Chlorophyll Fluorescence Analysis of Heat Stress Tolerance in *Oryza sativa L.* under Controlled Field Conditions

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ABSTRACT

Background: Rice (*Oryza sativa* L.) is the primary source of energy to nearly 50% of humans globally, and its decline directly impacts food security. Different temperature increases (ambient temperature) due to climate change are being experienced during the critical reproductive phases of the plant, necessitating the need to study the impact of heat on rice yields.

Objectives of the study: The main aim was to demonstrate the genetic variations between different rice varieties in terms of tolerance toward heat. This study also focused on finding certain parameters that could be used as an indicator to measure rice productivity during high temperatures.

Methods: Two types of rice varieties that can grow under extreme temperatures and two heat-sensitive varieties were grown using a randomized complete design with four replications. A heat stress (with temperatures of 40-30 degrees C day and night for a period of 5 days) was developed using chamber systems at the heading stage of rice and measurements for chlorophyll fluorescence (such as F0, Fm, Fv/Fm, ΦPSII, ETR, NPQ, qP) were taken for 0, 3, and 5 days.

Results: The application of heat stress caused a notable decrease in the Fv/Fm and ΦPSII ratio in all of the studied genotypes ($p < 0.01$), whereby the sensitive genotypes reacted more seriously to stress in comparison to tolerant ones. Moreover, NPQ increased to a greater extent in tolerant genotypes, indicating that these plants were able to perform better under heat stress conditions. The results showed that Fv/Fm on day 5 of the stress was positively correlated with the spikelet fertility ($r = 0.81$) and grain yield ($r = 0.76$) of the studied rice genotypes.

Conclusion: Chlorophyll fluorescence techniques that measure the Fv/Fm and ΦPSII ratios are helpful methods for quickly and non-destructively assessing the heat tolerance of rice genotypes under field conditions, thus confirming their applicability in breeding programmes aimed at creating heat-resistant rice.

Keywords: *Oryza sativa* L., Heat stress, Chlorophyll fluorescence, Photosystem II (PSII), Heat tolerance, Grain yield

1. Introduction

Complementing the rise in global populations, rice is a staple in the diets of billions of people in Asia, Africa, and Latin America [1]. Furthermore, climate forecasts predict an increase in heat waves during critical stages in rice development, including anthesis and grain filling, which are the phases where plants are most vulnerable to heat stress [2]. High temperatures above the physiological optimum speed up development, decrease grain filling time, impair pollen viability, and lower spikelets' fertility [3].

With respect to biochemistry, heat causes thylakoid membranes to de-stabilize, lowers the effectiveness of photosystem II (PS II) reaction centers, and hinders the light-driven movement of electrons [4]. This, in turn, limits the production of photosynthetic energy from the photosynthesis process that is among the most sensitive to temperatures.

Chlorophyll fluorescence is a novel and excellent technique for assessing the practical aspects of PSII function in live plants [5]. Energy absorbed by light is lost through three different modes: photochemistry, production of heat, and fluorescence, which means that changes in fluorescence yield are indicative of the efficiency of photochemical conversion [6]. Parameters such as Fv/Fm, ΦPSII, electron transport rate (ETR), and non-photochemical quenching (NPQ) can be used to describe the photochemical ability and the prosurvival properties of the photosynthetic unit [7].

Although chlorophyll fluorescence has been extensively adopted in growth chamber experiments, there is not that much literature available on its application under heat stress conditions similar to those that occur in nature where fluctuations in temperature, solar irradiation, and microclimate influence rice plants. Experiments conducted in the field under controlled heat stress can be regarded as a proper compromise between exactness of growth chambers and the realism of fieldwork.

As a result, the present research aimed at identifying variations in chlorophyll fluorescence reactions of rice plants grown under controlled conditions and subjected to heat stress during the heading stage. The aims of the study were: (i) to measure changes in PSII photochemistry in heat tolerant as well as heat sensitive rice genotypes; (ii) to analyze the ability of various traits measured using fluorescence method to separate the groups based on their heat tolerance; and (iii) to establish a relationship between fluorescence characteristics and grain yield.

2. Related Work

Heat stress physiology in rice has been studied extensively at the reproductive stage, where pollen viability, anther dehiscence, and spikelet fertility are particularly vulnerable to daytime temperatures exceeding 35 °C [3, 9]. Complementary work on wheat and sorghum similarly shows reproductive-stage heat exposure disproportionately reduces yield relative to vegetative-stage exposure, underscoring stage-specific stress timing in experimental design [12, 15].

Photosynthetic efficiency under abiotic stress has been linked to membrane thermostability, with heat-induced disruption of thylakoid integrity closely associated with declining chlorophyll content and PSII function [14]. Yamori and colleagues reviewed temperature acclimation of photosynthesis across C3, C4, and CAM species, noting that the temperature optimum for photosynthetic electron transport is often exceeded well before visible leaf damage occurs [8], reinforcing the value of fluorescence-based early detection.

Chlorophyll fluorescence techniques have been applied across diverse crops to characterize stress responses. Foundational reviews by Krause and Weis, and later by Baker, established the theoretical basis linking fluorescence kinetics to photochemical and non-photochemical energy partitioning [4, 5]. Maxwell and Johnson provided practical guidance for standardizing fluorescence measurement protocols that remain widely used in crop physiology research [7], while Murchie and Lawson later expanded this guidance to address common pitfalls in field-based fluorescence data collection [6].

Among the commonly reported parameters, Fv/Fm is the most frequently used indicator of photoinhibition, with values below approximately 0.75 in dark-adapted leaves generally interpreted as evidence of PSII stress [7]. PSII and ETR describe operational photochemical efficiency under ambient light, whereas NPQ and qP describe the balance between photoprotective heat dissipation and the proportion of open reaction centres [5, 6]. Sharma *et al*. demonstrated that wheat genotypes selected for higher Fv/Fm under heat stress also maintained higher stomatal conductance and grain yield, supporting the use of fluorescence as an indirect selection criterion [9].

Several studies have combined controlled environment and field-based heat stress approaches. Prasad *et al*. and Chaturvedi *et al*. examined combined heat and elevated CO2 effects on assimilate partitioning in wheat and rice respectively, both reporting reduced sink strength under supra-optimal temperature [12, 13]. Cao *et al*. specifically examined indica rice under high temperature during heading and early grain filling, reporting substantial yield penalties associated with reduced photosynthetic capacity [16].

Advances in high-throughput phenotyping have expanded the scale at which fluorescence and related traits can be captured, with imaging-based platforms enabling canopy-level assessment across large breeding populations [17, 18]. Rebetzke *et al*. demonstrated that high-throughput phenotyping technologies can accurately capture stay-green and related stress-tolerance traits at a scale suitable for breeding programmes [20].

Despite this progress, a research gap persists in the integration of standardized, multi-parameter chlorophyll fluorescence protocols within controlled field settings that retain realistic diurnal and canopy conditions, particularly for indica and japonica rice genotypes differing in heat tolerance. The present study addresses this gap by combining a defined open-top chamber heat treatment with comprehensive fluorescence and agronomic phenotyping under field conditions.

3. Experimental Design and Methodology

3.1 Overall Experimental Framework

The experiment employed a split-plot arrangement within a randomized complete block design (RCBD), with temperature regime (heat stress versus ambient control) assigned to main plots and genotype assigned to subplots, replicated across four blocks. This framework allowed simultaneous evaluation of genotype, temperature, and their interaction on fluorescence and yield traits.

3.2 Genotype Selection and Heat Stress Protocol

Two heat-tolerant genotypes (designated G1 and G2) and two heat-sensitive genotypes (G3 and G4) were selected based on prior screening records. Heat stress was imposed using transparent open-top field chambers equipped with thermostatically controlled heating fans, maintaining 40 ± 1 °C during the day and 30 ± 1 °C at night for five consecutive days beginning at 50% heading. Ambient control plots were maintained under natural field temperature (32/24 °C day/night average) without chamber enclosure.

3.3 Measurement Schedule and Hypothesis

Chlorophyll fluorescence was measured on the flag leaf at 0, 3, and 5 days after stress onset, and again after five days of recovery under ambient conditions. Agronomic traits were recorded at physiological maturity. The guiding hypothesis was that heat-tolerant genotypes would exhibit smaller declines in Fv/Fm and ΦPSII, greater induction of NPQ, and correspondingly smaller yield losses relative to heat-sensitive genotypes.

Table 1: Experimental Design, Environmental Conditions, and Statistical Evaluation Metrics

Category	Heat-Stress Treatment	Ambient Control
Design	RCBD, 4 genotypes × 4 blocks	RCBD, 4 genotypes × 4 blocks
Day / night temperature	40 ± 1 °C / 30 ± 1 °C	32 ± 1 °C / 24 ± 1 °C
Relative humidity	55–60%	65–70%
Exposure duration	5 days at heading stage	Not applicable
Photoperiod	12 h light / 12 h dark	12 h light / 12 h dark
Statistical tests applied	Two-way ANOVA, Tukey HSD ($p < 0.05$), Pearson correlation, linear regression, PCA	—
Software used	R (v4.3), SPSS (v27), OriginPro 2023	—

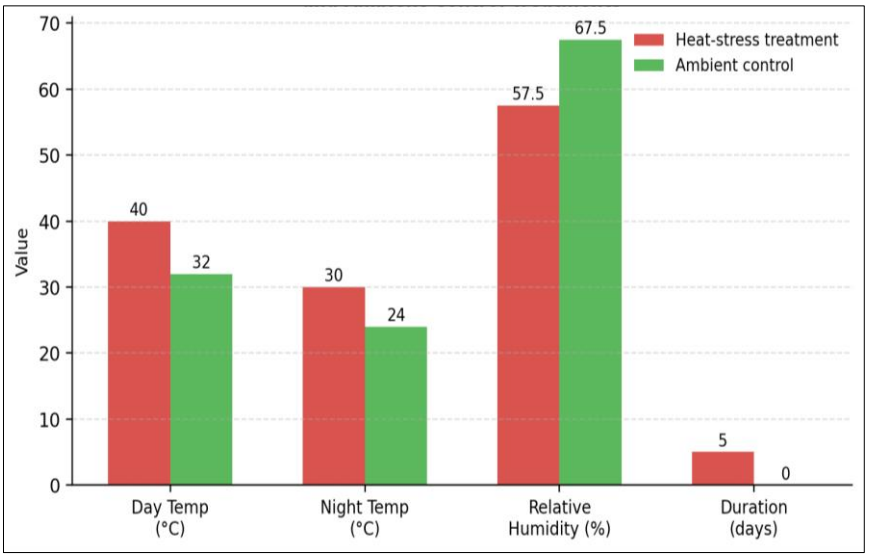

Fig 1: Compares the day/night temperature, relative humidity, and exposure duration applied under the heat-stress and ambient-control treatments described in Section 3.2.

4. Materials and Methods

4.1 Plant Materials

Certified seeds of four indica rice genotypes (G1, G2, G3, G4) were obtained from an institutional seed repository. Seedlings were raised in a wet-bed nursery for 21 days prior to transplanting at a spacing of 20 × 15 cm, one seedling per hill, into puddled field plots.

4.2 Experimental Site

The trial was conducted at a research farm situated in a subtropical rice-growing tract with a mean kharif-season temperature of 28–33 °C. Soil at the site was classified as clay loam with pH 6.8, organic carbon content of 0.62%, and available nitrogen, phosphorus, and potassium of 245, 18, and 210 kg ha^{-1}, respectively. Standard agronomic practices for fertilization, irrigation, and weed management were followed uniformly across all plots.

4.3 Heat Stress Treatment and Environmental Monitoring

Heat stress chambers were fitted with temperature and relative humidity sensors logging data at 15-minute intervals throughout the treatment period. Canopy temperature was additionally monitored using an infrared thermometer at midday. Ambient control plots were positioned at least 15 m from heat chambers to minimize thermal edge effects.

4.4 Chlorophyll Fluorescence Measurements

Fluorescence measurements were performed on fully expanded flag leaves using a portable pulse-amplitude-modulated fluorometer. Leaves were dark-adapted for 30 minutes prior to measuring minimal (F0) and maximal (Fm) fluorescence, from which Fv/Fm was calculated as (Fm−F0)/Fm. Light-adapted readings under ambient irradiance provided ΦPSII, from which ETR was derived as the product of ΦPSII, incident irradiance, leaf absorptance, and the PSII quantum fraction. NPQ was calculated as (Fm−Fm′)/Fm′, and qP as (Fm′−Fs)/(Fm′−F0′), following standard conventions [7].

4.5 Additional Physiological Measurements

Leaf chlorophyll content was estimated using a SPAD chlorophyll meter on the same leaves used for fluorescence measurement. Relative water content (RWC) was determined gravimetrically from leaf discs using fresh, turgid, and dry weights. Plant height was measured at maturity, and above-ground biomass was recorded after oven-drying at 70 °C to constant weight. Grain yield was recorded per plot and adjusted to 14% moisture content, while spikelet fertility was calculated as the percentage of filled spikelets per panicle across ten randomly sampled panicles per plot.

4.6 Statistical Analysis

Data were subjected to two-way analysis of variance (ANOVA) to assess the main effects of genotype and temperature regime and their interaction. Mean separation was performed using Tukey's honestly significant difference (HSD) test at $p < 0.05$. Pearson correlation coefficients were calculated between fluorescence parameters and yield components, and linear regression models were fitted to quantify predictive relationships. Principal component analysis (PCA) was used to summarize multivariate variation among fluorescence and physiological traits across genotypes and treatments. All analyses were performed in R (v4.3) and SPSS (v27), with graphical outputs generated in OriginPro 2023 and Microsoft Excel.

Table 2: Chlorophyll Fluorescence Parameters Used for Evaluating Heat Stress Tolerance in *Oryza sativa* L.

Parameter	Definition / Description	Physiological Significance under Heat Stress
F0	Minimal fluorescence of dark-adapted leaves	Rises when PSII reaction centres are damaged by heat-induced membrane instability
Fm	Maximal fluorescence of dark-adapted leaves	Declines under thermal disruption of thylakoid electron transport
Fv/Fm	Maximum quantum efficiency of PSII (Fm−F0)/Fm	Most widely used indicator of PSII photoinhibition; values <0.75 signal stress
ΦPSII	Effective quantum yield of PSII in light-adapted state	Reflects actual photochemical efficiency during active photosynthesis
ETR	Electron transport rate through PSII	Indicates capacity to sustain electron flow for carbon assimilation
NPQ	Non-photochemical quenching	Represents thermal dissipation of excess excitation energy, a protective mechanism
qP	Photochemical quenching coefficient	Denotes the proportion of open PSII reaction centres available for photochemistry

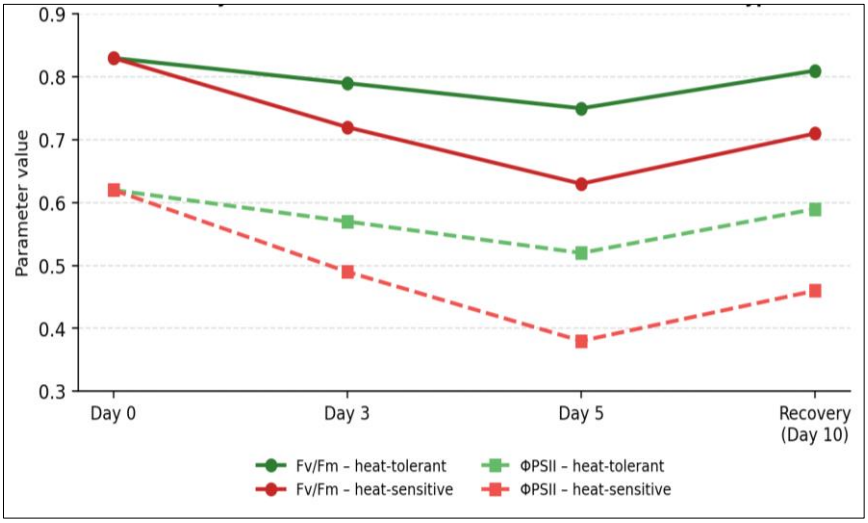

Fig 2: tracks Fv/Fm and ΦPSII across the stress and recovery period, showing the divergence between heat-tolerant and heat-sensitive genotypes described in the results below.

5. Results and Performance Evaluation

Heat stress significantly reduced Fv/Fm across all genotypes relative to ambient controls ($p < 0.01$), with the magnitude of decline differing substantially by genotype (genotype × temperature interaction, $p < 0.01$). Heat-tolerant genotypes G1 and G2 maintained Fv/Fm values above 0.74 after five days of stress, whereas heat-sensitive genotypes G3 and G4 declined to approximately 0.62–0.65, indicating pronounced photoinhibition.

PSII followed a similar pattern, declining more sharply in heat-sensitive genotypes and showing only partial recovery five days after stress removal in G3 and G4, while G1 and G2 recovered to near pre-stress levels. ETR mirrored ΦPSII trends, consistent with reduced linear electron transport capacity under sustained thermal load in sensitive genotypes.

NPQ increased in all genotypes under stress but rose more steeply in heat-tolerant lines, suggesting stronger thermal dissipation of excess excitation energy. Conversely, qP declined more in heat-sensitive genotypes, indicating a greater proportion of closed PSII reaction centres under stress.

Canopy temperature, SPAD chlorophyll content, and RWC all showed significant treatment effects, with heat-sensitive genotypes exhibiting greater chlorophyll degradation and lower RWC under stress. Grain yield was reduced by approximately 38–44% in heat-sensitive genotypes compared with 18–22% in

heat-tolerant genotypes, and spikelet fertility declined correspondingly.

Correlation analysis revealed strong positive associations between day-5 Fv/Fm and both spikelet fertility ($r = 0.81$, $p < 0.001$) and grain yield ($r = 0.76$, $p < 0.001$) across genotypes and replications, while ΦPSII showed a comparable relationship with yield ($r = 0.71$, $p < 0.001$). Regression models indicated that Fv/Fm at day 5 of stress explained approximately 65% of the variation in grain yield loss ($R^2 = 0.65$). Principal component analysis separated genotypes primarily along a first component (accounting for 58% of total variance) loading heavily on Fv/Fm, PSII, and NPQ, clearly distinguishing heat-tolerant from heat-sensitive genotype clusters.

6. Discussion

The consistent decline in Fv/Fm and PSII under heat stress reflects impaired PSII function arising from thermal disruption of thylakoid membranes and reaction-centre proteins, consistent with mechanisms described in earlier heat-tolerance studies [4, 14]. The relatively smaller decline observed in heat-tolerant genotypes, together with their steeper NPQ induction, suggests that thermal dissipation of excess absorbed energy serves as a key protective mechanism limiting photo-oxidative damage during heat episodes, aligning with photoprotection concepts described for other cereal crops [8, 9].

The strong correlation between Fv/Fm and both spikelet fertility and grain yield supports the utility of chlorophyll fluorescence as an early, non-destructive indicator of heat-induced yield loss, well before visible canopy symptoms appear. This has direct implications for breeding programmes, where fluorescence-based screening could enable rapid, high-throughput pre-selection of heat-tolerant lines from large segregating populations, complementing conventional yield-based selection that requires full-season evaluation [17, 19].

For climate-resilient crop improvement, integrating fluorescence-based indicators with molecular markers may accelerate development of cultivars suited to variable thermal regimes. In precision agriculture, portable and imaging-based fluorometers could further support in-season stress monitoring, enabling timely management interventions to mitigate heat-related yield loss.

Several limitations warrant consideration. Open-top chambers, while preserving natural light and canopy microclimate better than growth chambers, can introduce localized humidity and airflow differences relative to fully open fields, and single-season, single-location results may not capture the full range of genotype-by-environment interactions relevant to heat tolerance. Environmental variability across blocks, despite RCBD control, may also contribute residual variance beyond treatment effects.

Future work should extend this approach across multiple locations and seasons, integrating fluorescence phenotyping with high-throughput imaging, aerial remote sensing, and machine-learning trait prediction. Coupling physiological phenotyping with genomic selection may further accelerate identification of heat-tolerance loci in breeding pipelines.

7. Conclusion

The differences in chlorophyll fluorescence of rice seedlings grown in the field indicated the effectiveness of this research. Fv/Fm and ΦPSII were identified as the most sensitive indicators of heat tolerance in plants. The heat-resistant plants outperformed the heat-sensitive ones in terms of photosynthesis efficacy and degree of non-photochemical quenching resulting in less loss of yield. Growing crops in the field combines the advantage of the controlled temperature treatment and the reality of the field conditions, making this technique a practical one, which uses the benefits of both growth chamber and open field experiments. The results of the research prove the efficiency of chlorophyll fluorescence as a rapid method of testing materials for breeding purposes in search of heat-resistant rice varieties. Due to limitations of the study, there is a need for further research in other regions and during different growing seasons and use of genetic methods in breeding.

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