling involves a metabolic pathway converting sucrose into starch via a sequence of enzyme-driven reactions (Dong and Beckles 2019). It is widely accepted that SuSy (cleavage activity) and adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase) are pivotal in this sucrose-to-starch

metabolism process in rice (Fan et al. 2019). In this study, higher SuSy activity in C+B and S+B indicated its overall importance in providing carbon for actively growing tissue. SuSy activity was observed to increase progressively till the end of the booting stage, indicating its active involvement in sucrose cleavage and energy supply during rapid vegetative and early reproductive growth.

However, during the ripening stage, when there is a high demand for hexose sugars to support translocation from source tissues (leaves) to sink tissues (developing grains), the activity of SuSy increased in both varieties. This is because the enzyme plays a central role in the utilization of incoming sucrose in sink tissues. SuSy catalyzes the reversible cleavage of sucrose into UDP-glucose and fructose, which directly feed pathways essential for grain filling, including starch biosynthesis, cell wall formation, and energy production. As ripening grains have high demand for carbon skeletons and rapid starch accumulation, the plant upregulates SuSy to ensure efficient sucrose breakdown and channeling of carbon into storage compounds. Elevated SuSy activity during ripening is therefore considered a biochemical marker of strong sink strength and active assimilate unloading into developing grains.

While the reduction in SPS activity during ripening stage suggests that sucrose metabolism shifts toward enhanced sugar utilization for storage rather than synthesis or cleavage. SPS is primarily a source-strength enzyme, responsible for sucrose synthesis in photosynthetic tissues. Till early and mid-booting stages, SPS activity remains high to sustain carbon export from source leaves. However, at ripening, overall photosynthetic rates decline, leaf senescence accelerates, and carbon assimilation reduces. As a result, SPS activity is naturally downregulated because the plant's requirement for sucrose synthesis diminishes. Together, these changes reflect a coordinated shift, decreasing the biosynthesis of sucrose in senescing leaves with lower SPS while enhancing sucrose mobilization and utilization in grains by higher SuSy. These findings are in agreement with the observations of Kumari and Asthir (2016), wherein they reported a similar increase in SuSy and decrease in SPS activity in rice during the later stages of grain filling due to increased sugar-to-starch conversion.

5.8 Effect of biostimulant on metabolites/osmolytes accumulation

Osmotic adjustment is a fundamental mechanism adopted by plants to continue their growth under stress conditions. According to Paliwal et al. (2021), plants acquire osmotic balance by accumulating inorganic ions like K^+ , and synthesizing compatible solutes, such as free amino acids, soluble sugars, polyamines, and polyols. One of the consequences of abiotic stresses is the low accumulation of osmolytes and reduced osmotic potential, which limits plant access to soil water. This reduced osmolytes induce water shortage which can lead to loss of turgidity and dehydration of cells, and ultimately cause death of plants. The decrease in total free amino acids, sugars, proline, phenol and soluble protein content under various stress conditions suggests that stress-exposed rice seedlings were compromised without an efficient metabolism and synthesis of metabolites under excessive stress. Under nutrient-deficient conditions, the accumulation of amino acids was reduced which leads to a decrease in protein synthesis. The content of free amino acids was highly reduced due to nitrogen and molybdenum deficiency as reported by Sun et al. (2024). The decrease in sugar content in rice seedlings subjected to nutrient stress suggests that it could be due to reduced accumulation of carbohydrate which can be correlated with reduced chlorophyll, which affects energy harvest and accompanied water oxidation and electron transport through the photosystems, decreasing the production of ATP and NADPH required for the production of photosynthates.

Mineral salts are necessary for maintaining the osmotic pressure of the leaf cell sap along with many other osmolytes (Choudary et al. 2023). One of the consequences of nutrient deficiency is the low accumulation of inorganic ions and other osmolytes, resulting in reduced osmotic potential, which limits plant access to soil water. Moreover, deprivation of nitrogen has been shown to reduce the density of aquaporins and so the transport of water through the plant becomes more dependent on diffusion through the membrane again causing dehydration of cells due to slow water movement across cells. Moreover, the imbalance in potassium signals the plant to close the stomata and restrict water movement and also alters the distribution of aquaporins and thus reduces the uptake of water by the plant. This

creates a situation of dehydration in the cytoplasm, and thus generating osmotic stress. According to Imtiaz et al. (2023) potassium as well as nitrogen, deficiency induces dehydration of cells.

Biostimulant application maintain the osmoticum by the accumulation of osmolytes such as free amino acids, sugars, and proline when grown under nutrient deficiency condition. C+B and S+B assisted in the elevation of sugars and free amino acid levels which contributed to osmotolerance by improving water status, stabilizing membranes and protein complexes, and protecting various enzymes from denaturation. In the present study, the biostimulant containing yeast is known to contain trehalose-6-phosphate synthase, an enzyme responsible for the biosynthesis of trehalose, a crucial osmoprotectant that plays multiple protective roles under abiotic stress conditions (Kondrak et al. 2012). Trehalose helps in stabilizing thylakoid membranes, maintaining the integrity of chloroplast structures, and protecting photosystem II from photoinhibition and oxidative damage. It also prevents the denaturation of photosynthetic enzymes such as Rubisco by forming a protective hydration shell around proteins and membranes (Luo et al. 2021). In addition, trehalose modulates carbohydrate metabolism and energy balance, ensuring a steady supply of assimilates for growth and stress recovery. These combined effects help to sustain photosynthetic efficiency, resulting in higher sugar and amino acid accumulation in biostimulant-treated plants under nutrient deficient condition. While the presence of ascorbate and cysteine present in the biostimulant formulation contribute to maintaining cellular redox balance and protecting from oxidative damage.

Biostimulant treatment of rice seedlings (C+B and S+B), maintain the osmoticum by accumulation of osmolytes such as FAA and total protein when they were grown under nutrient deficient condition. These FAA and total soluble protein increased during booting and ripening stages that lead to a shift in the balance of carbon and nitrogen metabolism by enhancing starch biosynthesis, which can contribute to higher yield and plant tolerance. The results indicate that elevation of osmolytes in rice plants subjected to S+B condition from tillering to booting stages contributed towards osmotolerance by improving water status, stabilizing

membranes and protein complexes, and protecting various enzymes from denaturation. Tillering stage is characterized by active cell expansion, cell division and growth of tillers and yeast based biostimulant extract is known for cytokinins, stimulates cell division and cell expansion enabling enhanced growth. Moreover, the observed increase in free amino acids accumulation contribute to enhanced growth regulation and stress resilience. Similar positive effects were reported in maize plants where the osmolality was increased due to the increased exposure to carrot extract, a good source of cytokinin (Abdel Latef et al. 2019).

Proline is one of the most abundant amino acids known to frequently accumulate in plant cells in responses to a variety of abiotic stresses (Mansour and Salama 2020). In addition to its osmoprotective role, proline also acts as source of energy, ROS scavenger, and as signalling molecule (Ghosh et al. 2022). It is also observed that proline accumulation improved tolerance to salt stress by contributing to the osmotic adjustment under salt stress in *Cicer arietinum* and *Brassica napus* (Dadasoglu et al. 2022; Yan et al. 2024; Choudhury et al. 2025). It has also been observed that tolerant var. Hraswa accumulated higher levels of proline compared with sensitive var. Manu ratna, indicating a positive role of proline in stress acclimation. The enhanced level of proline in biostimulant treated seedlings on exposure to stress could be contributing toward the build-up of osmoticum and thus help in alleviating the stress. The increased accumulation of osmolytes in the seedlings raised from treated seeds could facilitate the increased uptake of water resulting in turgor which ultimately promotes the expansion of cells and thus promoting the growth.

5.9 Effect of biostimulant on oxidative stress and antioxidation phenomenon

Reactive oxygen species are partially reduced or activated forms of oxygen. The main end result of abiotic stresses is the formation of excessive ROS that can generate oxidative stress. The increased concentration of ROS can cause oxidative damage to membranes (lipid peroxidation), proteins, RNA and DNA molecules (Juan et al. 2021). The accumulation of ROS in different sites of plant cell (viz chloroplast, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic

reticulum and cell wall) increases during various stresses. In seed physiology, ROS are usually considered as toxic molecules, leading to cell injury with consequent problems in seed germination and development (Farook et al. 2021).

However, there is the increasing evidence that, at low concentrations, ROS can act as signalling molecules involved in a wide range of responses to various stimuli. The dual function of ROS in plants mainly relies to the cellular antioxidant machinery, which involves detoxifying enzymes and antioxidant (Hong et al. 2024). Such mechanisms can scavenge potentially toxic ROS, generally produced under stressful conditions, or rather tightly control ROS concentrations in order to regulate various signalling pathways. Among ROS, superoxide and hydrogen peroxide plays a key role in initiating signalling processes during germination, however, high levels of ROS can be toxic for the seeds. The ability of seeds to survive to this oxidative condition during germination phases is related, to their ability to activate different detoxification systems, including both the neo-synthesis of soluble antioxidants and the activation of gene expression of enzymatic defense (Farook et al. 2021).

In the present study, nutrient deprivation triggered the production of H_2O_2 and O_2^- in the rice seedlings under stress condition. The enhanced levels of these ROS also coincided with the higher lipid peroxidation rate which is regarded as a biochemical marker for the free radical mediated injury in plants. Nutrient deficient condition will result in over accumulation of ROS in seedling, owing to ion imbalance and hyperosmotic stress (Akhtar et al. 2023). Over production of ROS is a major indicator of the oxidative stress in rice seedlings. The phenomenon of oxidative injury modifies the structures of the membranes by the lipid peroxidation resulting in electrolyte leakage and ultimately results in loss of cell viability. These results are in line with previous researchers, who observed enhanced ROS-production and MDA content under nutrient deprivation such as N-deprivation (Hua et al. 2024), P-deprivation (Meng et al. 2021; Fu et al. 2023) and K- deprivation (Fu et al. 2023).

This study indicates that the level of O_2^- , H_2O_2 was reduced in biostimulant treated seeds, suggesting a possible role of the biostimulant in preventing the

accumulation of these reactive oxygen species. The reduced generation of O_2^- , H_2O_2 observed in biostimulant treated seeds can be linked to the enhanced activities of ROS-scavenging enzymes. Concomitant results were observed by Campobenedetto et al. (2020) in *Cucumis sativa* where liginin and amino acid derived biostimulant KIEM® led to increased antioxidant system under temperature stress.

Present study shows that higher MDA and superoxide (O_2^-) content was observed in tillering followed by booting and ripening stages in the leaves of non-treated plants which indicates the excess accumulation of reactive oxygen species, indicative of the imminent leaf senescence, which could hinder further plant growth and development process. Tillering is the beginning of vegetative phase where new leaf starts to emerge further followed by booting where microsporogenesis occur and deficiency of macro or any micro-nutrients directly affects these developmental processes. Similar results were reported by Zhang et al. (2023) in heat stress at booting stage, which affects the rice grain quality.

The biostimulant treatment in two most important stages (i.e., seedling and booting) recorded significantly lower accumulation of ROS and lipid peroxidation compared with stress condition which indicates that stress caused nutrient deprivation and seedling damage. Previously in rice, similar beneficial effects of seed priming have also been observed under drought (Waqas et al. 2022), UV (Thomas and Puthur 2019) and chilling stresses (Nie et al. 2022). The over produced ROS in stress conditions resulted in damaging the cell components whereas in the case of seedlings subjected to UV-B priming either at seed or seedling stage, the content of ROS produced was in limits and could even serve as a positive regulator of signalling cascade. A minor quantity of ROS could initiate a signalling cascade ultimately equipping the plant to effectively counter the stress. Researchers have reported that the moderate accumulation of ROS can act as signalling molecule for plant growth and development and various stress tolerance mechanisms (Huang et al. 2019).

As per Mishra et al. (2023), non-enzymatic antioxidants play a vital role in plant stress tolerance mechanisms, particularly in safeguarding against oxidative

stress under abiotic stress conditions. Non-enzymatic antioxidants such as AsA, GSH, and different phenolic compounds actively counteract ROS production and consequently aid in the non-enzymatic detoxification of ROS. Plants can respond to an increase in ROS production through the synthesis of soluble antioxidants. Among them, non-protein thiols are considered important molecules in counteracting the effect of oxidative stress (Salbitani et al. 2023). Thiols, such as glutathione (GSH) together with its regulatory role in redox signalling and defense processes, are important components for the stress tolerance. In this study, the levels of phenolic compounds, glutathione (GSH) and ascorbate (AsA) were severely reduced in the rice seedlings subjected to nutrient deficient condition. These results suggest that stress induced oxidative damage to rice seedlings were due to the absence of an efficient antioxidant system. Similarly, decreased non-enzymatic antioxidants levels were observed in rice under nutrient deprived condition (Lin et al. 2023).

On the other hand, biostimulant application improved antioxidant power of rice seedling under stress by enhancing the levels of AsA, GSH and total phenolic compounds, contributing to the reduction of oxidative state ensuring better tolerance to stress. The presence of cysteine in the biostimulant supports this protection by acting as a key precursor for glutathione synthesis. During seed germination, a high level of ROS is produced due to increased metabolic activity. Phenolic compounds, GSH and AsA play antioxidant roles by directly quenching the free radicals, limiting their reactivity towards membrane components (Rao and Zheng 2025). Moreover, phenolic compounds are also capable of stabilizing cellular membrane by reducing membrane fluidity, which result in decreased mobility of free radicals through the membranes, thereby restricting membrane peroxidation. As per the findings of this study, biostimulant-treated rice seedlings maintained higher antioxidant levels and lower MDA content, suggesting that the biostimulant played a protective role by strengthening the plant's redox defense system and mitigating oxidative damage under stress conditions.

Phenolic compounds function as strong scavenger of free radicals. Seed priming activates the phenolic biosynthetic pathways and enhances the accumulation of phenolic compounds (Yin et al. 2024). Phenolics compounds take multiple roles

in plants such as structural components of cell walls, involved in the modulation of growth and developmental processes as well as in the mechanisms of defence against abiotic stresses. Phenolic compounds protect the cells from oxidative damages, increase the cell membrane stability and mitigate the stress effects in seedlings treated with biostimulants. This was predominantly observed in S+B seedlings as compared to seedlings subjected to nutrient deficient condition. Additionally, phenolic compounds contribute to stabilizing cellular membranes by reducing membrane fluidity, which helps to limit membrane peroxidation together with high free radical scavenging activity and also acts as a cell wall constituent besides its antioxidant potential (Rao and Zheng 2025). Similar to phenolics, it was also found that an increase in other non-enzymatic antioxidants occurred indicating the activation of ascorbate-glutathione cycle, which helps to detoxify the ROS generated during germination and protects the cellular structures from oxidative damage. Glutathione and ascorbate are key antioxidant in plants. Biostimulants upregulate enzymes like glutathione reductase and glutathione peroxidase and promote sulfur metabolism, boosting cysteine levels, which are precursors for glutathione synthesis (Rao et al. 2025).

Ascorbate (AsA) is a water-soluble antioxidant and act as an enigmatic component of plant defence mechanism. Ascorbate has multiple functions in plants, influencing growth and development by metabolizing ROS and its products (Xiao et al. 2021). It is a non-enzymatic antioxidant which serves as electron donor in various enzymatic and non-enzymatic reactions. Ascorbate also plays major role in plant development such as regulation of cell division, cell cycle and cell elongation. It can also act as an alternative electron donor to PSII, when the oxygen evolving complex (OEC) is damaged during stress condition (Johnson et al. 2024). The enhancement in ascorbate accumulation provides an explanation of better PSII activity in UV-B irradiated seedlings (Liu et al. 2023). The increased content of ascorbate and glutathione in rice seedlings raised from biostimulant treated seeds shows a clear cut indication of their capacity to adapt to environmental stresses. The ascorbic acid directly scavenges the $^1\text{O}_2$ and $\text{OH}^\cdot$ and in addition removes the H_2O_2 through Asada Halliwell pathway and this antioxidant was over accumulated in

Vigna species on exposure to UV-B radiation (7.2 kJm^{-2}) (Dwivedi and Narayan 2024). The increased level of antioxidants confers the ability to plants for scavenging ROS and thus to effectively withstand the nutrient deficient stress condition. The protective role of ascorbate, glutathione and total phenolics against oxidative stress was well evident in many crop plants.

Glutathione is non-enzymatic antioxidant and it is involved in various cellular processes under stress and it also acts as a substrate for glutathione S transferase and glutathione peroxidase. Moreover, it can detoxify superoxide and hydroxyl radical and also function as non-enzymatic ROS scavenger (Raj et al. 2021). Respiration take a major role in the synthesis of GSH and at the time of respiration, different metabolites such as glycine are produced, that could be utilized for the synthesis of GSH. GSH also act as a substrate of the enzyme GR, so that the augmented GSH content can be correlated with decreased GR activity in stress conditions (Madhu et al. 2022). In this study, biostimulant application induced enhanced synthesis of ascorbate, glutathione and total phenolics in both rice varieties at booting and ripening stages. Biostimulants led to increased synthesis of AsA, GSH and total phenolics in both rice varieties during the booting and ripening stages. This enhancement aids in the detoxification of ROS, thereby playing a crucial role in effectively mitigating lipid peroxidation and electrolyte leakage, thus preserving membrane stability.

Plants elicit their enzymatic activity that is crucial to mitigate the ROS production even at low levels in the cells. The enzymes involved in the reduction of ROS are localized in different subcellular compartments like mitochondria, chloroplast, peroxisome and cytosol because they are the important sites of ROS production under stress. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydro ascorbate reductase (MDHAR), dehydro ascorbate reductase (DHAR), glutathione reductase (GR) and guaiacol peroxidase (GOPX) are the major components of enzymatic antioxidant system. Superoxide dismutase (SOD) is a metalloenzyme that initiates the first line of defense against ROS accumulation by rapid conversion of superoxides (O_2^-) into H_2O_2 and O_2 . Apart

from this, SOD has a key role in nuclear transcription, as a RNA binding protein, and a signal modulator in glucose metabolism (Su et al. 2021).

Catalase, a heme-containing redox enzyme found in all aerobic organisms, actively involves in the conversion of H_2O_2 . The conversion may be a direct one to H_2O and O_2 or indirectly by oxidizing substrates like methanol, ethanol, formaldehyde, formate, or nitrite by using hydrogen peroxide (You et al. 2022). Catalase plays a key role in early growth and establishment of seedlings by detoxifying H_2O_2 under stress. In antioxidant enzymatic defence system APX have major role in averting oxidative membrane damage and this is due to its higher affinity to hydrogen peroxide, converting it to water by utilizing thylakoid lumen ascorbate as electron donor (Corpas et al. 2024). GPOX is a heme containing enzyme and removes excess hydrogen peroxide during stress conditions. GPOX prefers guaiacol and pyragallol as electron donors. GPOX activity was higher in lettuce plants during UV-B stress condition and rice seedlings under drought stress conditions (Piccinic 2022). From the results obtained, it was clearly evident that treatment with biostimulant caused increase in activation of various free radical scavenging enzymes, such as SOD, CAT, APX and GPOX. Thus, biostimulant treatment enables the seedlings to counter the stress more rapidly and vigorously, by taking advantage of the enhanced activity of enzymatic antioxidants.

In var. Hraswa under nutrient deficient condition a decrease in SOD, CAT APX and GPOX activities were observed, indicating impairment in antioxidant system. Deficiency in zinc and iron causes an unbalanced functioning of SOD and it can be correlated with higher accumulation of superoxides. Similarly, as per the results of Khan et al. (2021), the SOD and POX activities under N or P deprivation were lower which were concomitant with ROS accumulation and indicate the impaired antioxidant system. The decreased activity of CAT can be an indication of potassium deficiency, as potassium plays an essential role in maintaining enzyme activation, osmotic balance, and stomatal regulation, all of which influence the plant's oxidative metabolism and it is consistent with the previous reports observed in rice (Jiaying et al. 2022). Under potassium-deficient conditions, the efficiency of antioxidant enzymes such as CAT often declines, leading to an imbalance in ROS

homeostasis. However, when different growth stages were compared under nutrient deficient condition, an increase in enzymatic activity was recorded in booting and ripening stages which signifies the strategy adopted by plant to mitigate the ROS accumulation to ensure normal growth at very important stages of development.

From the results, it was observed that both the varieties showed preferential pattern of antioxidation, where tolerant Hraswa seedlings recorded maximum activity of SOD, CAT and GPOX, whereas for sensitive Manu ratna, it was APX. Biostimulant stimulated the activities of antioxidants in both varieties of rice seedlings subjected to nutrient deficient stress and thereby contributed significantly towards the reduction of ROS toxicity, as indicated by a diminished level of MDA in treated rice seedlings. Also, biostimulant caused alteration in the synthesis pathway of SOD either directly or indirectly resulting in enhanced activity of the enzyme. When both varieties were compared, the tolerant var. Hraswa exhibited a stronger inherent or inbuilt genetic potential to withstand stress, reflected in its heightened antioxidant defense system compared to the sensitive variety Manu Ratna. This genetic advantage likely enables Hraswa to maintain more efficient regulation of ROS through the upregulation of key antioxidant enzymes such as SOD, CAT, and peroxidases, along with higher levels of non-enzymatic antioxidants like ascorbate and glutathione. The inbuilt genetic potential of Hraswa may also be linked to its superior osmotic adjustment, membrane stability, and more effective activation of stress-responsive genes involved in detoxification and signal transduction pathways. These coordinated responses collectively contribute to its ability to sustain cellular homeostasis and metabolic activity under adverse conditions.

Antioxidant activity exhibited a distinct pattern during the reproductive phase (booting and ripening stages) and was more pronounced compared to other growth stages. The reproductive stage represents the most crucial and sensitive phase of the plant life cycle, as it directly determines the final economic yield (Jiang et al. 2025). According to Huanhe et al. (2024), stress experienced during this phase can accelerate leaf senescence, reducing the supply of assimilates to developing grains and ultimately lowering productivity. In this context, the application of

biostimulants plays a significant role by enhancing the antioxidant defense system during the booting phase. Biostimulant-treated plants exhibited elevated levels of enzymatic antioxidants such as SOD, CAT, and APX, as well as non-enzymatic antioxidants like ascorbate, glutathione, and phenolics, which collectively mitigate oxidative stress. The bioactive compounds and signaling molecules present in the biostimulant formulations likely activate redox-regulated defense pathways and delay senescence, maintaining chlorophyll integrity and photosynthetic efficiency during grain filling.

5.10 Effect of biostimulant on photosynthetic processes

Chlorophyll *a* fluorescence is a tool to analyze the physiological state of photosystem II (PSII) components, electron transport chain components and the cooperation of light-dependent and light-independent biochemical mechanism (Jan et al. 2025). There are few reports on the effect of biostimulant treatment at different stages of rice under nutrient deficient condition on photochemistry. Nutrient deficiency at different developmental stages could reduce the function of photosynthetic apparatus, and the extent of the reduction depends not only on the severity of the stress, but also on the stage of plant development.

The efficiency of water splitting complex OEC (Fv/Fo) was reduced in both varieties of rice under nutrient deficient condition particularly in reproductive stage. Severe reduction in Fv/Fo was observed in the booting stage in tolerant var. Hraswa whereas in sensitive var. Manu ratna ripening stage was drastically affected. Fv/Fo reflects the efficiency of the primary photochemical reactions in photosystem II (PSII) and is a sensitive indicator of stress-induced damage to the photosynthetic apparatus. This reduction likely contributes to minimizing long-term photoinhibition and maintaining photosynthetic stability. Several studies examined the effect of stress at various growth stages and suggested that the reproductive stage was more adversely affected than the vegetative growth stage (Jiang et al. 2025). However, some studies have demonstrated that the period from the beginning of stem elongation to anthesis was most sensitive, in terms of yield penalties (Kim et al. 2024).

In the present study, booting was most susceptible stage, followed by ripening and grain filling stages. As booting is the most important stage characterized by the swelling of the flag leaf sheath which is caused by an increase in the size of the panicle, nitrogen deficiency can directly affect this critical growth stage (Zakari et al. 2021). The reduction in the function of OEC (F_v/F_o) led to the donor side photoinhibition of PSII and the result was significantly correlated with reduction in PSII activity under the nutrient stress condition. Deficiency in specific nutrients (N, P, K, Ca, Mg, S, and Fe) disrupts the functioning of the photosynthetic apparatus, decreasing PSII photochemical efficiency and modifies the values of Chl *a* fluorescence parameter (Latifinia and Eisevand 2022). Nitrogen (N) accessibility is the key factor limiting the growth of plants, being a component of all proteins, nucleic acids and other organic compounds. N deficiency modifies thylakoid membranes and disrupts their functioning, leading to acceleration of chloroplast aging and plastoglobule formation (Coulon et al. 2024).

The reduction in photosystem activities in the rice seedlings subjected to nutrient-deficient conditions was largely due to the inactivation of active reaction centers or the destruction of the photosynthetic apparatus. Such a situation was reflected through the decrease in F_m , F_v/F_o , Area, and $PI_{(abs)}$, eliciting a negative effect on the growth of seedlings. Very low F_m values in rice seedlings due to nutrient-deficient conditions indicate that the donor side of PSII, especially the oxygen-evolving complex was damaged and also there was an increase in the inactive PSII reaction centers due to the abiotic stress (Didaran et al. 2024). The reduction in these parameters indicates the decrease in efficiency of the water-splitting complex on the donor side of PSII in rice seedlings.

Under nutrient deficient condition, reduction of photochemistry was observed in both the varieties of *O. sativa* seedlings. This reduction in photosystem I and II activities under stressed condition may be also partly due to the decrease in chlorophyll pigment content which was discussed earlier. Moreover, the stress conditions causes inactivation of active reaction centers in the leaves, which directly reduces the photosynthetic activity of the seedlings, as evidenced from the chlorophyll *a* fluorescence data. The density of active reaction centres per cross section of PS II (RC/CS) was found to be decreased in the rice seedlings subjected to nutrient deficient condition, and also the dissipation of energy (DI/CS) was found

to increase in these seedlings. The low chlorophyll content in stressed plants might add up to the enhanced rate of energy dissipation because there are less chlorophyll molecules for harvesting the incident light energy. There are previous reports on the decline of PS I and PS II activities in plants under abiotic stresses. In wheat leaves, the number of PS II inactive centers increases upon NaCl treatment which ultimately leads to reduced PS II activity (Li et al. 2022). A reduced activity of C₃ cycle under drought stress was reported to result in increased production of superoxides affecting the acceptor side of PSI, ultimately reducing the PSI activity (Pandey et al. 2023).

The chlorophyll *a* fluorescence data reveals that biostimulants have influenced the increased distribution of reaction centres in a unit area and/or enhanced the activity of existing reaction centres. Due to the presence of ascorbate and cysteine in the biostimulant formulation it stabilizes chlorophyll and enhances photosynthetic performance by serving as a strong antioxidant and enzymatic cofactor in chloroplasts. Moreover, cysteine supports this protection by acting as a key precursor for glutathione synthesis. It not only improved the activity of reaction centres but also enhanced the count of open reaction centres and increased the efficiency of absorption, trapping, and transport of electrons per PS II cross-section. Biostimulant treatment (C+B & S+B) also decreased the dissipation of energy, thus maximizing the potential of reaction centers by more trapping and utilization of energy. It was already reported that seed priming with PEG and salicylic acid increased the photosynthetic activities of the seedlings in rice (Sen and Puthur 2020; Shan et al. 2024). Similarly, the efficiency of OEC complex and Fv/Fo was affected differently in biostimulant treated rice seeds subjected to nutrient deficient condition (S+B). This in turn, affected a lesser reduction in PSII activity of biostimulant treated seeds under nutrient deficient condition

5.11 Effect of biostimulant on photosynthetic gas exchange parameters

Abiotic stress substantially affects photosynthetic gas exchange parameters in rice, which directly translates into reductions in growth and productivity. In the present study, S treatment significantly hampered all major photosynthetic traits—net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), intercellular CO₂ concentration (Ci), and water use efficiency (WUE)—in both rice

varieties, with a notably more severe impact in the sensitive variety Manu ratna compared to the tolerant Hraswa. However, the application of biostimulant treatments (S+B and C+B) showed marked recovery and enhancement in photosynthetic performance, at different magnitudes across the two varieties.

Under nutrient stress condition, the decline in g_s was a prominent early response, with drastic reductions in var. Hraswa and Manu ratna, indicating a strong stomatal limitation. This is in accordance with previous findings, where nitrogen deficiency led to the reduction in g_s as a water-saving mechanism, consequently lowering P_n and C_i due to restricted CO_2 influx (Zhao et al. 2023). In the present study, the reduction in C_i under stress further supports the dominance of stomatal limitations over non-stomatal limitations.

Biostimulant application under stress (S+B) showed a significant ameliorative effect. Notably, in Manu ratna, g_s nearly doubled, resulting in improved gas exchange and internal CO_2 availability. This was paralleled by an improvement in P_n and an increase in WUE, suggesting that biostimulants effectively alleviated stomatal constraints and enhanced carbon assimilation. Similar recovery was observed in Hraswa, pointing to the inherent stress tolerance in the variety that responded moderately to biostimulant-mediated enhancement. Similarly, the transpiration rate (Tr) followed a trend consistent with g_s across treatments, with stress reducing Tr substantially and S+B recovering part of the loss. Notably, C+B treatment slightly decreased Tr in Manu ratna, suggesting improved water conservation under non-stress conditions, potentially through enhanced stomatal regulation.

The role of stomatal and non-stomatal factors in limiting photosynthesis under stress is well-established. Drought conditions often lead to stomatal closure, reducing CO_2 diffusion and hence lowering C_i and P_n (Arab et al. 2023). As per the results of this study, stress-induced decline in C_i was proportionate to the drop in g_s , affirming that stomatal limitations predominated in the observed photosynthetic reductions. Furthermore, increased C_i upon biostimulant application under stress suggests that enhancement of mesophyll conductance was involved in restoring

photosynthetic performance, a mechanism supported by previous silicon priming studies (Lahmaoui et al. 2025). Under drought stress, Si facilitates better gas exchange by enhancing mesophyll conductance, suggesting that Si priming aids in restoring photosynthetic performance through improved CO₂ diffusion and utilization.

Interestingly, under non-stress conditions (C+B), both varieties exhibited mild enhancements in all parameters, with Manu ratna showing a higher physiological responsiveness. An increase in g_s , WUE, and Pn in Manu ratna suggest that biostimulant treatment may optimize stomatal behavior and improve water-carbon assimilation efficiency even under optimal conditions. In contrast, Hraswa recorded only modest increases, indicating a ceiling effect due to its already efficient physiological baseline. The increased photosynthetic rate could be attributed to improved chloroplast activity or enhanced Rubisco function, possibly supported by better nutrient availability (such as Mg²⁺, Ca²⁺). The higher availability of these nutrients is discussed in later section on macro and micronutrient analysis. Overall, the gas exchange parameters emphasize that while abiotic stress severely reduces the photosynthetic activity, the application of biostimulants significantly alleviates these effects, particularly in the more stress-sensitive cultivar. This supports the concept of biostimulant application as an effective tool to mitigate the negative impact of stress on photosynthesis through improved stomatal regulation and CO₂ assimilation dynamics.

5.12 Effect of biostimulant on macro and micronutrient accumulation in leaves of booting stage

The elemental profiling done on leaves of rice varieties Hraswa and Manu ratna during the booting stage revealed distinct nutrient uptake patterns in response to stress and biostimulant treatments. Both macro- and micronutrients showed significant fluctuations under different treatments, with stress conditions reducing overall nutrient content, while biostimulant application largely mitigated these effects. The differential responses observed between the tolerant (Hraswa) and sensitive (Manu ratna) genotypes further highlight the role of genotype-specific

nutrient dynamics under abiotic stress. Stress (S) condition disrupts the homeostasis of macro, micro, and trace elements, while the application of C+B and S+B restored or improved element concentrations to a large extent. These findings are consistent with the research demonstrating that biostimulants directly or indirectly aid in rhizosphere interactions, membrane transport mechanisms, and root physiology to improve nutrient absorption and utilization efficiency (Cristofano et al. 2021; Staykov et al. 2025).

Potassium (K) exhibited treatment-dependent changes in var. Hraswa with higher recovery in S+B followed by C+B. Because of its essential function in osmotic adjustment, turgor maintenance, and stomatal regulation, potassium deficiency during stress is associated with decreased physiological function, and its replenishment is a sensitive marker of improvement by biostimulants. According to a number of mechanistic findings, biostimulants—in particular, microbial consortia and seaweed extracts—can increase K absorption by boosting transporter function and root surface area as well as by encouraging helpful rhizosphere microorganisms that mobilize K (Jacomassi et al. 2022). Magnesium (Mg) followed K trends, which is in line with biostimulant-mediated support of enzymatic cofactors and chlorophyll production. Applications of low-molecular-weight organic acids or seaweed extracts that promote Mg helps to maintain photosynthetic ability (Seregin and Kozhevnikova 2024). It stimulates cell division, facilitates the movement of phosphates and carbohydrates, stabilizes cell structures including membranes and ribosomes, and functions as a co-factor for enzymes involved in respiration and photosynthesis. Sufficient quantities of magnesium encourage strong root growth, enhance crop quality, and increase resistance to environmental stressors (Ishfaq et al. 2022). Calcium (Ca) responded most dramatically with highest accumulation in S+B followed by C+B in Hraswa. Ca act as a key secondary messenger in stress signalling and a stabilizer of cell walls and membranes. Higher Ca under S+B suggests that biostimulant treatment strengthens signal transduction and structural resilience at the booting stage — both vital during reproductive development. Reports show biostimulant treatments frequently increase Ca partitioning to shoots under suboptimal conditions, aligning with our observations (Baltazar et al. 2021).

In Manu ratna, the observed trends were directionally similar but less intense. While K, Mg, and Ca levels declined under stress by 15–20%, S+B treatment partially restored these nutrients, with improvements ranging from 20–50%. The relatively moderate response in Manu ratna compared to Hraswa suggests that stress-induced damage may be more difficult to reverse in sensitive genotypes, or that the efficiency of biostimulant uptake and translocation is inherently lower in these plants. This aligns with earlier reports indicating that stress hampers nutrient acquisition due to reduced transpiration and impaired root activity, while biostimulant use can restore physiological balance and improve nutrient transport (Rouphael et al. 2015; Di Mola et al. 2024).

Micronutrients accumulation was especially sensitive to stress and partially responsive to biostimulant application. In Hraswa, Mn rose by 30% (C+B) and 35% (S+B); Zn declined 30% under stress but recovered to 25% under S+B; Fe dropped 25% under S but increased 45% with S+B treatment. These recoveries are consistent with mechanisms where biostimulants increase micronutrient solubility (chelation), root uptake capacity, and phloem mobility — thereby reducing the photosynthetic penalties of micronutrient limitation. Some experimental studies indicate that both seaweed-based and microbial biostimulants often increase Fe, Zn and Mn availability and plant uptake under abiotic stress conditions (Abdel et al. 2020; Asif et al. 2023). Applying biostimulants during the end of tillering stage leads to interaction of biostimulants with soil particles and stimulating enzymes such as sucrase and protease, which increase the availability of essential micronutrients.

Trace and beneficial elements responded positively to biostimulants. In Hraswa, C+B increased Co, Ni, B, Mo and Se by 10–25%, while S+B produced even larger improvements over stress (Co +40%, Ni +30%, B +35%, Mo +30%, Se +40%). In Manu ratna, S+B increased these elements by 25–35% but absolute concentrations remained lower than in Hraswa. These trace element enhancements are physiologically meaningful because many of these elements act as cofactors in redox enzymes (Se), nitrogen metabolism (Mo), and cell wall/membrane integrity (B). The literature supports biostimulants' ability to enhance trace element

bioavailability via chelation, root exudation changes and stimulation of beneficial microbes that solubilize micronutrients (Cristofano et al. 2021).

Both varieties showed reductions in Al accumulation with biostimulant application under stress: Hraswa had a modest Al decline (15–20%) under S and S+B, while Manu ratna experienced a larger reduction (20–35%) under S+B. Aluminum toxicity negatively affects root elongation and nutrient uptake; therefore, lower tissue Al implies protective rhizosphere or internal detoxification responses. Mechanistically, Al exclusion is commonly achieved through enhanced secretion of low-molecular-weight organic acids (e.g., malate, citrate), improved chelation and stronger selective uptake of essential cations — processes that have been linked to biostimulant action and to shifts in root exudation and microbial composition. This highlights a potential protective mechanism offered by biostimulants—possibly through organic acid exudation, membrane stabilization, or Al chelation, thereby confer partial Al tolerance by promoting exclusion/detoxification pathways and limiting its phytotoxic effects (Ofoe et al. 2023).

5.13 Effect of biostimulant on yield traits and parameters

The positive effect of biostimulant application on yield was reflected in the increase of yield and yield components, which can be mainly attributed to the effect of biostimulant on the buildup of dry matter and enhanced translocation of photoassimilates from leaves (source) to reproductive structure. This plays a beneficial role in improving flower formation as well as enhanced accumulation of carbohydrate in the seeds. Earlier, Taha et al. (2020) indicated that the enhanced growth parameters, yield and yield attributes of *Lupinus termis* were observed when plants were treated with yeast extract.

Primed seeds gave an energetic head start and further crop establishment leading to earlier flowering (Yadav et al. 2023), which confirm with the findings of present study. Earlier and vigorous stand might reduce crop weed competition and increase absorption of water and nutrients by vigorous root system, and thereby produce increased number of branches, tillers/plant, pods/plant and seeds/ pod.

Biostimulant application enhanced the plant height under nutrient deficient condition due to enhanced rate of translocation of nutrients from culms to leaves enabling enhanced production of photosynthates, ensuring appropriate plant growth. Whereas under nutrient deficient condition, the reduction in plant height was due to lack of translocation of macro or micro nutrients to actively metabolizing regions. Moreover, the positive effects of dry yeast application, as a rich natural source of cytokinins can stimulate cell division and enlargement as well as the synthesis of proteins, nucleic acids and chlorophyll (Shalaby and Ramady 2014). According to Ebaid et al. (2025), the improvement of vegetative growth characters in response to the foliar application of active dry yeast may be attributed to its content of different nutrients, higher percentage of proteins, higher values of vitamins B which may play an important role in improving growth and controlling the incidence of fungi diseases. Also, Mannino et al. (2020) found that the positive effects caused by the addition of yeast suspension in improving shoots characteristics might be due to the development of the yeast and synthesis of wide groups of amino acids and vitamins. Likewise, the treatments of yeast suspension caused gradual but significant increase in plant height, number of aerial stem per plant, leaves area, total chlorophyll, and shoots dry matter percentage in potato.

Nutrient deficient condition during reproductive stage significantly reduces yield parameters in rice. Adverse environmental conditions such as N deficiency, drought, low solar radiation, low or high temperature, and blast disease can reduce panicle size and hence grain yield. A similar result of reduced reproductive development in rice plant was reported by Zhang et al. (2023). But the active response of yeast extract under nutrient deficient condition resulted in lesser reduction in the yield. The tolerant var. Hraswa responded in a faster and more active manner than the other, which allowed it to successfully minimize damage and sustain a relatively higher yield even in stressful conditions. Panicle size is influenced by nitrogen rate and also by genotypes. As current biostimulant used in the study is a combination of cysteine and ascorbate which maintains cellular redox balance and protect from oxidative stress, it helps to successfully overcome nutrient deficiency and the related physiological state. Increase in panicle density could be

due to the higher availability of nitrogen in yeast extract, which played a vital role in cell division.

Grain yield is due to successful spikelet filling. Spikelet filling or ripening stage in rice extends from flowering to physiological maturity and is also known as maturity growth stage. In this growth stage spikelet filling occurs, contributing towards the grain weight. Here the increase in grain weight was observed in biostimulant treatments (i.e., C+B and S+B) while stress treatment recorded reduction as compared to control. The dry weight of the caryopsis increases rapidly after flowering conditions. Some adverse environmental factors such as drought, low solar radiation, N deficiency, low or high temperatures, and panicle blast can increase spikelet sterility and consequently reduce grain yield. Spikelet sterility is also genetically controlled. When nutrients such as nitrogen, phosphorus, or potassium are limited, the photosynthetic features—particularly chlorophyll content, electron transport, and carbon fixation efficiency—is adversely affected. This leads to a decline in the production of assimilates and energy (ATP and NADPH), which are crucial for various metabolic pathways. As a result, physiological activities such as respiration, enzyme synthesis, and cellular division become less efficient, ultimately reducing biomass accumulation, delaying developmental processes, and causing a marked decrease in growth and yield (Chen et al. 2024).

Results of the present study revealed that significant increase occurred in unfilled grains under nutrient deficient condition in both varieties. It is apparent that yield components were reduced due to nutrient deficient condition compared to control. The greatest reduction was observed in the sensitive var. Manu ratna. The low grain yield could have occurred due to the direct effect of nutrient deficiency on carbohydrate accumulation. Nutrient deficiency stress has many features resembling that of water stress and the plant responses to both these stresses had similar characteristics (Muneer et al. 2024). Water stress affected wheat plants might have some reduction in very important physiological processes such as accumulation of osmolytes, antioxidant capacity, stomatal conductance and even in phytohormonal balance. Of all these situations, the yield components get reduced drastically due to

the imbalance in phytohormones. However, the same yield components could be increased when the level of hormones were significantly balanced.

Biostimulant application led to increase in the proportion of filled grains and reduced the count of unfilled grains, thereby enhancing the grain yield. Foliar spraying of wheat plants with protein hydrolysate based biostimulant significantly increased yield. And this was possible by the increase in the photosynthetic surfaces of plants that reflected positively in raising their yield components (Sun et al. 2024). The positive effect of yeast extract on yield and its components may be attributed to the fact that it contains cytokinins, vitamin B12 and minerals, which might play a role in translocation of metabolites from leaves into reproductive organs. Moreover, it may also play a role in enhanced synthesis of proteins and nucleic acids and at the same time minimize their degradation, which could lead to the improvement of yield in plants. These results are partially in agreement with the findings of humic acid based biostimulant application in rice by Izquierdo et al. (2024) and amino acid based biostimulant application in grape wine by Hallac and Kurt (2025).

Biostimulant application gave the highest mean value for grain weight for both varieties of rice. However, when both varieties were compared var. Hraswa had the highest significant mean value for grain weight compared to control. However, under nutrient deficient condition both varieties exhibited a decrease in grain weight and pronounced decrease was observed in var. Manu ratna. Similar results were recorded by Ebaid et al. (2025) and they support the fact that, the uses of dry yeast extract as a foliar spray leads to an increase of plant growth which causes significant increases in tuber weight per plant and total tuber yield of potato plant. A significant relationship between time to attain maturity, 100-seed weight, and seed yield were recorded which is consistent with the reported positive link between seedling vigor and total crop production (Mannino et al. 2020). Increase in grain weight and number of tubers could be due to the effect of plant hormones influencing grain weight or spikelet formation (Zhang et al. 2022). The microbial and non-microbial components promoted significantly better growth and supply of photosynthate enabling increased grain set even under stress conditions. Several studies have

convincingly demonstrated that the application of natural substances provides tolerance to various stress conditions and thus ensuring productivity.

Biostimulant application improved the yield parameters such as harvest index, spikelet fertility, seed setting and biological yield under nutrient deficient condition whereas these parameters were significantly decreased under nutrient deprived condition in both varieties of rice. Improved yield quality might be due to the yeast extract composition which is rich in protein, vitamin B12, antioxidants and minerals and other growth enhancing components (Abdel Lateef et al. 2019). Increase in biological yield was due to increased plant height from healthy and vigorous seedlings that happens due to improved crop growth rate and net assimilation rate (Rehman et al. 2024). Similarly increase in harvest index recorded in plants generated from treated seeds might be due to increased dry matter partitioning towards the growing seeds thereby increasing grain yield. Here the quality of the rice grain is determined by the key physiological processes such as microsporogenesis which occur at booting stage (Mohapatra et al. 2025). As yeast extract is a source of cytokinin which could delay the senescence related process and enhance photosynthesis, application of the same could make a great impact on the crop yield. Cytokinin deficiency resulted in diminished activity of the vegetative and floral shoot apical meristems and leaf primordia, indicating an absolute requirement for the hormone (Wu et al. 2021). And this deficiency could be successfully compensated by the cytokinin effect of the biostimulant.

Traits such as plant height, tiller proliferation, grain weight, and the number of filled grains are all influenced by stress. Here, a notable increase in yield and yield-related parameters was observed under S+B conditions, primarily attributed to the priming effect on the accumulation of dry matter and improved translocation of photosynthates from source to sink. This positively impacted flower formation and enhanced carbohydrate accumulation in the seeds. An early and robust stand can enhance water and nutrient absorption through a vigorous root system, resulting in increased branching, tillers per plant, pods per plant, and seeds per pod (Rehman et al. 2014).

Biostimulant application enhanced the plant height, panicle density, grain weight, number of filled grains under nutrient deficient condition due to enhanced rate of translocation of nutrients from culms to leaves enabling enhanced production of photosynthates, ensuring appropriate plant growth. Nutrient deficiency during the reproductive stage significantly reduces yield parameters such as 100-grain weight, grain size. Whereas, the application of biostimulants under nutrient-deficient conditions mitigated the decline in yield traits by increased activity of SuSy in reproductive stage, resulting in a comparatively lesser yield reduction and similar results were observed by Fan et al. (2019), wherein it was seen that SuSy enhances grain weight and size by modulating cell division and accumulation of starch in rice.

5.14 Effect of biostimulant on metabolomic profiling

Based on the various observations described alone, Manu ratna was selected for further in-depth metabolomic investigations, since it displayed greater sensitivity to nutrient deficiency but also exhibited marked improvement when treated with the biostimulant. Advanced analytical approaches including GC-MS/MS, HR-LCMS, and amino acids profiling were employed to characterize metabolic shifts induced by biostimulant application. These analyses revealed clear alterations in the abundance of primary metabolites, secondary compounds, and amino acid pools, underscoring the capacity of biostimulant treatment to reprogram central metabolic pathways under nutrient deficient conditions.

Metabolomic profiling of rice varieties under control, stress, and biostimulant treatments provided crucial insights into the biochemical reprogramming that accompanies stress adaptation and the ameliorative effects of biostimulants. Using complementary platforms (GC-MS/MS, HRLC-MS and amino acids profiling), a wide range of metabolites—including amino acids, organic acids, fatty acids, sugars, and secondary metabolites—were identified, revealing distinct signatures across treatments. The integration of multivariate analyses (PCA, PLS-DA, VIP scores, SAM) and pathway enrichment further highlighted treatment-specific metabolic regulation, underscoring the importance of biostimulants in restoring metabolic balance under treatments.

5.14.1 Total metabolic shifts and treatment-specific signatures

Nutrient stress treatment (S) caused a widespread downregulation of metabolites, with reductions noted across amino acids, organic acids, and sugars. This is at the metabolic cost of stress adaptation, where energy and resources are diverted to survival and defense, leading to suppressed primary metabolism. In contrast, biostimulant supplementation (C+B and S+B) significantly reversed these effects, with C+B and S+B treatments showing strong upregulation of metabolites related to energy metabolism, lipid remodeling, and antioxidant defense. Specifically, 164 metabolites were upregulated under S+B compared to only 32 in stress, highlighting the magnitude of stress alleviation under biostimulant influence.

Multivariate statistical analyses revealed clear group separation, with C+B and S+B forming distinct clusters away from C and S in PCA and PLS-DA plots. This indicates that biostimulants induced unique metabolic states, rather than simply reversing stress-induced declines. Similar findings have been reported in other crop systems where biostimulants modulated global metabolome signatures, driving a shift toward stress resilience and improved metabolic efficiency (Santaniello et al. 2017; Rouphael and Colla 2020a). Some key findings with respect to important metabolites are:

- Squalene, octadecanoic acid, oleic acid, hexadecanoic acid (methyl ester), and DL-arabinitol are positively correlated with PC1 and strongly associated with the S+B group, which appears in the same direction of the vectors. This suggests that, these metabolites are more abundant in S+B treated samples, potentially reflecting enhanced lipid metabolism, energy regulation, or stress-responsive biochemical pathways.
- Conversely, octadecanoic acid and 9-octadecenamide are aligned towards the C+B group, indicating their elevated levels under this treatment and suggesting treatment-specific fatty acid remodelling, which can cause membrane structure adjustments and this can be attributed solely to the biostimulant.

- The C and S treatments, found in the lower left quadrant, are located opposite to the main lipid-related metabolite vectors, implying a comparatively lower abundance of these compounds. This contrast could suggest downregulation of fatty acid biosynthesis or altered lipid turnover in these groups.
- Metabolites such as 4-hydroxy benzaldehyde and 9,12,15-octadecatrienoic acid methyl ester are also positioned in the direction of the S+B cluster, suggesting a possible link to oxidative signaling or aromatic compound metabolism in this treatment.

5.14.2 Lipid remodeling and stress amelioration

Several key metabolites identified by VIP analysis—including squalene, octadecanoic acid, oleic acid, and hexadecanoic acid—were significantly enriched in C+B and S+B groups. Lipid-related metabolites are central to membrane remodeling, stress signaling, and antioxidant functions (Henschel et al. 2024). Squalene, a triterpenoid precursor in sterol biosynthesis, has been associated with enhanced tolerance to oxidative stress due to its role as a radical scavenger. Similarly, the accumulation of saturated (stearic, palmitic) and unsaturated (oleic) fatty acids indicates shifts in membrane fluidity and lipid-derived signaling, which are crucial for maintaining membrane integrity and stress signaling under nutrient stress (Jacomassi et al. 2022; Zhang et al. 2025). Also, the differentiation between octadecanoic acid and palmitoleamide suggests non-redundant contributions of individual fatty acid derivatives as a result of treatment effects. The enrichment of lipid metabolism pathways in pathway analysis reinforces the role of biostimulants in stabilizing cellular membranes and promoting protective lipid turnover.

5.14.3 Amino acid metabolism and nitrogen reallocation

Nutrient stress (S) is characterized by elevated levels of stress-responsive amino acids such as arginine, phenylalanine, citrulline, and proline, consistent with their roles in osmoprotection, redox balance, and secondary metabolite precursors. However, S+B treatment induced a balanced recovery, with certain amino acids

(cysteine, glutamic acid, aspartic acid) showing restoration towards control levels, while others remained elevated to support stress adaptation. This suggests that biostimulants fine-tune amino acid metabolism to optimize nitrogen allocation between growth and defense.

Pathway enrichment analysis highlighted arginine biosynthesis, alanine-aspartate-glutamate metabolism, and glutathione metabolism as key pathways influenced by biostimulant application. The enhancement of arginine biosynthesis is particularly important, as arginine is a precursor of polyamines and nitric oxide, molecules central to stress signaling, while glutathione metabolism reflects enhanced antioxidant defense (Raj et al. 2021). Polyamines stabilize membranes, scavenge reactive oxygen species (ROS), and modulate ion channels, while NO acts as a signaling messenger regulating stomatal closure, antioxidant gene expression, and defense responses. Similarly, the activation of the alanine–aspartate–glutamate pathway reflects an upregulation of nitrogen metabolism and amino acid interconversion, which ensures the availability of carbon and nitrogen skeletons for biosynthetic processes during stress recovery. While enhanced glutathione metabolism further suggests an improvement in the plant's redox homeostasis and detoxification capacity. Glutathione (GSH), a tripeptide composed of glutamate, cysteine, and glycine, is one of the most crucial antioxidants that maintains the cellular redox balance by reducing H_2O_2 through the ascorbate–glutathione cycle (Raj et al. 2021). Increased GSH turnover and synthesis indicate the plant's strengthened antioxidant defense mechanism under biostimulant treatment. In addition, biostimulants are known to activate the phenylpropanoid pathway, leading to higher synthesis of anthocyanins, flavonoids, and phenolic acids—compounds that not only act as antioxidants but also contribute to structural integrity and stress resilience by protecting cells from oxidative and UV-induced damage. The upregulation of key enzymes such as chalcone synthase (CHS), phenylalanine ammonia-lyase (PAL), and cinnamate-4-hydroxylase (C4H) suggests enhanced secondary metabolism linked to defense. These findings align with previous reports where biostimulants promoted amino acid interconversion, polyamine biosynthesis,

and enhanced nitrogen use efficiency under stress (Ertani et al. 2017; Paul et al. 2019b).

5.14.4 Carbohydrate and energy metabolism

Carbohydrate metabolism was prominently enriched under biostimulant treatments. In C+B, starch and sucrose metabolism emerged as the most enriched pathway, reflecting enhanced carbon partitioning and energy storage. Under stress, carbohydrate metabolism was disrupted, but in S+B plants, starch and sucrose biosynthesis was restored, alongside modulation of TCA cycle intermediates (e.g., malic acid) and trehalose-6-phosphate. Trehalose signalling includes trehalose-6-phosphate which plays a key role in sugar sensing and stress tolerance, further supporting the protective role of biostimulants in carbohydrate reprogramming (Hassan et al. 2023). This suggests that biostimulants not only mitigate carbon depletion under stress but also activate signaling networks that prime plants for stress resilience.

5.14.5 Secondary metabolites and antioxidant pathways

Secondary metabolites such as flavonoids, catechins, and phenylpropanoid derivatives were enriched under S and S+B treatments, with a stronger accumulation under biostimulant supplementation. Pathway enrichment analysis confirmed that flavone/flavonol biosynthesis and phenylalanine-tyrosine-tryptophan pathways were significantly modulated, especially in S+B treatments. These pathways are linked to antioxidant activity, lignin biosynthesis, and stress defense. Elevated catechin levels, observed under stress and biostimulant treatments, corroborate enhanced ROS-scavenging capacity. These results are consistent with previous findings of other groups where biostimulants triggered secondary metabolite accumulation and improved antioxidant responses (Battacharyya et al. 2015; Bulgari et al. 2019).

5.14.6 Stress-specific metabolomic reprogramming under biostimulant treatment

Pathway enrichment uniquely highlighted butanoate metabolism and GABA shunt activation in S+B treatments. GABA, derived from glutamate, is increasingly

recognized as a signaling metabolite that regulates stress adaptation, carbon–nitrogen balance, and ROS detoxification (Rastegar and Sayyad 2025). Its induction under S+B indicates a targeted metabolic adjustment enhancing energy production through the TCA cycle, maintains cellular redox balance, and stabilizes metabolic homeostasis under stress conditions. The activation of the GABA shunt also facilitates rapid recycling of carbon and nitrogen intermediates, thereby sustaining metabolic flux and supporting continued growth and photosynthetic activity during stress exposure. Furthermore, enrichment of sphingolipid metabolism and one-carbon metabolism under S+B indicates that membrane stability and methylation-dependent regulation were strengthened, providing protective cover against stress damage. Taken together, the metabolomics results underscore the multifaceted role of biostimulants in modulating rice metabolism. They enhance energy metabolism, restore amino acid and nitrogen fluxes, strengthen antioxidant defense, promote lipid remodeling, and enrich protective secondary metabolites. The S+B treatment revealed that biostimulants do not merely restore control-like metabolism but actively reprogram metabolic networks towards a new, stress-resilient state. Such reprogramming involves both primary metabolism (sugars, amino acids, lipids) and secondary metabolism (flavonoids, terpenoids, phenylpropanoids), highlighting the holistic metabolic plasticity induced by biostimulants application.

5.15 Nutritional quality analysis of 1st generation seeds

Improving the amino acid content and elements of rice seeds is very important because rice is a major food for billions of people. The amino acids are important for human health, growth, and protein synthesis. By increasing amino acids in rice seeds, the nutritional quality of rice can be improved, helping to reduce malnutrition and provide a better-balanced diet for people who rely heavily on rice as their staple food. Traditionally, rice breeding focused mostly on increasing yield and stress tolerance, but now improving amino acids and other nutritional factors in seeds has become a key goal. Different approaches have been used to achieve this. Conventional breeding has produced rice varieties with higher amino acids and protein content, while modern molecular breeding and genetic engineering allow precise changes in genes that control amino acids. Agronomic methods, such as

using fertilizers, micronutrient sprays, and soil amendments, have also been applied to increase seed nutrients. Here in our study, biostimulants made of microbial and non-microbial components, have shown to bring along promising results in boosting proteins, amino acids, and other beneficial compounds in rice seeds. These approaches aim to produce rice that is not only high yielding but also more nutritious, helping to meet the dietary needs of the growing population.

5.15.1 Effect of biostimulant application on amino acid profiling of 1st generation seeds

To explore rice grain quality, first-generation seeds harvested from biostimulant-treated plants were analyzed for their amino acid composition and metabolic profile. The results revealed significant alterations in total and individual amino acid contents compared to seeds from untreated control plants. This observation indicates that the biochemical and physiological adjustments triggered by the biostimulant during the parental growth phase were not confined to those plants alone but were heritable to the progeny. Such inheritance may result from enhanced metabolic memory, or improved nutrient assimilation and storage processes established in the parent plants. The carry-over effects could enhance seed vigor, germination potential, and early seedling establishment, ultimately improving seed quality and stress adaptability in the next generation. This finding aligns with recent reports suggesting that biostimulant-induced metabolic reprogramming can exert long-lasting effects on plant physiology and yield traits across generations (Rouphael and Colla 2020b).

The amino acid profiling of first-generation rice seeds revealed a clear influence of both stress and biostimulant application on nutritional quality. Amino acids, being primary determinants of seed protein quality and metabolic health, provide an important indication of the physiological status of plants during grain filling and seed development (Amir et al. 2018). Stress reduced both amino acids and elemental composition of seeds, while biostimulant application significantly restored levels of essential amino acids (valine, leucine, isoleucine, phenylalanine) and central amino acids (glutamic acid, aspartic acid). This demonstrates a direct

role of biostimulants in improving not only plant stress resilience but also grain nutritional quality, thereby contributing to food security goals.

The C+B treatment demonstrated a pronounced enhancement in both essential and non-essential amino acids. Essential amino acids such as leucine, isoleucine, valine, and phenylalanine were elevated by 15–25%, while glutamic acid, aspartic acid, and alanine showed 20–30% increments. This enrichment points to improved nitrogen assimilation, amino acid biosynthesis, and carbon–nitrogen integration under biostimulant influence. Previous studies have also reported that biostimulants stimulate amino acid metabolism and protein synthesis, leading to amino acid accumulation of seeds (Yakhin et al. 2017; Rouphael and Colla 2020a). The observed increase in branched-chain amino acids further suggests a higher proteinogenic potential, directly contributing to enhanced seed nutritional quality.

In contrast, under stress conditions (S), a sharp reduction in amino acid pools was evident. Stress led to a 30–40% decline in glutamic and aspartic acids, amino acids central to nitrogen assimilation and having precursor roles in other metabolic pathways. Similarly, branched chain amino acids decreased by 25–35%, indicating impaired protein biosynthesis machinery. Aromatic amino acids (phenylalanine, tyrosine, tryptophan) also declined by 20–30%, suggesting disruption of the shikimate pathway and carbon–nitrogen flux under stress. Such reductions align with earlier findings where abiotic stress curtailed nitrogen metabolism, amino acid transport, and storage protein synthesis in seeds (Akhtar et al. 2024; Elbasan et al. 2025).

The S+B treatment clearly mitigated these stress-induced losses, with significant recovery in most amino acid classes. Glutamic and aspartic acids improved by 20–25% compared to stress, while branched chain amino acids showed a robust 30–35% recovery, nearly attaining control levels. Similarly, aromatic amino acids increased by 15–20%, reflecting a partial restoration of carbon–nitrogen balance. This recovery highlights the role of biostimulants in maintaining amino acid homeostasis under stress, likely through improved nitrogen uptake, stabilization of enzymatic activity, and modulation of stress-responsive metabolic pathways

(Calvo et al. 2014; Du Jardin 2015). Overall, these results indicate that biostimulant application enhanced amino acid biosynthesis in control conditions and significantly mitigated stress-induced reductions by restoring amino acid concentrations. This improvement in seed amino acid profile suggests an important role of biostimulants in promoting both nutritional quality and stress resilience in rice seeds.

5.15.2 Effect of biostimulant application on elemental profiling of 1st generation seeds

The elemental profiling of first-generation seeds revealed significant variation in both macro- and micronutrient accumulation across treatments, underscoring the dual influence of stress and biostimulant application on seed nutritional quality. Mineral nutrients are vital for seed development, functioning as cofactors for enzymes, structural components, and signaling molecules, and their differential accumulation directly reflects in the physiological state of the plant during grain filling.

Under C+B treatment, a consistent improvement in both macro- and micronutrient concentrations was observed relative to the control. Increases in K (12%), Mg (25%), and Ca (12%) highlight improved nutrient uptake and mobilization. These macronutrients are particularly important for enzyme activation, protein synthesis, and membrane stabilization (Tiwari et al. 2021). The marked enhancement in micronutrients—particularly Zn (33%) and Fe (16%)—suggests that biostimulant supplementation promoted efficient transport and deposition of essential minerals into developing seeds. Zn and Fe are crucial for seed nutritional value, given their roles in enzymatic activity, redox balance, and human dietary importance (Cakmak and Kutman 2018). The moderate increases in trace elements such as B, Ni, and Mo further point toward improved micronutrient assimilation, possibly mediated by biostimulant-induced stimulation of root activity and rhizosphere interactions (Bhupenchandra et al. 2022).

In contrast to the case of C+B, the S treatment drastically reduced mineral accumulation in seeds. K declined by nearly half (47%), Ca by 60%, and Mg by 32%. Such reductions are indicative of impaired nutrient uptake and xylem loading

under stress, as stress disrupts ion homeostasis and root membrane integrity (Hasanuzzaman et al. 2013). Micronutrient losses were also severe, with Zn, Mn, and Fe decreasing by 30–41%. These reductions compromise seed quality, as Zn and Fe deficiencies are directly linked to poor grain nutritional value and reduced stress tolerance (Welch and Graham 2004). The drastic decline in trace elements, particularly B (66%) and Mo (58%), underscores the vulnerability of secondary nutrient assimilation pathways under adverse conditions. These findings are consistent with previous reports that abiotic stress impairs mineral translocation, particularly into reproductive tissues (Kopecka et al. 2023).

Importantly, the S+B treatment demonstrated partial but significant recovery in nutrient accumulation compared to stress alone. K and Mg levels improved by 29% and 33%, respectively, while Ca recovered by 36%. Similarly Zn, Fe, and Mn exhibited recoveries in the range of 24–29%. Even trace elements such as B, Ni, and Mo showed notable recovery, with increases of 23–47% relative to seeds obtained from stressed plant. Although nutrient levels did not fully reach control values, the recovery trend highlights the protective and restorative effects of biostimulant supplementation under stress. Such improvements may be attributed to enhanced stabilization of membrane transporters, and activation of stress-protective signaling cascades induced by biostimulants (Bulgari et al. 2019; Rouphael and Colla 2020b).

Taken together, these findings demonstrate that while stress severely hampers nutrient deposition in seeds, biostimulants play a dual role: enhancing nutrient enrichment under optimal conditions and mitigating nutrient deficits under stress. This dual functionality is particularly significant for seed nutritional quality, as balanced accumulation of both macro- and micronutrients ensures improved physiological resilience of the seed and enhanced nutritional benefits for human consumption.

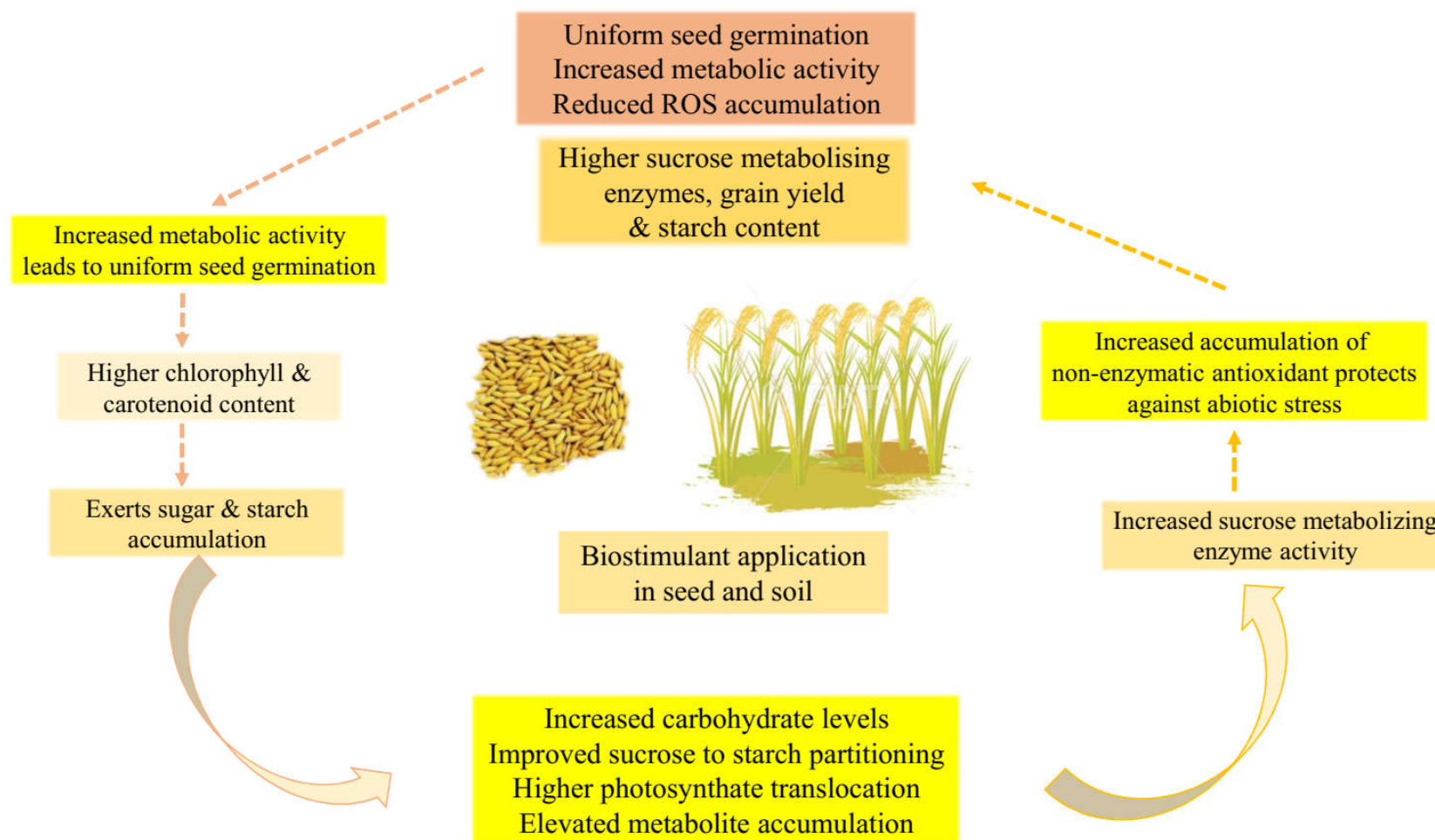


Fig. 73A: Mechanistic diagram representing the biostimulant action on rice plant

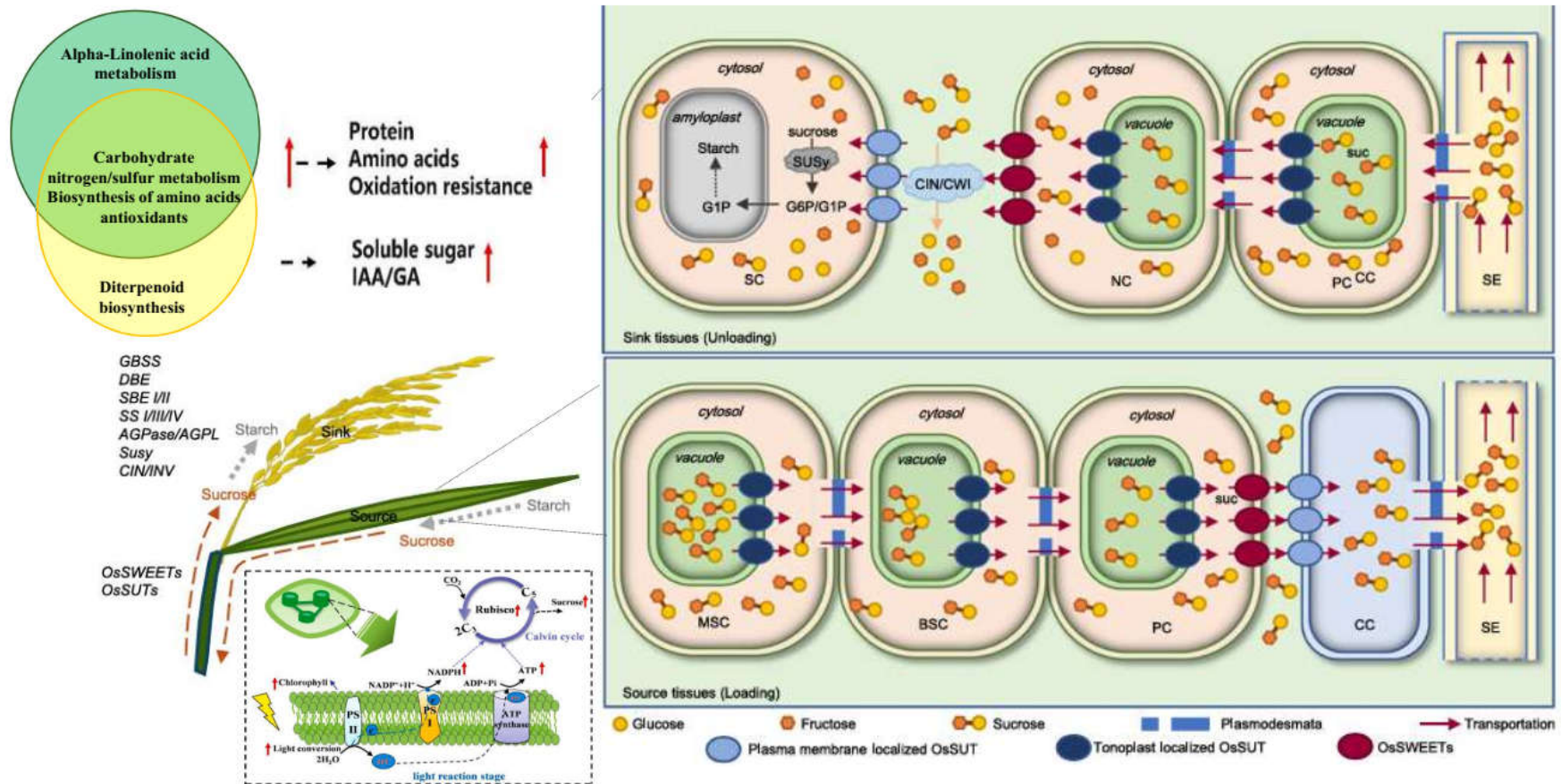


Fig. 73B: Mechanistic diagram representing the biostimulant action on rice plant

6. SUMMARY AND CONCLUSION

The present study is an attempt to enhance the nutrient deficiency tolerance potential of rice using a biostimulant formulation prepared from microbial and non-microbial components. Two rice varieties (Hraswa and Manu ratna) were selected for evaluation based on their performance in nutrient-deficient conditions in response to the application of different biostimulants. The experimental design was structured across four developmental stages—seedling, tillering, booting, and ripening—to capture dynamic changes in physiology and metabolism. At each stage, parameters such as carbohydrate translocation, metabolite allocation, reactive oxygen species (ROS) generation, antioxidant enzyme activity, and chlorophyll *a* fluorescence based physiological aspects were systematically assessed.

The stage-wise evaluation provided a comprehensive understanding of how biostimulant treatment modulated plant responses under stress at different stages of its lifecycle. Among the four stages, the booting phase emerged as particularly critical, showing pronounced changes in physiological performance and metabolic regulation. The booting stage marks a crucial shift from vegetative to reproductive growth in cereals like rice. The developmental sequences that ultimately determines spikelet fertility and yield potential occurs at this period, when the panicle primordia rapidly differentiate and elongate within the flag leaf sheath. Numerous studies have emphasized the physiological importance of this stage as plants are more vulnerable to abiotic stressors such as temperature extremes, water shortages, and nutritional deficiencies during the growth phase. Exposure to stress during booting can cause irreversible yield loss by interfering with pollen viability, assimilate partitioning, and floral organ development. Furthermore, the booting period marks the peak demand for mineral elements, especially potassium and nitrogen, which are essential for the transfer of carbohydrates and the synthesis of proteins. During this phase, lack of nutrients results in sterility and inadequate grain filling. Booting stage analyses may also be used as a diagnostic window to evaluate source-sink dynamics, translocation patterns, and nutrient absorption efficiency in both ideal and stressful conditions. Therefore, detailed analyses such as gas exchange measurements and

ICP-MS-based nutrient profiling were carried out at this stage. Subsequently, yield traits and FTIR spectroscopy were employed to examine grain development and functional group modifications of leaf metabolites subjected to biostimulant application revealed that among the both varieties studied, the var. Manu ratna appeared more sensitive to nutrient deficiency, however biostimulant treatment induced more structural and biochemical changes that improved its tolerance.

Based on the observations, Manu ratna was selected for further in-depth metabolomic investigations, since it displayed greater sensitivity to nutrient deficiency but also exhibited marked improvement when treated with the biostimulant. Advanced analytical approaches including GC-MS/MS, HR-LCMS, and amino acids profiling were employed to characterize metabolic shifts induced by biostimulant application. These analyses revealed clear alterations in the abundance of primary metabolites, secondary compounds, and amino acid pools, underscoring the capacity of biostimulant treatment to reprogram central metabolic pathways under nutrient deficient conditions.

Metabolomic profiling of rice under control, stress, and biostimulant treatments provided crucial insights into the biochemical reprogramming that accompanies stress adaptation and the ameliorative effects of biostimulants. Using complementary platforms (GC-MS/MS, HRLC-MS and amino acids profiling), a wide range of metabolites—including amino acids, organic acids, fatty acids, sugars, and secondary metabolites—were identified, revealing distinct signatures across treatments. The integration of multivariate analyses (PCA, PLS-DA, VIP scores, SAM) and pathway enrichment further highlighted treatment-specific metabolic regulation, underscoring the importance of biostimulants in restoring metabolic balance under treatments.

The major conclusions are summarized below:

1. Based on the characterization study of different biostimulant formulations, it was revealed that B1, PH and B showed optimal pH and electrical conductivity, ensuring better nutrient availability and stability of active compounds. The higher content of beneficial bioactive molecules, such as

amino acids, organic acids, and micronutrients, contributes to improved plant metabolism and growth. In addition, the presence of both microbial and non-microbial components in BI enhances nutrient assimilation and stress tolerance. These combined features make BI a more effective and balanced formulation compared to the others.

2. Among the different biostimulant formulations, BI consistently showed superior performance in enhancing seed germination, seedling growth, water uptake, seed vigor, and hydrolytic enzyme activities. The formulation not only improved α -amylase and protease activity but also increased soluble sugar content, contributing to better seed metabolism and early growth.
3. The comparative assessment of soil physiochemical properties among the four soil samples revealed significant variations in nearly all measured parameters. Sample 4 containing soil: sand (25:75) recorded a progressive decline in soil pH, electrical conductivity (EC), organic carbon, nitrogen content and secondary micronutrients suggests a substantial nutrient depletion pattern in soil fertility and thus taken as a nutrient deficient condition.
4. Based on the cumulative stress response index (CSRI), a clear distinction in response between the two rice varieties was observed under nutrient-deficient conditions. The var. Hraswa maintained stable growth and physiological performance with minimal reduction in shoot length, fresh weight, and dry weight, recording a higher CSRI 76,860 under stress. In contrast, Manu ratna exhibited a substantial decline in overall growth and physiological traits, with its CSRI 64499 under stress, indicating 60% growth retardation. Therefore, Hraswa was identified as the tolerant variety, demonstrating greater adaptability to nutrient deficiency, and Manu ratna as the sensitive variety due to its marked susceptibility under similar conditions.
5. Biostimulant priming at 25ppm for 12h in rice significantly improved various growth parameters like shoot length, root length, SVI, fresh weight and dry weight. Among both the varieties, tolerant var. Hraswa recorded

better improvement in growth parameters, followed by sensitive var. Manu ratna. The nutrient deficiency induced reduction in plant growth parameters was alleviated by biostimulant application through modification of metabolic processes.

6. The evaluation of biostimulant (200, 250, and 300 ppm) application in soil revealed significant effects on soil enzymatic activities and seedling vigor index (SVI). Among the tested concentrations, 250 ppm consistently exhibited the highest stimulatory effect on sucrase, protease, dehydrogenase, and urease activities, as well as on SVI values. At this concentration, enzyme activities increased by 40–95% over the control, indicating enhanced microbial activity and improved nutrient transformation processes in the soil. Conversely, higher concentrations (300 ppm) resulted in a mild decline in enzyme performance, suggesting potential inhibitory effects at elevated doses. Therefore, 250 ppm was identified as the optimal concentration for soil application, promoting maximum biochemical activity and seedling vigor.
7. The combined analysis of pigment composition, sucrose, and carbohydrate profiles indicates that nutrient deficiency negatively impacts photosynthetic efficiency and carbohydrate metabolism in rice. Both varieties exhibited reductions in chlorophyll, carotenoid, sucrose and starch contents under nutrient deficiency stress, reflecting impaired photosynthetic capacity and metabolic imbalance. However, biostimulant treatments (C+B and S+B) treatments markedly improved these parameters across developmental stages, particularly at the booting and ripening stages, which are critical for energy accumulation and grain filling.
8. Nutrient deficiency reduced leaf osmolality and disrupted osmotic balance in both rice varieties while, biostimulant treatments further enhanced these osmoprotectants, (proline, amino acids, and protein contents) supporting improved osmotic regulation and stress adaptation.

9. The sucrose metabolizing enzymes such as sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) showed enhanced activity indicating stimulation of carbohydrate metabolism and stress-adaptive enzyme systems. The concurrent increase in sucrose, total soluble sugars, reducing and non-reducing sugars, as well as starch accumulation, further demonstrate that biostimulant application enhanced carbon assimilation, translocation, and storage processes.
10. Nutrient deficiency interrupts the electron transport in chloroplast and resulted in higher ROS generation and damage to reaction centers which were recorded in Chl *a* fluorescence parameters. However, biostimulant application facilitates enhancement in the antioxidant system and thus helps to reduce the damage in the reaction center induced by ROS generated due to nutrient deficient condition highlighting improved oxidative stress management.
11. Plants subjected to nutrient deficient condition recorded higher reduction in chlorophyll *a* fluorescence parameter like F_v/F_o , PI_{ABS} , F_m , ϕE_o , and primary photochemistry of PSII as compared to biostimulant treated plants. The higher reduction in F_v/F_o in nutrient deficient condition indicated damage of the water-splitting complex on the donor side of PSII due to drought induced reduction in cellular water status and ROS generation. Increase in F_m value resulted in the lower efficiency of photosystems to convert light energy into chemical energy. Nutrient deficient condition-initiated ROS production and resulted in damage to photosystem proteins which lead to reduction in structural functional index (SFI). As a consequence of reduction in SFI, primary photochemistry and quantum electron yield also decrease. Reduction in F_v/F_o , F_m , ϕE_o and primary photochemistry of PSII parameters resulted in decrease of plant vitality decreased (PI_{ABS}). While these parameters were enhanced and improved by biostimulant treatment. Biostimulant treatment alleviates the donor side or acceptor side inhibition of PS II by reducing the accumulation of ROS.

12. The energy flux leaf model of Chl *a* fluorescence related parameters indicated that nutrient deficiency cause inactivation of RCs. The ABS/RC and TRo/RC decreased during stress condition and it might be due to the variation in antenna size and inefficiency of RC to utilize the trapped energy. The dissipation per reaction centre (Dio/RC) was increased in plants subjected to nutrient deficient condition and it indicates the incapability of RCs in trapping the light energy due to stress. This also led to a reduction in electron flow per RC (ETo/RC). Biostimulant treatments (C+B and S+B) effectively enhanced PSII performance by increasing energy absorption and trapping efficiency, improving electron transport, and reducing energy dissipation. The highest improvements were observed in the seedling and booting stages, indicating that biostimulants help to maintain photosynthetic efficiency and protect PSII under stress,
13. Biostimulant application significantly enhanced physiological performance of rice plants under both normal and stress conditions, reflected in improved photosynthetic efficiency and gas exchange parameters. Stress condition significantly reduced photosynthetic performance in both rice varieties, evidenced by decreased net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), intercellular CO₂ concentration (Ci), and water use efficiency (WUE). Biostimulant treatments (S+B and C+B) mitigated these effects by improving Pn, gs, Tr, Ci, and WUE, and a strong recovery was observed under S+B. The tolerant var. Hraswa showed moderate improvements, whereas the var. Manu ratna exhibited a prominent response, indicating that biostimulants enhance gas exchange and water use efficiency under stress, helping to maintain photosynthetic capacity.
14. The bioactive compounds and signaling molecules present in the biostimulant formulations likely activate redox-regulated defense pathways and delay senescence, maintaining chlorophyll integrity and photosynthetic efficiency during grain filling.

15. Biostimulant application enhanced the nutrient status of both soil and plants under control and stress conditions. In soil, biostimulants increased the levels of essential macronutrients (K, Ca, Mg) and micronutrients (Mn, Zn, B, Ni, Se, Mo) compared to control and nutrient-deficient treatments. In flag leaves, biostimulants improved macro- and micronutrient accumulation in both varieties, with Hraswa showing greater nutrient uptake than Manu Ratna. Stress condition reduced nutrient content, while S+B treatment restored and even enhanced the levels of essential elements, including beneficial trace elements, while partially limiting harmful Al accumulation. Overall, biostimulants positively influenced nutrient availability and uptake, helping plants to cope with stress and supporting better growth and metabolism.
16. Nutrient deficiency severely reduced agronomic traits and yield components in both rice varieties. Yield components improved significantly under biostimulant treatments, especially under stress. Biostimulant treatments (S+B and C+B) significantly improved plant height, panicle density, grain filling, grain weight, spikelet fertility, seed setting, biological yield, and harvest index in both varieties. The tolerant var. Hraswa consistently showed higher improvements, while sensitive var. Manu ratna exhibited larger relative yield responses, particularly under the S+B treatment, indicating a strong ameliorative and synergistic effect of biostimulants under stress.
17. Biostimulants reprogrammed the metabolome toward a growth-supportive and stress-mitigating profile, rather than purely stress-responsive accumulation. Metabolomics analysed by GC-MS/MS, HR-LCMS identified distinct enrichment of amino acids, amines, organic acids, sugars, sugar acids, sugar alcohols, fatty acids and secondary metabolites (flavonoids, phenolics, organic acids) in S+B treatments, linked to stress tolerance and signaling.
18. Nutrient stress caused a widespread downregulation of metabolites, with reductions noted across amino acids, organic acids, and sugars. This is at the metabolic cost of stress adaptation, where energy and resources are diverted

to survival and defense, leading to suppressed primary metabolism. In contrast, biostimulant supplementation (C+B and S+B) significantly reversed these effects, with C+B and S+B treatments showing strong upregulation of metabolites related to energy metabolism, lipid remodeling, and antioxidant defense.

19. In GC-MS/MS, a total of 305 metabolites were detected in all samples. Fold change analysis and volcano plot revealed the number of upregulated and downregulated metabolites in different treatments. In C+B treatment, 127 metabolites were significantly upregulated, 23 metabolites were significantly downregulated and 26 were found as insignificant metabolites as compared to control. While S treatment recorded 92 metabolites as significantly downregulated, 32 metabolites as significantly upregulated and 14 insignificant metabolites with respect to control. In S+B treatment, 164 metabolites were significantly upregulated, 27 metabolites were significantly downregulated and 13 were insignificant metabolites as compared to control.
20. Metabolites such as 9-octadecenamide, L-serine, catechin, L-cysteine, and DL-arabinitol were observed in S+B treatments suggesting altered nitrogen metabolism or oxidative stress response. Catechin, a polyphenolic compound with known antioxidant activity, was also elevated in S+B, reinforcing the possibility of an enhanced defense mechanism. Notably, trehalose-6-phosphate, a sugar signaling molecule, and malic acid, a TCA cycle intermediate, showed differential abundance across treatments, hinting at metabolic shifts in carbohydrate and energy pathways.
21. In HRLC-MS, a total of 1,410 metabolic features were detected and processed among which, 561 features were identified as statistically significant. In C+B treatment, 342 metabolites were significantly upregulated, 205 metabolites were significantly downregulated and 80 were found as insignificant metabolites. While S treatment recorded 232 metabolites as significantly upregulated, 307 metabolites as significantly

downregulated and 95 as insignificant metabolites. However, in S+B treatment, 293 metabolites were significantly upregulated, 186 metabolites were significantly downregulated and 112 insignificant metabolites were observed.

22. PCA and multivariate analyses clustered biostimulant treatments closer to control, showing that biostimulants effectively normalized stress responses. The stress treatments, found in the lower left quadrant, are located opposite to the main lipid-related metabolite vectors, implying a comparatively lower abundance of these compounds. This contrast could suggest downregulation of fatty acid biosynthesis or altered lipid turnover in these groups.
23. Squalene, octadecanoic acid, oleic acid, hexadecanoic acid (methyl ester), DL-arabinitol, benzaldehyde and 9,12,15-octadecatrienoic acid methyl ester are more abundant and positioned in the direction of the S+B cluster, potentially reflecting enhanced lipid metabolism, energy regulation, oxidative signaling and aromatic compound metabolism pathways. While, octadecanoic acid and 9-octadecenamide are aligned towards the C+B group, indicating fatty acid remodelling, which can cause membrane structure adjustments.
24. Pathway enrichment analysis revealed activation of stress-alleviating pathways, including antioxidant metabolism, amino acid biosynthesis, and energy metabolism in biostimulant-treated plants. Biostimulant application (C+B and S+B) profoundly modulates primary and secondary metabolism, enhancing plant growth, stress tolerance, and alleviating mechanisms. Under C+B, key pathways such as sphingolipid metabolism, amino acid metabolism (alanine, aspartate, glutamate), and purine metabolism were significantly enriched, reflecting enhanced membrane remodeling, nitrogen assimilation, nucleotide biosynthesis, and energy metabolism. Under S treatment, metabolic reprogramming prioritized survival, stress defense, and redox balance, with phenylalanine and sphingolipid metabolism, arginine biosynthesis, and energy-conserving pathways showing high significance.

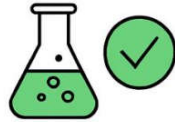
While S+B amplified these responses, promoting phenylpropanoid biosynthesis, nitrogen metabolism, amino acid interconversion, GABA production, folate cycling, glutathione metabolism, and membrane stabilization.

25. The amino acid profiling of first-generation seeds demonstrates that nutrient deficiency significantly reduces both essential and non-essential amino acid content, indicating impaired seed nutritional quality and also pointing towards the upregulation of stress-related amino acids (e.g., proline, glycine, serine) under stress. Nutritional quality of grains was enhanced, with higher protein, essential amino acids, and micronutrient content in S+B treatments. Biostimulant application (C+B) enhanced the accumulation of essential amino acids (leucine, isoleucine, valine, phenylalanine) and key non-essential amino acids (glutamic acid, aspartic acid, alanine), reflecting improved nitrogen metabolism and proteinogenic potential. While S+B treatment, effectively mitigated the adverse effects of nutrient deficiency, partially restoring amino acid levels toward or near control values.
26. The elemental composition of first-generation seeds reflects the strong impact of nutrient deficiency and the mitigating role of biostimulant application. Stress condition caused significant reductions in both macro (K, Mg, Ca) and micronutrients (Mn, Zn, Fe), as well as trace elements (B, Ni, Mo), indicating impaired nutrient uptake and deposition in seeds. In contrast, biostimulant application (C+B) enhanced nutrient accumulation across most elements, demonstrating its positive role in promoting nutrient enrichment and seed quality. In S+B, the biostimulant partially restored nutrient concentrations, with marked improvements in macro, micro, and trace elements relative to stressed seeds. This indicates that biostimulants can effectively alleviate stress-induced nutrient deficiencies, supporting better seed nutritional status and potentially improving seed vigor and overall plant performance.

27. Integration of metabolomics and physiological data confirmed that biostimulants act as metabolic modulators, rather than only providing nutrient supplementation. Physiological resilience, metabolic adjustments, and improved nutrient-use efficiency on biostimulant treatment contributed to sustainable yield advantage under stress.
28. Hraswa exhibited stronger physiological adaptability, while Manu ratna showed higher metabolic plasticity and this reflects that biostimulants influenced these two varieties differently.
29. Stress without biostimulant caused severe reductions in yield and nutrient content, reinforcing the necessity of biostimulant intervention. The combined data provide strong evidence that biostimulants are effective tools for improving rice performance under nutrient deficient and stress-prone environments.
30. Biostimulants reprogram physiological, biochemical, and metabolic networks in rice, enhancing stress resilience, nutrient efficiency, and yield quality—positioning them as sustainable alternatives for climate-smart agriculture.

SUMMARY OF THE WORK

Characterization of Biostimulant Formulations

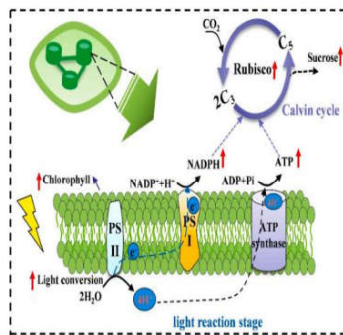


- Optimal pH, size and electrical conductivity
- Presence of beneficial bioactive molecules such as organic acids, amino acids and micronutrients

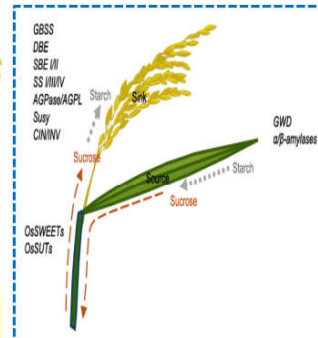
Plant Growth and Metabolism



- Superior performance in seed germination, seedling growth, water uptake, seedling vigor & hydrolytic enzyme activities



Higher yield & Nutritional quality



Soil and Plant Physiological Responses



- Enhanced soil enzymatic activities
- Improved photosynthetic efficiency
- Gas exchange parameters
- Tolerance to nutrient deficiency

Metabolomic Analysis



- Reprogramming of metabolic networks
- Enhanced stress resilience by promoting phenylpropanoid biosynthesis, nitrogen metabolism & amino acid interconversion

RECOMMENDATIONS

1. **Facilitating farmer training:** on the effective integration of biostimulants with sustainable agricultural practices.
2. **Multi-omics integration to decipher mode of action:** Future studies include integrated transcriptomic, metabolomic, and proteomic analyses to unravel the precise molecular targets of biostimulants.
3. **Microbiome biostimulant interactions:** Investigating how biostimulants influence rhizosphere microbiome composition and function under nutrient-deficient conditions would provide insights into plant–microbe–biostimulant interactions.
4. **Field scale validation across agro-climatic zones:** Greenhouse results must be validated through multi-location field trials across diverse soil types and climatic regions.
5. **Mechanistic studies on hormonal crosstalk:** Biostimulants often modulate endogenous hormones. Detailed research on crosstalk between auxin, cytokinin, ABA, ethylene, and SA pathways could clarify how biostimulants enhances stress tolerance.

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LIST OF PATENT, PUBLICATIONS AND PRESENTATIONS

Granted patent-1

Patent No. : 497761

Granted by : Indian patent office

Application No. : 202341030285 A

Date of Filing : 27/04/2023

Date of Grant : 11/01/2024

Name of Inventor(s) : 1. PUTHUR, Jos
2. JOHNSON, Riya

Articles -16

Sl No.	Articles	Authors	Journals	Impact factor
1.	Ascorbate, as a versatile regulator of plant development: practical implications for enhancing crop productivity, quality, and postharvest storage.	Johnson, R., Joel, J. M., Anjitha, K. S., Tóth, S. Z., & Puthur, J. T. (2024).	<i>Horticultural Plant Journal.</i>	6.2
2.	Biostimulants Enhance Photosynthate Partitioning, Translocation and Stress Tolerance in Rice.	Johnson, R., Joy M. Joel, and Jos T. Puthur. (2025)	<i>Journal of Soil Science and Plant Nutrition</i> 1-19.	3.6
3.	Biostimulant priming in <i>Oryza sativa</i> : A novel approach to reprogram the functional biology under nutrient-deficient soil.	Johnson, R., & Puthur, J. T. (2022).	<i>Cereal Research Communications</i> , 50 (1), 45-52.	1.9

4.	Biostimulants: The futuristic sustainable approach for alleviating crop productivity and abiotic stress tolerance.	Johnson, R., Joel, J. M., & Puthur, J. T. (2024).	<i>Journal of Plant Growth Regulation</i> , 43(3), 659-674.	4.9
5.	Seed priming as a cost effective technique for developing plants with cross tolerance to salinity stress.	Johnson, R., & Puthur, J. T. (2021).	<i>Plant physiology and biochemistry</i> 162, 247-257.	5.7
6.	Integrative application of AMF and CaO nanoparticles promote AMF symbiosis, Modulate Redox and Photosynthetic Pathways to Alleviate NaCl Stress Effects in Rice.	Joel, J. M., Johnson, R., & Puthur, J. T. (2026).	<i>Environmental Science: Nano</i>	5.1
7.	Potassium in plants: Growth regulation, signalling, and environmental stress tolerance.	Johnson, R., Vishwakarma, K., Hossen, M. S., Puthur, J. T., & Hasanuzzaman, M. (2022).	<i>Plant Physiology and Biochemistry</i> , 172, 56-69.	5.7
8.	Co-application of arbuscular mycorrhizal fungi and engineered nanomaterials: a promising strategy for crop resilience against abiotic stresses.	Joel, J. M., Johnson, R., & Puthur, J. T. (2023).	<i>South African Journal of Botany</i> , 162, 314-323.	2.7
9.	Biopriming with a native microbial consortium favourably modulates the growth dynamics and yield of <i>Amaranthus tricolor</i> and <i>Oryza sativa</i>	Raj, R., Johnson, R., Joel, J. M., Nair, S. G., Cherian, E., Job, J., & Puthur, J. T. (2024).	<i>Journal of Plant Growth Regulation</i> , 1-14.	4.9
10.	Modulations in chlorophyll a fluorescence based on intensity and spectral	Janeeshma, E., Johnson, R., Amritha, M. S., Noble, L., &	<i>International Journal of Molecular Sciences</i> , 23(10), 5599.	4.9

	variations of light.	Puthur, J. T. (2022).		
11.	Paradoxical effects of nanomaterials on plants: Phytohormonal perspective exposes hidden risks amidst potential benefits.	Faseela, P., Joel, J. M., Johnson, R. , Janeeshma, E., Sameena, P. P., Sen, A., & Puthur, J. T. (2024).	<i>Plant Physiology and Biochemistry</i> , 210, 108603.	5.7
12.	Effect of cultivation practices on productivity and nutrient dynamics of wheat varieties under northern hill zone of India	Saini, A., Johnson, R Sahoo, C., Singh, A., ... & Fayeizadeh, M. R. (2025).	<i>BMC Plant Biology</i> , 25(1), 497.	5.4
13.	Assessing the effect of soil cultivation methods and genotypes on crop yield components, yield and soil properties in wheat (<i>Triticum aestivum</i> L.) and Rice (<i>Oryza sativa</i> L.) cropping system.	Saini, A., Manuja, S., Upadhyay, R. G., Manhas, S., Johnson, R. , Sahoo, C., Singh, G., ... & Fayeizadeh, M. R. (2024).	<i>BMC Plant Biology</i> , 24(1), 349	5.4
14.	Assessing the impact of tillage practices and nutrient levels on the growth and productivity of Ethiopian mustard (<i>Brassica carinata</i> L.)-soybean (<i>Glycine max</i> (L.) Merr.) cropping system.	Manhas, S., Johnson, R ..,Saini, A., Kumawat, R. (2024).	<i>BMC Plant Biology</i> , 24(1), 1059.	5.4
14.	Metal/metalloid-based nanomaterials for plant abiotic stress tolerance: an overview of the mechanisms.	Sarraf, M., Johnson, R. , ... & Hasanuzzaman, M. (2022).	<i>Plants</i> , 11(3), 316.	4.5
15.	Modulation of plant photosynthetic processes during metal and metalloid stress, and strategies for	Shomali, A., Johnson, R. , Janeeshma, E., Kumar, V., ... & Hasanuzzaman,	<i>Plant Physiology and Biochemistry</i> , 206, 108211.	5.7

	manipulating photosynthesis-related traits.	M. (2024).		
16.	Revelations on photosystem II, thermoluminescence, and artificial photosynthesis: a retrospective of Govindjee from fundamentals to applications.	Veena, M., Sarath, N., Johnson, R & Puthur, J. T. (2023).	<i>Physiology and Molecular Biology of Plants</i> , 29(9), 1225-1238.	3.9

Book chapters -10

1. **Johnson, R.**, Joel, J. M., & Puthur, J. T. (2025). Bacterial/fungal inoculant application as biostimulants toward sustainable agricultural system. In *Microbial Inoculants* (pp. 61-71). Academic Press.
2. **Johnson, R.**, Joel, J. M., Anjitha, K. S., Noble, L., Faseela, P., & Puthur, J. T. (2025). GABA-Mediated Salt Stress Tolerance Through Physiological and Molecular Mechanisms. *GABA in Plants: Biosynthesis, Plant Development, and Food Security*, 287-303.
3. **Johnson, R.**, Joel, J. M., & Puthur, J. T. (2025). Effect of biostimulants on yield and fruit quality of different crops. In *Biostimulants for Improving Reproductive Growth and Crop Yield* (pp. 289-304). Academic Press.
4. **Johnson, R.**, Joel, J. M., Janeeshma, E., & Puthur, J. T. (2024). Biostimulants mediated imprints on seed physiology in crop plants. In *Biostimulants in Plant Protection and Performance* (pp. 221-236). Elsevier.
5. Sen, A., **Johnson, R.**, & Puthur, J. T. (2021). Seed priming: a cost-effective strategy to impart abiotic stress tolerance. In *Plant performance under environmental stress: hormones, biostimulants and sustainable plant growth management* (pp. 459-480). Cham: Springer International Publishing.
6. Joel, J. M., **Johnson, R.**, & Puthur, J. T. (2025). Rhizomicrobiome interactions: Fundamentals and recent advances. *Rhizomicrobiome in Sustainable Agriculture and Environment*, 93-106.
7. Joel, M. J., **Johnson, R.**, Janeeshma, E., Mirshad, P. P., & Puthur, J. T. (2023). Myconanotechnology: a sustainable means for multiple applications in environment protection. In *Myconanotechnology and Application of Nanoparticles in Biology* (pp. 119-137). Academic Press.

8. Janeeshma, E., Joel, J. M., Shackira, A. M., **Johnson, R.**, & Dhanya, T. T. (2023). Role of Arbuscular Mycorrhizal Fungi in the Alleviation of Heavy Metal Stress in Rice. In *Arbuscular Mycorrhizal Fungi* (pp. 83-90). CRC Press.
9. Shackira, A. M., Sarath, N. G., Veena, M., **Johnson, R.**, Jeyaraj, S., Raj, K. A., & Puthur, J. T. (2023). Resistance Identification and Implementation: Genomics-Assisted Use of Genetic Resources for Breeding against Abiotic Stress. In *Cereal Crops* (pp. 141-156). CRC Press.
10. Janeeshma, E., Sen, A., Aswathi, K. R., **Johnson, R.**, Dhankher, O. P., & Puthur, J. T. (2022). Reclamation and Phytoremediation of heavy metal contaminated land. In *Bioenergy crops* (pp. 187-203). CRC Press.

Awards & Paper Presentations

- Awarded with 2nd best oral paper presentation with paper presentation entitled “**Sustainable rice growth and yield: The influence of biostimulant (YCA) on key physiological traits**” at International Phytotechnology Conference organized by International Phytotechnology Society and University of Calicut on Oct 22-25, 2024 at Kerala.
- Paper presentation entitled “**Biostimulant Mediated Enhanced Photosynthesis in Rice During Tillering and Booting Stages Under Nutrient-Deficit Soil**” at the 3rd International Conference on Agriculture, Biosystems and Technology themed “AGRICULTURE FORESIGHT” Harnessing Innovative Digital for Sustainable and Resilient Agri-Food, Forest, and Bio-based Systems” on December 11-12, 2023 at Tarlac Agricultural University, Malacampa, Camiling, Tarlac.
- Paper presentation entitled “**Boosted photosynthetic machinery via biostimulant amid nutrient scarce soil in rice seedlings**” in DBT-SERB sponsored national conference on Advancements in Plant Sciences on January 05, 2024 at St. Berchman’s College.
- Paper presentation entitled “**Biostimulant application improves the antioxidant and photosynthetic machinery at seedling stage of rice exposed to long term nutrient deficient soil condition**” in University of Hyderabad on January 12, 2023 at Hyderabad.
- Paper presentation entitled “**A Biostimulant formulation for crop growth and uses thereof**” in Agri tech Show on August 02, 2024 at C-CAMP, Bangalore.