GREEN BIOTECHNOLOGY FOR FUTURE AGRICULTURE

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Nodar Sulashvili

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TABLE OF CONTENTS

PREFACE

In the face of rapidly growing global food demand and the escalating impacts of climate change, agriculture must evolve towards new and sustainable directions. Green biotechnology represents a transformative pathway—offering smart solutions to increase productivity while preserving soil health, biodiversity, and ecological balance. By prioritizing innovation and responsibility, it enables us to rethink traditional agricultural approaches and prepare for the challenges of the future.

This book brings together leading researchers and diverse scientific contributions from different parts of the world, all united by a common goal: to explore biotechnological tools that can make agriculture more efficient, resilient, and sustainable. Each chapter presents both theoretical insights and practical applications—from biofertilisers and rice biofortification to advanced extraction techniques—demonstrating how science can directly contribute to real-world agricultural solutions.

We hope that this volume will serve not only as an academic resource but also as an inspiration for policymakers, researchers, students, and practitioners working in the field of agricultural sciences. By promoting collaboration between disciplines and encouraging innovation, we believe that green biotechnology can play a decisive role in shaping the future of agriculture and securing food sustainability for the next generations.

Editorial Team
November 21, 2025
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CHAPTER 1
SCREENING PHOSPHATE SOLUBILIZING RHIZOBACTERIA (PSB) FROM UMYU AGRICULTURAL SOILS, KATSINA, NIGERIA FOR POTENTIAL APPLICATION AS PHOSPHATE-BASED BIOFERTILISERS (PBB)

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INTRODUCTION

Phosphorus is an essential nutrient for plant growth, and its availability in soil is often limited due to its insolubility. Phosphate-solubilizing bacteria (PSB) play a vital role in making phosphorus more accessible to plants by converting insoluble phosphates into soluble forms (Sharma & Fraga, 2015). The ability of PSB to solubilize phosphate is attributed to their production of organic acids, chelating agents, and enzymes that help in the dissolution of sparingly soluble phosphates (Vargas et al., 2013).

PSB have been found to belong to various genera including Bacillus, Pseudomonas, Enterobacter, and Rhizobium, among others (Rajput et al., 2013). Their presence in the soil is beneficial for plant growth and development due to their ability to release phosphorus from organic and inorganic sources (Rajput et al., 2013). This, in turn, can lead to a reduced requirement for chemical fertilizers, which can have detrimental effects on soil health and the environment (Liang et al., 2006). As a result, PSB have garnered significant attention due to their potential application in sustainable agricultural practices and environmental remediation (Richardson et al., 2009).

Phosphate solubilization by bacteria occurs through various mechanisms, including the production of organic acids, chelation, acidification, and the release of phosphatase enzymes. These mechanisms serve to dissolve insoluble mineral phosphates and make the phosphorus available for plant uptake (Rajput et al., 2013).

This research was aimed at isolating and screening phosphate solubilizing bacteria (PSB) from Umaru Musa Yar'adua University botanical garden.

1. MATERIALS AND METHODS

This study was conducted in Umaru Musa Yar'adua botanical garden located in Batagarawa, Katsina, Nigeria. Batagarawa is a Local Government Area in Katsina State, Northern Nigeria, located on latitude (7034'30E, 7035'0E) and longitude (12053'30N, 12054'0N). Katsina is located some 280 km away from Sokoto and 135 kilometers (84 mi) northwest of Kano, close to the border with Niger. In 2016, Batagarawa's estimated population was 429,000.

The city is a centre of agriculture producing groundnuts, cotton, hides, millet and guinea corn and also has mills for producing peanut oil and steel. It is also an educational hub, being the location of many tertiary institutions of learning. Batagarawa is predominantly populated by Fulani and Hausa ethnic groups.

Root nodules of three (3) different Vigna unguiculata and soil samples were collected from the garden using a hand trowel, at a depth of ten centimeters (10cm). The soil samples were scooped, transferred into a clean and sterile polythene bag and transported to the Microbiology Laboratory, Umaru Musa Yar'adua University, Katsina for analysis. The nodules were washed in running tap water, separated and surface sterilized in 0.01% HgCl for 5 minutes followed by washing in sterilized distilled water, before dipped in 70% ethyl alcohol for 3 minutes. They were then washed in slow running tap water, and then crushed with a sterile glass rod in a small aliquot of sterile water (Lubna et al., 2018).

pH Analysis of the Soil

Soil pH was analyzed by measuring 15g of the soil samples followed by the addition of 30 mL of deionized water. The soil was swirled to homogenize, the meter was inserted and the displayed result was recorded (Lubna et al., 2018).

Serial Dilution of the Soil Samples

One gram (1g) of the soil sample was measured and dispensed into a test tube containing 9 mL of fresh sterile distilled water to make the 10-1 dilution. From this dilution, 1 mL was taken and further added to 9 mL of sterile distilled water in a separate test tube. This process continued until the (10-12) dilution was achieved (Fentahun et al., 2013). Each dilution was properly mixed using a vortex mixer to ensure even distribution of the microbial cells. All glassware and pipettes used in the process were sterilized to avoid contamination. The prepared serial dilutions were then used for subsequent microbial isolation and enumeration.

Isolation of Rhizobia from Botanic Garden Soil and Root Nodules

One (1) mL from the 10-11 and 10-12 dilutions of the samples was inoculated on to the prepared plate count agar using pour plate technique, agitated to ensure the absolute homogeneity of the media, allowed to solidify and incubated at 30 0C for 24 hours (Fentahun et al., 2013). The colonies were then transferred onto the Congo Red Yeast Extract Mannitol agar (YEMA) medium enriched with Bromothymol Blue (BTB) which could detect the production of an acid growth reaction with yellow colonies (Kucuk et al., 2013).

Total Rhizospheric Count

Colonies from the incubated plate count agar (PCA) were counted and recorded as:

$$\text{No. of cells/mL} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of inoculum}}$$

Morphological Characteristics

The morphological traits, comprising: mucous production, colony morphology, pH change of the medium during growth of the isolates and growth rate were evaluated. Mucous production analysis was done based on the type, consistency and appearance; while colony morphology parameters were based on diameter, form, elevation and optics (Ahmad et al., 2013).

Growth Promoting Activities

- **Production of Indole Acetic Acid (IAA):** IAA production in all the bacterial isolates suspected to be PGPRs strains was detected according to Ahmed et al. (2013).
- **Siderophore Production:** Siderophore production was detected according to the method of Silini et al. (2012)
- **Phosphate Solubilization** :Phosphate solubilization was detected using the protocol of Mehta et al. (2015).
- **Production of Ammonia :** Ammonia production was determined using the protocol of Salman (2013).

Stress Tolerance Assays

Salt tolerance test was conducted using different concentration of NaCl in nutrient broth (1.0%, 2.0%, 4.0%, 6.0%, 8.0% and 10.0%). The isolates were inoculated in the media containing the concentrations and incubated at 28±2 0C for 5 days. One (1) milliter of each of the concentrations were inoculated into a fresh nutrient broth and incubated for 24 hours. The colonies were counted and reported as:

$$\text{No. of cells/mL} = \frac{\text{No. colonies} \times \text{Salt concentrations}}{\text{Volume of inoculum}}$$

pH Tolerance

pH tolerance test was conducted using Luria Bertani broth medium adjusted to alkaline and acidic conditions using NaOH and HCl, at pH of 5, 7 and 9, respectively. Rhizobial isolates were inoculated into test tubes containing the medium at these pH values followed by incubation for 5 days. The growth was recorded using a spectrophotometer (wavelength of 600nm).

Temperature Tolerance Test

Fresh isolates were inoculated into nutrient broth at different temperatures of 200C-500C followed by incubation at 28 ± 20C for 5 days. Growth was monitored by observing the turbidity in each of the tubes. Later, 1 mL of the broth was transferred into fresh nutrient agar medium and incubated at 28±20C for 24 hours. The number colonies per milliliter was counted and recorded (Fentahun et al., 2013).

2. RESULTS AND DISCUSSION

The results of the number of bacteria from each of the soil samples showed varying counts: rhizospheric counts ranged from M1 (129.0 ± 4.24 ×10-11 CFU/mL to 26.5 ±3.54× 10-11 CFU/mL) to M2 (37.5±4.95 ×10-12 - 13.5±6.36 × 10-12 CFU/mL), respectively, as shown in table 1. Statistically there is a significant difference between the colony count of the different samples (P = 0.00013, P= 0.05, F crit= 9.276). Similarly, nodulation count also ranged from 36 to 97 from plants 1 and 3, respectively, as presented in Table 1.

Table 1. Soil pH and Nodulation Count Obtained from the Root

Soil/Plants Samples	pH	Nodulation Count per Cluster
S1/P1	6.96±0.2	36
S2/P2	6.90±0.12	44
S3/P3	7.20±0.2	97

The results of the pH tolerance assay were reported in Table 2, and they show little to no changes in the optical density. Statistical analysis shows that there is no significant difference between the isolates (P-value = 0.288, F crit =3.190, P=0.05, S1-S3 =Soil samples; P1-P3= Plant samples).

Table 2. The pH Tolerance Test of the Selected Rhizobium Isolates

Isolates ID	pH 5				pH 7				pH 9			
	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
M3	0.347	0.361	0.779	0.963	0.273	0.591	0.897	1.290	0.201	0.524	0.811	1.040
M5	0.311	0.530	0.682	0.788	0.212	0.731	0.953	1.327	0.184	0.396	0.551	0.686
M9	0.344	0.418	0.793	1.277	0.351	0.487	0.823	1.172	0.221	0.328	0.774	1.000
M11	0.398	0.582	0.881	1.038	0.229	0.518	0.769	1.276	0.258	0.417	0.931	1.263
M18	0.379	0.760	0.940	1.139	0.279	0.421	0.683	0.856	0.229	0.368	0.996	1.356
Control	0.349	0.353	0.368	0.381	0.204	0.211	0.236	0.271	0.113	0.131	0.144	0.156

The salt tolerance test results showed that the number of bacterial colonies recovered from each concentration ranged from 128-281 CFU/g obtained from M18 and M5, 89-194 CFU/g in M18 and M9, 72-112 CFU/g in M18 and M9, 52-73 CFU/g in M18 and M11, 21-57 CFU/g in M3 and M5 and finally 9-19 CFU/g in M5 and M9, in the 1%, 2%, 4%, 6%, 8% and 10% concentrations, respectively. Lastly, the control had 203-354 CFU/g, respectively, as presented in Table 2.

Table 3. Salt Tolerance by Rhizobia Isolated from UMYU Botanical Garden

ISOLATES ID	1%	2%	4%	6%	8%	10%	Controls
M3	226	142	82	64	21	13	294
M5	281	156	98	59	57	09	342
M9	246	194	112	67	24	0	347
M11	214	114	109	73	49	16	286
M18	128	89	72	52	34	14	229

From the temperature tolerance test conducted over the range of 100C, 200C, 300C, 400C and 500C, it can be seen that there is a differences in temperature tolerance for the isolates, as seen in Table 3. Isolate M13 has the highest temperature tolerance when compared to M23, with counts of 93±2.0 CFU/mL and 26±1.0, at 200C. Meanwhile, M15 had a count of 202.5 ± 0.5 CFU/mL and M21 had 89±2.0 CFU/mL, at 300C. Further, at 400C, M3, with 114±1.0 CFU/mL and 28±1.5 CFU/mL had the highest count. Finally, M8 had a range of 53±0.5 cfu/mL and in M2, no any visible colony was obtained. Statistically, there is a significant difference in terms of the temperature tolerance exhibited by the isolates. This variation indicates that different isolates possess distinct adaptive mechanisms to temperature stress. The ability of M13 and M15 to survive higher temperatures suggests potential thermotolerance, which may enhance their field performance under fluctuating environmental conditions. Conversely, the poor growth of M2 implies sensitivity to temperature changes. These differences could be linked to the physiological and enzymatic composition of each isolate. Overall, the findings highlight the importance of temperature as a critical factor influencing microbial survival and phosphate solubilization efficiency. These results emphasize the need to select isolates with high temperature tolerance for applications in diverse agro-climatic regions. Further studies on the molecular and genetic basis of thermotolerance could provide insights into their adaptive strategies. Such knowledge may contribute to developing more resilient microbial inoculants for sustainable agriculture.

Table 4. Temperature Tolerance Test

Isolates ID	20^0C (CFU/mL)	30^0C (CFU/mL)	40^0C (CFU/mL)	50^0C (CFU/mL)
M1	49±1.5	156±1.5	58±2.5	04±1.5
M2	28±0.5	201±2.0	91±1.0	00±0.0
M3	71.5±3.0	187±0.5	114±1.0	41±1.0
M4	52±1.0	119.5±1.5	62±0.0	23±1.0
M5	26±1.0	156±2.0	98±2.0	11±2.0
M6	61.5±1.0	196.51.0	79.5±3.0	48±2.5
M7	84±1.5	173.5±1.0	41±3.5	20±0.0
M8	55±0.0	129±0.0	76±2.5	53±0.5
M9	49.52.5	194±2.5	112±2.0	21±0.5
M10	26±0.0	148.5±1.5	69±2.0	56±0.0
M11	25±1.0	114±2.0	95±1.0	42±1.5
M12	63±1.0	173.5±2.0	91.5±1.0	21.5±2.5
M13	72.5±3.5	118±2.0	71±2.0	34.5±2.0
M14	93±2.0	126±0.0	49.50.0	43±0.0
M15	68±0.0	202.5±0.5	67±0.5	18±0.5
M16	19.5±1.5	94±0.5	67±0.0	44±1.0
M17	46±1.0	105±1.5	55±0.5	31±1.5
M18	33±0.5	149±1.0	28±1.5	23±1.0
M19	68±0.5	182±1.0	39±0.0	19±1.0
M20	41±1.0	93±1.5	61±0.5	32.5±2.5
M21	27±0.0	89±2.0	33±4.0	28±0.5
M22	59±0.0	137±0.5	49±1.0	33±0.0
M23	26±1.0	91±1.5	39±1.5	19±0.5
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Result of ammonia, siderophore and indole acetic acid productions were presented in Table 4. Isolates M3, M5, M6, M9 and M13 produced ammonia, siderophore and indole acetic acid. However, the rest of the isolates were positive to either one or two of the tests. This indicates that these isolates possess multiple plant growth-promoting traits. The combined production of these metabolites enhances nutrient availability and supports plant root development. Such multifunctional isolates could therefore serve as promising candidates for biofertilizer formulation.

Table 5. Ammonia and Siderophore Production by Plant Growth Promoting Rhizobium (PGPR)

Sample ID	Siderophore production	Ammonia (NH_3) Production	IAA Production
M1	+	-	-
M2	-	-	-
M3	+	+	+
M4	-	-	-
M5	+	+	+
M6	+	+	+
M7	-	-	-
M8	-	-	-
M9	+	+	+
M10	-	-	-
M11	+	+	+
M12	+	+	+
M13	-	-	+
M14	+	+	-
M15	-	+	-
M16	-	-	+
M17	-	-	-
M18	+	+	+
M19	-	-	+
M20	-	-	+
M21	+	-	-
M22	+	-	-
M23	+	-	+
Controls	-	-	-

The results of the phosphate solubilization tests from the isolates showed that the average zone of inhibition produced by the isolates, as shown in Figure 1, is highly variable ranging from 8.75 mm to 18 mm. (Key = + = Presence of yellow to brownish colour in Ammonia -= Absence of pink color in Ammonia, Presence of flourescent pigment indicates siderophore production)

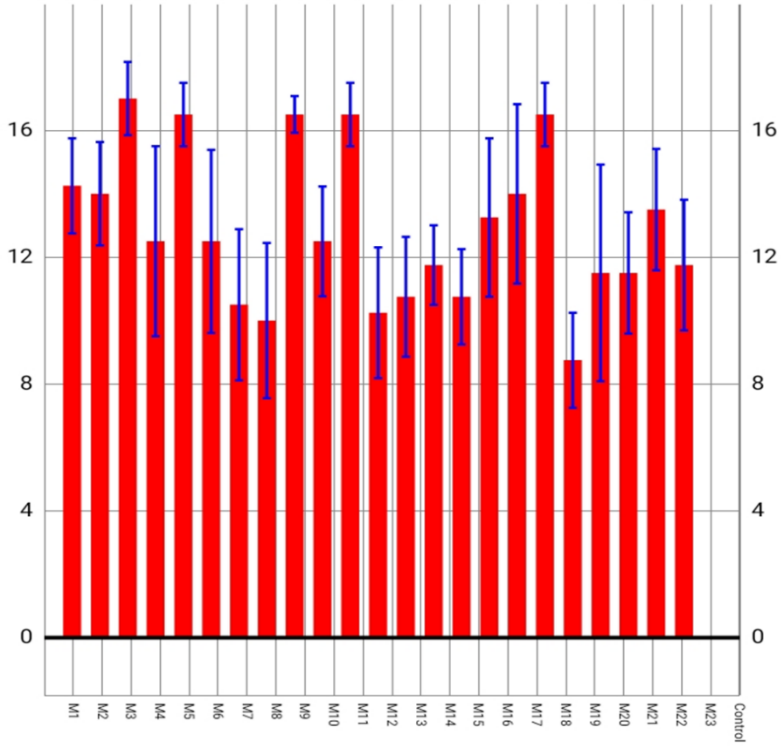

Figure 1. Mean value of phosphate solubilization by PSB

The isolates in this study were preliminarily identified based on their ability to absorb Congo Red on CRYEMA, their morphology on YEMA, and the colour formation on YEMA supplemented with Bromothymol Blue. These preliminary inferences indicated that the PSB in this research were suspected to be Bacillus spp., R. leguminosaurum, K. pneumoniae and Pseudomonas spp. as shown in Table 5.

Specifically, the best isolates in the study, namely: M3, M5, M6, M9, M11, M12, M14, M18 and M23, were specifically identified as R. leguminosaurum, Pseudomonas spp., R. leguminosaurum, K. pneumoniae, K. pneumoniae, Pseudomonas spp., K. pneumoniae, Bacillus spp., and K. pneumoniae. Therefore, from the foregoing, most of the isolates were K. pneumoniae (4), followed by Pseudomonas spp. and R. leguminosaurum, with two isolates each. Bacillus spp. appeared only once.

Table 6. Characteristic Features of Rhizobium Isolates on Different Media

ID	CRYEMA	YEMA	YEMA + BTB	Inference
M1	Absorbed Congo red	Mucoid colonies that are white	Blue but later turns into yellow	*Bacillus* spp.
M2	Absorbed Congo red	Mucoid colonies that are white	Yellow colonies that are round	*Bacillus* spp.
M3	No congo red	Small surface-adherent colonies	Blue colonies	*R.leguminosarum*
M4	Absorbed Congo red	Mucoid colonies that are white	Yellow colonies	*K.pneumoniae*
M5	Chalky Congo red colonies.	Mucoid colonies that are white	Pink chalky colonies	*Pseudomonas* spp.
M6	No congo red	Colorless mucoid	Blue colonies	*R.leguminosarum*
M7		Colorless mucoid colonies		*Pseudomonas* spp.
M8	Absorbed Congo red	milky mucoid colonies	Blue colonies	*R.leguminosarum*
M9	Absorbed Congo red	Colorless mucoid colonies	Yellow colonies	*K.pneumoniae*
S10	Absorbed Congo red	Milky colonies that are elevated.	Yellow colonies	*K.pneumoniae*
M11	Absorbed Congo red	Mucoid colonies that are milky	Yellow colonies	*K.pneumoniae*
M12	No congo red absorption	Colorless mucoid colonies	Blue colonies	*Pseudomonas* spp.
M13	-	-	Yellow colonies	*K.pneumoniae*
M14	Absorbed Congo red	Mucoid colonies that are white	Yellow colonies	*K.pneumoniae*
M15	Absorbed Congo red	Milky colonies that are elevated.	Blue colonies	*R.leguminosarum*
M16	No congo red	Colorless mucoid	Blue colonies	*R.leguminosarum*
M17	Absorbed Congo red	Mucoid colonies that are white	Yellow colonies	*K.pneumoniae*
M18	No congo red	Colorless mucoid	Blue colonies	*Bacillus* spp.
M19	Absorbed congo	Colorless mucoid	Yellow colonies	*K.pneumoniae*
M20	Absorbed congo	Colorless mucoid	Yellow colonies	*K.pneumoniae*
M21	Absorbed congo red	Milky colonies that is raised	Yellow colonies	*K.pneumoniae*
M22	Chalky Congo red	Small milky colonies	Yellow colonies	*K.pneumoniae*
M23	Absorbed congo	Colorless mucoid	Yellow colonies	*K.pneumoniae*

3. DISCUSSION

The study was aimed at isolating and screening the Phosphate Solubilizing Rhizobacteria for their potential as phosphate solubilizing bacteria from Umaru Musa Yar'adua University botanic garden soil. The total rhizospheric bacteria refer to the population of microorganisms that occupy the soil surrounding the roots of plants. This microbial community plays a vital role in plant growth and health. Studies have reported a wide range of total rhizospheric bacteria, from as low as 25 CFU/g to as high as 129 CFU/g of bacteria, and these values are linked to the nodulation counts of the plants (Glick, 2012).

One of the important mechanisms through which PSB act as biofertilisers is linked to soil pH. Soil pH plays a crucial role in plant growth and development. Most plant nutrients are available at a pH range of 6-7. However, soil pH can be acidic, alkaline or neutral depending on various factors like soil type, climate, and agricultural practices. During this this research, optical density results showed that the bacteria thrive in different pH levels, in line with the optimum conditions for bacterial proliferation. This is concomitant with the submission of Kavamura et al. (2013), who reported that Proteus mirabilis could produce organic acids that can solubilize phosphorus under acidic conditions. Furthermore, Proteus mirabilis can secrete urease, which can hydrolyze urea and release ammonia that increases the soil pH. Sun et al. (2020) also reported that Proteus mirabilis could produce indole-3-acetic acid, which can promote plant growth under acidic conditions. Therefore, the survival of the isolates in this study at varying pH indicates their ability to release nutrients in the soil even beyond the normal pH for the release of soil nutrients. These findings suggest that the isolates could be effective in diverse soil environments with fluctuating pH levels. Their adaptability may enhance nutrient availability and support sustainable crop production. Future research could focus on field trials to confirm their performance under real agricultural conditions.

Rhizospheric bacteria are known to promote plant growth by fixing nitrogen, solubilizing phosphorus, and producing plant growth-promoting hormones (Glick, 2012).

The population of rhizospheric bacteria is influenced by several factors, including soil type, plant species, and agricultural practices. From this study presence of this rhizospheric which also is in line with the recommended level was attributed to the agricultural activities taken place in the study area and also in line with the one conducted on the rhizosphere of maize plants reported a total bacterial count of 25-48 CFU/g soil (Kumar et al., 2014). Another study on rhizospheric soil of legumes reported a higher total bacterial count of 81-129 CFU/g soil (Ahmad et al., 2016).

Plant-growth promoting rhizobacteria (PGPR) can produce ammonia in the soil through nitrogen fixation or by the activity of different soil microbes that can produce ammonia, which is essential for plant growth and reproduction (Singh et al., 2016). From the results of this study, it was shown that the isolates have the potential of ammonia production, and this is an indicator of nitrogen fixation, as reported in a study of Singh et al. (2016), Zhang et al. (2018), Tariq et al. (2019) and Glick et al. (2021); who investigated the effect of PGPR ammonia production on wheat and rice (Oryza sativa), maize (Zea mays) and tomato (Solanum lycopersicum).

Siderophores are low molecular compounds produced by micro-organisms, including PGPR to chelate iron from the surrounding environment. They play significant roles in nutrition, growth and protection of the plants from biotic and abiotic stress (Kumar et al., 2018). Production of siderophores from some of the screened isolates corresponds with the study of Sindhu (2009) who observed that Pseudomonas aeruginosa, Citrobacter freundii and Proteus mirabilis produce siderophores called pyoverdines, aerobactin and enterobactin. Pyoverdines have been shown to play important roles in virulence, biofilm formation, and survival of P. aeruginosa in various ecologies. Aerobactin synthesis is regulated by the Fur protein, which senses the intracellular levels of iron and enterobactin is synthesized by a multi-enzymatic pathway and is regulated by multiple factors, including iron concentration, temperature, and pH. This is similar to the findings of Shukla et al. (2009), and Saxena et al. (2020).

Indole acetic acid (IAA) is one of the most important plant growth regulators involved in various physiological and developmental processes. Bacteria are known to be capable of producing IAA.

During this research, some of these bacteria were observed to exhibit positive IAA production, indicated by the development of pink color on Salkowski's reagent, as similarly reported by Sindhu (2009), who reported that P. aeruginosa showed maximum IAA production under nitrogen-deficient conditions. Similarly, Shukla et al. (2009) reported that C. freundii exhibited maximum IAA production under phosphate-deficient conditions. P. aeruginosa produces IAA through tryptophan-dependent and tryptophan-independent pathways, while C. freundii and P.mirabilis synthesize IAA via the indole-3-pyruvic acid (IPyA) pathway (Saxena et al., 2020).

The use of Plant Growth-Promoting Rhizobacteria (PGPR) has gained immense popularity due to their potential to induce plant growth and alleviate the negative effects of different environmental stresses on plants. In the present study, the potential of PGPR was evaluated at different temperatures, ranging from 100C, 200C, 300C, 400C, and 500C, with C. freundii, Proteus mirabilis and Pseudomonas aeruginosa as selected strains for temperature stress optimization. The results agreed with what was obtained in the study of Raza et al. (2020), who reported that C. freundii improved the growth and stress tolerance of cucumber plants under high-temperature stress. The study attributed the positive effects of C. freundii to the production of various secondary metabolites and modulation of the plant's antioxidant system. Similarly, Proteus mirabilis has been reported to alleviate the negative effects of salinity stress on wheat plants. A study conducted by Tu et al. (2020) reported that Proteus mirabilis enhanced the growth, photosynthesis, and antioxidant activity of wheat plants under salinity stress by regulating the endogenous hormonal balance and increasing the plant's antioxidant capacity. In another similar study, Kim et al. (2018) evaluated the effects of Pseudomonas aeruginosa on the growth and yield of tomato plants under high-temperature stress and reported that P. aeruginosa significantly improved plant growth, yield, and fruit quality compared to the control group. These findings collectively highlight the versatile role of PGPR in enhancing plant resilience under diverse abiotic stresses. The selection of appropriate PGPR strains can therefore be crucial for developing sustainable agricultural practices.

CONCLUSION

Phosphate-solubilizing bacteria (PSB) represent a valuable resource in sustainable agriculture and environmental remediation. Their ability to solubilize insoluble phosphate compounds and promote plant growth makes them promising candidates for biofertilizer development and application. This study reported the presence of nine isolates namely: M3, M5, M6, M9, M11, M12, M14, M18 and M23, preliminary identified as R. leguminosaurum, Pseudomonas spp., R. leguminosaurum, K. pneumoniae, K. pneumoniae, Pseudomonas spp., K. pneumoniae, Bacillus spp., and K. pneumoniae; as stress tolerant PSB with potential of being used as PBB in sustainable agriculture.

Extensive research on the mechanisms of phosphate solubilization by PSB and their beneficial effects on plant nutrition and soil remediation is necessary to highlight their potential for practical implementation in agricultural systems.

Further studies and field trials are necessary to fully elucidate the efficacy of PSB inoculants across different agro-ecosystems and environmental conditions.

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CHAPTER 2
IMPROVEMENT OF A MODERN RICE VARIETY FOR HIGH-YIELDING UNDER WATER DEFICIT CONDITION THROUGH MORPHOLOGICAL MARKER BACKCROSS SELECTION

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INTRODUCTION

Rice is a domesticated, annual, self-pollinated, semi-aquatic, monocotyledonous tropical C3 grass with low-radiant habitat (Harushima, et al., 1998). In Asia, the people get 30-80% of the daily calories from rice in the form of breakfast cereals, noodles and alcoholic beverages (Narciso and Hossain, 2002). Different parts of the world grow the rice crop in a wide range of conditions. Therefore, the impact of drought on rice production is connected with the relationship between variable rainfall and drought. There is a significant impact of drought stress on global rice production in rain-fed low and upland regions. This is because the supply of water is decreasing, and the intensity of the changing climate condition is increasing. Drought is the most imperative limiting factor of rice growth and its production amongst many abiotic factors in rainfed ecosystems (Nelson, et al., 2014, Pandey and Shukla, 2015). Shamsudin, et al., (2016) is reported the annual yield loss of 13–35% by drought stress in nearly 42 mha of rice grown under rainfed lowlands and uplands. In world's total rice cultivated area, impact of drought stress is calculated to 33% (9.9 × 107 hm2), 25% (6.0 × 107 hm2) and 42% (12.6 × 107 hm2) in countries belong to developing, developed and under developed level, respectively (Rijsberman, 2006). In Asia alone, about 34 million hectares of rainfed lowland and 8 million hectares of upland rice-cultivating areas experience drought stress (Singh et al., 2016). In India, 68% of the total agricultural land is rainfed and of these, approximately 40 Mha rice-cultivating areas relying on rainfall are prone to drought stress (Agricultural Statistics at a Glance, 2015).

It is also important to note that seasonal drought stress in rain-fed regions poses a significant risk to rice agriculture from the stage of seed germination to the flowering stage. There has been a recent worrying decline in the amount of water resources available around the world in several developing countries. Rice experts have been making consistent efforts over the past two decades to identify new sources of rice that are able to withstand drought. This is something that should be kept in mind. Last but not least, a successful breeding approach known as selection for grain yield under drought stress condition has been put into practice (Kumar, et al. 2014; Dixit, et al. 2014b).

In addition, numerous significant effective QTLs have been recorded all over the world for this characteristic (Bernier, et al., 2007; Venuprasad, et al., 2009; Vikram, et al., 2011; Ghimire et al., 2012; Mishra, et al., 2013; Palanog, et al., 2014; Sandhu, et al., 2010; Dixit, et al., 2014b). Through the utilisation of these quantitative trait loci (QTLs), numerous high-yielding rice cultivars have been enhanced for their ability to withstand drought during the reproductive stage all over the world (Arvind Kumar et al., 2014; Vikram, et al., 2011; Venuprasad, et al., 2009). Very recent study also, new rice genotypes are reported as drought stress tolerant donors for climate-resilient rice development (Chakraborty, et al., 2023). In Tamil Nadu state of India, rice agriculture is extensively practised in the Cauvery delta region. However, there is a significant loss of yield that is caused by drought stress as a result of the early withdrawal of the monsoon or protracted dry phases during the reproductive period. One of the supporting documents discussed the decrease in the total amount of rainfall that occurred in this region between the years 1974 and 2014. In 2016, this region received a total of only 168 millimetres, which is much less than the annual average of 1000 millimetres. Therefore, the only option to effectively control drought in agriculture and fortify agricultural production is through the genetic modification of crops to increase their resistance to drought. In this connection, several traits at morphological, physiological and molecular level such as plant height, panicle number, root volume, fertile spikelets, plant biomass, leaf area development, root/shoot ratio, grain yield, chlorophyll, starch, soluble sugar and proline contents have been used for drought screening in previous studies (Maisura, et al., 2014). Moreover, the morphological marker is a cheaper and easy way to identify prominent characteristics of a specific crop plant. Recently, Ghorbanzadeh, et al., (2023) have reported several metabolic markers in shallow-rooting, drought-susceptible genotype, IR64 and a drought-tolerant and deep-rooting genotype, Azucena. Hence, keeping above considerations in mind a modern popular short-term rice variety, ADT36 which is widely cultivated in Cauvery delta region was improved for drought tolerance at flowering stage through incorporation of DTY1.1 quantitative trait locus (QTL) by backcrossing on plant height (PH) character as morphological marker.

1. MATERIALS AND METHODS

A small quantity of rice seeds of ADT36 from Tamilnadu Rice Research Institute (TRRI), Aduthurai, Tamilnadu state and CR Dhan 801 from National Rice Research Institute (NRRI), Cuttack, Odisha state were sourced. In this study, rice varieties, ADT36 as female parent (recipient) for improving drought tolerance at reproductive stage and CR Dhan 801 harboring qDTY1.1 as male parent (Donor) were used.

1.1 Isolation of Genomic DNA and PCR Amplification

Genomic DNA was isolated from fresh leaf of ADT36 and CR Dhan 801 parental line using CTAB method with minor modifications (Murray, et al 1980) and the quality of DNA was checked on 0.8% agarose gel using horizontal gel electrophorosis unit. About 200 mg of leaf samples were cut into small pieces with the help of sterile scissors and transferred to sterile mortar. Leaf tissues were ground and extracted with 800 µl of CTAB buffer and incubated at 65°C in water bath with occasional mixing for 30 minutes. The tubes were removed from the water bath and 400 µl of chloroform : Isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversion for 15 minutes. It was centrifuged at 10,000 rpm for 20 minutes at room temperature. The clear aqueous phase was transferred to a new sterile eppendorf tube. A 400 µl of ice-cold isopropanol was added and mixed gently by inversion and then they were kept in the freezer until DNA was precipitated out. After incubation, it was centrifuged at 10,000 rpm for 15 minutes at 4°C to pellet out the DNA. DNA pellet was washed with 70% ethanol and air dried at room temperature completely. Then the DNA pellet was dissolved in 100 µl of sterile water (depends on the pellet size) and stored at -20°C. Isolated genomic DNA was quantified using Nanodrop spectrophotometer (ND-1000) Spectrophotometer, M/s. NanoDrop Technologies, USA). About 1 µl of DNA sample was kept in the Nanodrop spectrophotometer and the absorbance was read at 260 nm. Amount of DNA in the samples was calculated by using the standard reading of 1 OD at 260 nm is equivalent to a DNA concentration of 50 ng/µl.

Genomic DNA was diluted to a final concentration of 25 ng/µl and stored at -20°C for further use. Assessment of DNA quality was carried out by resolving the genomic DNA through 0.8% agarose gel electrophoresis and visualized under UV light in the gel documentation system (M/s. Syngene Ltd., UK). PCR was done with the volume of 10µl containing 20-30ng template DNA, 5 pmol of each primer (Forward- 5'-tcctgcgaactgaagagttg-3' and reverse-5'-agagcaaaaccctggttcac-3') of SSR marker, RM431linked with qDTY1.1 Vikram, et al. (2011) 0.05 mM dNTPs, 10x PCR buffer (10mM Tris, pH 8.4, 50 mM KCl and 1.8 mM MgCl2) and 0.5U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bengaluru, India.

1.2 Phenotypic Selection

In this study, big plastic pots filled with rice field soil mixed with cow dung were used in this study to identify positive plants based on plant height (PH) at vegetative stage (Vikram, et al., 2011), in Net house, Kandaswami Kandar's College (11.1202ºN, 78.0031°E), Velur, Namakkal (District), Tamil Nadu, India. For screening of BC1, BC2 and BC3 progenies along with their both parental lines (ADT36 and CR Dhan 801), BC progenies and both parental lines were raised in plastic cups separately for 21 days and then, they were transplanted to big plastic pots. Seedlings were allowed for 30 days to establish and then, they were grown under moisture condition. Each pot consists of 10 rice progenies along with their both parental lines. For control, parental lines were grown under flooding. Plant height of the BC progenies was measured from the ground level to the top of each plant at booting stage and taller plant than RP were identified for backcrossing. For studying the seed setting percentage (SS%) of BC2F3 rice lines at reproductive stage under aerobic condition at field level, one seed per hill from each rice line was planted in soil of the plot and the plot soil was maintained with moisture up to harvesting. Seed sowing was done in plot with 10 x 15 cm gap. At the time of harvesting, plant height (PH) and seed setting percentage (SS%) were recorded. Scoring for SS% was done according to IRRI's scale (2002) (Scale 1-More than 80%; 3-61-80%; 5-41-60%; 7-11-40%; 9-Less than 11%).

1.3 Development of Near Isogenic Lines (Nıls) Of ADT36 Rice Variety

Hybridization was done between ADT36 and CR Dhan 801 in Nethouse, Kandaswami Kandar's College, Velur, Namakkal (District), Tamil Nadu, during Kharif season-2019. F1 seeds derived from ADT36/CR dhan 801 cross did backcross using ADT36 rice variety as recurrent parent (RP) to generate BC1F1 seeds during Kharif - 2020. Progenies were selected from BC1F1 population based on plant height under moisture condition. The progenies with increased plant height over the recipient parent were selected as positive plant. Thus selected positive plants were backcrossed with RP to generate BC2F1 seeds during Kharif – 2021. BC2F1 progenies were screened for selecting tall progenies and selected progenies were used to backcross with RP to produce BC3F1 seeds during Kharif - 2022. From BC3F1 population, positive progenies were selected through genotypic selection using foreground marker, RM431 linked with qDTY1.1 during Kharif – 2023 and genotypically selected positive plants were allowed for self-pollination to produce BC3F2 seeds. During Rabi-2023-24, near isogenic lines of ADT36 rice variety were evaluated along with RP parent in greenhouse under moisture condition. In experimental plots, 21 days old seedlings of parental lines and NIL population were transplanted at 5.0 cm standing water. Seedlings were planted with 10 x 15 cm distance in plot of 2.0 m2 area which was nourished with cow dung and green manure. In plots, only one seedling per hill was planted and the plots were applied with chemical fertilizers after 15 days of transplantation according to the formula NPK 75-30-30 at a rate of 65 kg of DAP, 29 kg of urea, and 50 kg of K2O per ha (Swapna and Shylaraj, 2017). For maintaining the moisture condition, plot was irrigated for 3 days once. Drought stress was imposed on rice plants when the flag leaf started to initiate by withholding water irrigation. In control plot, the water level was maintained to 5.0cm until the seed harvesting (Vikram, et al., 2011). NILs were evaluated under moisture condition for the agronomic characters of plant height (PH), root length (RL), number of tiller (NT), number of spikelet per panicle (NSP) and number of fertile seeds (NFS). Plant height was measured from the ground level to tip of the tallest panicle at the time of maturity and expressed in centimeter (Nguyen, et al., 2004).

Root length was measured from the ground level to tip of root at the time of maturity and expressed in centimeter (Nguyen, et al., 2004). The total number of tillers in a hill including both productive and vegetative tillers were counted and recorded at maturity stage. The mean percentage of spikelet fertility was obtained from percentage of filled grains to the total number of grains in the primary panicle of the selected plants as per the below mentioned formula:

$$\text{Spikelet fertility (\%)} = \frac{\text{Number of filled grains per panicle}}{\text{Total number of grains per panicle}} \times 100$$

2. RESULTS AND DISCUSSION

Unlike other cereals, rice is very sensitive to drought stress since it is a hydrophillic crop (Panda, et al., 2021). Hence, grain yield in rice can decrease by up to 90% depending on drought intensity, its duration and plant growth stage is decreased grain yield in rice by up to 90% (Basnayake, et al., 2006; Venuprasad, et al., 2007). Drought is associated with insufficient rainfall or higher evaporation rates caused by water deficit conditions (Rollins, et al., 2013; Upadhaya and Panda, 2019). It is difficult to predict intensity and severity of drought because it is linked with many factors such as rainfall frequency, evaporation rate and soil moisture content (Rijsberman, 2006; Hao, et al., 2018; Oladosu, et al., 2019). Drought stress is a major constrain to rice growth at any stage of its growth cycle (early seedling, vegetative and reproductive stage). Growth of rice is hampered by limited leaf growth (Zhu, et al., 2020), reduced leaf area, leaf rolling, leaf drying, thickened leaf size, early senescence, stomatal closure and cutinized layer on the leaf surface (Mishra and Panda, 2017; Hussain, et al., 2018; Panda, et al., 2021) due to water stress. Particularly, drought stress at reproductive stage results in decreased or no grain-yielding depends on drought intensity. With this connection, many studies are carried out and suggested direct selection for grain yield under drought stress based on practical approach and the feasibility of combing high yield genetic source for grain yield has been reported (Venuprasad, et al., 2008).

Until now, several promising breeding lines linked with drought tolerance at reproductive stage for rainfed lowland and upland have been reported and they are used for improvement of popular rice varieties at morpho-physiological and growth level (Verulkar, et al., 2010; Mandal, et al., 2010; Vikram, et al., 2011; Dixit et al., 2017; Vinod, et al., 2019). According to Panda, et al. (2023), the identification of rice lines with superior morphological and physiological characters enhances the breeding programme. In the present study, the recipient and donor variety responded as susceptible (scale 7) and moderately resistant (scale 3) for grain-yield under drought stress at reproductive stage, respectively. In PCR screening, we found a polymorphic banding pattern between donor and recipient variety with RM431 linked with qDTY1.1 for drought tolerance at reproductive stage. Similarly, polymorphism of Nagina 22 (N22), the drought-tolerant donor harbouring qDTY1.1 was detected with Swarna, IR64, and MTU1010 using RM431 (Vikram, et al., 2011). Thus, we confirmed the susceptibility of ADT36 rice variety to drought tolerance at reproductive stage at phenotypic and genotypic level.

In the cross-pollination between ADT36 and CR dhan801 rice variety, we derived a number of forty three F1 seeds. In the foreground selection, we detected 6 positive plants (plant # 2, 4, 5, 6, 8, 10) with heterozygous allele for qDTY1.1 out of 10 plants using RM431 marker. This marker is helped in the introgression of qDTY1.1 since it exhibited polymorphic banding pattern between N22, drought tolerant rice variety and mega rice varieties such as IR64, Swarna, MTU1010, etc (Dhawan, et al., 2021; Vikram, et al., 2011). Only 3 F1 positive plants were backcrossed using ADT36 as recurrent parent (RP) and a number of eighty seven BC1F1 seeds were produced. In screening of BC1F1 population, the PH of the progenies was noted in the range of -36.5-8.9% higher rate to that of RP (71.0cm). From these progenies, 5 progenies (plant # ADT36-F12.6; ADT36-F14.14; ADT36-F15.1, ADT36-F15.13, ADT36-F15.16) with higher value for PH in the range of 5.3-8.9% were selected. Selected progenies were backcrossed with its RP and produced 75 seeds. In BC2F1 population, we found higher and lower value for PH (-34.6 to 10.2 %) to RP (70.0cm). From these, plant # 36-F15-13.5, 6; 16.2, 5, 9 with higher value from 5.3 to 10.2 % for PH were selected for backcrossing and 105 seeds for BC3F1 generation were produced.

In BC3F1 population screening, we found variations in the range of -29.2 to 9.2% for PH with RP (70.0 cm) (Fig,1c). From these, 7 progenies (plant # 36-F15-13-5.1, #36-F15-13-6.3, #36-F15-13-6.6, #36-F15-16-2.5, #36-F15-16-2.6, #36-F15-16-2.7, #36-F15-16-5.8) showing higher value of PH (from 5.3 to 8.9 %) than RP were selected for selfing and BC3F2 generation was derived (Fig.1). Higher and lower value of PH among BC population is associated with drought tolerance and intolerance under drought stress (Vikram, et al., 2011). In a study, increased plant height is reported in N22 drought tolerant variety and reduced growth in drought susceptible rice variety, Swarna-Sub1 under drought condition. Reduced plant height is associated with hampering of cell division and cell elongation activity due to drought stress (Sonam, et al., 2018). Thus, we could advance the breeding programme up to BC3 generation using PH character as morphological marker. In earlier attempts to improve grain-yield under reproductive stage stress, selection of best rice lines was based on root architecture, leaf water potential, panicle water potential, osmotic adjustment and relative water content but they did not give expected results (Fukai, et al., 1999; Pantuwan, et al., 2002). Here, PH character was used for introgression of DTY1.1 into ADT36 popular rice variety to improve grain yield at reproductive stage stress. In the screening, we considered only PH but not the character of day to flowering (DTF) since it is made no difference in grain yield under drought stress (Vikram, et al., 2011). Supportively, qDTY1.1 showed more effect on PH and lead to more grain-yield under drought stress at reproductive stage in a previous study (Vikram, et al., 2011) since this QTL is tightly linked to sd1 loci controlling plant height in rice (Vikram, et al., 2015). Therefore, the PH trait can serve as a reliable morphological marker for the selection and introgression of drought-tolerant genes. Additionally, the tight linkage of the DTY1.1 QTL to the sd1 locus reinforces the positive relationship between plant height and grain yield. In future studies, this approach could be applied to other high-yielding and drought-susceptible varieties to enhance reproductive-stage drought tolerance.

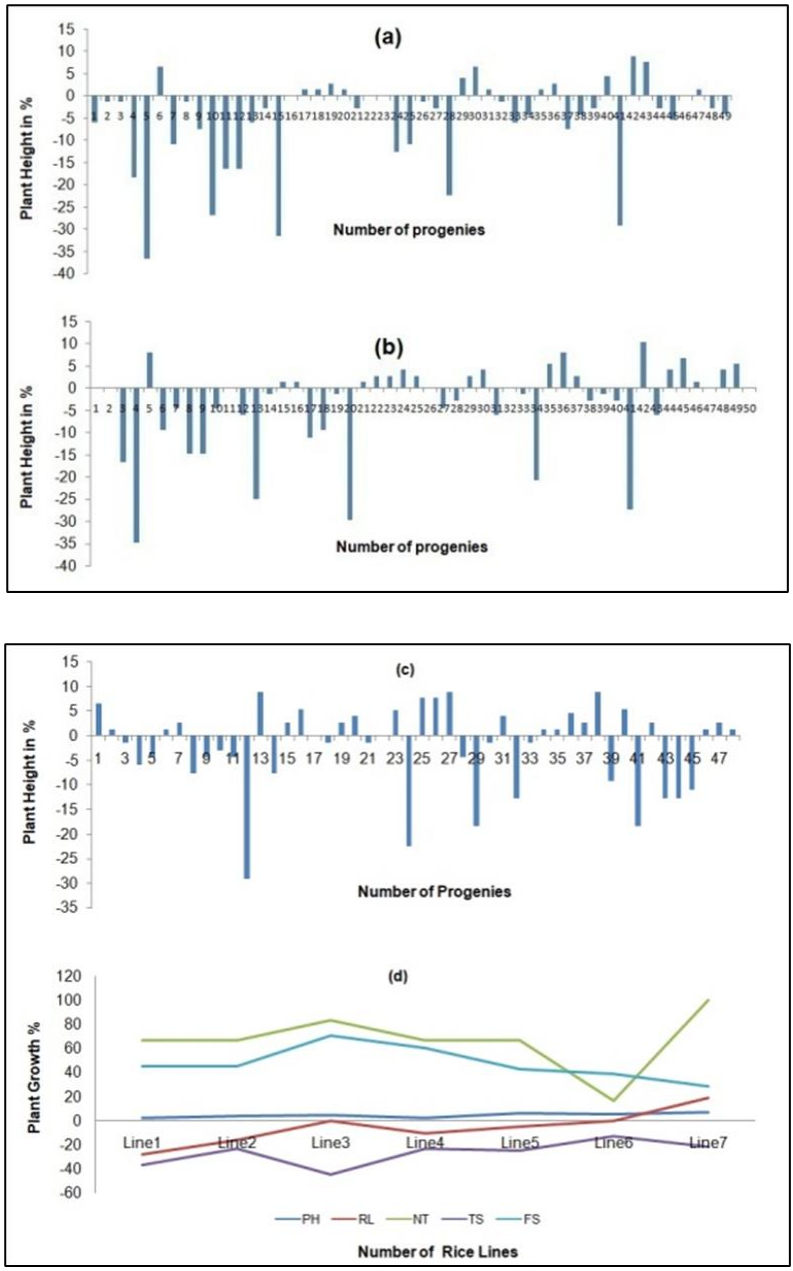

Figure 1. Selection of positive plants from BC populations at phenotypic level. (a) BC1F1; (b) BC2F1; (c) BC3F1 population and (d) BC3F2 rice lines

Growth of RP found to be reduced at 19.3%, 52%, 80%, 32.2%, 24.8% for the trait of PH, RL, NT, SP and FS under moisture condition respectively, when compare to the control condition. In the evaluation of 7 NILs under moisture condition, minimum and maximum rate was noted for PH, RL, NT, NSP and NSS at 1.8 % and 6.9%, -27.9 % and 19.2%, 0% and 83.3%, -44.9% and -13.3%, 28.2% and 70.5%, respectively (Fig.1d). Here, we found positive effect of qDTY1.1 on PH, NT and NFS but negative effect on RL and NSP. Similarly, many studies have been carried out with N22 (harboring qDTY1.1) under drought stress condition (Yang *et al*., 2019; Rahman, *et al*., 2002). In previous studies, qDTY1.1 exhibited a major positive effect on grain yield under drought stress at reproductive stage as well as control condition consistently in different genetic backgrounds of mega rice varieties (Vikram, *et al*., 2011). Moreover, qDTY1.1 in N22(drought tolerant) is associated with more number of fertile seeds through increasing the level of chlorophyll, total phenolics and proline contents, antioxidant potential, lipid peroxidation and 5-methylcytosine (5-mC) under drought stress condition (Nitin kumar, *et al*., 2019; Kumar, et al., 2023).

CONCLUSION

In this study, a short duration modern rice variety (ADT36), cultivated widely across Tamil Nadu was confirmed as susceptible to grain-yield character at both phenotypic and genotypic level. In hybridization process, positive plants were selected based on the plant height character as morphological marker up to BC_3F_1 generation. At final stage, phenotypically selected rice progenies were confirmed the presence of qDTY1.1 using foreground marker in PCR amplification. In field evaluation, some rice lines have showed increased grain yield under moisture condition over the RP under moisture condition. Introgression of QTL associated with morphological character was cheaper and more effective rather than marker-assisted selection to address the problem under intensification of climate change. These improved lines also exhibited better agronomic performance, such as enhanced tillering and panicle length, under drought stress. The study highlights the potential of using plant height as a simple and reliable selection criterion in breeding programs. Future research

could focus on combining qDTY1.1 with other drought-tolerant QTLs to further enhance grain yield stability under varying environmental conditions.

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CHAPTER 3
SUSTAINABLE PROCESSES FOR CUPUAÇU OIL EXTRACTION: KINETIC, THERMODYNAMIC, AND BIOACTIVE ASPECTS

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INTRODUCTION

Cupuaçu (Theobroma grandiflorum), belonging to the Malvaceae family, is a fruit native to the Amazon region that has been traditionally domesticated by Indigenous peoples over the past 8,000 years, with the specific purpose of utilizing its pulp as a food resource (Colli-Silva et al., 2023). Its fruit holds significant socioeconomic importance, particularly for small-scale producers in Northern Brazil. According to Pereira et al. (2025), cupuaçu cultivation in Brazil is characterized as extractive; the pulp is commercialized in frozen portions of 100 g or 1 kg. Cupuaçu pulp is widely used in the production of juices, sweets, creams, and ice creams, while the seeds, previously regarded as waste, have gained recognition as a strategic raw material for obtaining a high value-added vegetable oil, known as cupuaçu butter (Santos et al., 2018; Mota, Serrufo, & Rocha, 2020; Benchimol, Silva, & Melo, 2025).

The cupuaçu butter, extracted from the seeds, is rich in unsaturated fatty acids and triglycerides, with applications in both the food and cosmetic industries. Its high saponification index and emollient properties further reinforce the growing industrial demand. Moreover, the presence of phenolic compounds provides antioxidant activity, enhancing its functional potential (Santos et al., 2018; Costa et al., 2020).

The phenolic compounds present in the seeds can be classified into two main groups: flavonoids and non-flavonoids. Flavonoids constitute a diverse class of natural pigments with complex chemical structures, while non-flavonoids exhibit antioxidant activity dependent on the position of hydroxyl groups and the proximity of the carboxyl group relative to the phenyl group, thereby increasing their antioxidant efficacy (Singhal et al., 2019). In a study conducted by Costa et al. (2020), these compounds were identified and quantified in cupuaçu fat (Figure 1) using high-performance liquid chromatography (HPLC) coupled with a photodiode array detector. This study reported that T. grandiflorum seeds contain gallic acid, protocatechuic acid, coumaric acid, epicatechin, epigallocatechin gallate, quercetin, and glycosylated quercetin as the main phenolic compounds.

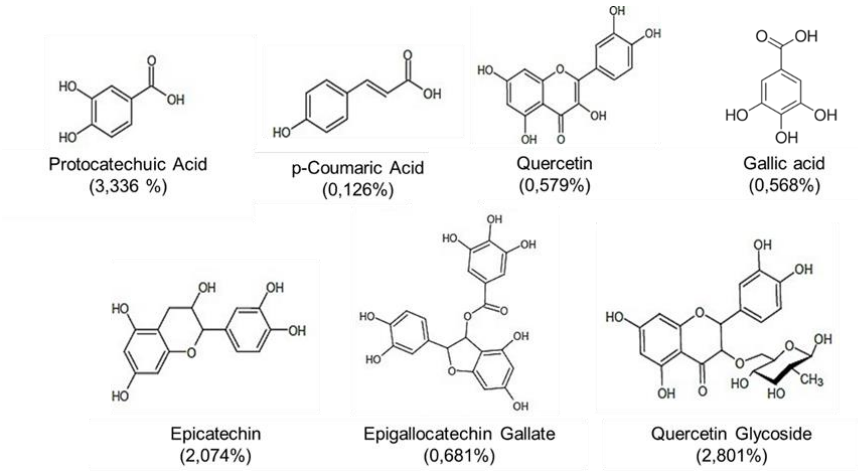

Figure 1. Bioactive compounds present in cupuaçu

Traditionally, hexane has been the most widely used solvent for extracting oil from various oilseeds, owing to its high efficiency in solubilizing lipophilic compounds and its superior extraction yields when compared with other methods, such as mechanical pressing and supercritical CO_2 extraction, as reported in the literature (Yilmaz & Güneşer, 2017; Schoss & Kočevar Glavač, 2024; Sharma, Kumar, & Singh, 2023; Eddaoudi et al., 2025).

Hexane, or n-hexane, is a hydrocarbon produced from crude petroleum and a component of many industrial solvents. Most commercial/industrial hexanes consist of a mixture of hexane isomers and aliphatic hydrocarbons, with one of its primary applications being the extraction of vegetable oils in closed systems, particularly from crops such as soybeans (ATSDR, 2022). However, besides being a non-renewable solvent (petroleum-derived), its use raises environmental and toxicological concerns, especially due to its neurotoxicity in occupational settings (Shubhakaran, Lalwani, & Yadav, 2025), which motivates the search for more sustainable alternatives (Cravotto et al., 2022). Recent studies (Prasad et al., 2022; Shejawale et al., 2024; Ribas, Gasparetto, & Salau, 2025) have demonstrated the effectiveness of alternative solvents, such as ethyl acetate and ethanol, which can replace hexane and even improve the quality of the extracted oil in terms of the presence of bioactive compounds (Cravotto, et al., 2022; Lee et al., 2024).

The present study aims to evaluate alternative solvents for sustainable extraction of oil or cupuaçu butter, through ultrasound-assisted extraction (UAE), analyzing mass transfer, kinetic and thermodynamic parameters of the process, as well as the content of phenolic compounds and the antioxidant activity of the obtained oil.

1. METHODOLOGY

For the application of the present methodology, the seed preparation was carried out as adapted from Shi et al. (2022). Theobroma grandiflorum seeds were previously dehydrated in an oven at 40°C until reaching constant weight. After drying, the seeds were ground to different particle sizes and used in the two main stages of the study: the kinetic and thermodynamic analysis of the process and the evaluation of the antioxidant properties and phenolic content of the oil.

1.1 Kinetic and Thermodynamic Analysis of Cupuaçu Oil Extraction

In this stage, the previously prepared seeds were crushed in a blender and subsequently sieved using a Mesh 16 sieve to ensure uniform particle size. For the extraction process, 5 g aliquots of the crushed seeds were weighed into 250 mL Erlenmeyer flasks, and ethanol was added as the solvent in a sample-to-solvent ratio of 1:3.

1.1.1 Evaluation of Kinetic Constants

The oil extraction was performed using an ultrasound bath (UAE) at a frequency of 40 Hz, with extraction times varying between 5 and 90 minutes, and temperatures of 30°C, 40°C, and 50°C. After extraction, the solid particles were separated from the oil/solvent mixture by simple filtration using a funnel and analytical filter paper.

The filtrate was collected in a round-bottom flask, and the oil was then separated from the solvent by rotary evaporation to obtain cupuaçu seed oil. The yield was calculated according to Equation 1, which was also used in the analysis of the influence of solvent mixtures on the antioxidant activity and phenolic content of the oil.

$$\%\ of\ oil\ extracted = \frac{Mass\ of\ oil\ extracted}{Mass\ of\ initial\ seed}\ .100 \quad (1)$$

- According to Pacola (2018), the oil extraction process occurs in two simultaneous steps: solvent rinsing of the seed and solvent diffusion into the seed pores. Each step has a distinct mass transfer coefficient (k_1 and k_2):
- Rinsing: Approximately 85% of the oil is removed during this stage, characterized by the highest transfer rate from the solid surface to the solvent, predominating at the beginning of extraction.
- Diffusion: The remaining 15% of the oil exhibits a lower transfer rate, corresponding to the diffusion of the solvent into the interior of the seed.

In this work, two mathematical models were evaluated. The first model considers both the rinsing and internal diffusion stages, while the second focuses solely on the internal diffusion stage. The equations describing these models are presented below, with Equation 2 representing both stages (rinsing and internal diffusion) and Equation 3 representing only internal diffusion.

$$q = q^{\infty}\ [1 - f.e^{-k1.t} - (1 - f)e^{-k2.t}] \quad (2)$$

$$q = q^{\infty}\ [1 - (1 - f)e^{-k2t}] \quad (3)$$

Where:

- $q\infty$ = oil yield at saturation;
- k2 (min-1) = extraction coefficient for internal diffusion;
- k1 (min-1) = extraction coefficient for rinsing;
- f = fraction of oil extracted by rinsing.

To determine the kinetic constants, nonlinear regression was applied to both equations. Regression analysis was performed using the least squares method, comparing experimental yields with calculated values. Microsoft Office Excel™ Solver was employed, applying the GRG Nonlinear optimization method to fit the parameters.

1.1.2 Evaluation of Thermodynamic Constants

According to Pacola (2018), to calculate the energies involved in solid-liquid extraction, Equations 4 and 5 were used. Equation 4, corresponding to the Van't Hoff equation, describes the thermodynamic equilibrium, while Equation 5 was used to calculate the Gibbs free energy (ΔG).

$$ln\ ln\ K\ = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

Where:

- K = thermodynamic equilibrium constant;
- ΔH = enthalpy change during extraction;
- ΔS = entropy change during cupuaçu oil extraction;
- R ($J \cdot mol^{-1} \cdot K^{-1}$) = universal gas constant;
- T (K)= temperature.

Thermodynamic equilibrium constants from Equation 4 were obtained using linear regression, following the same procedure as in the kinetic analysis.

1.2 Analysis of the Influence of Solvent Mixtures on Antioxidant Activity and Phenolic Compounds

For this stage, particles retained on a Mesh 20 sieve were selected to facilitate filtration and enable comparison with the particle size used in Section 2.1. Subsequently, 3 g of ground seeds were added to the respective solvents at a ratio of 1:10 (sample mass/solvent). The mixture was subjected to ultrasonic treatment for 1 hour at 35°C and 40 kHz. After extraction, the seed/solvent suspension was filtered, and the solvent was separated from the oil via rotary evaporation. Oil yield was calculated according to Equation 1, as the ratio between extracted oil mass and sample mass, multiplied by 100. The mixture was subjected to ultrasonic treatment for 1 hour at 35°C and 40 kHz. The ultrasonic waves help disrupt the seed matrix, improving solvent penetration and oil release.

Experiments were conducted according to a simplex-lattice mixture design with mid- and central points, considering seven experimentais points: three points with each pure solvent, three points with binary solvent mixtures, and one central point with a ternary mixture. This design allowed the evaluation of the statistical significance of main factors and their interactions on the responses studied (oil yield, antioxidant capacity, and phenolic content).

Antioxidant activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. DPPH is a stable free radical due to electron delocalization across its structure, resulting in a purple color with a maximum absorption at 516 nm. When an antioxidant or radical species reacts with DPPH, a reduction occurs, forming 2,2-diphenyl-picrylhydrazine, which exhibits a yellow color. Antioxidant activity was quantified using a standard antioxidant, such as ascorbic acid. Solutions of ascorbic acid (20–200 mg/L) were prepared, and a calibration curve allowed the expression of results in mg/L AAE (ascorbic acid equivalent).

Total phenolic content was determined using the Folin–Ciocalteu method, which involves the reaction of phosphomolybdic and phosphotungstic acids (both in oxidation state IV) that produce a yellow color. In the presence of reducing agents, such as phenolic compounds, the metals are oxidized to states V and VI. Phenolic compounds, in basic medium, are deprotonated to form carboxylate anions that react with the metals, turning the solution blue (Oliveira et al., 2015).

Analysis was conducted by comparing the extract to a calibration curve prepared with pure gallic acid, obtaining results in mg gallic acid equivalents (mg GAE). Absorbance was measured at 765 nm, using a reaction mixture of 2.75 mL Folin–Ciocalteu reagent (3%), 0.25 mL of diluted extract (2 mL pure extract diluted to 5 mL), and 0.25 mL saturated sodium carbonate solution. After mixing in a test tube, the mixture was incubated for 1 hour at 35°C before reading. For the calibration curve, six gallic acid dilutions were prepared (200, 100, 80, 60, 40, 20 mg/L) using the extraction solvent (Oliveira et al., 2015). All assays were performed in triplicate. The calibration curve showed good linearity within the tested concentration range. Results were expressed as mean values ± standard deviation to ensure analytical reliability.

2. RESULTS AND DISCUSSION

The results obtained from the extraction and analytical procedures provide valuable insights into the influence of solvent composition and extraction conditions on the yield and bioactive properties of the seed oils. These findings are discussed in detail below, emphasizing the relationships between extraction efficiency, antioxidant activity, and total phenolic content.

2.1 Kinetic Analysis

Tables 1, 2, and 3 present the yield values of cupuaçu oil extracted at times ranging from 5 to 90 minutes, for the temperatures of 30, 40, and 50°C, respectively.

Table 1. Yield data obtained at 30°C

Time (min)	Oil extraction yield (%)
5	6,5401
10	6,8123
30	7,1323
60	8,2710
90	8,2727

Table 2. Yield data obtained at 40°C

Time (min)	Oil extraction yield (%)
5	7,4414
10	7,4536
30	8,4028
60	8,3325
90	9,1211

Table 3. Yield data obtained at 50°C

Time (min)	Oil extraction yield (%)
5	8,1406
10	8,8753
30	8,2766
60	9,6242
90	10,0980

The yield data of cupuaçu oil as a function of time (Tables 1–3) showed a rapid extraction in the initial minutes, followed by a progressive decrease in the extraction rate until reaching saturation, a typical characteristic of processes controlled by internal diffusion. This behavior is consistent with observations in other oilseeds, such as Attalea tessmannii kernels, where a fast initial phase is followed by a diffusion-limited stage (Ahmad et al., 2025) and ghanaian cashew kernels (Baidoo et al., 2024) also reported similar patterns, supporting the idea that oil extraction in oilseeds follows an internal diffusion-controlled dynamic after an initial rapid extraction phase.

According to Singhal et al. (2019), increasing temperature and extraction time enhances the amount of oil extracted due to the intensification of mass transfer phenomena and the increased solubility of lipid components in the solvent. Figure 2 illustrates the extraction yield distribution as a function of time for cupuaçu oil using ethanol as solvent at different temperatures (30, 40, and 50 °C), considering only the internal diffusion stage.

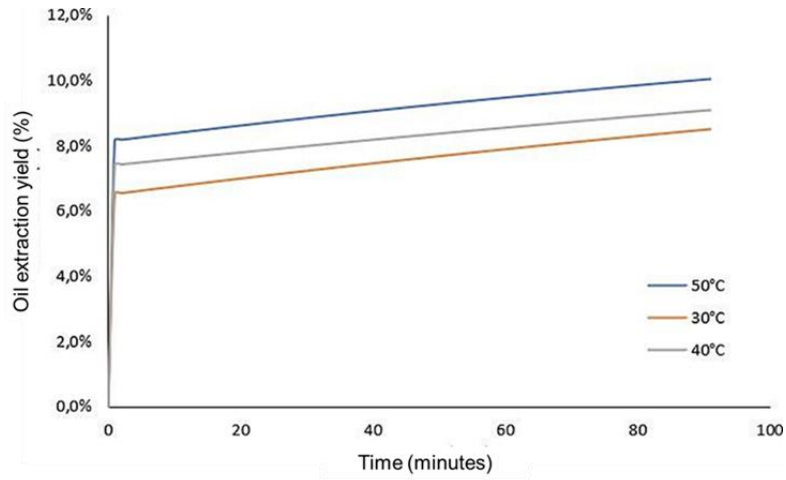

Figure 2. Extraction yield distribution as a function of time at different temperatures, considering only the internal diffusion stage

According to Kostić (2014) the high extraction rate at the beginning of the process, followed by the progressive decrease over time observed in Figure 2, occurs because saturation represents the point at which the solvent is no longer able to dissolve the oil present within the oilseed.

At this stage, the system reaches equilibrium, and mass transfer from the solid to the solvent is interrupted. Tables 4 and 5 present the kinetic constant values for the mathematical models described by Equations (2) and (3), respectively.

Table 4. Kinetic constants considering the rinsing and internal diffusion stages

Temperature (°C)	$q\infty$ (%)	k_2 (min-1)	k_1 (min-1)	f
30	14,10	2,36E-03	5,47E-01	0,4875
40	15,66	2,43E-03	8,29E-01	0,4758
50	12,94	4,95E-03	6,57E-01	0,6407

Table 5. Kinetic constants considering only the internal diffusion stage

Temperature (°C)	$q\infty$ (%)	k2 (min-1)	f
30	13,90	3,48E-03	0,4676
40	13,55	3,57E-03	0,5448
50	13,20	5,15E-03	0,6162

The analysis of the kinetic models, considering both rinsing and internal diffusion (Equation 2) and only internal diffusion (Equation 3), showed that the k_2 values were very similar between the two models, whereas k_1 was considerably higher. This indicates that the rinsing step occurs rapidly and does not limit the overall extraction process, while the internal diffusion of oil from plant cells into the solvent represents the rate-limiting step. The gradual decrease in extraction rate over time reflects the approach to equilibrium, as solvent saturation eventually prevents further mass transfer. Furthermore, increasing temperature was observed to enhance diffusion, resulting in higher k_2 values without changing the limiting role of internal diffusion. This behavior is consistent with observations in other plant oils, such as Irvingia gabonensis kernels, where higher temperatures significantly improved oil yield by promoting mass transfer and increasing lipid solubility (Agu et al., 2021).

2.2 Thermodynamic Analysis of Extraction

The Figure 3 presents the data obtained from Equation 4, used to determine the enthalpy and entropy variation of the system.

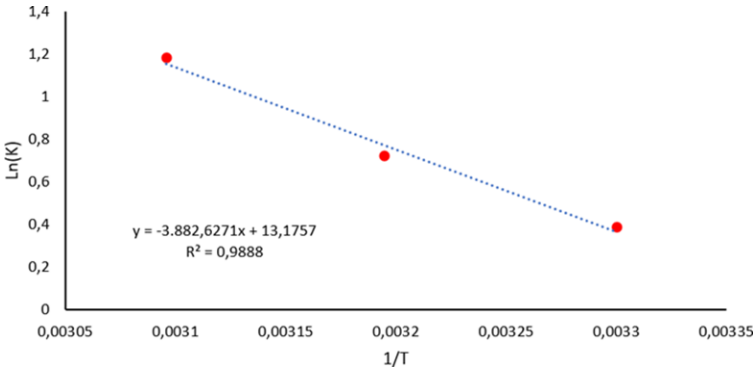

Figure 3. Temperature distribution based on the Van't Hoff equation

From the values obtained from the linear equation outlined in Figure 3, the enthalpy and entropy variations were determined. In addition, the Gibbs free energy was calculated, as presented in Table 6.

Table 6. Energy values involved in the extraction process

Temperature (°C)	Δ H (J/mol)	Δ S (J/(mol.K))	Δ G (J/mol)
30	466,9987	1,5848	-44,8790
40			-13,1838
50			-29,0314

The data in Table 6 shows a positive enthalpy change (ΔH) and a negative Gibbs free energy (ΔG), indicating that the energy input favors the diffusion of oil from the vegetal seed cells of cupuaçu into the solvent (ethanol), characterizing the process as endothermic. According to Baidoo et al. (2024), positive ΔH values signify that the extraction process absorbs energy, supporting the endothermic nature of the process. The negative ΔG values confirm the spontaneity of the extraction, as a negative Gibbs free energy indicates a thermodynamically favorable process under constant temperature and pressure conditions. In addition, the positive entropy change (ΔS) demonstrates an increase in entropy during extraction.

According to Smith et al. (2020), positive ΔS values indicate greater molecular disorder, a condition commonly observed in irreversible processes and essential for explaining the spontaneity of physical and chemical transformations. Therefore, process analyzed can be characterized as both spontaneous and irreversible.

2.3 Analysis of the Influence of Solvents and Solvent Mixtures on Antioxidant Activity and Phenolic Compounds

To evaluate the influence of different solvents on the antioxidant activity and phenolic compound content of cupuaçu oil, a mixture experimental design was employed. The experiments allowed the determination of oil yield, antioxidant capacity, and total phenolic content, the results of which are presented in Table 7.

Table 7. Experimental results – Yield, Antioxidant Activity, and Total Phenolic Content

Experiment	Solvent mass fractions			Oil extraction yield (%)	AA (mg/L AAE)	CFT (mg/L EAG)
	Hexane	Ethyl acetate	Ethanol			
1	1	0	0	20,08	5,79	7,13
2	0	1	0	20,09	11,03	13,83
3	0	0	1	5,12	181,75	191,65
4	0,5	0,5	0	18,07	7,76	10,04
5	0,5	0	0,5	17,11	60,79	62,65
6	0	0,5	0,5	17,26	85,29	94,42
7	0,33	0,33	0,33	19,72	78,76	86,26

The calibration curves, both for the antioxidant activity assay and for the total phenolic content determination, showed good reliability, as illustrated in Figure 4. High correlation coefficients confirmed the linearity of the analytical response within the tested concentration range. This ensured accurate quantification of the antioxidant and phenolic levels in the analyzed samples.

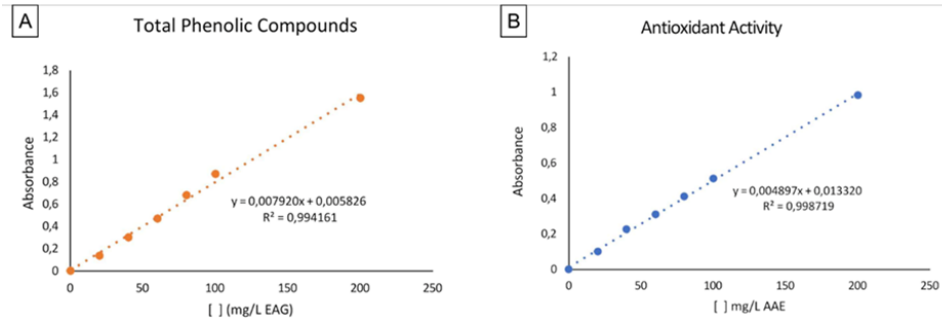

Figure 4. A – Calibration curve for Total Phenolic Content; B- Calibration curve for Antioxidant Activity

Figure 4 shows the calibration curves for Total Phenolic Compounds (Figure 4-A) and Antioxidant Activity (Figure 4-B), both exhibiting high linearity, as evidenced by determination coefficients close to 1.0 ($R^2 \geq 0.994$). Using the results from Table 7, response surfaces for total phenolic content, antioxidant activity, and oil extraction yield were plotted using Statistica 10.0 software (Figure 5). Additionally, the Pareto chart was used to evaluate which coefficients were statistically significant at a 95% confidence interval.

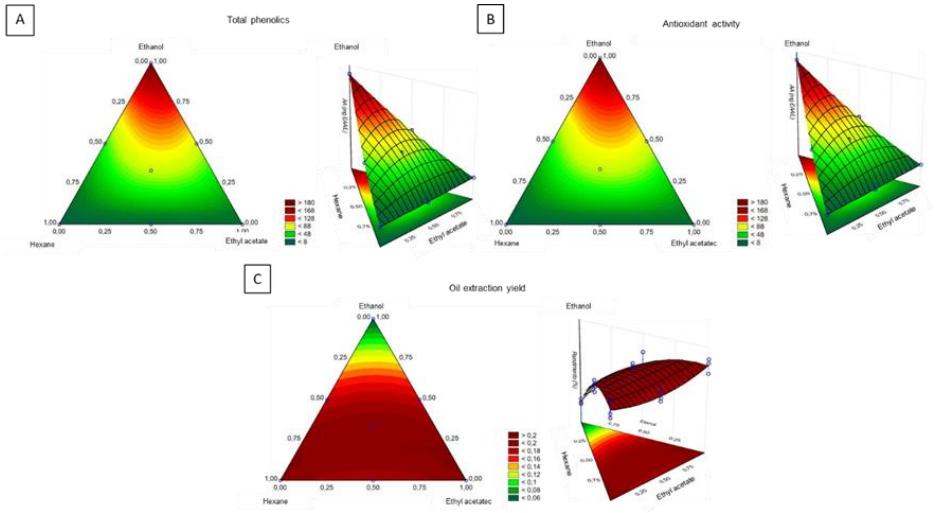

Figure 5. A – Response surfaces for total phenolic content; B – Response surfaces for antioxidant activity; C – Response surfaces for oil extraction yield

The response surface for oil yield (Figure 5-A) shows that regions with a higher proportion of hexane exhibit the highest yield values (represented by darker coloring, close to 0.2). This aligns with the fact that hexane is traditionally an efficient solvent for lipid extraction (Schoss & Kočevar Glavač, 2024; Eddaoudi et al., 2025) explaining its good performance in oil yield evidenced by the response surface. However, as the proportion of ethanol and ethyl acetate increases, the yield gradually decreases, although there are still zones with reasonable yields in binary mixtures, particularly in the combination of hexane with ethanol and ethyl acetate with ethanol. These synergistic interactions suggest that hexane can be partially replaced by these solvents while maintaining extraction efficiency.

In the response surface for antioxidant activity (Figure 5-B), a different pattern is observed. Regions with higher antioxidant activity are concentrated where ethanol predominates, indicating that this solvent favors the extraction of compounds with antioxidant properties. These results are consistent with previous studies, which highlighted ethanol as an effective and safe solvent for extracting phenolic and other bioactive compounds from plant matrices (Gil-Martín et al., 2022; Lohvina; Sándor; Wink, 2021; Alara; Abdurahman; Ukaegbu, 2021). Regions with higher hexane concentrations show lower antioxidant activity, suggesting that while hexane is effective for oil extraction, it is less suitable for extracting bioactive compounds. However, the combination of all three solvents appears to present a synergistic effect, yielding intermediate antioxidant activity, which could be advantageous for optimizing both bioactive compound extraction and oil yield.

The response surface for total phenolic compound contente (Figure 5-C), follows a pattern very similar to that of antioxidant activity, with the highest concentrations located in the region where ethanol predominates. This reinforces the role of ethanol as an effective solvent for extracting phenolic compounds. The combination of hexane with ethanol and ethyl acetate with ethanol also results in considerable phenolic concentrations, although at levels lower than those obtained with pure ethanol. According to Lohvina, Sándor, and Wink (2022), this efficiency can be further optimized if, instead of pure ethanol, the extraction is performed using 70% ethanol containing 30% water.

The effects of the solvents and their interactions on oil extraction yield, antioxidant activity, and total phenolic content are illustrated in the Pareto charts (Figure 6).

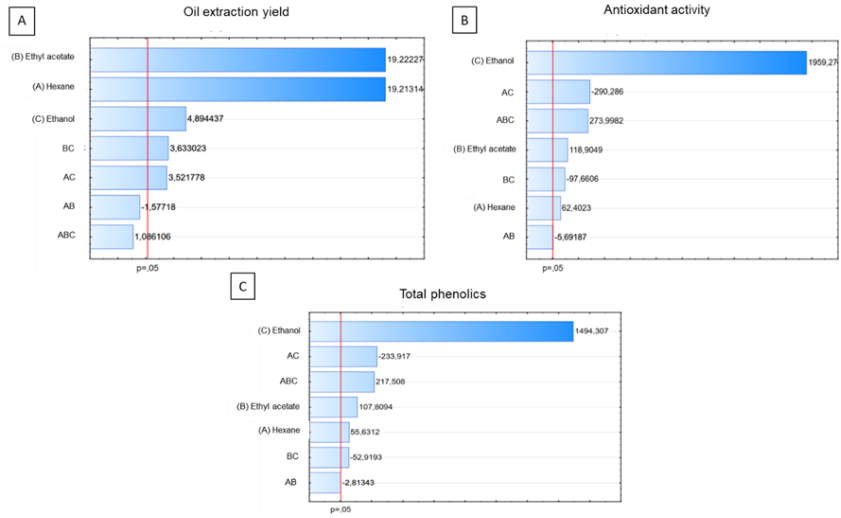

Figure 6. A – Pareto chart for oil extraction yield; B – Pareto chart for antioxidant activity; C – Pareto chart for total phenolic contente

Regarding extraction yield (Figure 6-A), it can be observed that the linear effects associated with hexane (A) and ethyl acetate (B) are significantly relevant, highlighting the importance of these solvents in the lipid extraction process. Ethanol (C), although contributing positively to a lesser extent, is also significant. The higher efficiency of oil extraction using hexane compared to etanol, corroborates findings from literature, Magalhães et al. (2023), who employed sequential counter-current continuous extraction to compare ethanol and hexane for oil extraction from peanut press cake. Hexane achieved a yield of 86 ± 2% in only two stages at 55 °C with a solid-to-solvent ratio of 1:4, whereas ethanol reached 87 ± 4% in three stages at 75 °C with a 1:5 ratio, requiring higher energy consumption, more solvent, and an additional contact stage to achieve a similar yield.

Additionally, the quadratic interactions between ethyl acetate and ethanol (BC), as well as between hexane and ethanol (AC), are synergistic and significant, suggesting that the combined use of these solvents can maintain adequate yield even with a reduced proportion of hexane. On the other hand, the interaction between hexane and ethyl acetate (AB) shows an antagonistic effect, as it falls below the significance threshold. The cubic interaction was not significant, indicating that the combination of all three solvents does not have a relevant impact on extraction yield.

In the evaluation of both the oil's antioxidant activity (Figure 6-B) and total phenolic content (Figure 6-C), ethanol (C) stands out as the most influential factor, being primarily responsible for the extraction of these bioactive compounds. This pronounced effect is attributed to its polarity, which enhances the solubility of phenolic and other polar compounds, as noted by Lee et al. (2024), who reported that polar solvents like ethanol are more effective than non-polar ones in extracting antioxidants from plant matrices. In contrast, hexane (A) and ethyl acetate (B), although important for overall oil yield, contribute minimally to antioxidant and phenolic extraction. The quadratic interaction between hexane and ethanol (AC) has a significant negative effect, indicating that this combination is unfavorable for extracting these compounds. Conversely, the cubic term (ABC) is significant in both cases, suggesting a possible synergistic effect when all three solvents are combined, although its impact is less pronounced than that of ethanol alone. Other quadratic interaction (AB) were not significant, confirming their minimal contribution to phenolic extraction.

CONCLUSIONS

The solid–liquid extraction of cupuaçu oil/butter proved to be effective, allowing a comprehensive characterization of the system from physicochemical, kinetic, and thermodynamic perspectives. The positive enthalpy (ΔH), negative Gibbs free energy (ΔG), and increased entropy (ΔS) confirmed that the process is spontaneous, endothermic, and associated with greater system disorder. Kinetic analysis indicated that internal diffusion is the rate-limiting step, highlighting the need to optimize this stage to improve overall extraction efficiency.

The study also demonstrated that solvent choice strongly influences both oil yield and bioactive compound recovery. Hexane was most effective in maximizing oil extraction, while ethanol excelled in extracting antioxidants and phenolic compounds due to its polarity. Synergistic interactions between hexane, ethanol, and ethyl acetate indicate that partial replacement of hexane is feasible, maintaining satisfactory oil yields while enhancing the functional quality of the oil.

Although ethanol does not provide the highest oil yield, it is highly effective in extracting bioactive compounds, particularly antioxidants and phenolic constituents, from cupuaçu seeds. The use of ethanol a green and renewable solventnot only preserves the functional properties of cupuaçu butter but also adds value to derived products. This approach can stimulate local economies dependent on cupuaçu processing to explore new opportunities, reinforcing the socio-economic importance of this Amazonian fruit and supporting the creation of employment and income for these communities.

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CHAPTER 4
AGRONOMIC BIOFORTIFICATION OF RICE WITH FOLATE

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INTRODUCTION

Rice is a staple food which feeds over half of the world's population. And about one fifth of the calories consumed by humans come from rice. Rice is commonly consumed in Asia and the Pacific, in North and South America as well as in Africa. This makes rice the most important grain utilized to satisfy human nutrition and caloric consumption (Munasingha & Napagoda, 2021). Although Asia produces the largest percentage of rice it is grown in all continents except Antarctica. There are mainly two kinds of rice—Oryza sativa and Oryza glaberrima— among the 22 wild species of rice in the world. While Oryza glaberrima has been grown in West Africa for the past 3500 years, Oryza sativa originated in the humid tropics of Asia and is now grown all over the world. Rice grains can be eaten as it is or parboiled to further increase its nutrient bioavailability. Rice is naturally gluten-free which makes it an ideal alternative to wheat for those with gluten intolerance. In Sri Lanka, rice is called 'dhanya' which means sustainer of the human race alluding to its historical and religious significance in some countries. Rice is available in many varieties, each with different levels of macro and micro nutrients. It contains a wide variety of minerals and vitamins such as vitamin E and B5 as well as carbohydrates, thiamine, calcium, folate, and iron. Red rice in particular contains iron and zinc which supports haemoglobin production and enzymatic processes (Boue et al., 2016). Overall, red rice and traditional rice tend to have a higher nutritional profile than white rice (Kulasinghe et al., 2017).

1. ROLE OF FOLATE IN HUMAN HEALTH

Folate is a B vitamin for the synthesis of purine and thymidine nucleotides and for the synthesis of methionine from homocysteine. It is also identified as pteroylglutamate acid and is a cofactor in enzymes in the process of one-carbon metabolism. This vitamin is available in plants since they are able to synthesize and store it in a form that can be absorbed and digested by humans. Folate cannot be synthesized by mammals, therefore they are dependent on plants to obtain this micronutrient (Hanson & Gregory, 2002). This vitamin plays a crucial role in the prevention of neural tube defects in the unborn which makes it an important nutrient to be taken even before conception.

Folate is a water soluble vitamin and is a co-enzyme in DNA synthesis. Folate co-enzymes are also key players in purine and pyrimidine synthesis. It is also involved in amino acid interconversions such as the interconversion of serine to glycine and homocysteine to methionine (Stover, 2004). There are reported associations of folate intake with cardiovascular disease, cancer and cognitive dysfunction (Bo et al., 2020) which claim that folate intake produces beneficial effects on these conditions.

2. DEFICIENCY OF FOLATE IN HUMAN

Remethylation of homocysteine to methionine, purine and pyrimidine synthesis, and methylation of cytosine in DNA and histones are some functions of folate in humans. Therefore, folate deficiency can give rise to chronic diseases due to certain abnormalities of protein synthesis which in turn affects DNA synthesis and gene expression (Ebara, 2017). Folate deficiency has been significantly associated with risk of preterm birth (Lazar et al., 2024) and Neural Tube Defects (NTDs) which give rise to defects of the skull (Santoso et al. 2006). And some studies also indicate that low maternal folate concentrations are associated with altered brain development during childhood (Zou et al., 2021). In addition to this, folate deficiency has also been linked to female fertility in a study done by Kadir et al. 2022 where a positive association has been observed between dietary folate intake and the ovarian reserves of women diagnosed with infertility (Kadir et al., 2022). Deficiency in this vitamin can also go as far as to disrupt the normal proliferation and growth of lymphocytes, a type of white blood cell which plays a key role in the immune system (Abe et al., 2013). The link between lymphocytes and folate levels is in the process called DNA methylation which in this case is the decrease in methylation of lymphocytes that happens during folate deficiency (Jacob et al., 1998). The recommended daily intake of folate is 400 micrograms of folate per day for both males and females which is equivalent to about 200 grams of beef liver or 2 cups of boiled black eyed peas (cowpeas). Folate deficiency can be easily mitigated by consuming a variety of folate sources, or in the case of critical conditions, through supplementation.

3. BIOFORTIFICATION OF RICE

Biofortification in particular pertains mostly to genetic engineering, whereas agronomic biofortification is a much less complicated process. Agronomic biofortification is the application of certain fertilizers or nutrients into the soil to facilitate the growth and the nutrient stores of the plant, which in this case is rice. Two common minerals used for agronomic biofortification are iron (Ramaswamy et al., 2022) and zinc (De Moura et al., 2024). The vitamin most commonly used for agronomic biofortification is Vitamin A (Majumder et al., 2019). Biofortification through agronomic applications to rice has emerged as an ideal solution for the poor and rural communities to ensure they also meet their daily nutrient requirements. Agronomic biofortification has shown to be one of the most sustainable, practical and cost-effective ways of incorporating nutrients into the edible portion of rice plants i.e. the rice grain consumed by humans (Zhang et al., 2018). As the palatability, softness and digestibility is enhanced when rice is milled, this process removes most of the bran layer in rice where the majority of the B vitamins stored therein are lost. Therefore agronomic biofortification of rice is an essential addition to rice cultivation (Tiozon Jr et al., 2021).

4. FOLATE SYNTHESIS AND DISTRIBUTION IN RICE

Folate levels in rice are typically very low ranging from 0.03 to 0.16 μg/g in the frequently consumed polished rice (Zhao et al., 2020). Therefore agronomic fortification of rice with folate can lead to higher daily intake, as rice usually occupies the largest portion of diet in populations where it is a staple food. Folate occurs in rice bran naturally as a group of tetrahydrofolate (THF) derivatives, the most of them being polyglutamated folates (Anukul et al., 2010). These polyglutamated forms of rice are produced by an enzyme called folypolyglutamate synthetase (Anukul et al., 2010). It is produced by the plant using a de novo biosynthetic pathway including multiple reactions in three cell components: cytosol, mitochondria and plastids (chloroplasts). This pathway is also known as folate polyglutamylation and it takes place during rice seed development (Anukul et al., 2010). The synthesis of folate in rice necessitates the precursors GTP (Guanosine Triphosphate), chorismate and glutamate (Dong et al., 2014).

Folate biosynthesis occurs in the leaves, seed (Anukul et al., 2010) and bran (Zarei et al., 2017) layers of the rice plant. It is then stored in the germ, bran layer and aleurone layer of the rice grain as shown in Figure 1. However during the milling process the aleurone and bran layers are removed, taking with it a significant portion of the folate in rice grains (Dong et al. 2011).

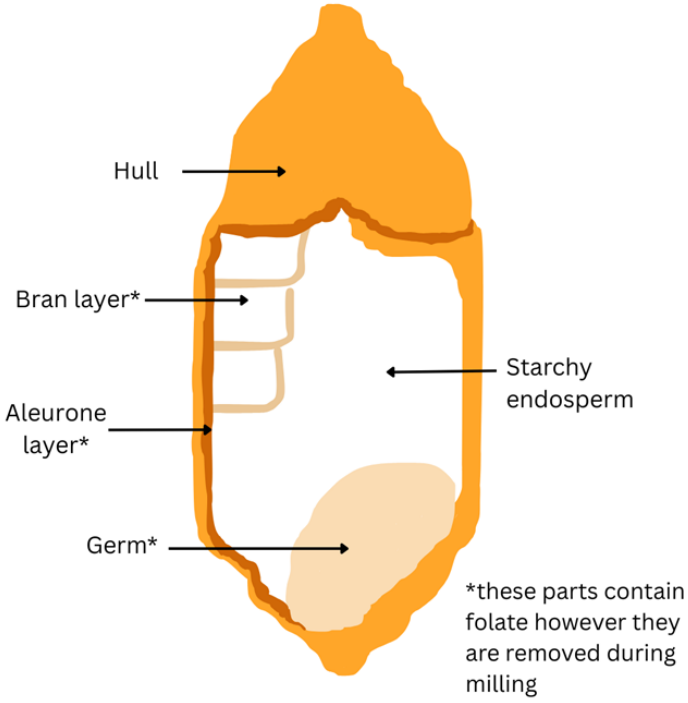

Figure 1. Anatomy of a rice grain

5. FOLATE SOURCES FOR RICE PLANTS

Since rice contains relatively low amounts of folate, the folate levels in rice can be improved using different techniques such as metabolic engineering, foliar application and conventional breeding. However an important point to consider would be that the rice plant does not get its folate from the external environment, it synthesizes its folate therefore other factors that contribute to or facilitate this synthesis pathway can be applied to the plant.

Therefore biofortification of rice can be done to generate rice that can even withstand conditions such as milling and cooking, conditions that bring about loss of folate from rice as shown in a study done by Tiozon Jr et al. 2020 (Tiozon Jr et al., 2020). Three common techniques used in the agronomic biofortification of rice are discussed with an emphasis on the advantages and disadvantages of each in Table 1.

5.1 Foliar Application

Folic acid is the synthetically produced form of folate used in the foliar application of rice plants. Foliar application is performed by application of folic acid onto the leaves of the rice plant using spraying gadgets. It can be done during any stage of the rice plant development, nevertheless this is commonly done during three stages: mid-tillering, panicle initiation and mid-booting (Omar et al., 2020). In the mid-tillering stage most of the tillers i.e. side shoots have emerged and the rice plant is preparing for panicle initiation i.e. when grain production occurs (Mohapatra & Sahu, 2022). The mid-booting stage is before flowering and is the point where the panicle is fully grown and covered with the flag leaf sheath (boot) (Mohapatra & Sahu, 2022). By foliar application of folic acid to rice plants, the deficiencies of folate in rice can be remedied.

Although the targeted benefits of foliar application of folic acid is intended for humans, the plant also derives benefits from this technique. Folic acid has gained attention in the field of agronomic biofortification as a technique which can make nutrients available to plants during high stress periods, mainly during stresses from drought (Ibrahim et al 2021). There could be many rationales for this form of protection given to the plant during drought which negatively affects the stomatal activity, CO2 absorption and photosynthetic rates. Some theories are that folic acid lowers the ROS (Reactive Oxygen Species) produced during this type of stress and also regulates the protein and nucleic acid synthesis leading to a boost in cell division and expansion (Khan et al., 2022).

5.2 Metabolic Engineering

Another method of agronomic biofortification is metabolic engineering where genes related to the rice plant's metabolic pathways are altered to increase folate production. It does this by managing and reprogramming plant genetic pathways using gene editing tools for precision breeding to improve nutritional value of plant edible parts (Kaya, 2024). The precise and strategic manipulation of the genes corresponding to the biosynthesis of folate have shown to increase folate concentrations in rice and will fortify rice with folate. Folate (vitamin B9) is made from: a pterin moiety which is from GTP, p-Aminobenzoate (pABA) from chorismate and glutamate residues from glutamate, for polyglutamylation. Polyglutamylation leads to polyglutamated folates which is the form of folate stored in the rice plant (Dong et al., 2014). The rate limiting steps in this process are particularly those involved in synthesizing its two main precursors: Pterin moiety (from GTP) and p-Aminobenzoate (pABA) (from chorismate). The pterin branch synthesis happens in the cytosol, then the pABA branch is synthesized in the plastids and are finally assembled and reduced in the mitochondria (Gorelova et al., 2019). And these are altered using three common mechanisms namely, overexpression, stacking or editing to optimize their function in the cytosol or the plastids (Storozhenko et al., 2007, Blancquaert et al., 2015). Studies done by Blacquaert et al. 2015 state that metabolic engineering techniques targeting gene overexpression have multiplied folate concentrations by up to 150 folds compared to the wild rice varieties tested (Blancquaert et al., 2015).

5.3 Conventional Breeding

Conventional breeding can increase certain nutrients in rice by cross pollination among nutrient yielding varieties and stress resistant varieties of rice to breed offspring that, as a result, produces those desired traits. Conventional breeding targeting folate levels in rice has not used the traditional cross pollination method since rice is self-pollinating (Hu et al., 2025) which makes the probability of cross pollination very low.

Another reason cross pollination technique is not used in increasing folate concentrations in rice is because the folate biosynthesis pathway is polygenomic i.e. it constitutes several genes thereby necessitating the need to use sophisticated breeding mechanisms and genomic selection (Wei et al., 2014).

Among the sophisticated breeding methods, screening for: rice germs with high folate concentrations (Dong et al., 2011) rice genotypes which help sustain folate levels during processing, storage and cooking are done (Dong et al., 2014). Other than that, focusing on total folate levels in rice such as methyl tetrahydrofolate (MTHF) and also breeding pigmented rice varieties, proven to have higher folate levels than the other varieties, are also advanced breeding techniques (Ashokkumar et al., 2018).

5.4 Advantages vs Disadvantages of Three Agronomic Biofortification Methods

Foliar Application (FA) Advantages

- Foliar spray is easy to incorporate into the agronomic procedures in rice cultivation especially and is less costly than genetic engineering
- FA improves plant growth characteristics significantly (Lahijani et al., 2020)
- FA inhibits the negative impact of drought stress on rice plants by combatting ROS (Reactive Oxygen Species (Ibrahim et al., 2021)

Foliar Application (FA) Disadvantages

- Some studies like one done by Fernández et al., 2021 suggest that uptake by foliage in plants is exclusive to water and previous research has not completely deciphered the uptake of any dissolved molecules (like folic acid) by leaves (Fernández et al., 2021)
- The form in which folic acid is stored if and when uptake takes place is still unknown
- Folic acid may degrade even before it is taken up by the plant leaves since it is unstable and is sensitive to light (Lahijani et al., 2020), heat (Ramakrishnan, 2016) and oxidation (Delchier et al., 2014)

Metabolic Engineering Advantages

- Improves concentration of folate in germ (Storozhenko et al., 2007) and improve stability of folate in rice during processing (Blancquaert et al., 2015)
- Can meet the required level of folate production in rice to address deficiency surpassing that from conventional breeding (Straeten et al., 2020)
- Genetic modifications can be used to add agronomically beneficial traits (such as the ability to withstand stressors) along with increased folate production to engineered rice plants, a concept known as trait stacking (Straeten et al., 2020)

Metabolic Engineering Disadvantages

- Overexpression of GTP cyclohydrolase I enzyme in pterin biosynthesis, a common strategy in genetic engineering, can lead to accumulation of pterins, which do not increase folate production after a certain saturation point (Houssain et al., 2004)
- Research and development cost is high
- Some rice plants that are genetically engineered may accumulate non bioavailable forms of folate i.e. oxidized forms (Zamberlan, 2024)

Conventional Breeding Advantages

- Simple and easy to practice for all levels of farmers, educated and uneducated alike
- Lower cost than metabolic engineering, and requires less technology
- Allows the combination of multiple genes (resistant genes and folate synthesis genes)by a process known as gene pyramiding, which makes pathogens difficult to attack, rather than single genes (Palloix et al., 2009)

Conventional Breeding Disadvantages

- It is time-consuming to obtain desired outcomes

- It is limited to existing gene pools which is not found in metabolic engineering which allows the use of foreign genes (Zhou & Xu, 2024)
- Lacks precision when compared with metabolic engineering and has negative effects like linkage drag i.e. deleterious genes linked to the beneficial gene are passed on during breeding (Huang et al., 2023)

6. SOIL ENVIRONMENT AFFECTING AVAILABILITY OF FOLATE FOR RICE

In rice cultivation, clay loam or silty clay are the commonly used soil types because of their slightly acidic pH, high water retention capacity and high nutrient content (high in N, P, K, Fe and Zn) (Dou et al., 2016). Effects of soil environment on folate levels is an often overlooked area but is crucial since most of the micronutrients essential for proper enzyme production are absorbed by the roots (Alaoui et al., 2022). On that note, the soil environment of rice plants can influence the availability of folate in indirect and direct ways. It can affect how available folate is in rice and its accumulation. And while soil rich in micronutrients, having optimal pH for root function and beneficial nitrogen fixing bacteria can improve folate production, storage and stability, soil stress conditions can reduce folate levels in rice (Khan et al., 2017). Soil conditions also impact the stability of folate during processing and storage, by determining how folate complexes in the rice plant i.e. by folate-binding proteins or polyglutamylation.

6.1 Soil Micronutrient Content

Micronutrients play a vital role in the growth and development of rice plants and impact the plant's ability to perform photosynthesis, produce rice grains and combat stressors. Furthermore, micronutrients are also essential in the proper functioning of several key enzymes in the folate biosynthesis pathway. Table 1. illustrates this dependency between enzymes and micronutrients like iron, zinc, magnesium, copper and sulfur.

Table 1. Functions of essential micronutrients in folate biosynthesis

Micronutrient	Function in folate biosynthesis
Iron (Fe)	It is vital for mitochondrial metabolism (Richardson *et al*., 2010). May indirectly affect enzyme DHFR (dihydrofolate reductase) (Sun *at al*., 2021)
Zinc (Zn)	Acts as a structural cofactor and may help maintain structural integrity of GTP cyclohydrolase I (Auerbach *et al* 2000). Transcription factors that are dependent on zinc may regulate folate pathway gene expression (Hu *et al*., 2024)
Magnesium (Mg)	Most enzymes that are kinases and synthetases involved in folate biosynthesis need Mg^{2+} (Scrimgeour, 1986; Rebeille *et al*., 1997) Magnesium is required for ATP dependent reactions and folate biosynthesis is ATP dependent in multiple steps (Williams, 2000)
Sulfur (S)	Sulfur deficiency may impair folate recycling, a process by which cells rebuild active folate cofactors from their depleted or oxidized forms. Since it is essential for the methionine synthetase enzyme in the production of Tetrahydrofolate (Lyon *et al*., 2020)

6.2 Soil pH

One of the biggest misconceptions when it comes to soil pH is the belief that we are talking about the pH of the soil, the solid when in reality we are measuring the pH of the liquid that comes into contact with the soil. The pH of a clay soil is given as 7.5- 10 and it is the ideal and most common soil type used in rice cultivation. When it comes to charge, clay soil which is primarily used for rice cultivation contains negative charges (Barrow & Hartemink, 2023), thereby attracting positively charged ions essential to the plant such as Mg2+, Fe2+, Zn2+ etc. Soil pH makes the aforementioned micronutrients available for absorption by the roots of rice plants. And while acidic soil pH can make these nutrients more available for the plant, alkali pH can deplete their levels (Jackson & Meetei, 2018). Supporting nutrient availability and plant growth eventually helps in folate biosynthesis in rice plants while extreme pH might compromise the availability of cofactors necessary for folate biosynthesis.

Clay soil, being negatively charged also shows higher cation exchange capacity, which means the ability to hold onto positively charged ions keeping essential ions from leaching (Dey et al., 2023). The positively charged nutrient minerals are loosely attached to the clay surface making them easily absorbed by the root ends which in turn, makes the clay release positively charged ions to balance the charge loss (cation exchange) (Meychik et al., 2021).

6.3 Soil Texture and Structure

Soil texture can influence folate production indirectly by regulating water retention, aeration, nutrient availability, and microbial activity, all of which affect the biochemical pathways which pave the way for optimal folate production (Ye et al., 2024). Soil texture can affect water retention capacity and the soil's ability to hold onto essential mineral ions required for enzymes in folate biosynthesis (Alghamdi et al., 2023). Water retention can also help combat stresses from sudden drought which can slow rate limiting enzymatic reactions (Farooq et al., 2009). The soil texture of clay and sandy have balanced to high aeration which boosts mitochondrial function thereby increasing ATP, an essential energy source in the folate biosynthesis pathway (Vera-Vives et al., 2024; Kirk, 2003). Clay and loam textured soils favor diverse and high microbial biomass which support nitrogen fixation in rice plants and can modulate plant gene expression of secondary metabolites like folate (Garza-Aguilar et al., 2024; Cipriano et al., 2021).

6.4 Microbial Activity

Microbes can affect folate biosynthesis by facilitating hormonal signaling, gene regulation, and even precursor supply. Microbes can enhance soil micronutrient availability by micronutrient solubilization as shown in a study done by Prathap et al. 2021, where zinc solubilization is increased by zinc solubilising bacteria (Prathap et al., 2021). And in this research by Kartik et al. 2023, where iron is solubilized by iron solubilizing bacteria applied to soil (Kartik et al., 2023). Both iron and zinc are crucial nutrients in forming enzymes in the folate biosynthesis pathway.

Microbes which assist in nitrogen fixation help in the synthesis of glutamate and p-aminobenzoic acid, both of which are important precursors in folate biosynthesis (Aguilar et al., 2024; Kan et al., 2015).

7. MANAGEMENT PRACTICES THAT ENHANCE FOLATE LEVELS IN RICE

Incorporating management practices in order to biofortify rice plants with folate must be done strategically to address the current folate deficiency. The right management scheme can enhance and protect folate biosynthesis while sustaining their levels during postharvest handling. All steps leading the rice from farm to fork must be analyzed with scrutiny to ensure that farmers give food that delivers its intended benefits. There can be awareness workshops to train farmers on how to implement management practices into their farming procedure while making resources accessible to farmers. Moreover research on the area of agronomic biofortification must be encouraged with funds to bring lab findings to the farm to be tested. On this regard, some management strategies for the agronomic biofortification of rice with folate are discussed.

7.1 Soil Nutrient Management

Soil nutrient management is a commonly used management practice and involves adding mineral precursors to soil in order to promote enzymes related to folate biosynthesis. Minerals such as iron, zinc, sulfur and magnesium are added to soil in the form of FeSO4, ZnSO4 and Magnesium superphosphate. These micronutrients can even be introduced to the plant even by foliar application other than the soil. However this method is less efficient since there is a lack of research supporting the complete foliar uptake mechanisms and the existing research fails to prove the route of uptake into the leaf (Barrow & Hartemink, 2023).

7.2 Plant Growth Promoting Microorganisms (Rhizobacteria And Mycorrhizal Fungi)

Studies done by Cakmakci et al. 2006 have shown that many bacteria come under the category of plant growth promoting rhizobacteria (PGPR), that work together with the host plant to stimulate growth (Cakmakci et al., 2006).

These bacteria work by increasing the mobility, uptake and enrichment of nutrients in the plant (Hossain, 2024). Research by Rana et al. 2012 shows that PGPR increases the availability of nutrients in the rhizosphere region of the plant (Rana et al., 2012). In addition to rhizobacteria which provide the rice plant with these benefits, arbuscular mycorrhizal fungi (AMF) have been shown to enhance nutrient uptake through roots of upland plants due to increased surface area for soil exploration (Smith & Read, 1997). Although these bacteria will not directly enhance folate biosynthesis in plants, farmers can use them to fortify plant micronutrient levels and maintain optimal functioning of their metabolic pathways which can increase folate production and yields. This is important because a proper supply of essential cofactors for folate biosynthesis such as iron and zinc assures the pathways are being carried out without disturbances.

7.3 pH Management

pH management of soil is important when it comes to nutrient availability, as it must not be either too low or too high. The optimal pH range for paddy rice is 5.5-6.5 (Delgoda et al., 2023) and a study by Abdul Halim et al. 2018 implies that the optimal pH for upland rice is 6.0-6.8 (Abdul Halim et al., 2018). Low pH levels can be harmful for rice plants because it can lead to manganese and aluminium toxicity as stated in Li et al. 2012 and in Thawarwong and Van Diest, 1974 (Li et al., 2012; Thawarwong & Van Diest, 1974). It can also result in phosphorus fixation (Liu et al., 2023) making phosphorus unavailable for the plant. On the other hand, too high pH levels can lead to iron, phosphorus, and zinc deficiencies in the plant by limiting their uptake (Saleem et al., 2023). High pH levels are also not ideal for microbial activity (Koga et al., 2003) leading to lower breakdown of organic matter.

7.4 Water Management

Aerobic conditions in rice cultivation are scenarios where rice is grown in non-flooded non-puddled soil whose moisture is managed. Contrastingly, anaerobic conditions in rice cultivation is when water is in excessive amounts thus flooding the rice plants.

Aerobic conditions in rice plants such as during flooding (Farooq et al., 2011) and anaerobic conditions can both negatively affect rice (Fageria, 2003). However some studies suggest otherwise, like the study done by Liu et al., 2019, which indicates that lowland rice cultivars could make better use of the rainfall because of a longer growth period and a higher growth potential (Liu et al., 2019). Another study by Bouman, 2007 states that higher water productivity i.e. the amount of output or benefit gained per unit of water consumed, gives a higher grain yield (Bouman, 2007). Aerobic conditions also save water used for cultivation and can train rice plants to adapt to periods of drought (Sandhu et al., 2012; Melandri et al., 2021). Similarly, anaerobic conditions also favor higher yield as shown in a study by Dou et al. 2016 where they demonstrated that continuous flooding treatments had greater panicle numbers (Dou et al., 2016). The study however stated that when implementing water supply for rice cultivation, the soil texture and cultivar must be taken into account. Research suggests that lowland varieties can be more suited for anaerobic conditions (Sandhu et al., 2012). This may be because flooding eliminated weeds in the field and many rice cultivations were trained to adapt in flooded situations.

7.5 Management of Postharvest Handling

Metabolic engineering techniques have emerged to protect folate in rice during storage and handling postharvest since folate stored in rice is relatively unstable. One such technique is the binding of folate to folate-binding proteins to increase its concentration. And a study done by Blancquaert et al. 2015 demonstrated a 150 fold increase in yield of folate concentrations in rice by employing this technique (Blacquaert et al., 2015). Other studies like the one done by Akhter et al. 2023, state that polishing depletes folate levels in rice indicating that unpolished, brown or parboiled rice have higher folate concentrations (Akhter et al., 2023).

8. CONDITIONS WHICH DEPLETE FOLATE STORES IN RICE

Folates are sensitive to heat, light and air and they readily soluble in water, which makes them prone to degradation while stored in the bran layer of rice (Gazzali et al., 2016).

While "folate" refers to the several tetrahydrofolate derivatives found naturally in foods, "folic acid" is frequently used to refer to the fully oxidized synthetic molecule found in dietary supplements. However, there is no distinction between "natural" and "synthetic" folic acid. According to Marchetti et al. (2014), it is the same molecule (Marchetti et al., 2014).

8.1 High Carbon Dioxide Levels

A study by Smith and Myers, 2019 discovered that the average loss of B vitamins, including folate, were 17-30% in increased CO2 concentrations (Smith & Myers, 2019). They also found that as a result of this effect alone, risk of folate deficiency could rise by 1.5 percentage points (95% confidence interval: 0.6 - 2.6), corresponding to 132 million (57–239 million) people. The exact mechanism by which this takes place is still under investigation although there have been some theories. Smith and Myers, 2019 once again suggests that there is a 'dilution effect' from the increase in production of starch coming from the increase in photosynthesis (Smith & Myers, 2019). Another study by Myers et al. 2014 showed that increased CO2 concentrations reduces iron and zinc levels in rice crops which in turn reduce folate levels since they are essential micronutrients for the folate biosynthesis pathway (Myers et al., 2014).

8.2 High Environmental Temperature

Higher environmental temperature can be due to many reasons, some of them being during storage, hot climate and cooking. A study by Dong et al. 2014 stated that the losses of folate in rice due to cooking and storage were 23% and 48.3% in the four rice cultivars from China which were studied (Dong et al., 2014). However the studies on how heat stress affects rice during growth is less discussed and most studies emphasize the effects of storage temperature on harvested rice grains on folate levels.

CONCLUSION

Rice is ideal to be used as a dietary supplement to support folate deficiency since it is largely consumed around the globe. If half of the world's population consumes rice it has been found that in contrast, there is also a prevalence of folate deficiency in half of the world's population.

Folate biofortification in rice using agronomic practices is an impactful step that can address this issue since it enables the farmer to be in control of the quality and level of folate of his produce. From high end metabolic engineering to simple practices like foliar application, farmers from all economic levels can participate in this mission to eradicate folate deficiency. By understanding where this nutrient is compromised in the journey from farm to fork, farmers and researchers can strategically fine tune the procedure to yield rice with higher folate concentrations. This review hopes to shed a light on the factors which must be considered to enhance rice folate levels using agronomic biofortification.

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