HIGH TEMPERATURE MEDIATED CHANGES IN SUGAR SIGNALING PATHWAY AND IDENTIFICATION OF ASSOCIATED MICROSATELLITE MARKERS IN RICE (*Oryza sativa* L.)

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(2018-21-053)

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By

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THESIS

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2022

DECLARATION

I, hereby declare that this thesis entitled, **"High temperature mediated changes in sugar signaling pathway and identification of associated microsatellite markers in rice (*Oryza sativa* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled, **"High temperature mediated changes in sugar signaling pathway and identification of associated microsatellite markers in rice (*Oryza sativa* L.)"** is a record of research work done independently by Mr. Stephen Kukkamudi under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of Mr. Stephen Kukkamudi (2018-21-053), a candidate for the degree of **Doctor of Philosophy in Agriculture** with major in Plant Physiology, agree that the thesis entitled **"High temperature mediated changes in sugar signaling pathway and identification of associated microsatellite markers in rice (*Oryza sativa* L.)"** may be submitted by Mr. Stephen Kukkamudi, in partial fulfillment of the requirement for the degree.

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EXTERNAL EXAMINER

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Stephen

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LIST OF ABBREVIATIONS

CO_2	carbon-dioxide
ppm	parts per million
%	percent
˚C	degree celsius
cm	centimeters
m	meter
nm	nano meters
g	gram
kg	kilogram
µg	microgram
µl	microliter
ml	milliliter
Fv/Fm	variable to maximal fluorescence
NPQ	non-photochemical quenching
kDa	kilo daltons
rpm	rotations per minute
kPa	kilo pascals
µmol	micro-moles
mmol	milli-moles
s	seconds
mol	moles
h	hours

Introduction

1. INTRODUCTION

It is quite well known that more than half the world's population consume rice as their staple food (Muthayya *et al*., 2014). India is the second largest producer of rice with a production capacity of more than 140 million tonnes per year (Dawe *et al*., 2010). Rising global temperatures as a result of climate change and global warming is a serious impediment for achieving higher productivity in rice cultivation. In the crop modelling studies on rice production, Saseendran *et al*. (2000) predicted a significant reduction in crop yield in a scenario of 1.5 ˚C rise in temperature by 2040-2049 compared to 1980's in India's southern State of Kerala, as a result of reduced growth period and early maturation.

As a result of ambient temperature increase of 1.6 ˚C - 2.6 ˚C from the 1986-2005 period, the average crop productivity is estimated to decrease significantly in tropical regions such as South Asia. The risks are mainly posed by limitation of water resources and failure of crop adaptation (O'niell *et al*., 2017). The intensity of higher temperatures in the form of heat waves is projected to decisively reach a high risk point by the year 2035 (Change, 2013). This provides a background highlighting the importance of assessing crop performance under high temperature stress and the need for identifying better adaptation mechanisms in plants.

One of the important mechanisms to impart tolerance to heat stress is by regulating the sugar metabolism. Sugars such as sucrose, fructose and trehalose function as osmo-protectants in regulating the osmotic adjustment, membrane protection and scavenging toxic reactive oxygen species (ROS) against various kinds of stresses (Singh *et al*., 2015). Sugars control the expression of several functional plant genes and thereby many metabolic and developmental processes (Koch, 1996). A large number of stress responsive genes have been reported to be induced by glucose, indicating the role of sugars in environmental responses (Price *et al*., 2004). Sugars are involved in the regulation of growth activities by modulation of gene expression and enzyme activities in source and sink tissues ensuring the optimal synthesis and use of carbon and energy resources (Corruzzi and Bush, 2001).

Sugars have a dual role as they can function as both a metabolite and a signaling molecule in a manner similar to hormones. The two functions are distinct from one another as evidenced by partially metabolizable or non-metabolizable analogs of sucrose or hexose analogs to induce gene expression without the requirement of sugar catabolism indicating signal sensing and transduction mechanism (Rolland *et al*., 2002). Sugars such as glucose, fructose, sucrose or trehalose, when acting as signaling molecules play a significant role in linking the energy or carbon nutrient status of the cells or tissues to plant growth and development responses (Smeekens *et al*., 2010). The major metabolic sensors involved in sensing the sugar signals or the glycolytic flux are hexokinases (*HXK*) which sense glucose content; Trehalose Phosphate Synthase (*TPS*) which produces trehalose-6-phosphate; the Target of Rapamycin (*TOR*) kinase system and Sucrose non-fermenting 1- related protein kinase (*SnRK1*). *HXK, TPS* and *TOR* are involved in growth promoting functions while *SnRK1* is specific to growth inhibitory functions.

As the sugar signaling pathways and components have yet to be fully elucidated, the research on the gene expression of the important regulatory components in the sugar signaling mechanism especially under stress is of vital interest. Therefore, in the current study, the sugar sensing orthologs reported in rice plant viz. *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR* have been investigated under high temperature conditions.

The sugar signaling mechanism is key to regulating the allocation, partitioning and assimilation of the photo-assimilates in the source and sink organs of the plant. This knowledge has significant applications in agriculture by modulating the components of the sugar signaling mechanism or by selecting varieties that are more efficient in their signaling pathways, thereby, contributing to higher yield.

As heat stress is a serious impediment for optimum crop growth and development, it is crucial to identify genomic regions with Quantitative Trait Loci (QTL) that are associated with tolerance traits. In this regard, bulked segregant analysis (BSA) is used to rapidly identify molecular markers that are closely linked to QTLs of interest (Zou *et al*., 2016). Marker-assisted selection (MAS) for developing improved cultivars can be

achieved by using micro-satellite markers in combination with BSA (Barakat *et al*., 2011). Currently, Simple Sequence Repeats (SSR) also known as micro-satellite markers are the most widely used markers in MAS as they are cheap, easily available and require a simple technique while providing a higher polymorphism rate (Gao *et al*., 2016).

Therefore, the present investigation entitled, "High temperature mediated changes in sugar signaling pathway and identification of associated microsatellite markers in rice (*Oryza sativa* L.)" was conducted with the afore-mentioned concerns about the impacts of heat stress with the following objectives-

1) To study the effect of high temperature on sugar signaling pathway.
2) To identify the molecular markers associated with high temperature tolerance in rice using bulked segregant analysis.

Review of Literature

2. REVIEW OF LITERATURE

The current review focusses on the changes in climate occurring due to global warming and the effects of high temperature stress on plant growth and functions. It also covers the mechanism of sugar signaling and the advances made regarding marker-assisted selection (MAS). As the present investigation is greatly dependent on understanding the above aspects, the research findings regarding these topics is presented through the following review of scientific literature.

2.1 Climate Change

Climatological data analyzed in the State of Kerala over the past 49 years prior to the year 2008 revealed that on average, the maximum day temperature increased by 0.64 ˚C and night temperature by 0.23 ˚C. It is expected to continue increasing to about 3 ˚C by the year 2100 (Rao *et al*., 2009). The aberrations in the temperature and rainfall patterns can cause unpredictable extreme weather phenomenon such as droughts, floods and heat waves causing a drastic decline in the level of food production.

Kumar *et al*. (2011) in their assessment of climate data have estimated that temperature in southern Kerala are likely to be increased by about 1.5 ˚C by the year 2030 taking the years 1969-1990 as the baseline. The rainfed rice production is expected to be reduced by about 10 percent in such a scenario. It is to be noted that the observed rate of climate change is greater than what is predicted currently (Vermuelen *et al*., 2012).

2.2 Effect of high temperature on plant characteristics

2.2.1 Plant height

The shoot growth of rice seedlings, grown at 40/35 ˚C (day/night temperature), was inhibited by 20-21 percent compared to the control conditions (30/25 ˚C). The root growth was also found to be inhibited by 14-18 percent under higher temperatures (Kumar *et al*., 2012). Evaluation of rice genotypes N22, NH219 and IR64 noted that under heat stress conditions (average 44.6 ˚C – booting stage till maturity), the plant height was increased

in all three genotypes. The increase in height was greater in the mutant line, NH219 (12.82 %) than in N22 (4.59 %) compared to the plants grown in ambient conditions (Poli *et al*., 2013).

A combined stress of drought and high temperature (- 50 kPa, 33.9 ˚C - 34.5 ˚C) in the rice varieties N22, Dular and Anjali caused a significant ($p<0.001$) reduction in the plant height in all three genotypes (Lawas *et al*., 2018). In a study that evaluated 48 stable rice lines under high temperature conditions (44.3 ˚C- stress and 30 ˚C - control), it was revealed that the mean plant height was significantly increased (by 9.55%) under stress compared to control conditions (Prasanth *et al*., 2017).

The above studies are an indication that plant height is inhibited mostly as a result of water limitation rather than through the sole effect of a rise in the tissue temperature. The studies on the individual effects of heat reveal that plants possess mechanisms that can adapt by increasing the plant height rather than by decreasing it.

2.2.2 Tiller number

Aghamolki *et al*. (2014) in their study on four rice varieties, Hovaze, Hashemi, Fajr and MR219 noted that heat stress (38 ± 2 ˚C at booting, flowering and ripening) had no significant effect on the tiller number compared to the plants subjected to ambient conditions (32 ± 2 ˚C). They indicated that the stage at which the crop is subjected to heat stress would determine the intensity of the effect concluding that heat stress at the later stages of reproductive development had no influence on tillering.

In a study on the transgenic thermotolerant variant (T-Oa-19) of Rubisco activase (Rca) showed that the recombinant plants grown at higher temperatures of 45 ˚C (early tillering till panicle initiation) recorded higher panicle number. However, the tiller number measured at maturity was equal in both the genotypes. This proves thermotolerance mechanisms that improve the yield through reproductive traits are different from the contribution through vegetative traits such as tiller number (Scafaro *et al*., 2018). Gaballah and Abu El-Ezz (2019) in their study that evaluated 13 rice genotypes and reported that

under high temperature conditions (38 – 42 ˚C) during the active tillering stage, there was a significant reduction in the tiller number. They also noted a negative correlation of the number of tillers per plant with the yield index, however, they were positively correlated with the number of panicles per plant underlining their contribution to plant yield.

The tiller number of rice genotypes N22 and CO51 was found to be increased under elevated temperature (+ 5 ˚C of ambient). However, they were negatively correlated with yield pointing in the direction that lesser number of tillers contribute to mitigation of heat stress through remobilization of reserves, thereby maintaining yield (Vinitha *et al*., 2020).

2.2.3 Flowering Characteristics

2.2.3.1 Days to flowering

Raju *et al*. (2013) evaluated 43 rice genotypes in three environmental conditions and reported that there was incomplete exertion of panicles and earlier days to flowering in the plants subjected to higher temperatures (39.2 ˚C). In a study conducted by Beena *et al*. (2018), 29 rice genotypes were evaluated under high temperature conditions (6-8 ˚C above ambient) at panicle initiation. On an average, under stress, the days to 50 percent flowering was advanced by 2-7 days compared to the non-stressed treatment.

Julia and Dingkuhn (2012) in their study evaluating different varieties in four varying climatic environments found that plants grown in southern France (28.7 – 29.7 ˚C) had longer duration from sowing to flowering (128.5 days) compared to the plants grown in the hot-dry season in Senegal (35.6 ˚C) which had a mean of 84.4 days to flowering. They also studied the time of anthesis and noted that it was dependent on the air temperature and relative humidity especially 7 days prior to anthesis. They recorded that the time of anthesis was delayed by lower minimum temperature whereas, under lower vapour pressure deficit (VPD), it was advanced.

Using transgenic lines that were modified to overexpress DTH8 (QTL for days to heading) in rice, Mishra *et al.* (2022) have indicated that the physiological mechanism of the flowering period can be controlled through molecular approaches.

2.2.3.2 Time of anthesis

Hirabayashi *et al*. (2014) identified a novel QTL, *qEMF3* for the early-morning flowering trait which could advance the flower opening time (FOT) by 1.5 – 2.0 h such that the plant can avoid higher temperatures (> 35 ˚C) by completing anthesis earlier. Near-isogenic lines (NILs) of IR64 carrying the *qEMF3* QTL (IR64 + *qEMF3*) on exposure to temperatures above 36.1 ˚C recorded lower spikelet sterility which is attributed to the earlier flower opening time (Ishimaru *et al*., 2022). This points to the uncharted area of research that can utilize the early flowering character of plants as a robust mechanism to ensure reproductive success leading to increased yield under heat stress.

2.2.3.3 Pollen viability

Kumar *et al*. (2015) studied the pollen characteristics of 11 rice genotypes and noted that at temperatures > 35 ˚C, a reduction in the pollen viability was observed in the range of < 15 percent for genotypes IET21415, IET21577 and PA6444, whereas it was in the range of 15 – 50 percent for the remaining genotypes. They also observed that under heat stress, the pollen grains had smaller diameter (31.4 µm) compared to control (36.1 µm).

Assessing 11 Korean rice cultivars under high temperature stress, Thuy *et al*. (2020) reported that there was a significant reduction in pollen viability at 33 ˚C (57.9 – 72.1 %) compared to the plants grown at 24, 28 and 30 ˚C which recorded >70 percent pollen viability. They also noted that plants subjected to heat stress experienced anthesis earlier than plants subjected to lower temperature. A similar trend was observed in the investigation by Zhang *et al*. (2016), wherein the pollen viability in the susceptible rice genotype, GT937 was reduced by 53.38 percent under higher temperatures (40 ˚C) compared to the tolerant genotype, N22 which was reduced by only 2.55 percent.

2.2.4 Membrane stability index (MSI)

In an experiment involving 6 rice genotypes subjected to elevated temperatures of >39 ˚C, it was reported that the membrane stability index of plants subjected to elevated

temperature was reduced significantly compared to the control treatment (27.8 – 34.4 ˚C). The tolerant varieties (IET2577, IET21404) recorded lesser reduction in MSI (5-11%) compared to other varieties (>20%). The decrease in MSI was attributed to the lipid peroxidation of the membranes resulting in membrane leakage (Kumar *et al*., 2016). Cell membrane stability index is one of the methods that can quickly and reliably measure the damage caused to the membranes under stress conditions. Some other methods that can be associated with MSI are lipid peroxidation (malondialdehyde content) (Zafar *et al*., 2020), membrane leakage test (Karwa *et al*., 2020) and cell membrane thermostability.

Zafar *et al*. (2017) evaluated 46 rice genotypes under higher temperature (46 ˚C) and based on relative cell membrane thermostability (CMTS) could identify 15 genotypes as heat tolerant and HTT-1 as heat sensitive genotype. They denoted Kashmir basmati and HTT-114 as heat tolerant checks at early growth stage.

2.2.5 Gas exchange and fluorescence-related parameters

In a study by Thussagunpanit *et al*. (2015), high temperature stress (40/30 ˚C, day/night) was reported to cause a significant reduction in net photosynthetic rate (Pn) in rice. Comparitively, plants treated with 24-epibrassinolide (EBR) could maintain higher Pn. Similar drastic reduction in the stomatal conductance (Gs), evapotranspiration (E) and water-use efficiency was noted in plants subjected to heat stress. The fluorescence parameters Fv/Fm and Fv'/Fm' were significantly higher in EBR treated plants compared to the non-treated plants. However, ΦPSII did not show any significant variation between the treatments although NPQ was increased significantly in the control.

Zhao *et al*. (2009) reported that under elevated temperature (38 ˚C), the non-transgenic rice genotype showed a 4.7 percent decrease in the net photosynthetic rate compared to the transgenics (with Glutathione-S-transferase (GST) and Catalase 1 (CAT)). The improved performance of the transgenics under stress is attributed to the better scavenging mechanisms of the encoded anti-oxidants.

In their investigation on the effects of heat stress (40 ˚C) on the spikelets and flag leaves in rice, Zhang *et al*. (2015) noted that although the difference in the temperature of the spikelets of the two rice genotypes, N22 (tolerant) and GT937 (susceptible) was not significant, the damage to the spikelets of GT937 was greater than that of N22 under heat stress. This points to the suggestion that control of organ temperature is dependent on transpirational cooling, whereas, the alleviation of the heat stress effects is through the anti-oxidative capacity of the genotypes. The transpiration rate of the spikelets was recorded to be higher in the heat stressed plants.

Islam *et al*. (2011) conducted an experiment under elevated temperature (34 ˚C) on five aromatic rice varieties and recorded that the mean photosynthetic rate was significantly lower at booting stage (16.7 µmol m^{-2} s^{-1}) and at grain filling stage (13.5 µmol m^{-2} s^{-1}) compared to the plants grown under non-stressed conditions (23.2 µmol m^{-2} s^{-1}). Contrastingly, the stomatal conductance was increased in plants grown at 34 ˚C (0.38 mol m^{-2} s^{-1}) compared to that of ambient conditions (0.29 mol m^{-2} s^{-1}). The transpiration rate also followed a similar trend with an increase under stress at booting stage (5.26 mol m^{-2} s^{-1}) and grain filling stage (5.82 mol m^{-2} s^{-1}) while it was significantly lower (3.81 mol m^{-2} s^{-1}) under non-stressed conditions.

In a similar experiment, evaluating four rice genotypes, N22, IR52, IR20 and IR46 under different temperature conditions (29, 33, 37 and 41 ˚C), it was noted that N22 could maintain a higher transpiration rate, which was greater than the other varieties at 41 ˚C indicating that its mechanism of heat tolerance was through transpirational cooling (Egeh *et al*., 1992). A study by Xiong *et al*. (2014) also noted a comparative trend in their observations on three rice cultivars, Koshikari, ZS97 and N22 under elevated (35 ˚C) and ambient (28 ˚C) temperatures. Even under sufficient nitrogen supply (0.538 g urea kg^{-1} soil), there was a significant reduction in the photosynthetic rate in the plants subjected to stress in all three varieties while the stomatal conductance and transpiration rate were increased only in ZS97 and N22. On correlation of parameters with nitrogen content and yield, it was observed that 1000 grain weight was only marginally decreased in N22 (23.7 g – ambient & 22.8 g – stress), whereas it was significantly decreased in the other two

cultivars. Strong correlation was obtained between the grain yield and increased transpiration rate in N22 under heat stress confirming it as a tolerance mechanism for that variety.

Vivitha *et al*. (2018) investigated RILs of rice variety IWP introgressed with QTLs qHTSF 1.1 or qHTSF 4.1, which were related to spikelet fertility. They noted that the positive lines showed better Fv/Fm values (0.636, 0.672) under high temperature stress (37 – 42 ˚C) which resulted in higher photosynthetic rate (26 – 28.53 µmol CO_2 m^{-2} s^{-1}) as the PSII was protected from damage due to heat stress. The higher stomatal conductance (0.59 – 0.75 mol H_2O m^{-2} s^{-1}) was strongly correlated to the photosynthetic rate, indicating its contribution to the crop yield. The positive RILs were also recorded to maintain lower transpiration rates (6.27 – 6.83 mmol H_2O m^{-2} s^{-1}) under elevated temperature.

Chandrakala *et al*. (2013) reported on the amelioration of the effects of heat stress on the photosynthetic characteristics of two contrasting rice genotypes viz. Pusa Sugandh 5 (susceptible) and NERICA L-44 (tolerant) by spraying different concentrations of three signaling molecules (24-epibrassinolide, calcium chloride and salicylic acid). The treated plants exposed to high temperature stress (36 ± 1.7 ˚C) for two weeks at the pre-anthesis stage showed higher photosynthetic rate of stomatal conductance and Fv/Fm ratio (PSII efficiency). However, the transpirational loss of water was recorded to be higher under high temperature stress. The ameliorative effects were more pronounced in Pusa Sugandhi 5. Hermann and Gabriel (2013) evaluated two rice genotypes, F50 and F733 under heat stress (40 ˚C, $2^{1/2}$ h for 5 days) to assess the impact of short duration stress on plants. They reported that under stress conditions, the photosynthetic rate was significantly reduced along with stomatal conductance at both the panicle initiation phase (IP) and grain-filling (GF) phases compared to plants grown in normal conditions.

Caine *et al*. (2019) genetically engineered the rice cultivar IR64 to produce lesser number of stomata in order to conserve water. When the transgenic plants were subjected to high temperature stress of 40 ˚C, it was observed that the transpirational water loss was reduced significantly. However, the stomatal conductance was maintained at the level of

the control treated plants (30 ˚C) by increasing the size of the stomatal pore. The transformed plants could maintain plant yield under heat stress on par with plants grown under non-stressed conditions, although the photosynthesis was observed to be decreased. This implies that the stomatal conductance and stomatal density have a crucial role to play in increasing crop yield under stress conditions.

2.2.6 Leaf temperature

On exposure to elevated temperatures (>33 ˚C), Yun-Ying *et al*. (2008) in their study on 4 rice genotypes recorded significant rise in the leaf temperature. The difference in the leaf temperature between the heat stress treatment and the control treatment was recorded to be higher in the heat sensitive genotypes, Shangui 1 and T219 (3.5 ˚C and 6.8 ˚C) whereas it was comparatively lower in the tolerant genotypes, Huanghuazhan and T226 (1.9 ˚C and 2.5 ˚C).

Yan *et al*. (2010) studied the effect of heat stress (32.2 – 35.2 ˚C) on 21 *indica* and 29 *japonica* rice varieties and recorded significant difference in the leaf temperature (3.8 – 5.5 ˚C) under stress across all genotypes. The temperature difference of the leaves was in the decreasing order of flag leaf>second leaf>third leaf indicating that the upper organs of the plant have higher temperatures compared to the lower parts.

Zhang *et al*. (2012) in their study evaluating various early maturing rice varieties for heat stress tolerance reported that the leaf temperature was increased with increasing air temperature across all varieties, although the leaf temperature was slightly lower than the surrounding air temperature. It was also noted that with rising air temperatures, the stomatal conductance was also increased upto 38 ˚C. Based on these parameters, the variety Yuexianzhan was categorized as heat tolerant.

Li *et al*. (2020) conducted an investigation involving the wild-type (WT) of rice variety Nipponbare and its mutant with semi-rolled leaves and susceptibility to heat stress (*hts*). When subjected to high temperature stress (45 ˚C), the leaf temperature was found to be increased by 1.5 ˚C and 2.5 ˚C in *hts* mutant compared to WT plants under control

(30 ˚C) and stress conditions respectively. The increase in leaf temperature is implicated to be the major cause of damage under heat stress as it leads to increased rate of respiration and consumption of carbohydrates. The stomatal conductance was significantly lower in the susceptible *hts* mutant under both temperature conditions.

2.2.7 Panicle length

Zhen *et al*. (2019) subjected the japonica rice varieties, Nanjing 41 and Wuyunjing 24 to short term heat stress at the booting stage and noted that compared to treatments of lower temperature (27 ˚C), the temperature treatment (39 ˚C) recorded a significant reduction in the panicle length by 16.67 percent in Nanjing 41 and 20.65 percent in Wuyunjing 24. Ramadan *et al*. (2021) reported that there was significant correlation between panicle length and spikelet fertility percentage in plants grown at elevated temperatures (37.4 – 45.6 ˚C). Among the 25 rice genotypes evaluated, the varieties Bala (14.03 %), Dular (14.89 %) and N22 (14.95 %) recorded the least reduction in panicle length compared to the plants grown at lower temperatures (29.7 – 34.7 ˚C). Susceptible varieties such as GZ10101-5-1-1-1 and CO39 recorded a reduction of 35.49 percent and 35 percent respectively under stress.

In contrast, significant difference in the length of the panicle was not observed between the stress treated (39 ˚C) plants and the control treated (31 ˚C) plants in the rice genotypes N22 and IR64 (Jagadish *et al*., 2013). Lawas *et al*. (2018) also reported that the panicle length of rice sheathed panicle (SP) phenotype (cv. Sathi) at high temperature (38 ˚C) was not significantly affected compared to plants grown under ambient temperature (29 ˚C). The variation observed between the various studies regarding panicle length could be due to the difference in the intensity and duration of the stress or the cultivars being investigated.

2.2.8 Spikelet fertility

The tolerant rice cultivars M9962 and M7988 were reported to maintain higher spikelet fertility under heat stress. This has been attributed to better anther dehiscence,

higher percentage of pollen viability and pollen germination, all of which were found to be decreased in the susceptible genotype Sinlek (Malumpong *et al*., 2019). Based on the seed-setting rate, Cheabu *et al*. (2018) selected 11 rice accessions out of 169 exotic germplasm tested under higher temperatures (40-45 ˚C). The varieties N22, AUS17 and M9962 were identified as most tolerant as they could maintain a spikelet fertility of >80 percent.

Rice introgression lines evaluated for heat tolerance revealed significant association between spikelt fertility and yield per plant under elevated temperature conditions based on heat susceptibility index (HSI) as criteria. The SSR markers RM229, RM430 and RM210 were reported to be significantly associated with spikelet fertility under heat stress conditions (Prasanth *et al*., 2016). Auxins have been implicated in preventing the inhibition of pollen tube elongation in plants subjected to heat stress. Zhang *et al*. (2018) noted that spikelet sterility was significantly lower in the rice genotype Nipponbare under high temperature stress (40 ˚C) when sprayed with 1- naphthalene acetic acid. A relationship between reactive oxygen species (ROS) signaling and peroxidase (POD) activity was also implicated in the mechanism of heat tolerance.

Spikelet fertility is a reliable screening tool that can be used to characterize heat tolerance in rice. Prasad *et al*. (2006) reported that lower spikelet fertility was caused by decreased pollen viability and failure of pollen germination on stigma. Of the 14 rice cultivars evaluated under high temperature stress, WAB-12, CG-14, L-204 were identified as highly heat-susceptible and IR-8 and IR-72 as moderately susceptible to heat. Using QTL-seq approach, Nubankoh *et al*. (2020) identified three QTLs, qSF1, qSF2 and qSF3, that were associated with spikelet fertility under heat stress (40-45 ˚C). Potential candidate genes that were specific to the anthers were identified in the QTLs.

2.2.9 1000 grain weight

The reduction in the grain yield of the heat sensitive cultivar, Shanggui (35.3 – 39.5 %) was significantly higher than the heat tolerant cultivar, Huanghuazhan (21.7 – 24.5 %) under high temperature stress (>35 ˚C). The decrease in 1000 grain weight under stress

was the resultant of poor anther dehiscence which affected pollen viability percentage and lowered the spikelet fertility (Yun-Ying *et al*. 2008).

Chen *et al*. (2021) reported significant reduction in the 1000 grain weight of the rice plants subjected to heat stress (40 ˚C). The negative effects of high temperature stress was found to be alleviated to a good extent on spraying spermidine (1.5 mM). The gene *OsSAP5* was found to be up-regulated significantly which might be involved in the heat tolerance mechanism mediated by spermidine.

A QTL associated with 1000-grain weight was found to be located on chromosome 6 in rice, which explained 30.6 percent of the total phenotypic variance. The QTL which was located at the locus of RM103 was identified by screening BC_2F_2 lines (OM5930 x N22) under high temperature stress conditions (36 – 44 ˚C) at flowering (Buu *et al*., 2014).

2.3 Mechanism of sugar signaling

2.3.1 Role of sugars in plants

Sugars/carbohydrates are generally considered as resources or building blocks for structural integrity, metabolism, energy production, intermediary molecules or storage functions. Apart from being organic components in metabolism, they are also involved in altering the gene expression during several growth and developmental processes. Usually hormones are generally the standard examples of signaling that are effective strictly at micro-molar concentrations. However adequate evidence has been provided to prove that sugars also act as signaling molecules, albeit at milli-molar concentrations. The dual function of sugars as signaling molecules and metabolites have separate mechanisms.

The mechanism of sugar signaling majorly involves components such as sensors/receptors, signaling molecules and the transduction pathway. The sugars that have been widely implicated in the signaling process are sucrose, glucose, fructose, maltose, mannose and trehalose. In the current study, four sensors have been investigated for their role in regulating sugar signals under high temperature stress viz. hexokinase (*HXK*), sucrose non-fermenting related kinase-1 (*SnRK1*), target of rapamycin (*TOR*), trehalose

phosphate (*TPS*). Sugar signals may be generated through metabolic fluxes, enzyme-derived, carbohydrate concentrations relative to other metabolites. The sugars may regulate the gene expression through signal transduction cascades either at the transcriptional or post-transcriptional level (Sheen *et al*., 1999). The secondary messengers involved in the signaling cascades of protein kinases are cytosolic Ca^{2+}, cAMP, cGMP, inositol phosphate etc.

Extensive interactions have been found between sugar regulation and plant hormones. Hexose signaling reportedly regulates the balance between auxin and cytokinin which is essential for root/shoot development and embryogenesis. Strong regulatory relationship exists between sugars and nitrogen as evidenced by the control of gene expression of nitrate reductase, nitrate transporters, asparagine synthase (*ASN2*) and glutamine synthase (Jang and Sheen, 1997; Lam *et al*., 1998; Lejay *et al*., 1999).

Soluble sugars have been reported to increase the tolerance of plants to oxidative stress through various direct and indirect mechanisms (Couee *et al*., 2006). Sugar accumulation through variations in environmental conditions is expected to initiate leaf senescence. The sensing of low nitrogen content under these conditions is made possible through hexokinase-mediated sugar signaling (Wingler *et al*., 2006)

2.3.1.1 Hexokinase (HXK)

HXK is a dual function enzyme that has a major role in metabolism of glucose through glycolysis. As a sensor, it is known to sense the signal of glucose-6-phosphate (Jang *et al*., 1997). *HXK* indicates sugar availability in the cells or tissues. In a manner that is similar to other receptors, conformational changes in *HXK* occur through the binding of a ligand leading to modification in the protein-protein interaction of the receptor producing a signaling cascade. *HXK* is implicated to play a role in multiple signaling cascades in the cells. *HXK*s are reported to down-regulate photosynthesis. Hexokinases have also been noted to be involved in catalyzing enzymatic reactions in the sink organs in various crops through their signaling functions (Aguilero-Alveredo and Sanchez-Nieto, 2017).

The evidence for the unique signaling function of hexokinase is provided by Pego *et al*. (1999) who found that mannose (glucose analog) could inhibit seed germination at low levels without being phosphorylated by hexokinase. They also reported that mannoheptulose (competitive inhibitor of hexokinase) caused the de-repression of germination, thereby proving the critical role of hexokinase in the signaling mechanism,

Experiments on the *gin2* (glucose insensitive) mutants have offered ample evidence that the glucose sensor, At*HXK*1 integrates nutrient, light and hormonal signals to co-ordinate the plant responses to the glucose content (Rolland and Sheen, 2005). In yeast, the glucose sensor *HXK*2 is involved in inactivating the key protein kinase, Snf1 (Sucrose non-fermenting 1) through de-phosphorylation by interacting with Glc7-Reg1 protein phosphatase 1 (PP1) complex. This mechanism is indicated to be the main pathway for repression of several genes involved in gluconeogenesis and respiration (Rolland *et al*., 2006). *OsHXK2*, a type-B hexokinase was reported to be localized on the mitochondrial membrane indicating its role in regulating the balance between anabolic and catabolic functions (Cheng *et al*., 2011).

2.3.1.2 SnRK1

Sucrose non-fermentation 1-related protein kinase (*SnRK1*) is an energy sensor protein that is critically involved in low energy status in plants by regulating gene expression. *SnRK1* is known to be localized in the nucleus where it induces transcriptional networks to activate genes involved in stress responses thereby maintaining an energy homeostasis in the plants (Cho *et al*., 2012). *SnRK1* is essential for maintaining the plant metabolism and their normal development under carbon starvation and dark conditions. Shortening of lifespan is a characteristic feature that was noted in plants with *SnRK1* knockdown. It has also been well corroborated as a central regulator integrating carbon, lipid and nitrogen metabolism as well as hormonal signals and energy status of the plants (Emanuelle *et al*., 2015). Radchuk *et al*. (2006) reported that repression of *SnRK1* was associated with a decreased accumulation of cytokinin and ABA indicating a strong correlation between sugar and hormonal signals.

Under abundant sugar status, *SnRK1* activates ADP-glucose phosphorylase (AGPase) which is an important enzyme for starch biosynthesis. The repression of *SnRK1* was reported to decrease the starch content in pea embryos (Radchuk *et al*., 2010) while over-expression of *SnRK1* resulted in the enhanced starch accumulation in potato and barley (Mckibbin *et al*., 2006; Zhang *et al*., 2001). Luo *et al*. (2020) reported that *SnRK1* was involved in regulation of sucrose metabolism. The overexpression of *FaSnRK1α* gene in strawberry was found to significantly increase the sucrose content by upregulating *FaSUS1* and *FaSUS3* (Sucrose synthase) along with sucrose transporter genes, FaSUT1 and FaSUT5. In another study, antisense *SnRK1* expression in plants was found to have impaired invertase activity leading to the inability of pollen grains to utilize the available sucrose resulting in failure of pollen development. *SnRK1* is activated when the glucose content is low or by a higher content of sucrose (Halford *et al*., 2003).

Hexokinases interact with SnRKs in a unique manner. The surface receptor protein (Mig1) of Snf1 is phosphorylated to *HXK*2 in the nucleus under low glucose conditions. Upon phosphorylation, Mig1 moves out from the nucleus resulting in the de-repression of genes repressed by glucose (Ahuatzi *et al*., 2007). *SnRK1* and *TOR* have an antagonistic relationship with regards to sensing nutrient and energy status of the plants, as *SnRK1* is activated under low energy conditions caused by starvation or hypoxia, while *TOR* is activated under ample or abundant nutrient status in the plant cells and tissues (Robaglia *et al*., 2012). On the other hand, T6P also indicates high sugar levels contrastingly to the functions of *SnRK1*, but is reported to have an indirect relationship in activating or deactivating the conserved protein kinase *SnRK1* (Zhang *et al*., 2009a).

Signals generated by *SnRK1* under decreasing energy conditions which cause repression of anabolic processes, are blocked by sugars such as T6P under stress (Baena-Gonzalez *et al*., 2007; Nunes *et al*., 2013a). Crepin and Rolland (2019) were of the opinion that rather than being activated under low energy conditions, *SnRK1* seems to be repressed by T6P through high energy signals. Sugar phosphates such as T6P, glucose-6-phosphate and glucose-1-phosphate are well known to inhibit the activity of *SnRK1* (Zhang *et al*., 2009a).

2.3.1.3 TOR

Target of Rapamycin (*TOR*) is an evolutionarily conserved Ser/Thr protein kinase that acts as a central hub/master regulator integrating energy, nutrient, environmental and hormonal signals. *TOR* senses the glucose signals produced from the vegetative parts of the plant and transduces these signals in relation to the photosynthesis-mitochondria generated energy balance to regulate genes that are important for cell cycle regulation, synthesis of DNA, transcription, signaling , proliferation of root meristem, oxidative pentose phosphate pathway and, nucleotides, lipids and amino acid synthesis (Xiong *et al*., 2013). Baena-Gonzalez *et al*. (2007) reported that the genes that are essential for the degradation of lipids, amino acids and proteins are repressed through the Glc-*TOR* pathway. This might be counter-productive under stress conditions as these are important coping mechanisms for the plant during starvation conditions.

In seedlings of Arabidopsis, the transition to photosynthetic autonomy produces glucose signals which activate *TOR* which in turn represses the genes involved in lipid biosynthesis and storage, such as TAG lipase and acyl-CoA oxidase. This redirection between starch and lipids indicates the versatility of *TOR* in adjusting plant growth and development or stress tolerance (Xiong *et al*., 2013; Caldana *et al*., 2013). The genome-wide expression analysis (GWAS) of *TOR* revealed that *TOR* negatively regulates genes involved in catabolic processes such as autophagy, senescence and lipid metabolism while it positively regulates genes involved in anabolic processes such as photosynthesis, carbon and nitrogen utilization etc. Apart from these, *TOR* is also noted to modulate genes involved in stress signaling and hormone metabolism (Xiong and Sheen, 2014).

TOR is reported to control protein synthesis through ribosome biogenesis and translation of proteins. *TOR* is found to regulate the transcription of ribosomal RNA through its kinase domain. Ren *et al*. (2012) reported that transgenic Arabidopsis lines overexpressing *TOR* had an increased rate of protein synthesis. Disruption of *TOR* signaling or reducing the expression of *TOR* results in accumulation of high levels of amino

acids, starch and triacylglycerides (TAG), intermediates of TCA cycle and numerous secondary metabolites (Deprost *et al*., 2007; Moreau *et al*., 2012). Jewell *et al*. (2013) reported that the process of autophagy was negatively regulated by *TOR* through phosphorylation of Autophagy related 1 (ATG1) kinase complex by *TORC1*.

TOR is regulated through phosphorylation of its RAPTOR complex by protein kinases that are activated under stress conditions (Fu *et al*., 2020). van Leene *et al*. (2019) conducted interactome and phospho-proteomic analysis to identify 215 proteins interacting with *TOR* complex and 83 phospho-proteins that were regulated by *TOR*. About 20 percent of these proteins were found to be involved in stress responses, notably the La-related protein 1 involved in mRNA degradation that is triggered by heat stress (Merret *et al*., 2013). In a study by Sharma *et al*. (2019) on the role of *TOR* in conferring thermo-tolerance, it was reported that over-expression of *TOR* led to increased survival rates and higher expression of heat shock proteins in seedlings subjected to heat stress conditions. Dong *et al*. (2015) noted that genes involved in stress-hormone (ABA, jasmonic acid etc.) signaling pathways were activated as a result of the down-regulation of *TOR*. However, tolerance or susceptibility response of the plants through modulation of *TOR* expression was found to be dependent on the type of stress conditions and was not standard (Dong *et al*., 2019; Bakshi *et al*., 2017; Wang *et al*., 2017a).

The hormone auxin was found to be involved in the *TOR-S6K1* pathway that resulted in resuming the translation of plant mRNAs of upstream open reading frames (Schepetilinikov *et al*., 2013). The studies on the cross-talk between ABA and *TOR* reveal that ABA which is produced under stress causes transduction cascades that result in the inhibition of PP2C protein which in turn allows for the activation of SnRK2 resulting in the activation of ABA-responsive genes or stomatal closure (Chen *et al*., 2020). The activated SnRK2 phosphorylates Raptor B of *TOR* complex, effectively inhibiting the activity of *TOR* kinase and initiating the stress adaptation responses. Alternately, Wang *et al*. (2018) reported that under nutrient rich or un-stressed conditions, *TOR* phosphorylates the receptors of ABA thereby inhibiting ABA signaling leading to promotion of growth and development.

2.3.1.4 TPS

Trehalose is a non-reducing disaccharide that is known to provide stress tolerance in plants. The biosynthesis of trehalose is catalyzed by trehalose-6-phosphate synthase (*TPS*) which transfers glucose from UDP-glucose (UDPG) to glucose-6-phosphate (G6P) to form trehalose-6-phosphate (T6P) and uridine di-phosphate (UDP). T6P is further de-phosphorylated to trehalose by trehalose-6-phosphate phosphatase (*TPP*). The intermediate in the trehalsoe biosynthesis pathway, T6P has a signaling function that is crucial in co-ordinating the metabolism of the plants for maintaining the energy-resource utilization balance (Paul, 2007).

T6P signaling has been widely implicated in various developmental processes in plants such as cell cycle activity (Gomez *et al*., 2006), embryo development (Gomez *et al*., 2010), flowering time (Dijken *et al*., 2004), cell shape defects (Chary *et al*., 2008), branching of inflorescence (Satoh-Nagasawa *et al*., 2006), tuber development (Debast *et al*., 2011) among others. *TPS*1 is predicted to be localized mainly in the cytoplasm (Kolbe *et al*., 2005). The T6P signal thus produced mediates the sucrose level in the cytoplasm to starch synthesis by AGPase activation in the chloroplasts (Schleupmann *et al*., 2004). T6P level is an indirect indicator of sucrose level as it is synthesized by G6P and UDP-glucose (Ponnu *et al*., 2011).

The increasing or decreasing levels of T6P in plants is proportional to the level of available sucrose. In case of abundance of sucrose, T6P promotes biosynthetic processes by inhibiting *SnRK1*, whereas under sucrose-limiting conditions, T6P levels also are lowered causing de-repression of *SnRK1* that would allow for induction of genes involved in photosynthesis to provide more carbon to the cells (Delatte *et al*., 2011). High T6P levels promote the growth and development related biosynthetic pathways whereas low T6P levels direct the carbon remobilization towards stress responses (Paul *et al*., 2018). Nunes *et al*., (2013b) noted that at low endogenous levels of sucrose, the expression of *TPS*1 and sucrose content had a linear relationship, thereby indicating the regulation of sucrose.

The mechanism of T6P signaling is clearly associated with activating or deactivating the function of *SnRK1*. Zhang *et al*. (2009a) noted that T6P specifically inhibits activity of *SnRK1* which was evidenced by the fact that most of the genes that were upregulated in AKIN10 (subunits of *SnRK1*) over-expressed plants were down-regulated in plants over-expressing *TPS*1. It was also observed that in all the actively growing tissues of maize, *SnRK1* was inhibited by T6P, except in mature leaves where there was no correlation. Van Djiken *et al*. (2004) reported that At*TPS*1 was constitutively expressed throughout the plant and not limited to specific organs or tissues.

A study by Pellny *et al*. (2004) demonstrated that T6P levels were instrumental in regulating the photosynthetic capacity of plants by modulating the sugar signaling mechanisms. The photosynthetic capacity per unit leaf area was thus found to be increased. Wahl *et al*. (2013) in their study showed that induction of T6P signal by *TPS*1 was essential in inducing flowering through regulation of Flowering locus T (*FLT*) and Shoot apical meristem (SAM). The T6P here acts as a physiological signal that signals the minimum required quantity of carbohydrate that is needed for the transition from vegetative phase to flowering phase. T6P was found to coordinate with other signaling pathways about the carbohydrate status to regulate the flowering time in Arabidopsis.

Nuccio *et al*. (2015) decreased the T6P levels in maize kernels by expressing *TPP* gene and observed that concentration of sucrose was increased in developing maize ears leading to increased kernel set and yield due to starch accumulation regardless of the drought conditions. Decreased T6P levels were also found to promote starch mobilization through amylose activation in rice seedlings under anaerobic conditions. A gene *TPP*7 was identified by Kretzchmar *et al*. (2015) in the genomic region qAG-9-2 that is associated with this mechanism. Over-expression of *OsTPS1* in rice was found to improve the tolerance of seedlings to drought, salinity and cold stress treatments (Li *et al*., 2011).

2.4 Quantitative real-time PCR

Real-time PCR is one of the methods in biotechnology that is used to measure the amplification of the PCR product at each cycle of the PCR reaction. It is a technique that

can quantify the amount of starting material precisely (Gachon *et al*., 2004). Some of the advantageous features of the qRT-PCR are the rapidity with which the data can be analyzed, the sensitivity with which the DNA or RNA can be detected (Bustin, 2000), the high specificity of the hybridized probe being analyzed and the seven-fold higher quantification range compared to normal PCR (Filon *et al*., 2003).

Lovdal and Lillo (2009) examined 8 putative constitutively expressed genes in tomato under nitrogen, cold and light stress for their potential application as a reference genes and reported that none of the genes could express a constant level under all three stress conditions. This highlights the importance of selecting and normalizing reference genes that are specific to the particular crop and specific condition. In another investigation, the real-time PCR technique was used to detect the transgenic rice variety TT51-1 that was being planted illegally. The study utilized the specificity of the qRT-PCR technique to identify the transgenic event (Wu *et al*., 2010).

2.5 Marker-assisted selection (MAS)

The research on the genetic mechanism of heat tolerance is getting more and more important to the utilization of heat tolerant genes in the development of new rice varieties with heat tolerance. Using DNA markers for identification of QTL's was a breakthrough in the characterization of quantitative traits. Molecular markers has been validated and adopted globally as an effective and appropriate tool for primary and practical studies addressing physiological traits. The advantages associated with the markers include speed, consistency, efficiency and biosafety. SSR markers associated with multiple QTL's conferring heat tolerance especially at flowering stage in rice can be deployed in initiating MAS for pyramiding genes to breed for high temperature tolerant varieties (Ye *et al*., 2015).

Advances in rice genomics research and completion of the rice genome sequence have made it possible to identify and map precisely a number of genes through linkage to DNA markers. By determining the allele of a DNA marker, plants that possess particular genes or quantitative trait loci (QTL's) may be identified based on their genotype (Foolad

and Shama, 2005). Vivitha *et al*. (2018) working on rice reported that the physiological basis of the introgressed QTLs controls the spikelet fertility by maintaining the photosynthetic rate and chlorophyll fluorescence and minimizing the transpiration rate under high temperature stress. MAS is the current technique being used to improve the efficiency of plant breeding (Zafar *et al*., 2018).

Many stress resistance genes which are tightly linked to SNP's, SSRs and STS markers are available (Das *et al*., 2017). Marker assisted selection can integrate these genes in breeding populations by combining with conventional breeding approaches. Prasanth *et al*. (2017) performed single marker analysis with 49 selected SSR markers and 18 agronomic and yield traits to find out significant ($p<0.05$) marker-trait associations. They reported 45 candidate genes, close to nine markers significantly associated with six traits under heat stress conditions in both wet and dry seasons. Identifying QTLs related to different traits involved in heat tolerance can be useful in incorporating them in breeding programmes. SSR markers linked with different heat tolerance characters were used in marker assisted selection among 25 wheat genotypes for heat tolerance (Sadat *et al*., 2013).

2.5.1 Bulked segregant Analysis

Bulked segregant analysis (BSA) is used to rapidly identify markers that are tightly linked to the genes for a given phenotype (Zou *et al*., 2016). With the release of sequenced genomes, the combined application of BSA and next-generation sequencing technology represents a new way to accelerate the identification of candidate genes controlling important agronomic traits in various crops (Tiwari *et al*., 2016). Barakat *et al*. (2011) conducted a study which demonstrated that SSR markers combined with bulked segregant analysis (BSA) could be used to identify molecular markers linked to the grain filling rate as indicator for heat tolerance in wheat and suggested that marker-assisted selection with microsatellite primers might be useful for developing improved cultivars.

A genome-wide analysis of single nucleotide polymorphisms (SNPs) using BSA enabled the detection of a genomic region harbouring the dwarfism gene in watermelon (Dong *et al*., 2018). Li *et al*. (2018) conducted BSA and fine-mapping to narrow *PSH*, a

dominant gene for purple leaf sheath in maize from inbred line T877, to a 304.2 kb interval. Waghmare *et al*. (2021) identified 41 parental polymorphic markers between N-22 (tolerant parent) and Uma (susceptible parent). These were used for the genotyping of tolerant bulk, susceptible bulk, tolerant parent (N-22) and susceptible parent (Uma). Among these, RM5749 on chromosome number 4 showed polymorphism between tolerant bulk and susceptible bulk during BSA.

Development of reliable markers for genes conferring biotic and abiotic stress related trait genes and the effective utilization of marker technology in crop improvement has been highlighted as one of the current and future thrust areas in rice production technologies in Kerala (Kumari, 2010). As heat-stress responses are generally governed by QTL/thermotolerance genes, concerted efforts should be made to understand the tolerance mechanisms at molecular and physiological levels (Priya *et al*., 2018).

2.5.2 Markers and QTLs linked to heat stress

Using 200 SSR markers, Zhang *et al*. (2009b) reported a 30 percent polymorphism between the parents 996 (tolerant) and 4628 (susceptible) in their attempt to assess QTLs associated with heat tolerance at flowering stage in rice. Single marker analysis (SMA) revealed that RM3735 (Chromosome 4) and RM3586 (Chromosome 3) which accounted for 17 percent and 3 percent respectively of variation towards heat tolerance among the 279 F_2 lines assessed through bulked segregant analysis. Buu *et al*. (2014) evaluated 310 BC_2F_2 lines from a cross of rice genotypes OM5390/N22 using 264 polymorphic SSR markers at flowering stage for heat tolerance and identified that RM160 contributed 17.1 percent while RM3568 contributed 36.2 percent among others toward phenotypic variation. RM103 on chromosome 6 was also found to be associated with 1000 grain weight which could explain 30.6 percent of the total phenotypic variation.

Bulked segregant analysis of F_3 progenies from a cross of Uma (heat susceptible) and N22 (heat tolerant) for association with heat tolerance traits revealed that RM5749 (Chromosome 4) co-segregated with the tolerance regarding spikelet fertility trait. Reported polymorphic markers from earlier studies did not seem to have much linkage on

the tolerance traits suggesting each population needs specifically validated markers while employing in breeding programming (Waghmare *et al*., 2021). Bharathkumar *et al*. (2014) screened seven rice germplasm using the SSR marker RM6100, which is reported to be linked to heat tolerance at flowering stage and identified that the genotype 'Marishal' to possess tolerance characteristics.

Assessing the performance of 240 rice germplasm lines under high temperature conditions, Pradhan *et al*. (2016) classified the entire population into 3 sub-groups using the 'STRUCTURE' analysis based on the spikelet fertility percentage. Several QTLs associated with heat tolerance were reported in the study. Apart from this, the SSR markers RM547 was found to be strongly associated with spikelet fertility along with RM228, RM247, RM205, RM242, RM314 and INDEL3 that were indirectly associated with the tolerance traits. Ravikiran *et al*. (2020) reported that the SSR marker RM401 showed significant association with spikelet fertility under heat stress in a population of recombinant inbred lines (RILs) derived from the rice genotype NL-44. The marker was linked to the QTL responsible for spikelet fertility, qSF4.

Materials and Methods

3. MATERIALS AND METHODS

The present investigation entitled 'High temperature mediated changes in sugar signaling pathway and identification of associated microsattelite markers in rice (*Oryza sativa* L.)' was carried out at the College of Agriculture, Vellayani, Thiruvananthapuram, during the years 2019 – 2021. The investigation was conducted in a series of experiments. The first experiment deals with the effect of high temperature on the sugar signaling pathway whereas the second, third and fourth experiments are interconnected to deal with the identification of polymorphic SSR markers associated with heat tolerance in rice. The details of the materials used and techniques employed during the course of investigation are presented in this chapter.

3.1 Experimental Conditions

3.1.1 Location

The research area is located at the College of Agriculture, Vellayani which is present in the southern Indian state of Kerala (8.44° N, 76.99° E). The climate is typically humid tropical with average summer temperature reaching around 35° C and the average winter temperatures about 20° C.

3.1.2 Planting materials

The experiments were conducted as a pot culture studies. The pot size was 25×15 cm and each pot was filled with soil and farm yard manure in 2:1 ratio. The average weight of the pot after filling was about 6 kg. Rice varieties Uma, Vandana, NERICA L-44 (New Rice for Africa – Lowland 44) were selected for the study. Uma (MO-16) is a high-yielding variety developed by the Kerala Agricultural University, and has been reported to be susceptible to high temperature conditions (Waghmare *et al*., 2018). Vandana (ICAR-National Rice Research Institute, Cuttack, Odisha) has also been reported as heat susceptible variety (Prasanth *et al*., 2017) while NERICA L-44 (NL-44), an interspecific hybrid, was reported as a heat tolerant variety (Bahuguna *et al*., 2015; Ravikiran *et al*.,

2020). The seedlings were raised in a nursery and transplanted to the pots after 18 days. The fertilizer dosage followed (100:60:40 kg ha^{-1} of N:P:K) was rationalized and supplied in the form of complex fertilizer (19:19:19) with 10 grams basal in each pot before transplanting and the remaining amount was applied as top dressing at 15 days after transplanting, maximum tillering and booting stage.

3.2 Experiment I

Effect of high temperature on sugar signaling pathway

3.2.1 Treatments

Factor I: Heat stress levels -2

1) Control (T1): 26-34 ˚C
2) High temperature (T2): 38-42 ˚C

Factor II: Varieties – 2

i. NL-44 (V1)
ii. Vandana (V2)

Total number of treatments: 2 x 2 = 4

Replications: 4

Design of experiment: Completely Randomized Design (CRD)

Season: Mundakan, 2019

3.2.2 Methodology

The two rice varieties were evaluated under control and high temperature conditions. The plants were grown as in lowland conditions i.e. with standing water for most of the crop duration except for mid-season drainage at 40 and 60 days. Until maximum tillering stage, the plants were kept in normal environmental conditions (plate 1) with the average daytime temperature ranging around 30-32° C and night temperature

Plate 1. General view of experimental area - ambient condition

Plate 2. Induction of stress in high temperature polyhouse (genotypes: NL-44 and Vandana)

around 24-26° C. The stress treatment plants were moved to a high temperature poly-house (plate 2) where the average temperature was around 42-43° C during the day. The plants were kept in the high temperature conditions until their harvest. The physiological parameters were recorded at the flowering phase and yield parameters were recorded at the grain-filling phase.

The leaf samples of both the varieties of the two stress levels were collected at the vegetative stage and the grain filling stage after the inducement of stress. Two biological replications and three technical replications were considered for studying the gene expression using quantitative real-time PCR.

3.2.3 Parameters recorded

3.2.3.1 Physiological and yield parameters

3.2.3.1.1 Plant height (cm)

The height of the plant was considered by measuring the length from the base of the plant till the tip of the the primary panicle and expressed in centimeters.

3.2.3.1.2 Tiller number

The number of tillers was counted at the harvest stage

3.2.3.1.3 Productive tiller number

The number of tillers that produced panicles were considered as productive tillers and the count was taken at the harvest stage.

3.2.3.1.4 Days to flowering

The number of days taken from sowing till the emergence of the first panicle was considered as the days to flowering.

3.2.3.1.5 Time of anthesis

The time of day when the matured spikelets cause anther dehiscence was considered as the time of anthesis.

3.2.3.1.6 Pollen viability (%)

The pollen viability percentage was calculated by the Iodine-Potassium iodide method, where 1% Iodine Potassium iodide solution was used to stain the pollen grains placed on a glass slide and observed under a microscope (Lx300, Labomed). The iodine-potassium iodide solution was prepared by dissolving 1 g of potassium iodide and 0.5 g of iodine in 100 ml of distilled water (Sulusoglu and Cavusoglu *et al*., 2014). The dark, stained pollen grains were counted as viable and the unstained pollen grains as non-viable. The pollen viability percentage was calculated by the formula:

$$Pollen\ viability\ (\%) = \frac{number\ of\ stained\ pollen\ grains}{Total\ number\ of\ pollen\ grains} \times 100$$

3.2.3.1.7 Cell membrane stability index (%)

The cell membrane stability was measured according to the procedure given by Sairam *et al*. (1997). The membrane stability index (MSI) was calculated using the formula:

$$MSI = \left[1 - \left({}^{C1}/_{C2} \right)\right] \times 100$$

where C1 and C2 are the initial and final electrical conductance measured when leaf discs (100 mg) were heated in a water bath at 40° C (30 min) and 100° C (10 min.) respectively.

3.2.3.1.8 Photosynthetic rate (µmol cm^{-2} s^{-1})

The photosynthetic rate was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in µmol cm^{-2} s^{-1}.

3.2.3.1.9 Transpiration rate (mmol m^{-2} s^{-1})

The transpiration rate was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in mmol m^{-2} s^{-1}.

3.2.3.1.10 Stomatal conductance (mol m^{-2} s^{-1})

The stomatal conductance was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in mol m^{-2} s^{-1}.

3.2.3.1.11 Leaf temperature (^{0}C)

The leaf temperature was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in µmol cm^{-2} s^{-1}.

3.2.3.1.12 Fv/Fm ratio

The Fv/Fm ratio was measured in dark adapted leaves using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA).

3.2.3.1.13 ϕPSII

The ϕPSII was measured in dark adapted leaves using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA).

3.2.3.1.14 Electron transport rate (ETR)

The electron transport rate was measured in dark adapted leaves using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA).

3.2.3.1.15 Water-Use efficiency (WUE)

The water-use efficiency was calculated by dividing the photosynthetic rate (A) with the transpiration rate (E). This measurement is the instantaneous value of water-use efficiency as it is based on the physiological parameters that were measured using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA).

3.2.3.1.16 Panicle length (cm)

The length of the panicle was measured from the base of the panicle to the tip and expressed in centimeters.

3.2.3.1.17 Spikelet fertility (%)

An average of three panicles in each replication were considered to calculate the spikelet fertility percentage. The number of fertile spikelets in each panicle were counted and the spikelet fertility percentage was calculated using the formula:

$$Spikelet\ fertility\ (\%) = \left(\frac{number\ of\ fertile\ spikelets}{total\ number\ of\ spikelets}\right) \times 100$$

3.2.3.1.18 1000 grain weight (g)

A total number of 1000 grains after harvest and drying were counted whose weight was then measured using a weighing balance and expressed in grams.

3.2.3.2 Expression profile of genes

The expression levels of the four genes, *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR,* which are the major genes involved in the sugar signaling pathways along with the reference gene *OsActin*, were quantified using the technique of quantitative real-time PCR (qRT-PCR). The gene expression profile was analysed at two stages viz. the vegetative stage (maximum tillering) and the grain-filling stage. Two biological replicates and three technical replicates were used for the study. The details are presented as follows:

Number of varieties: 2

i. NL-44

ii. Vandana

Number of heat stress levels: 2

1. Control (T1): 26-34 ˚C
2. High temperature (T2): 38-42 ˚C

Total number of samples: 2 x 2 = 4

Number of biological replications: 2

Number of genes: 5

1) *OsHXK2*
2) *OsSnRK1*
3) *OsTPS1*
4) *OsTOR*
5) *OsActin*

Total number of reactions: 4 x 2 x 5 = 40

Number of technical replications: 3 (for each reaction)

3.2.3.2.1 Isolation of RNA

All the glassware and plastic-ware was necessary for the procedure treated with 0.1% di-ethyl pyrocarbonate (DEPC) for few hours after which they were dried in the oven overnight at a temperature of 37˚ C. Later, they were autoclaved to eliminate any trace of DEPC so as to not interfere with the reactions. All the solutions used in the procedure were prepared using DEPC treated water i.e. double-autoclaved 0.1% DEPC water. The 0.1% DEPC solution was prepared by adding 0.1 ml of DEPC using a micropipette to one litre of double distilled water and stirred overnight on a magnetic stirrer.

The leaf samples for RNA isolation were collected in an aluminum foil early in the morning and transported to the lab carefully in a flask of liquid nitrogen. The leaf samples of 100 mg were ground in a pre-chilled mortar and pestle to fine powder using liquid nitrogen. 1 ml of Trizol reagent was added to each of the samples, mixed lightly and kept at room temperature for 5 minutes. The homogenate was then transferred to a 2 ml pre-chilled micro-centrifuge tube. 0.2 ml of chloroform was added to each of the tubes, shaken vigorously for 15 seconds and incubated at room temperature for 5 minutes. It was later

kept on ice for 10 minutes, after which it was centrifuged at 12000 x g for 15 minutes at 4˚ C. The top aqueous phase was transferred to a fresh tube, to which 0.5 ml of ice-cold isopropanol (100%) was added at room temperature for 10 minutes. The tubes were then inverted constantly in order to mix the solutions. After a few minutes of inversion, light milky white strands of RNA can be observed. To precipitate the RNA into a solid pellet, the tubes are centrifuged at 12000 x g for 10 minutes at 4˚ C. The supernatant is discarded and the pellet is washed with 1 ml of 75 % alcohol. The sample is then briefly vortexed and spun at 7500 x g for 5 minutes at 4˚ C, after which it is air dried for 30-40 minutes. Later, it is dissolved in 30 µl RNAase free water and incubated at 55-60˚ C for 10 minutes.

3.2.3.2.2 Determination of quality and quantity of RNA

The quality of the isolated RNA was visualized by checking the banding pattern through agarose gel electrophoresis. 5 µl of RNA sample was mixed with 2 µl of 6X gel loading dye and loaded into the wells along with RNA ladder. The presence of clear bands indicating 18s and 26s RNA size bands were deemed as sufficient quality of RNA. The bands were visualized in the gel documentation system.

The purity of RNA was also estimated by the ratio of absorbance measured at 260 nm and 280 nm using a spectrophotometer. A ratio of 1.8 to 2.0 was considered to have the least contamination of proteins or DNA.

The concentration of RNA present in the sample was calculated using the formula:

$$\text{Concentration of RNA (ng } \mu l^{-1}) = A260 \times 40 \times \text{dilution factor}$$

where, A260 is the absorbance measured at 260 nm. An absorbance value of 1.0 is an indication that 40 ng μl^{-1} is present in the sample. The absorbance was taken by adding 5 µl of RNA sample to 2995 µl of sterile water. Therefore, the dilution factor is determined as 600.

3.2.3.2.3 Synthesis of cDNA

The isolated RNA was converted to complementary DNA (cDNA) using the cDNA synthesis kit (Verso cDNA synthesis kit, Thermo Fisher Scientific). The components in the kit include oligo dT primer, verso reverse transcriptase enzyme, random hexamer, RNase inhibitor, and RT enhancer.

Table 1: Composition of reaction mixture (20 µl) used for synthesizing cDNA

S.no.	Components	Quantity
1	5x cDNA buffer	4 µl
2	dNTP mix	2 µl
3	Oligo dT primer	0.5 µl
4	Random Hexamer	0.5 µl
5	RT enhancer	1 µl
6	Verso enzyme mix	1 µl
7	RNA	4µl
8	Nuclease free water	7 µl
	Total volume	**20 µl**

After all the components were added in a 0.2 ml PCR tube, the contents were mixed well and incubated at 42° C for 30 min followed by 92° C for 2 min. The synthesized cDNA samples were stored at -20° C.

3.2.3.2.4 Differential expression analysis using real-time quantitative PCR

The qRT-PCR (Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction) was done on the BIO-RAD CFX^{TM} Touch Real time detection system with SYBR Green Master mix (Origin Diagnostics & Research, India).The data on threshold cycle value (Cq) of the four target genes viz. *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR* were retrieved using BIO-RAD CFX Maestro 1.0 software (Plates 3,4). Actin (*OsActin*)

was used as internal reference and non-template (without cDNA) were kept as control. The real-time quantitative PCR was carried out in a reaction mixture of 20 µL.

Table 2: Components of reaction mixture used for qRT-PCR

S.no.	Components	Quantity
1	2x Real time PCR Smart Mix	10 µl
2	Forward primer (10 pmol/µl)	1 µl
3	Reverse primer (10 pmol/µl)	1 µl
4	Template cDNA	1 µl
5	Nuclease free water	7 µl
	Total	**20 µl**

The reactions were performed with three technical replicates (to avoid pipetting errors) with amplification conditions as follows: initial denaturation at 95 ˚C for 3 minutes, followed by 40 cycles of denaturation at 95 ˚C for 10 seconds, annealing at 60 ˚C for 30 seconds and extension at 65 ˚C for 5 seconds. The final extension was carried out at 95 ˚C for 50 seconds. The list of primers used for qRT-PCR is given in table 3.

The gene expression was calculated as relative fold change using the Livak's method / $2^{-\delta\delta Ct}$ method using the control condition as baseline. Each reaction of each sample was replicated three times (technical replications) along with non-template control (NTC). The house-keeping gene *OsActin* was used as the reference gene for normalisation of qRT-PCR data. The threshold cycle (Cq) values were determined for all the reactions using BIO-RAD CFX Maestro 1.0 software. Threshold cycle (Cq) value is the number of the reaction cycle at which the fluorescence value of a sample reaches an arbitrary threshold fluorescence.

The difference between Cq values of target gene and reference gene of a particular sample (control/stress) was considered as ΔCq value.

$$\Delta Cq = Cq \text{ (target gene)} - Cq \text{ (Reference gene)}$$

The difference between ΔCq values of control and stress samples of the particular target gene were considered as ΔΔCq value.

. $$\Delta\Delta Cq = \Delta Cq \text{ (Stress Sample)} - \Delta Cq \text{ (Control)}$$

The fold change in the expression of that gene was calculated using the formula:

$$\text{Relative fold change} = 2^{-(\Delta\Delta Cq)}$$

The results were expressed as relative fold change in expression. The gene expression of the control treatment was normalized to 1. A gene with relative fold change of less than 1 was considered to be down-regulated whereas the fold change greater than 1 was considered to be up-regulated (Livak and Schmittgen, 2001).

Table 3. List of primers used for qRT-PCR

Gene	Forward primer	Reverse primer
OsHXK2	5'-TATACTGGGAACAGGTACTAATGC-3'	5'-CCATCTTTAATAGGACTCTACGAA-3'
OsSnRK1	5' CGAATCACTTCACAAGAGACTG 3'	5' CTGGAGTTACTTGAGCGAGAG 3'
OsTPS1	5' TTGAAGTTCGGTCTGTCG 3'	5' CTGCCTATCCAAGAACATG 3'
OsTOR	5' CAAATCGTATGGGAGGAGCTA 3'	5' GCAGCCATAAGAAGTTTCTCCA 3'
OsActin	5**'** CTCCCCCATGCTATCCTTCG 3'	5' TGAATGAGTAACCACGCTCCG 3'

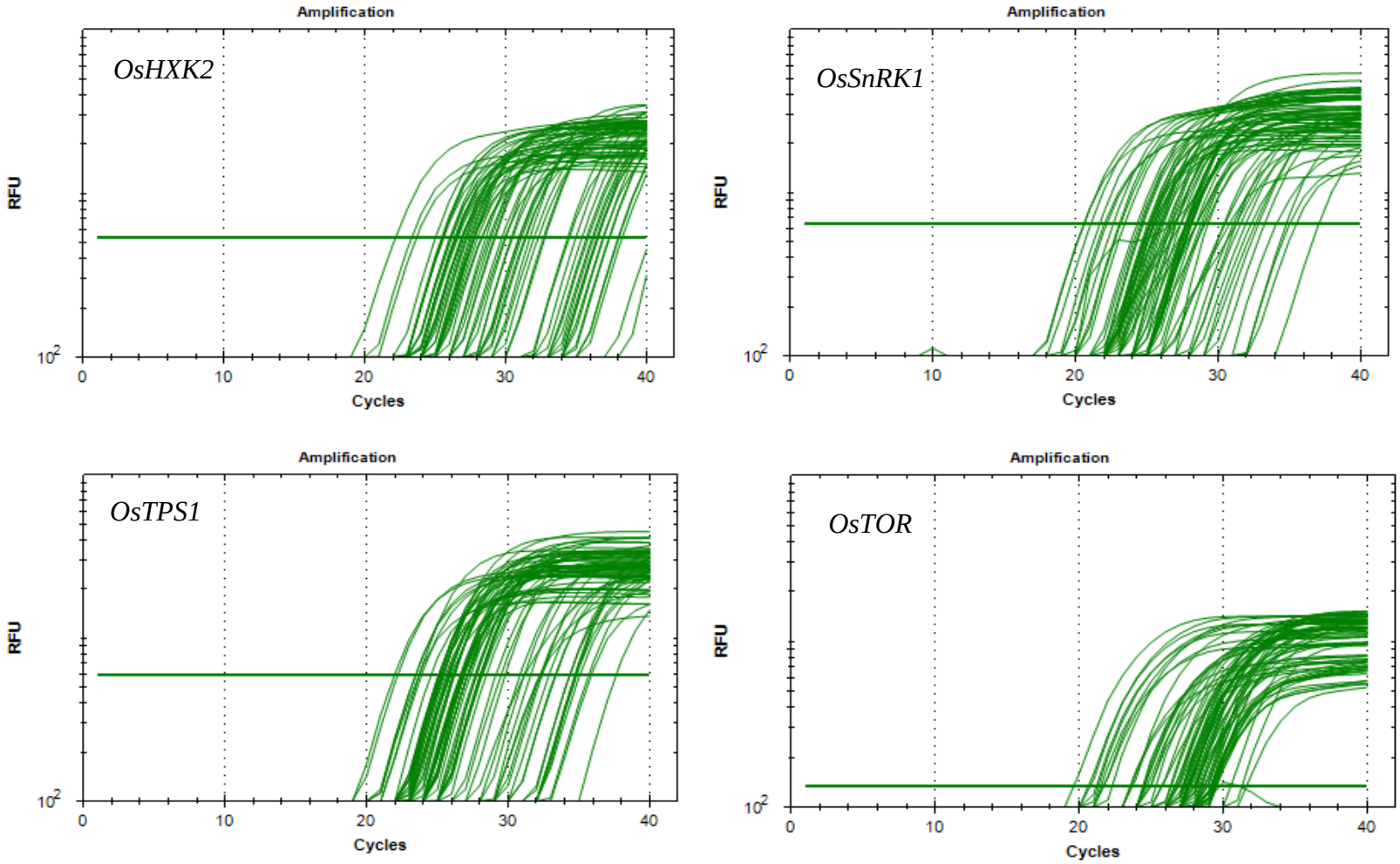
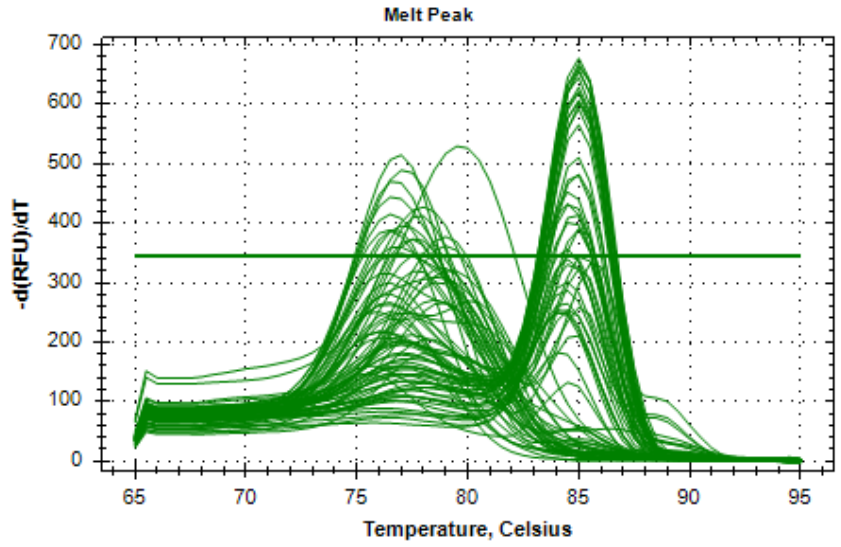
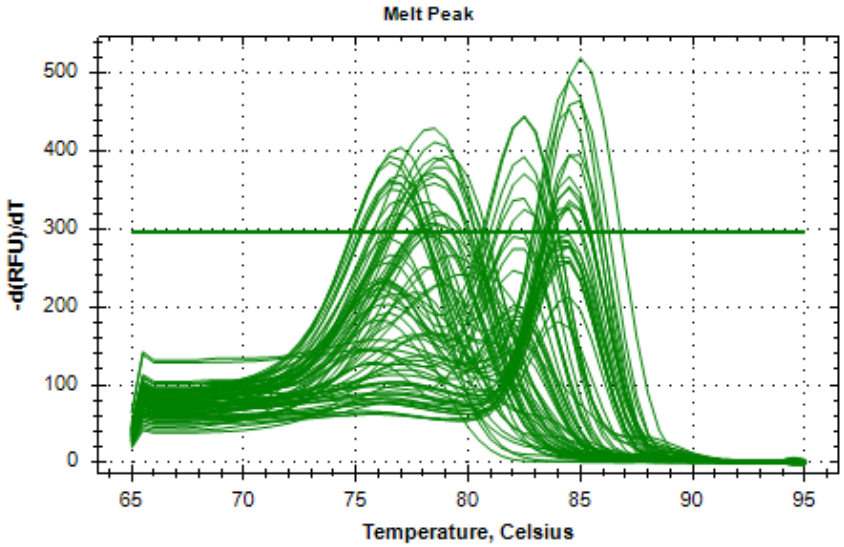


Plate 3: Amplification plot of *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR*



A) *OsHXK2 and OsSnRK1*



B) *OsTPS1 and OsTOR*

Plate 4: Melt curve analysis of *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR*

Experiment II

Laying of crossing blocks to produce F_1 and F_2 seeds

3.3.1 Crossing Block 1 – Crossing of NERICA-L-44 with Uma to obtain F_1 seeds

Varieties: 2

1. NL-44
2. Uma

Season: Mundakan, 2019

3.3.1.1 Methodology

The two rice varieties, NL-44 (heat tolerant) and Uma (heat susceptible) were sown in a staggered planting pattern (plate 5) which is a method of germinating seeds of two varieties on different dates so that their varying flowering times can coincide. In the current experiment, the plants of the two varieties were raised with three different dates of sowing as NL-44 had an earlier flowering phenology (65 days) compared to Uma (78 days). Standard crop management was followed according to the package of practices (KAU, 2016), at ambient temperature of 26 – 34 ˚C without any stress during the crop growth period.

Reciprocal crosses of the two varieties was conducted at the flowering stage. The plant of the variety selected as the male parent was emasculated in the evening, a day earlier before the opening of the spikelets. The spikelets were emasculated using the clipping method (plate 6), where the top and bottom one third of the spikelets on the panicle were removed with a scissor to retain only the spikelets on the mature middle portion of the spikelets. The top portion of the remaining spikelets were clipped to remove half or one third part of the spikelets so as to expose the stamens. The six stamens were then removed carefully using forceps or needle and only the stigma was retained. The emasculated panicles were tagged with the details of the male and female parents along with the date of emasculation and covered with well with a butter paper bag (plate 7) to prevent self-pollination from plants of the same parent. On the following morning, the butter paper bag

Plate 5. Staggered planting of genotypes NL-44 and Uma in crossing block-I

Clipping Emasculation Pollen dusting

Plate 6. Hybridisation using clipping method

Plate 7. Bagging of panicles after pollination

Plate 8. Production of hybrid seeds (F_1 generation)

was removed and the emasculated panicle was dusted with pollen collected from the plant selected as the female parent. After ensuring sufficient dusting of pollen, the panicles are bagged once again to enable the success of the cross. After a few days the paper bag was removed to check for the number of spikelets in which the cross pollination had taken place. Several such crosses were conducted to ensure a good number of F_1 seeds (plate 8).

3.3.2 Crossing block 2 – Selfing of F_1 seeds to produce F_2 progeny

Season: Mundakan, 2019

3.3.2.1 Methodology

The F_1 seeds that were produced in the crossing block 1 from the reciprocal cross of NL-44 and Uma were raised to produce F_2 seeds (plates 9, 10). Standard crop management was followed according to the package of practices (KAU, 2016), at ambient temperature of 26 – 34 ˚C without any stress during the crop growth period. The panicles with F_2 seeds produced by selfing the F_1 plants were carefully collected in brown paper, dried to sufficient moisture content and seeds separated from the panicles.

3.3 Experiment III

Phenotypic evaluation of F_2 population of NL-44 x Uma for high temperature tolerance

3.4.1 Treatment details

Crop population: F_2 progeny (NL-44 x Uma)
Replications: Un-replicated trial
Season: Mundakan, 2020

3.4.2 Methodology

The F_2 seeds produced from the F_1 progeny of the cross between the rice varieties NL-44 and Uma were germinated and raised following standard crop management according to the package of practices (KAU, 2016). The plants were grown as in lowland conditions i.e. with standing water for most of the crop duration except for mid-season

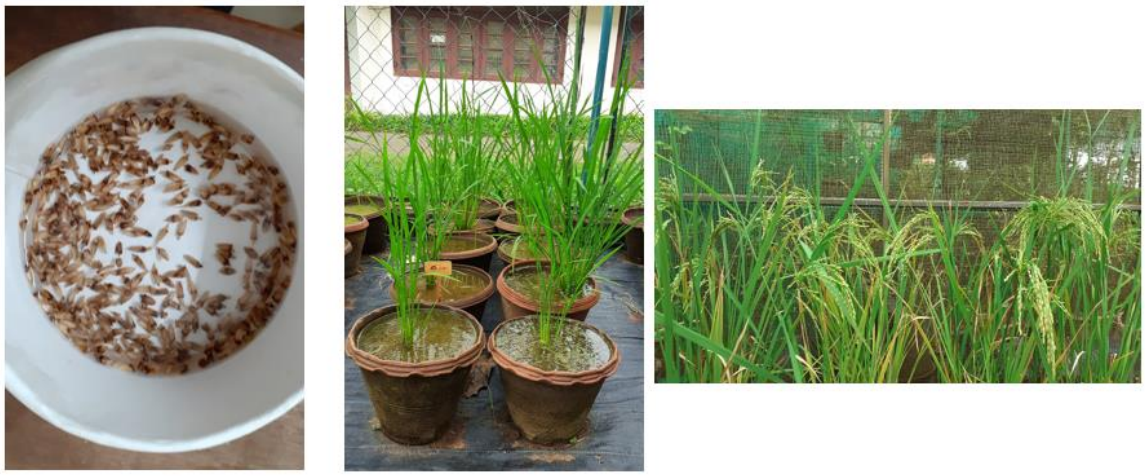


Plate 9. Selfing of F_1 hybrids in crossing block II

Plate 10. Production of F_2 generation seeds

Plate 11. Raising F_2 generation plants under ambient conditions

Plate 12. Induction of high temperature stress in polyhouse (F_2 generation)

drainage at 40 and 60 days. Until maximum tillering stage (plate 11), the plants were kept in normal environmental conditions with the average daytime temperature ranging around 30-32° C and night temperature around 24-26° C. The stress treatment plants were moved to a high temperature poly-house (plate 12) where the average temperature was around 38-42° C during the day. The plants were kept in the high temperature conditions until their harvest. The physiological parameters were recorded at the flowering phase and yield parameters were recorded at the grain-filling phase.

The F_2 lines were evaluated along with the two parents NL-44 and Uma by calculating the mean and the deviation of each line from the mean. The range of the data was estimated by dividing the data into quartiles and the shape of the normal curve for each parameter was determined.

3.3.3 Parameters recorded

3.4.3.1 Plant height (cm)

The height of the plant was considered by measuring the length from the base of the plant till the tip of the the primary panicle and expressed in centimeters.

3.4.3.2 Tiller number

The number of tillers was counted at the harvest stage

3.4.3.3 Productive tiller number

The number of tillers that produced panicles were considered as productive tillers and the count was taken at the harvest stage.

3.4.3.4 Days to flowering

The number of days taken from sowing till the emergence of the first panicle was considered as the days to flowering.

3.4.3.5 Time of anthesis

The time of day when the matured spikelets cause anther dehiscence was considered as the time of anthesis.

3.4.3.6 Pollen viability (%)

The pollen viability percentage was calculated by the Iodine-Potassium iodide method, where 1% Iodine Potassium iodide solution was used to stain the pollen grains placed on a glass slide and observed under a microscope (Lx300, Labomed). The dark, stained pollen grains were counted as viable and the unstained pollen grains as non-viable. The pollen viability percentage was calculated by the formula:

$$Pollen\ viability\ (\%) = \frac{number\ of\ stained\ pollen\ grains}{Total\ number\ of\ pollen\ grains} \times 100$$

3.4.3.7 Cell membrane stability index (%)

The cell membrane stability was measured according to the procedure given by Sairam *et al*. (1997). The membrane stability index (MSI) was calculated using the formula:

$$MSI = \left[1 - \left({}^{C1}/_{C2} \right)\right] \times 100$$

where C1 and C2 are the initial and final electrical conductance measured when leaf discs (100 mg) were heated in a water bath at 40° C (30 min) and 100° C (10 min.) respectively.

3.4.3.8 Photosynthetic rate (µmol cm^{-2} s^{-1})

The photosynthetic rate was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in µmol cm^{-2} s^{-1}.

3.4.3.9 Transpiration rate (mmol m^{-2} s^{-1})

The transpiration rate was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in mmol m^{-2} s^{-1}.

3.4.3.10 Stomatal conductance (mol m^{-2} s^{-1})

The stomatal conductance was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in mol m^{-2} s^{-1}.

3.4.3.11 Leaf temperature (ºC)

The leaf temperature was measured from 8:30 am to 11:00 am using the Infra-Red Gas Analyzer (LI-COR 6400XT, USA) and expressed in µmol cm^{-2} s^{-1}.

3.4.3.12 Panicle length (cm)

The length of the panicle was measured from the base of the panicle to the tip and expressed in centimeters.

3.4.3.13 Spikelet fertility (%)

An average of three panicles in each replication were considered to calculate the spikelet fertility percentage. The number of fertile spikelets in each panicle were counted and the spikelet fertility percentage was calculated using the formula:

$$Spikelet\ fertility\ (\%) = \left(\frac{number\ of\ fertile\ spikelets}{total\ number\ of\ spikelets}\right) \times 100$$

3.4.3.14 1000 grain weight (g)

A total number of 1000 grains after harvest and drying were counted whose weight was then measured using a weighing balance and expressed in grams.

3.4 Experiment IV

Identification of molecular markers linked to high temperature tolerance in rice using Bulked Segregant Analysis

3.5.1 Methodology

Based upon the phenotypic evaluation of the F_2 lines from experiment III, ten extremely tolerant and ten extremely susceptible lines were selected. The DNA was extracted from the selected 10 heat tolerant and 10 susceptible lines. The DNA from 10 heat tolerant and heat susceptible segregants was taken and and equal quantities were

pooled into heat tolerant and susceptible bulks. The bulked DNA samples were screened using 100 Simple Sequence Repeat (SSR) primers. The putative polymorphic markers between the bulks was checked among the parents as well as the individual lines constituting the tolerant and susceptible bulks.

3.5.2 Molecular profile characteristics

3.5.2.1 Isolation of DNA

100 mg of plant tissue was ground into a fine powder using liquid nitrogen with a mortar and pestle. 750 µl of extraction buffer (CTAB + β mercapto-ethanol) was added to the ground samples, slightly mixed and transferred into a 2 ml centrifuge tube. The contents in the tube were inverted for a few seconds for proper homogenisation and incubated in a water-bath at 65 ˚C for 30 minutes. After removing from the water-bath, 750 µl of chloroform: isoamyl alcohol (24:1) solution was added to the centrifuge tube. The contents were slightly mixed by inversion for a few seconds and centrifuged at 13000 rpm for 10 minutes. The separated top phase obtained after centrifugation was transferred to a new 2 ml centrifuge tube.

The chloroform: isoamyl alcohol step was repeated one more time and the top phase was again transferred to a fresh tube. 450 µl of ice-cold isopropanol was added to this and mixed by inversion. After inverting for a few seconds, the sample was centrifuged at 13000 rpm for 5 minutes. The supernatant was discarded and the pellet of DNA obtained was washed with 500 µl of 70 % ice-cold ethanol. Later, it was subjected to centrifugation at 13000 rpm for 5 minutes and the ethanol was carefully pipetted out. The DNA pellet was then air-dried at room temperature or in a speed vacuum. After drying, the pellet was resuspended in 30 µl sterile water and stored at – 20 ˚C.

3.5.2.2 Quality of DNA isolated

The quality of the isolated DNA was visualized as bands on 0.8% agarose gel using electrophoresis technique. The presence of a clear band without a smear is indicative of good quality DNA as a smear indicates that the sample has contamination by proteins. The

purity of the DNA was also checked by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) and the value of their ratio was considered. A ratio (A_{260}/A_{280}) of 1.8 – 2.0 is considered to be good quality DNA. A ratio below that indicates protein contamination.

3.5.2.3 Quality check of PCR products

The PCR reaction to check for DNA polymorphism was carried out by preparing a standard mixture having the following components along with DNA template (1 µl/reaction)

Table 4: Components of reaction mixture to check for DNA polymorphism

S.no.	Component	Quantity (5 reactions)
1.	Sterile water	63.5 µl
2.	10x Taq buffer	10 µl
3.	Forward primer	3.75 µl
4.	Reverse primer	3.75 µl
5.	dNTP mix	7.5 µl
6.	Taq polymerase	1 µl/5 reactions

The thermal profile of the PCR cycling conditions were standard except for the annealing temperature which was specific to the individual markers.

Table 5: The cycling conditions for PCR reaction

Step	Stage	Temperature (°C)	Duration	
1	Initial denaturation	95° C	5 min.	
2	Denaturation	94° C	1 min.	x 30 cycles
3	Annealing	57° C	1 min.	
4	Extension	72° C	2 min.	
5	Final extension	72° C	5 min.	

The quality of the PCR products was checked by separating them a 3 % agarose gel using the electrophoresis technique. 10 µl of the PCR product was mixed with 2 µl of 6X gel loading dye and loaded into the wells. Clear visualization of the bands in the gel documentation system and determination of their sizes using a DNA ladder (100 bp) was deemed to be crucial for good quality PCR product.

3.5.2.4 Polymorphism between the tolerant and susceptible bulks

Bulked segregant analysis (BSA) technique was utilized to identify the polymorphic markers between the tolerant and the susceptible bulks. The markers which differentiated between the tolerant and susceptible bulks through the difference in the size of bands of the PCR products that were separated through gel electrophoresis were considered to be polymorphic.

3.5.2.5 Segregation pattern of polymorphic markers among the selected lines

The polymorphic markers that showed polymorphism between the tolerant and susceptible bulks through BSA were used to study the segregation of the alleles in the individual lines constituting the tolerant and susceptible bulks. PCR reactions were carried out using the DNA of the individual lines as a template and the PCR products were separated on a 3% agarose gel through gel electrophoresis. The list of SSR markers is given in table 6.

Table 6. List of SSR markers

S.no.	Marker	Forward primer	Reverse primer
1)	RM1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
2)	RM3	ACACTGTAGCGGCCACTG	CCTCCACTGCTCCACATCTT
3)	RM5	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG
4)	RM11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
5)	RM19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA
6)	RM20A	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG
7)	RM23	CATTGGAGTGGAGGCTGG	GTCAGGCTTCTGCCATTCTC
8)	RM25	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC
9)	RM70	GTGGACTTCATTTCAACTCG	GATGTATAAGATAGTCCC
10)	RM103	CTTCCAATTCAGGCCGGCTGGC	CGCCACAGCTGACCATGCATGC
11)	RM122	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC
12)	RM127	GTGGGATAGCTGCGTCGCGTCG	AGGCCAGGGTGTTGGCATGCTG
13)	RM140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG
14)	RM142	CTCGCTATCGCCATCGCCATCG	TCGAGCCATCGCTGGATGGAGG
15)	RM166	GGTCCTGGGTCAATAATTGGGTTACC	TTGCTGCATGATCCTAAACCGG
16)	RM167	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC
17)	RM200	CGCTAGGGAATTTGGATTGA	CGATGAGCAGGTATCGATGAGAAG

18)	RM205	CTGGTTCTGTATGGGAGCAG	CTGGCCCTTCACGTTTCAGTG
19)	RM210	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG
20)	RM212	CCACTTTCAGCTACTACCAG	CACCCATTTGTCTCTCATTATG
21)	RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG
22)	RM220	GGAAGGTAACTGTTTCCAAC	GAAATGCTTCCCACATGTCT
23)	RM222	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG
24)	RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
25)	RM228	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
26)	RM229	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT
27)	RM231	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG
28)	RM237	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC
29)	RM240	CCTTAATGGGTAGTGTGCAC	TGTAACCATTCCTTCCATCC
30)	RM241	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG
31)	RM242	GGCCAACGTGTGTATGTCTC	TATATGCCAAGACGGATGGG
32)	RM247	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG
33)	RM248	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG
34)	RM249	GGCGTAAAGGTTTTGCATGT	ATGATGCCATGAAGGTCAGC
35)	RM251	GAATGGCAATGGCGCTAG	ATGCGGTTCAAGATTCGATC
36)	RM259	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT

37)	RM260	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG
38)	RM261	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC
39)	RM263	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG
40)	RM264	GTTGCGTCCTACTGCTACTTC	GATCCGTGTCGATGATTAGC
41)	RM273	GAAGCCGTCGTGAAGTTACC	GTTTCCTACCTGATCGCGAC
42)	RM279	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG
43)	RM282	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG
44)	RM284	ATCTCTGATACTCCATCCATCC	CCTGTACGTTGATCCGAAGC
45)	RM286	GGCTTCATCTTTGGCGAC	CCGGATTCACGAGATAAACTC
46)	RM287	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
47)	RM289	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG
48)	RM302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC
49)	RM310	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC
50)	RM314	CTAGCAGGAACTCCTTTCAGG	AACATTCCACACACACACGC
51)	RM316	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
52)	RM320	CAACGTGATCGAGGATAGATC	GGATTTGCTTACCACAGCTC
53)	RM336	CTTACAGAGAAACGGCATCG	GCTGGTTTGTTTCAGGTTCG
54)	RM337	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC
55)	RM348	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC

56)	RM351	CCATCCTCCACCGCCTCTCG	TGGAGGAAGGAAAGGGGACG
57)	RM413	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC
58)	RM418	TCGCGTATCGTCATGCATAG	GAGCACATATGCCACGTACG
59)	RM429	TCCCTCCAGCAATGTCTTTC	CCTTCATCTTGCTTTCCACC
60)	RM461	GAGACCGGAGAGACAACTGC	TGATGCGGTTTGACTGCTAC
61)	RM468	CCCTTCCTTGTTGTGGCTAC	TGATTTCTGAGAGCCAACCC
62)	RM471	ACGCACAAGCAGATGATGAG	GGGAGAAGACGAATGTTTGC
63)	RM472	CCATGGCCTGAGAGAGAGAG	AGCTAAATGGCCATACGGTG
64)	RM473	TATCCTCGTCTCCATCGCTC	AAGGATGTGGCGGTAGAATG
65)	RM474	AAGATGTACGGGTGGCATTC	TATGAGCTGGTGAGCAATGG
66)	RM480	GCTCAAGCATTCTGCAGTTG	GCGCTTCTGCTTATTGGAAG
67)	RM490	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
68)	RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
69)	RM495	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC
70)	RM528	GGCATCCAATTTTACCCCTC	AAATGGAGCATGGAGGTCAC
71)	RM547	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG
72)	RM552	CGCAGTTGTGGATTTCAGTG	TGCTCAACGTTTGACTGTCC
73)	RM554	GTTCGTCCGTCTCTCGTCTC	CCCAAAAATCTGTGCCTCTC
74)	RM556	ACTCCAAACCTCACTGCACC	TAGCACACTGAACAGCTGGC

75)	RM566	ACCCAACTACGATCAGCTCG	CTCCAGGAACACGCTCTTTC
76)	RM570	GTTCTTCAACTCCCAGTGCG	TGACGATGTGGAAGAGCAAG
77)	RM592	TCTTTGGTATGAGGAACACC	AGAGATCCGGTTTGTTGTAA
78)	RM1287	GTGAAGAAAGCATGGTAAATG	CTCAGCTTGCTTGTGGTTAG
79)	RM3412	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
80)	RM3475	GTCGGTTTGCCTAGTTGAGC	TTCCTCGGTGTATGGGTCTC
81)	RM3586	GAAGAGAGAGCCAGAGCCAG	ACACGATCGAGCTAGAAGACG
82)	RM3735	GCGACCGATCAGCTAGCTAG	ATAACTCCTCCCTTGCTGCC
83)	RM3808	CGTTAGCGAAACGAACAGTG	CAGTGGCTCGGTAATCGC
84)	RM5687	GATCGCTGGCGATTGATC	GACTTGTGGGGTGGTTTTTG
85)	RM5749	GTGACCACATCTATATCGCTCG	ATGGCAAGGTTGGATCAGTC
86)	RM6100	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC
87)	RM6132	CCGCCATCTCTCTTCAGTTC	CAGTGCATAGAGGAGAGGACG
88)	RM6306	CACCGGTCTAAGTCGACTCC	CCACTCGTTGTCGTCGTATG
89)	RM7364	TTCGTGGATGGAGGGAGTAC	AGCGTTTGTAGGAGTGCCAC
90)	RM7365	GCTTTTTTCAGGAACTAAAC	TGGATGGTTCATAACAAATA
91)	RM8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
92)	RM10745	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
93)	RM10793	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC

94)	RM14140	CCTCCCTCTCCAAACACATTGC	TCATCAGCAGACAGAATGTTGACC
95)	RM25181	AAAGAGCTTCCCTAATGGCTTCG	GAGAGAATGACCTCTCCCAAGACC
96)	RM26212	GTCGCTCCTCTCCTCCAATCC	GCTCGCTGCTTCTAATCTCTTGC
97)	RM27920	AAAGCGAGAAATCCGGAGATGG	TCCTCTCTCAAATCTCCTCGAAGC
98)	RM27933	TCCTCTGTCATATGGCTGTAAACG	GGACAAGGAGGAACTATTGATTGG
99)	RM27962	GGGAGTCGTGGATTCTGAGACG	ATCCCACGCCAGGAGATAATAAGG
100)	RM27981	CTGGATGGCATCACTCCTATTGC	TCCCTCCCTAGCTCCAAGATCC

Results

4. Results

The objective of the present investigation entitled 'High temperature mediated changes in sugar signaling pathway and identification of associated microsattelite markers in rice (*Oryza sativa* L.)' was to study the effect of high temperature on the sugar signaling pathway and to identify the polymorphic SSR markers associated with heat tolerance in rice. The results of the investigation were analyzed statistically and presented in the following sections.

4.1 Experiment I - Effect of high temperature on sugar signaling pathway

The two rice varieties NL-44 and Vandana were evaluated under control and high temperature conditions. Their gene expression of the four target genes *OsHXK2, OsSnRK1, OsTPS1* and *OsTOR* was assessed using quantitative real-time PCR.

4.1.1 Physiological and yield parameters

4.1.1.1 *Plant height*

The data on the effect of high temperature stress on plant height is presented in table 7. The mean height of NL-44 was 106.09 cm while Vandana variety had a higher mean height of 129.6 cm. Both the varieties expressed significantly increased height under high temperature stress compared to control conditions.

4.1.1.2 *Tiller number*

The data on the tiller number which was measured at the harvest stage is presented in table 7. The number of tillers was significantly reduced in plants grown under the high temperature stress conditions. On average, Vandana variety characteristically produced almost twice the number of tillers (10.33) compared to NL-44 (5.66).

Table 7. Effect of high temperature stress on plant height and tiller number of rice genotypes NL-44 and Vandana

Plant height (cm)				Tiller number			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	104.96 c	107.23 c	**106.09**	**NL-44**	6.66 c	4.66 d	**5.66**
Vandana	126.5 b	132.03 a	**129.26**	**Vandana**	11.33 a	9.33 b	**10.33**
Mean	**115.73**	**119.63**		**Mean**	**8.995**	**6.995**	
C.D. ($p \leq 0.05$)	2.558			C.D. ($p \leq 0.05$)	1.104		
S.E.(m)	0.772			S.E.(m)	0.333		
C.V.	1.137			C.V.	7.217		

4.1.1.3 *Productive tiller number*

The statistical analysis of the results on the number of productive tillers is given in table 8. Similar to the tiller number, the productive tiller number showed a decreasing trend under the stress conditions for both the varieties compared to the un-stressed plants. The number of productive tillers of NL-44 variety under stress (4.33) and control (5.33) conditions did not vary significantly and were statistically on par.

4.1.1.4 *Cell membrane stability index*

The results of the cell membrane stability index is presented in table 8. The cell membrane stability index under control conditions was 82.7% and 76.7% for NL-44 and Vandana respectively. The MSI was significantly decreased in both the varieties recording 65.93% (NL-44) and 55.66% (Vandana) under stress (Figure 3).

Table 8. Effect of high temperature stress on productive tiller number and cell membrane stability index of rice genotypes NL-44 and Vandana

Productive tiller number				Cell membrane stability index			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	5.33 c	4.33 c	**4.83**	**NL-44**	82.7 a	65.93 c	**74.315**
Vandana	9.67 a	8.33 b	**9**	**Vandana**	76.7 b	55.93 d	**66.315**
Mean	**7.5**	**6.33**		**Mean**	**79.7**	**60.93**	
C.D. ($p \leq 0.05$)	1.104			C.D. ($p \leq 0.05$)	2.104		
S.E.(m)	0.333			S.E.(m)	0.645		
C.V.	8.347			C.V.	1.591		

4.1.1.5 *Days to flowering*

The data on the days to flowering, presented in table 9 shows that under normal environmental conditions, Vandana variety expressed earlier flowering habit (54 days) compared to NL-44 (66 days). Under heat stressed conditions, the plants of both the varieties took more number of days to come to flowering with regards to the plants under normal conditions which was statistically significant.

4.1.1.7 *Pollen viability*

In table 9, it can be observed that Vandana variety showed a statistically greater decrease in pollen viability (-30.23%) under high temperature conditions. The decrease under the same conditions was not so much in NL-44 which was only -7.79% although statistically significant compared to its control conditions.

Table 9. Effect of high temperature stress on days to flowering and pollen viability of rice genotypes NL-44 and Vandana

Days to flowering				Pollen viability (%)			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	66.33 b	72.67 a	**69.5**	**NL-44**	94 b	86.67 c	**90.33**
Vandana	54 d	58.33 c	**56.165**	**Vandana**	97 a	67.67 d	**82.33**
Mean	**60.165**	**65.5**		**Mean**	**95.5**	**77.17**	
C.D. ($p \leq 0.05$)	2.529			C.D. ($p \leq 0.05$)	2.814		
S.E.(m)	0.764			S.E.(m)	0.85		
C.V.	1.08			C.V.	1.705		

4.1.1.6 *Time and duration of anthesis*

The data on the time and duration of anthesis is presented in table 10. Under ambient temperature conditions, the time of anthesis for both Vandana and NL-44 started at 10:00 am. The difference lay in the duration of anthesis which was two hours for NL-44 whereas it was only one hour for Vandana. Under stress conditions, Vandana showed earlier anthesis time beginning at 9:30 am continuing upto one hour. Contrastingly, NL-44 showed a delay in anthesis time beginning at 10:30 am however, the duration of anthesis was comparatively reduced to one hour.

Table 10. Effect of high temperature stress on time of anthesis and duration of anthesis of rice genotypes NL-44 and Vandana

Time of anthesis (am)			Duration of anthesis (hrs)		
	Control	**Stress**		**Control**	**Stress**
NL-44	10:00 – 12:00	10:30 – 11:00	**NL-44**	2	1
Vandana	10:00 – 11:00	9:30 – 10:30	**Vandana**	1	1

4.1.1.8 *Panicle length*

The data on the panicle length, observed in table 11 reveals that the mean panicle length of NL-44 (27.66 cm) was much greater than that of Vandana (24.44 cm). The difference in the panicle length between the stress and control plants of NL-44 was not significant, however, significant difference was observed between the plants of the two conditions of Vandana variety with lower panicle length (23.26 cm) recorded in the stressed plants.

Table 11. Effect of high temperature stress on panicle length of rice genotypes NL-44 and Vandana

Panicle length (cm)			
	Control	**Stress**	**Mean**
NL-44	27.2 a	28.13 a	**27.665**
Vandana	25.63 b	23.26 c	**24.445**
Mean	**26.415**	**25.695**	
C.D. ($p \leq 0.05$)	1.103		
S.E.(m)	0.333		
C.V.	2.213		

4.1.1.9 *Photosynthetic rate*

The effect of high temperature stress on the photosynthetic rate is presented in table 12. The photosynthetic rate of NL-44 (30.15 µmol $cm^{-2}s^{-1}$) and Vandana (31.73 µmol $cm^{-2}s^{-1}$) was found to be on par under un-stressed conditions whereas they were significantly reduced under heat stress conditions with the least value recorded in Vandana (21.12 µmol $cm^{-2}s^{-1}$).

4.1.1.10 *Stomatal conductance*

The results of stomatal conductance, presented in table 12 reveals that Vandana variety recorded the highest stomatal conductance (0.278 mol $m^{-2}s^{-1}$) compared to NL-44 (0.201 mol $m^{-2}s^{-1}$) under control conditions. Under higher temperatures, the two varieties exhibited significantly contrasting trends with Vandana recording a decrease (0.227 mol $m^{-2}s^{-1}$) while NL-44 showed an increase (0.254 mol $m^{-2}s^{-1}$) in stomatal conductance compared to their respective controls.

Table 12. Effect of high temperature stress on photosynthetic rate and stomatal conductance of rice genotypes NL-44 and Vandana

Photosynthetic rate (µmol $cm^{-2}s^{-1}$)				**Stomatal conductance (mol $m^{-2}s^{-1}$)**			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	30.15 a	25.87 b	**28.01**	**NL-44**	0.2 c	0.254 ab	**0.227**
Vandana	31.73 a	21.12 c	**26.425**	**Vandana**	0.278 a	0.227 bc	**0.252**
Mean	**30.94**	**23.495**		**Mean**	**0.239**	**0.24**	
C.D. ($p \leq 0.05$)	2.155			C.D. ($p \leq 0.05$)	0.037		
S.E.(m)	0.651			S.E.(m)	0.011		
C.V.	4.14			C.V.	8.012		

4.1.1.11 *Transpiration rate*

The effect of high temperature on the rate of transpiration is shown in table 13. Under ambient temperature, NL-44 had lower rate of transpiration (5.89 mmol $m^{-2}s^{-1}$) compared to that of Vandana (7.82 mmol $m^{-2}s^{-1}$). However, under heat stress conditions, significantly increased transpiration rate was recorded in NL-44 (6.74 mmol $m^{-2}s^{-1}$) while there was a significant decrease in the rate of transpiration in Vandana (6.82 mmol $m^{-2}s^{-1}$) in relation to their corresponding controls.

4.1.1.12 *Leaf temperature*

The results on the leaf temperature analyzed and presented in table 13 show that the leaf temperature of the two varieties under ambient conditions was on par with a mean of 36.5° C. The leaf temperature was significantly decreased under stress treatment with a mean of 35° C although the difference between the varieties was not significant.

Table 13. Effect of high temperature stress on transpiration rate and leaf temperature of rice genotypes NL-44 and Vandana

Transpiration rate (mmol $m^{-2}s^{-1}$)				**Leaf temperature (˚C)**			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	5.89 c	6.74 b	**6.315**	**NL-44**	36.23 a	35.04 b	**35.635**
Vandana	7.82 a	6.82 b	**7.32**	**Vandana**	36.82 a	35 b	**35.91**
Mean	**6.855**	**6.78**		**Mean**	**36.525**	**35.02**	
C.D. (p≤0.05)	0.437			C.D. (p≤0.05)	1.102		
S.E.(m)	0.132			S.E.(m)	0.333		
C.V.	3.347			C.V.	1.611		

4.1.1.13 *Water-Use Efficiency (WUE)*

The data on the water-use efficiency presented in table 14 reveals that the WUE of NL-44 was significantly higher (5.11) under control conditions. The WUE of Vandana (3.09) and NL-44 (3.83) under stress were significantly lower compared to their corresponding non-stressed treatments.

4.1.1.14 *Fv/Fm ratio*

The effect of heat stress on the Fv/Fm ratio is presented in table 14. The Fv/Fm ratio is used as an estimate of the maximal yield of photochemical efficiency. The Fv/Fm

ratio of Vandana (0.763) was found to be significantly higher than NL-44 (0.688) under normal temperature conditions. The Fv/Fm ratio of NL-44 was significantly increased during high temperature (0.799). Contrastingly, there was non-significant decrease in the Fv/Fm ratio in Vandana (0.736) under stress.

Table 14. Effect of high temperature stress on water-use efficiency and Fv/Fm ratio of rice genotypes NL-44 and Vandana

Water-Use Efficiency				**Fv/Fm**			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	5.14 a	3.84 c	**4.49**	**NL-44**	0.681 c	0.799 a	**0.74**
Vandana	4.05 b	3.09 d	**3.57**	**Vandana**	0.763 ab	0.736 bc	**0.749**
Mean	**4.595**	**3.465**		**Mean**	**0.722**	**0.7675**	
C.D. ($p \leq 0.05$)	0.577			C.D. ($p \leq 0.05$)	0.059		
S.E.(m)	0.177			S.E.(m)	0.018		
C.V.	7.61			C.V.	4.155		

4.1.1.15 *ΦPSII*

The results of ϕPSII under high temperature stress conditions is presented in table 15. The effective quantum yield of Photosystem-II is denoted as ϕPSII. The ϕPSII of NL-44 (0.151) was significantly higher than Vandana (0.126) under ambient temperatures. The ϕPSII of both the varieties was decreased under increased temperature; however, the difference was not statistically significant.

4.1.1.16 *Electron transport rate (ETR)*

The effect of heat stress on the rate of electron transport is given in table 15. It was observed that the electron transport rate of NL-44 (99.05) was higher than Vandana (83.25) under un-stressed conditions. The ETR was significantly reduced under heat stress conditions for both the varieties.

Table 15. Effect of high temperature stress on ΦPSII and electron transport rate of rice genotypes NL-44 and Vandana

ΦPSII				ETR			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	0.151 a	0.131 ab	**0.141**	**NL-44**	99.05 a	86.56 b	**92.805**
Vandana	0.126 b	0.111 b	**0.118**	**Vandana**	83.25 c	73.29 d	**78.27**
Mean	**0.1385**	**0.121**		**Mean**	**91.15**	**79.925**	
C.D. (p≤0.05)	0.024			C.D. (p≤0.05)	15.35		
S.E.(m)	0.007			S.E.(m)	4.709		
C.V.	9.624			C.V.	9.535		

4.1.1.17 *Spikelet fertility*

The data on the spikelet fertility under heat stress conditions is given in table 16. The spikelet fertility percentage was found to be highest in Vandana (90.63%) with NL-44 recording 85.73% under non-stressed conditions. The extreme stress condition that the plants were subjected to was evident in the drastic reduction in the spikelet fertility percentage with NL-44 recording 30.83% and the least being observed in Vandana (11.73%).

4.1.1.18 *Thousand grain weight (TGW)*

The effect of high temperature on the thousand grain weight is presented in table 16. Under control conditions, the highest TGW was recorded in NL-44 (24.16 g) while Vandana had a weight of 20.86 g. The reduction in the grain weight under the high temperature stress was not statistically significant in the variety NL-44 (23.1 g). However, there was significant reduction in the TGW of Vandana (18.67 g) under the stress conditions.

Table 16. Effect of high temperature stress on spikelet fertility and 1000 grain weight of rice genotypes NL-44 and Vandana

Spikelet fertility (%)				1000 grain weight (g)			
	Control	**Stress**	**Mean**		**Control**	**Stress**	**Mean**
NL-44	85.73 b	30.83 c	**58.28**	**NL-44**	23.1 a	24.16 a	**23.63**
Vandana	90.63 a	11.73 d	**51.18**	**Vandana**	20.86 b	18.66 c	**19.76**
Mean	**88.18**	**21.28**		**Mean**	**21.98**	**21.41**	
C.D. ($p \leq 0.05$)	1.96			C.D. ($p \leq 0.05$)	1.239		
S.E.(m)	0.592			S.E.(m)	0.374		
C.V.	1.873			C.V.	2.987		

4.1.1.19 *Correlation Analysis*

Correlation analysis between the various morphological, physiological, gas exchange and yield related traits were conducted and presented in Figure 1. The data analysed revealed that significant positive correlation for the 1000 grain weight was observed with water use efficiency (WUE), electron transport rate (ETR), ϕPSII and pollen viability at 0.05 level, days to flowering at 0.01 level and panicle length at 0.001 level. Strong positive correlation was also obtained at 0.001 level between spikelet fertility and pollen viability, photosynthetic rate, leaf temperature and membrane stability index, and at 0.01 level with WUE.

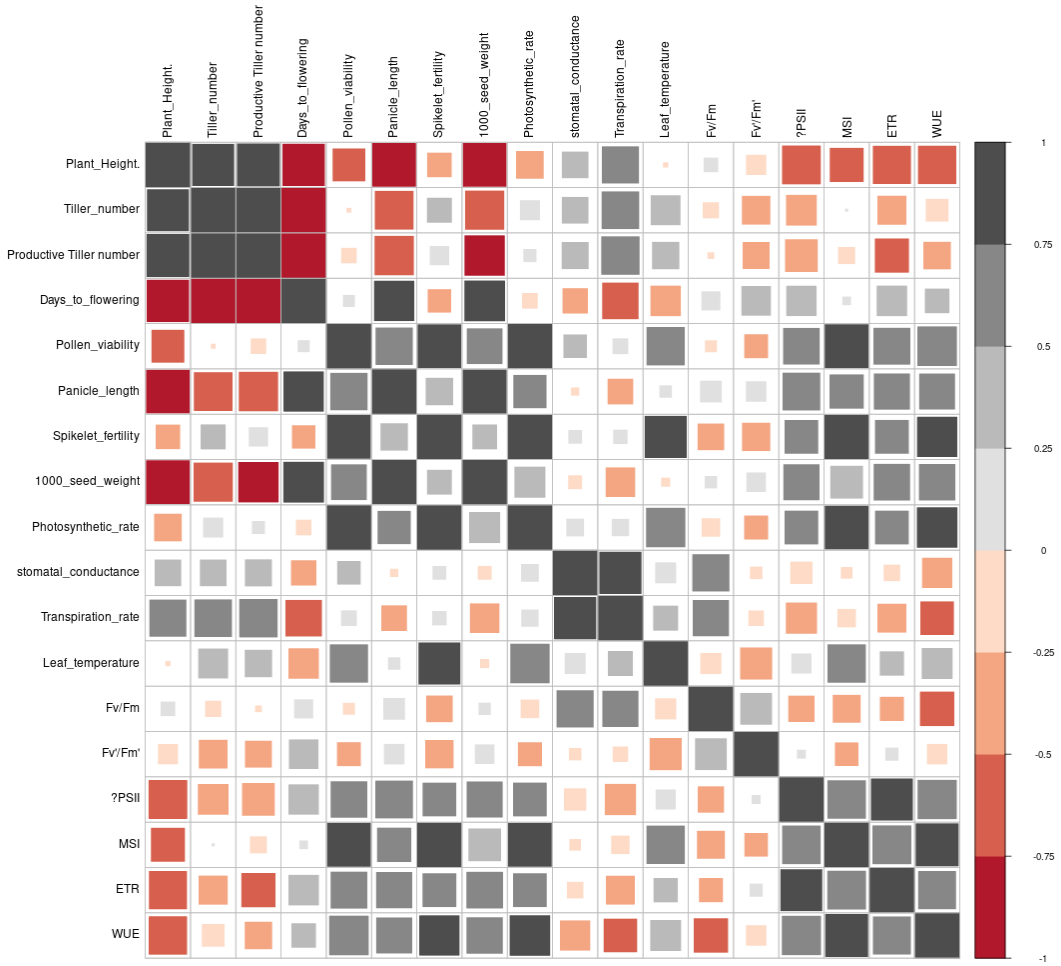


Figure 1. Correlation analysis of various parameters under the effect of high temperature stress on NL-44 and Vandana

4.1.2 Expression profile of genes

4.1.2.1 Vegetative phase

The data on the gene expression level under high temperature conditions during the vegetative phase is presented in table 17. The relative fold change value below 1.0 is taken as down-regulated expression and above 1.0 is considered as upregulation of genes. The genes *OsHXK2* and *OsSnRK1* were up-regulated in both the varieties. Their expression

levels were significantly higher in Vandana (28 and 7.31 respectively) compared to NL-44 (9.7 and 2.79 respectively). The expression of *OsTPS1* was down-regulated in both the varieties. The gene expression of *OsTOR* was down-regulated in NL-44 (0.23) and up-regulated in Vandana (1.29).

Table 17. Expression levels of genes during vegetative phase

Vegetative phase		***OsHXK2***	***OsSnRK1***	***OsTPS1***	***OsTOR***
NL-44	**Control**	1 c	1 c	1 a	1 b
	Stress	9.71 b	2.79 b	0.07 c	0.23 c
Vandana	**Control**	1 c	1 c	1 a	1 b
	Stress	28.64 a	7.31 a	0.84 b	1.29 a

4.1.2.2 Grain filling

The data on the expression levels of the target genes during the grain-filling stage is presented in table 18. It was noted that *OsHXK2* was up-regulated in the variety NL-44 (1.06) whereas it was down-regulated in Vandana (0.44). The genes *OsSnRK1*, *OsTPS1* and *OsTOR* were upregulated in both the varieties. The expression level of *OsSnRK1* was on par in both Vandana (1.52) and NL-44 (1.5). The expression level of *OsTPS1* in Vandana (3.3) was greater than in NL-44 (1.68) however, the reverse was true in case of *OsTOR* expression with Vandana (1.97) level lower than NL-44 (8.44).

Table 18. Expression levels of genes during grain-filling phase

Grain-filling phase		***OsHXK2***	***OsSnRK1***	***OsTPS1***	***OsTOR***
NL-44	**Control**	1 a	1 b	1 c	1 c
	Stress	1.06 a	1.5 a	1.68 b	8.44 a
Vandana	**Control**	1 a	1 b	1 c	1 c
	Stress	0.44 b	1.52 a	3.3 a	1.97 b

4.2 Experiment II – Production of F_1 hybrids and their F_2 progeny

4.2.1 Characteristics of parents

The two rice varieties, Uma and NL-44 were evaluated for their general growth characteristics and utilized in the current crossing programme. The experiment was undertaken at ambient environmental conditions with the maximum temperature of 32-34˚ C. Uma variety is characterized as a medium duration genotype with 120 days growth duration with a medium length, bold grain character with flowering time of 78 days after sowing. The variety NERICA L-44 (NL-44) is a short duration genotype having 95 days duration exhibiting a grain character of long, slender type and flowering time of 65 days after sowing.

4.2.2 Production of F_1 hybrids

The two varieties were sown at different times to adjust their flowering times to carry out hybridization. The two varieties were crossed reciprocally with one another using the clipping method of hybridization. The plants with the potential hybrid seeds were transferred to a protected polyhouse until the maturation of grains. All the F_1 generation hybrid seeds thus produced were collected carefully into paper bags until the moisture content in the seeds was sufficiently decreased for the next generation.

4.2.3 Production of F_2 generation seeds

The F_1 hybrid seeds (plate 11) produced from crossing the parents Uma and NL-44 were raised at ambient environmental conditions similar to that of previous generation. The F_1 progeny plants were allowed to undergo selfing and carefully nurtured till maturity using standard agronomic practices without any stress imposition. The F_2 generation seeds thus produced were harvested and stored in paper bags for drying to sufficient moisture level. The F_2 generation seeds exhibited characteristics with the length and thickness intermediate to that of both the parents (plate 12).

4.3 Experiment III - Phenotypic evaluation of F_2 population of NL-44 x Uma for high temperature tolerance

The F_2 generation seeds produced in the previous experiment were raised in a nursery and transplanted. The 144 F_2 plants which germinated and were established successfully were evaluated along with the two parents, Uma and NL-44 under heat stress conditions. The experiment was conducted as an un-replicated trial. The phenotypic evaluation was carried out using various physiological and yield-related parameters.

4.3.1 Plant height

The frequency distribution of the plant height of the F_2 progeny is shown in Figure 2 and the data is summarized in table 19. The mean plant height of the population was 99.09 cm with the minimum height being 64 cm and the maximum recorded as 121 cm. The plant height of Uma variety was recorded as 96.73 cm while NL-44 recorded 105.62 cm. The normal distribution curve of the population data had a kurtosis of 0.66. The curve had a negative skewness of -0.31.

4.3.2 Number of tillers

The data on the number of tillers presented in table 19 summarizes the performance of the F_2 progeny under heat stress (Figure 3). The average number of tillers was 10.17 with the highest number of tillers recorded as 17 and the least being 5. The number of tillers recorded in Uma was 11 while the NL-44 produced 8 tillers. The curve of the the normal distribution of the population was platykurtic (-0.34) and had a positive skewness (0.44).

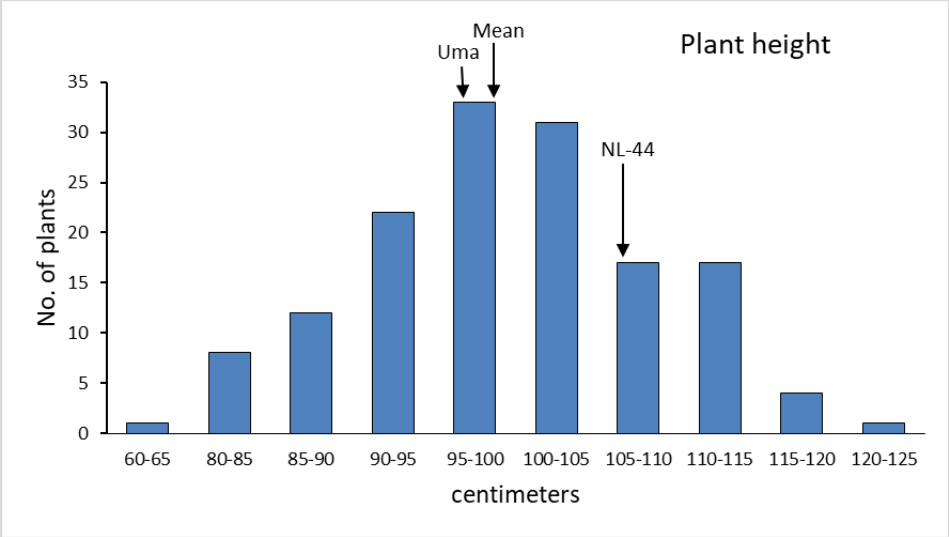


Figure 2. Frequency distribution of plant height under the effect of high temperature stress among the F_2 population

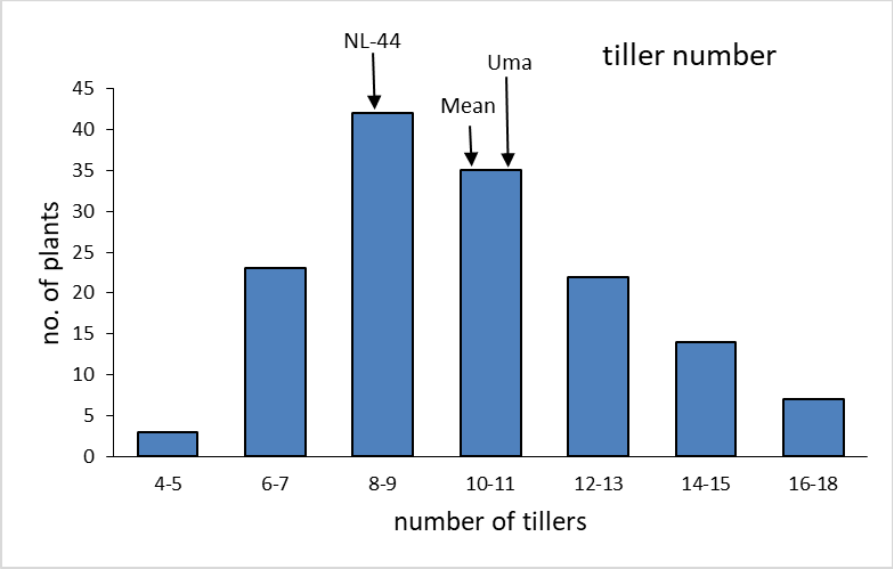


Figure 3. Frequency distribution of tiller number under the effect of high temperature stress among the F2 population

Table 19. Evaluation of plant height and tiller number under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	*Plant height (cm)*	*tiller number*
Mean	99.09	10.17
Standard Error	0.74	0.23
Median	99	10
Mode	104	9
Standard Deviation	9.01	2.83
Sample Variance	81.20	8.03
Kurtosis	0.66	-0.34
Skewness	-0.31	0.44
Range	57	12
Minimum	64	5
Maximum	121	17
Count	146	146
Confidence Level (95.0%)	1.47	0.46

4.3.3 Number of productive tillers

The descriptive statistics of F_2 population based on the number of productive tillers is presented in table 20. The average number of productive tillers was 5.58 with the lowest number being 2 while the maximum productive tiller number was 11. Uma variety recorded 8 productive tillers under heat stress whereas NL-44 recorded 6 tillers (Figure 4). The normal distribution of the population data recorded a platykurtic curve with a value of -0.05. The positive skewness value of 0.61 is indicative of a right leaning curve.

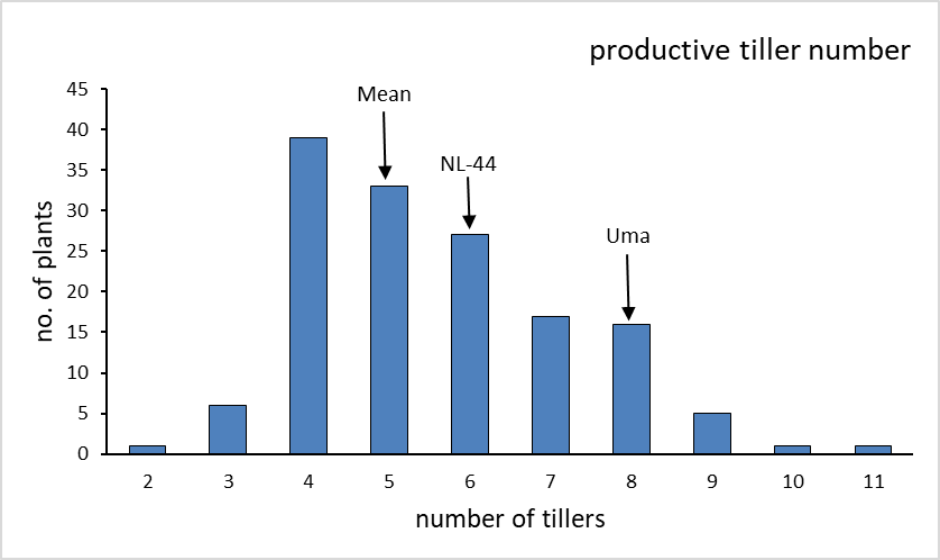


Figure 4. Frequency distribution of productive tiller number under the effect of high temperature stress among the F_2 population

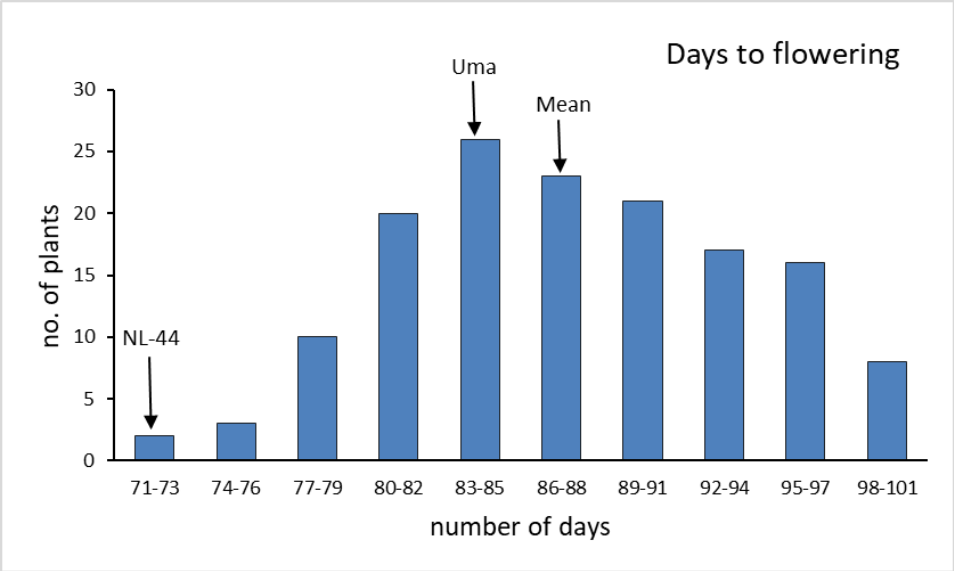


Figure 5. Frequency distribution of days to flowering under the effect of high temperature stress among the F_2 population

4.3.4 Days to flowering

The data on the number of days taken to flowering by the F_2 progeny is presented in table 20. The mean of the population was calculated as 87.5 days having a range of 30 days with the least being 71 days and the maximum being 101 days. The variety Uma was recorded to flower at around 83 days while NL-44 has taken 71 days to flower (Figure 5). The curve of the normal distribution of the data was platykurtic (-0.64) with an almost perfect normal curve having a skewness of 0.01.

Table 20. Evaluation of productive tiller number and Days to flowering under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	***Productive tiller number***	***Days to flowering***
Mean	5.58	87.5
Standard Error	0.13	0.53
Median	5	87
Mode	4	91
Standard Deviation	1.66	6.41
Sample Variance	2.76	41.20
Kurtosis	-0.05	-0.64
Skewness	0.61	0.01
Range	9	30
Minimum	2	71
Maximum	11	101
Count	146	146
Confidence Level (95.0%)	0.27	1.04

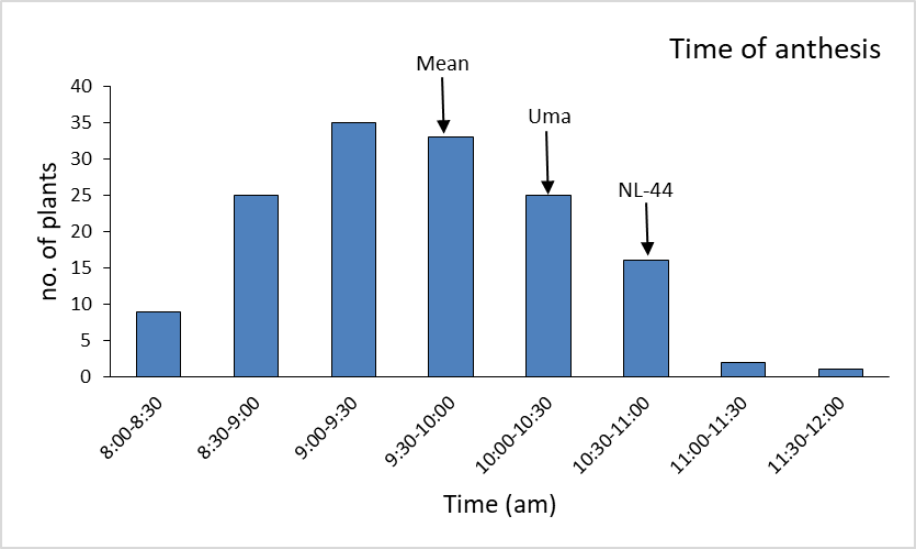


Figure 6. Frequency distribution of time of anthesis under the effect of high temperature stress among the F_2 population

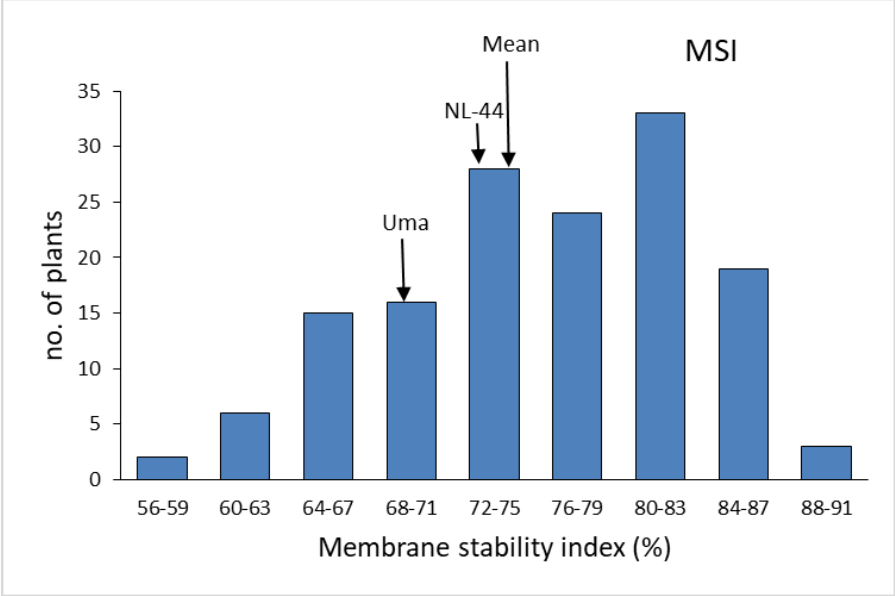


Figure 7. Frequency distribution of membrane stability index under the effect of high temperature stress among the F_2 population

4.3.5 Time of anthesis

The analysis of data on the time of anthesis under the effect of heat stress is presented in table 21. The mean time of anthesis of the F_2 population is 9:47 am having a range of 3 hours and 45 minutes with the earliest anthesis occurring at 8:00 am while the most delayed anthesis occurring at 11:45 am. The time of anthesis of the Uma variety was recorded at 10:15 am while that of NL-44 was 10:45 am (Figure 6). The normal distribution curve was platykurtic (-0.52) with a skewness of 0.08.

4.3.6 Membrane stability index (MSI)

The effect of heat stress on the membrane stability index of the F_2 population is analysed and presented in table 21. The mean MSI of the population was calculated as 75.84 with the minimum MSI of 56 and a maximum MSI of 90. The MSI of Uma variety was 68 while that of NL-44 was 79 (Figure 7). The normal distribution curve was platykurtic (-0.58) with a negative skewness of -0.40.

Table 21. Evaluation of time of anthesis and membrane stability index (MSI) under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	*Time of anthesis (a.m.)*	*MSI (%)*
Mean	9:47	75.84
Standard Error	0.06	0.60
Median	9:47	76.5
Mode	10:00	81
Standard Deviation	0.75	7.30
Sample Variance	0.56	53.38
Kurtosis	-0.52	-0.58
Skewness	0.08	-0.40
Range	3:45	34
Minimum	8:00	56
Maximum	11:45	90
Count	146	146
Confidence Level (95.0%)	0.12	1.19

4.3.7 Photosynthetic rate (Pn)

The photosynthetic rate of the F_2 population under the effect of high temperature stress has been analyzed and presented in table 22. The mean photosynthetic rate (Pn) of the population was calculated as 24.35 µmol $cm^{-2}s^{-1}$ with the minimum Pn of 15.6 µmol $cm^{-2}s^{-1}$ and a maximum Pn of 29.7 µmol $cm^{-2}s^{-1}$. The Pn of Uma variety was 20.68 µmol $cm^{-2}s^{-1}$ while that of NL-44 was 27.21 µmol $cm^{-2}s^{-1}$ (Figure 8). The normal distribution curve was platykurtic (-0.51) with a negative skewness of -0.64.

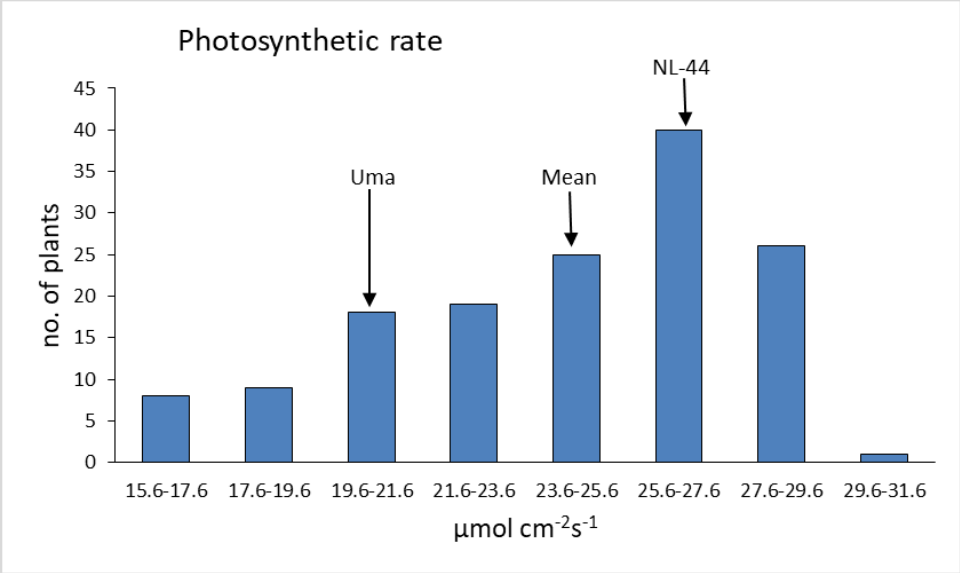


Figure 8. Frequency distribution of photosynthetic rate under the effect of high temperature stress among the F_2 population

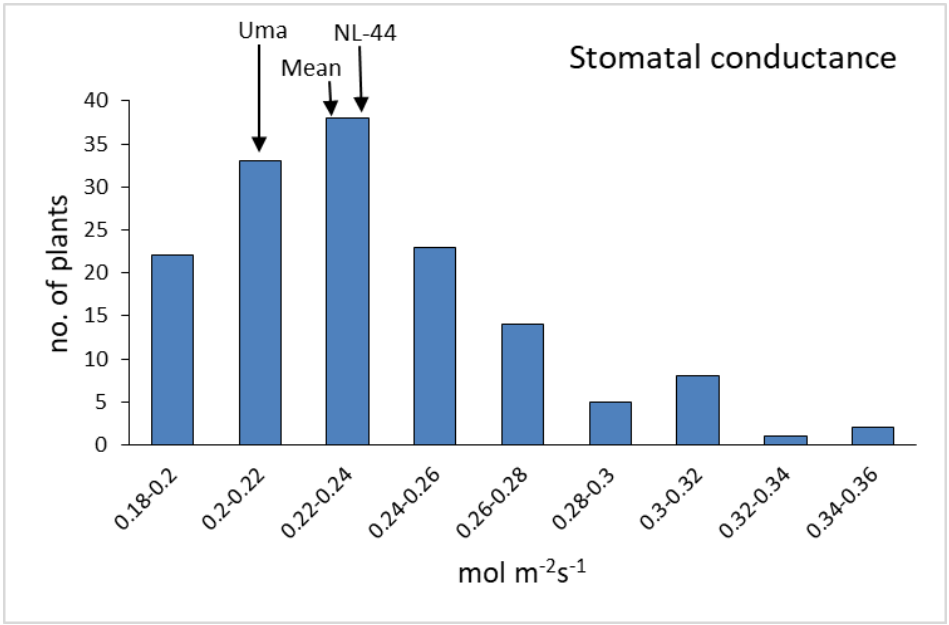


Figure 9. Frequency distribution of stomatal conductance under the effect of high temperature stress among the F_2 population

4.3.8 Stomatal conductance (Gs)

The descriptive statistics on the stomatal conductance of the F_2 population under the effect of high temperature stress is presented in table 22. The mean stomatal conductance of the F_2 population was 0.234 mol $m^{-2}s^{-1}$ having a range of 0.164 mol $m^{-2}s^{-1}$ where the maximum Gs was recorded as 0.345 mol $m^{-2}s^{-1}$ and the minimum Gs was 0.181 mol $m^{-2}s^{-1}$. The Gs of Uma variety was recorded to be 0.213 mol $m^{-2}s^{-1}$ while that of NL-44 was 0.236 mol $m^{-2}s^{-1}$ (Figure 9). The normal distribution curve was leptokurtic (0.37) with a positive skewness of 0.79.

Table 22. Evaluation of photosynthetic rate and stomatal conductance under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	***Photosynthetic rate*** **(µmol $cm^{-2}s^{-1}$)**	***Stomatal conductance*** **(mol $m^{-2}s^{-1}$)**
Mean	24.35	0.23
Standard Error	0.28	0.0029
Median	25.3	0.23
Mode	23.4	0.234
Standard Deviation	3.47	0.035
Sample Variance	12.08	0.0012
Kurtosis	-0.51	0.37
Skewness	-0.64	0.79
Range	14.1	0.164
Minimum	15.6	0.181
Maximum	29.7	0.345
Count	146	146
Confidence Level (95.0%)	0.56	0.0058

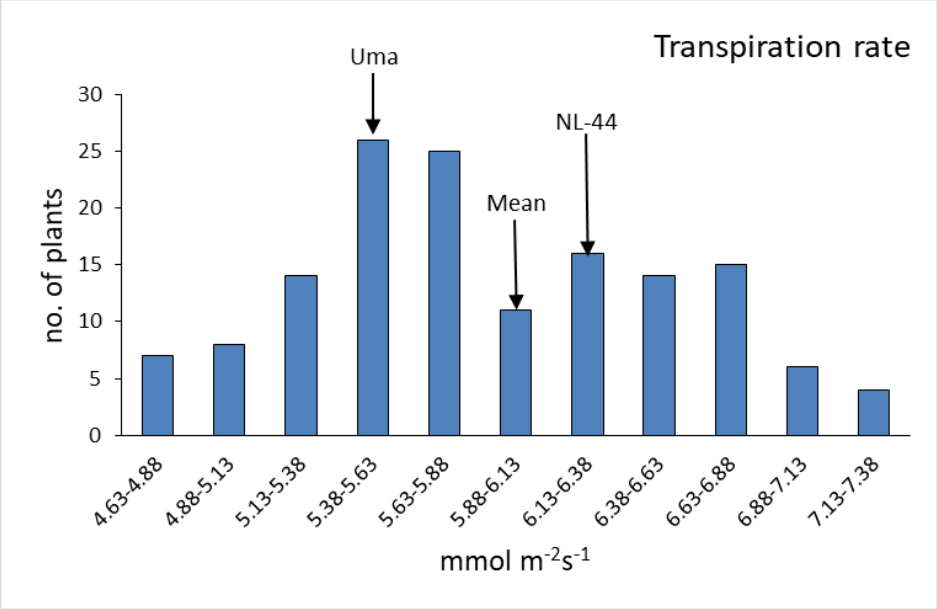


Figure 10. Frequency distribution of transpiration rate under the effect of high temperature stress among the F_2 population

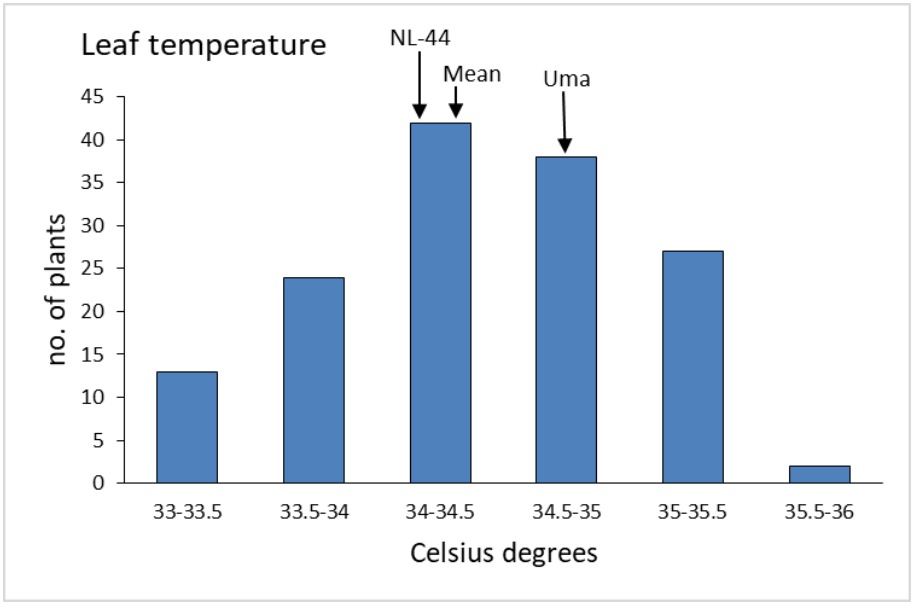


Figure 11. Frequency distribution of leaf temperature under the effect of high temperature stress among the F_2 population

4.3.9 Transpiration rate

The descriptive statistics on the stomatal conductance of the F_2 population under the effect of high temperature stress is presented in table 23. The mean transpiration rate of the F_2 population was 5.91 mmol $m^{-2}s^{-1}$ having a range of 2.52 mmol $m^{-2}s^{-1}$ where the maximum transpiration rate was recorded as 7.15 mmol $m^{-2}s^{-1}$ and the minimum was 4.63 mmol $m^{-2}s^{-1}$. The transpiration rate of Uma variety was recorded to be 5.45 mmol $m^{-2}s^{-1}$ while that of NL-44 was 6.24 mmol $m^{-2}s^{-1}$ (Figure 10). The normal distribution curve was platykurtic (-0.86) with a positive skewness of 0.16.

Table 23. Evaluation of transpiration rate and leaf temperature under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	***Transpiration rate (mmol $m^{-2}s^{-1}$)***	***Leaf temperature*** (˚C)
Mean	5.91	34.41
Standard Error	0.051	0.05
Median	5.82	34.43
Mode	5.14	34.91
Standard Deviation	0.62	0.60
Sample Variance	0.39	0.36
Kurtosis	-0.86	-0.74
Skewness	0.16	-0.15
Range	2.52	2.67
Minimum	4.63	33.14
Maximum	7.15	35.81
Count	146	146
Confidence Level (95.0%)	0.10	0.098

4.3.10 Leaf temperature

The data on the leaf temperature of the F_2 population is provided in table 23. The mean of the leaf temperature was calculated to be 34.4 ˚C having a range difference of 2.6 ˚C with the maximum leaf temperature of 35.8 ˚C and minimum of 33.1 ˚C. The leaf temperature of the Uma variety was recorded as 34.87 ˚C and that of NL-44 as 34.16 ˚C (Figure 11). The normal distribution curve of the data was platykurtic (-0.74) with a negative skewness of -0.15.

4.3.11 Pollen Viability

The analysis of the data on the pollen viability of the F_2 population under the effect of heat stress is presented in table 24. The mean of the population was calculated as 77.76 % having a range of 32 % with the least pollen viability percentage of 59 % and the highest pollen viability of 91 %. The pollen viability of the Uma variety was recorded as 74 % and that of NL-44 was 86.67 % (Figure 12). The normal distribution of the population data revealed a mesokurtic curve with a value of 0.03 and a negative skewness of -0.62.

4.3.12 Panicle length

The analysis of the data on the panicle length of the F_2 population under the effect of heat stress is presented in table 24. The mean of the population was calculated as 22.94 cm having a range of 10.2 cm with the least panicle length of 17.6 cm and the highest panicle length of 27.8 cm. The panicle length of the Uma variety was recorded as 23.4 cm and that of NL-44 was 27.62 cm (Figure 13). The normal distribution of the population data revealed a platykurtic curve with a value of -0.32 and a negative skewness of -0.18.

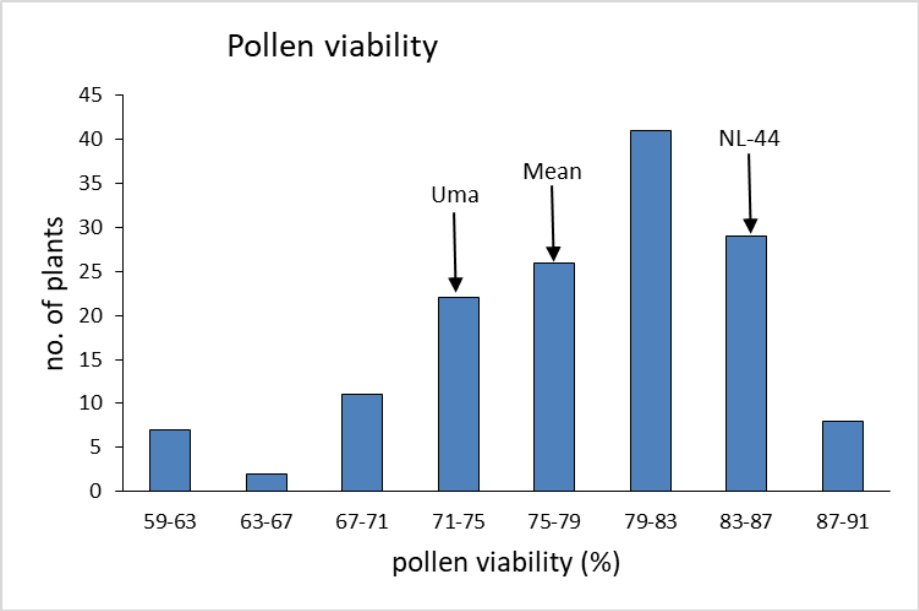


Figure 12. Frequency distribution of pollen viability under the effect of high temperature stress among the F_2 population

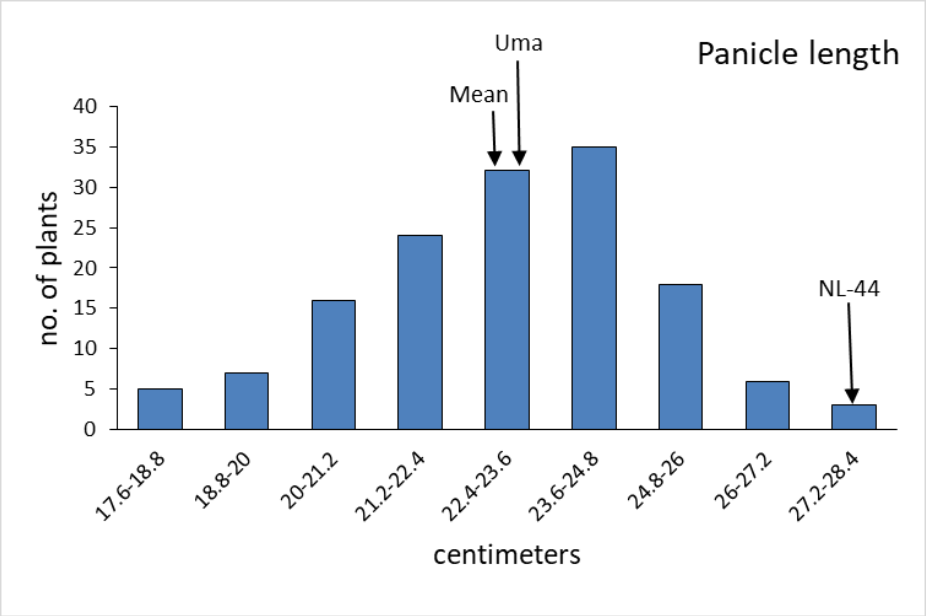


Figure 13. Frequency distribution of panicle length under the effect of high temperature stress among the F_2 population

Table 24. Evaluation of pollen viability and panicle length under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	*Pollen viability (%)*	*Panicle length (cm)*
Mean	77.76	22.94
Standard Error	0.56	0.17
Median	79	23.1
Mode	81	24.2
Standard Deviation	6.82	2.07
Sample Variance	46.53	4.31
Kurtosis	0.035	-0.32
Skewness	-0.62	-0.18
Range	32	10.2
Minimum	59	17.6
Maximum	91	27.8
Count	146	146
Confidence Level (95.0%)	1.11	0.33

4.3.13 Spikelet fertility

The effect of heat stress on the spikelet fertility percentage of the F_2 progeny is summarized in table 25. The mean spikelet fertility percentage of the population was 41.54 % with the minimum spikelet fertility of 15.17 % and the maximum recorded as 75.27 %. The spikelet fertility of Uma variety was calculated as 35.16 % while that of NL-44 was 56.15 % (Figure 14). The normal distribution curve of the population data had a kurtosis of -0.05, which is mesokurtic and a positive skewness of 0.33.

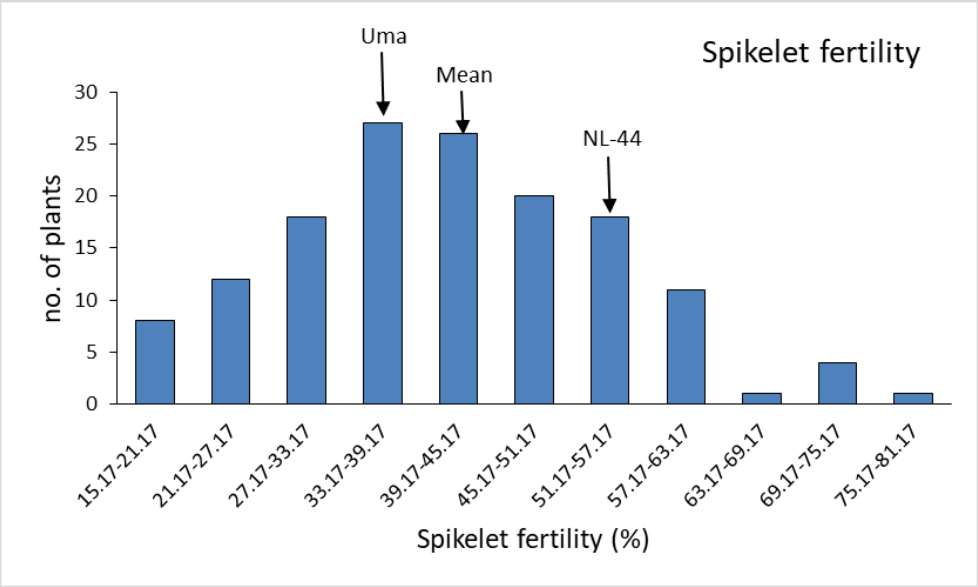


Figure 14. Frequency distribution of spikelet fertility under the effect of high temperature stress among the F_2 population

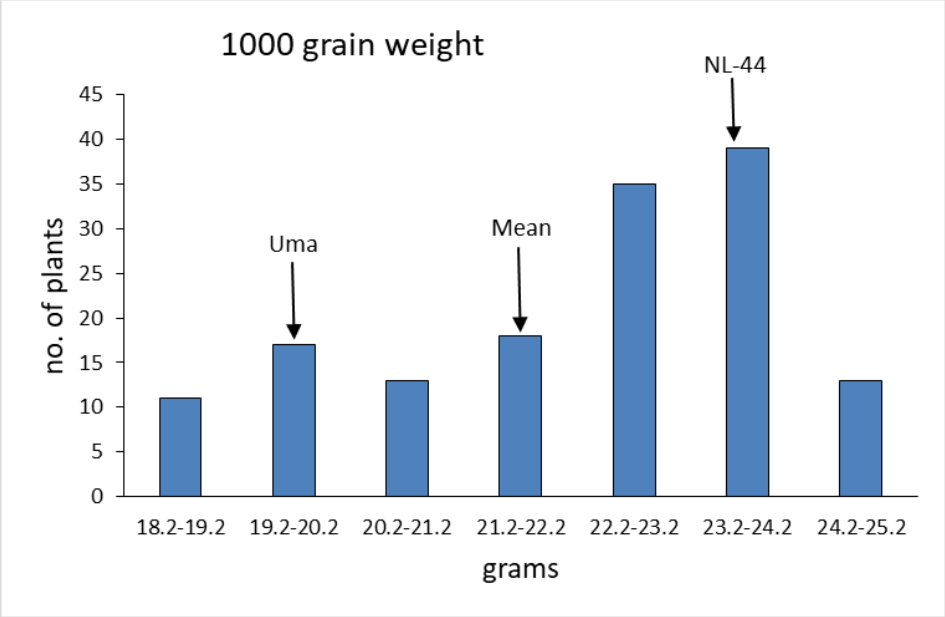


Figure 15. Frequency distribution of 1000 grain weight under the effect of high temperature stress among the F_2 population

4.3.14 Thousand grain weight (TGW)

The effect of heat stress on the thousand grain weight of the F_2 progeny is summarized in table 25. The mean TGW of the population was 22.11 g with the least TGW of 18.2 g and the maximum recorded as 24.8 g. The TGW of Uma variety was recorded to be 20.14 g while that of NL-44 was 23.27 g (Figure 15). The normal distribution curve of the population data had a kurtosis of -0.76, which is platykurtic and a negative skewness of -0.58.

Table 25. Evaluation of spikelet fertility percentage under the effect of heat stress on the F_2 population of NL-44 x Uma

Descriptive statistical parameter	***Spikelet fertility (%)***	***1000 grain weight (g)***
Mean	41.54	22.11
Standard Error	1.05	0.14
Median	40.90	22.5
Mode	46.53	22.5
Standard Deviation	12.69	1.74
Sample Variance	161.07	3.053
Kurtosis	-0.05	-0.76
Skewness	0.33	-0.58
Range	60.1	6.6
Minimum	15.17	18.2
Maximum	75.27	24.8
Count	146	146
Confidence Level (95.0%)	2.075	0.28

4.3.15 Correlation Analysis

The correlation matrix of the parameters assessed in the F_2 population under high temperature stress is presented in table 26. The tiller number was positively correlated with the productive tiller number at 0.001 level of significance and with days to flowering and and pollen viability at 0.01 level of significance. The productive tiller number was found to be correlated positively with membrane stability index. The time of anthesis was negatively correlated with spikelet fertility and 1000 grain weight at 0.05 level of significance. The membrane stability index was significantly correlated with the pollen viability, spikelet fertility and 1000 grain weight in a positive manner. The pollen viability was also found to have a significant positive correlation with spikelet fertility and 1000 seed weight whereas it was negatively correlated with the leaf temperature. The correlation between spikelet fertility and 1000 grain weight was positive at ($p \leq 0.001$) level. The parameters of photosynthetic rate, evapotranspiration rate, stomatal conductance and leaf temperature were found to be strongly correlated to one another at 0.001 level of significance.

Table 26. Correlation matrix of F_2 population under high temperature stress

	Plant Height	No. of tillers	Productive tiller no.	Days to flowering	Time of anthesis	MSI	Pollen Viability	Panicle length (cm)	Spikelet fertility	1000 seed weight	Pn	E	Gs	T
Plant Height	1	0.073	0.157	-0.014	0.023	-0.002	-0.074	0.095	0.022	-0.069	0.006	-0.039	0.054	0.041
No. of tillers	0.073	1	0.789***	0.195*	0.151	0.136	0.171*	0.035	0.079	0.071	-0.197*	-0.15	-0.151	-0.246**
Productive tiller no.	0.157	0.789***	1	0.017	0.102	0.177*	0.161	0.002	0.115	0.112	-0.209*	-0.178*	-0.139	-0.261**
Days to flowering	-0.014	0.195*	0.017	1	0.023	0.133	-0.007	-0.001	-0.004	0.041	0.096	0.061	0.133	0.035
Time of anthesis	0.023	0.151	0.102	0.023	1	-0.111	-0.151	-0.009	-0.165*	-0.184*	0.022	0.047	0.051	0.007
MSI	-0.002	0.136	0.177*	0.133	-0.111	1	0.458***	0.06	0.324***	0.274***	0.039	-0.008	0.035	-0.073
Pollen Viability	-0.074	0.171*	0.161	-0.007	-0.151	0.458***	1	0.074	0.315***	0.298***	-0.116	-0.161	-0.158	-0.253**
Panicle length (cm)	0.095	0.035	0.002	-0.001	-0.009	0.06	0.074	1	0.001	-0.052	-0.144	-0.165*	-0.037	-0.226**
Spikelet fertility	0.022	0.079	0.115	-0.004	-0.165*	0.324***	0.315***	0.001	1	0.861***	0.036	-0.015	-0.055	-0.107
1000 seed weight	-0.069	0.071	0.112	0.041	-0.184*	0.274***	0.298***	-0.052	0.861***	1	-0.026	-0.064	-0.115	-0.151
Pn	0.006	-0.197*	-0.209*	0.096	0.022	0.039	-0.116	-0.144	0.036	-0.026	1	0.896***	0.679***	0.764***
E	-0.039	-0.15	-0.178*	0.061	0.047	-0.008	-0.161	-0.165*	-0.015	-0.064	0.896***	1	0.706***	0.835***
Gs	0.054	-0.151	-0.139	0.133	0.051	0.035	-0.158	-0.037	-0.055	-0.115	0.679***	0.706***	1	0.66***
T	0.041	-0.246**	-0.261**	0.035	0.007	-0.073	-0.253**	-0.226**	-0.107	-0.151	0.764***	0.835***	0.66***	1

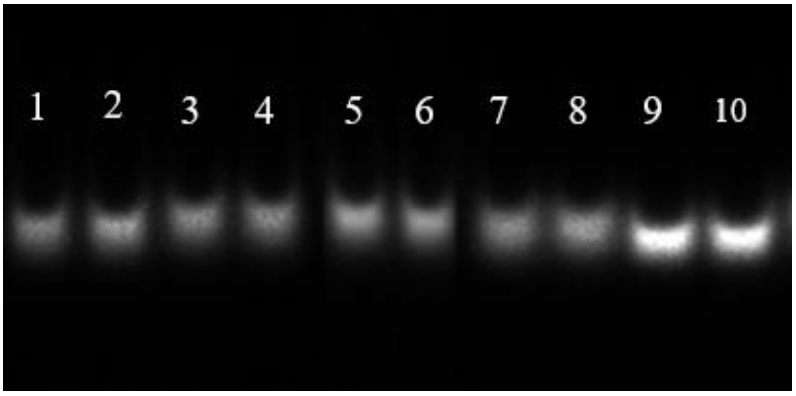
(*** indicates significance at 0.001 level (two tailed), ** indicates significance at 0.01 level (two tailed) and * indicates significance at 0.05 level (two tailed))

4.4 Experiment IV - Identification of molecular markers linked to high temperature tolerance in rice using Bulked Segregant Analysis

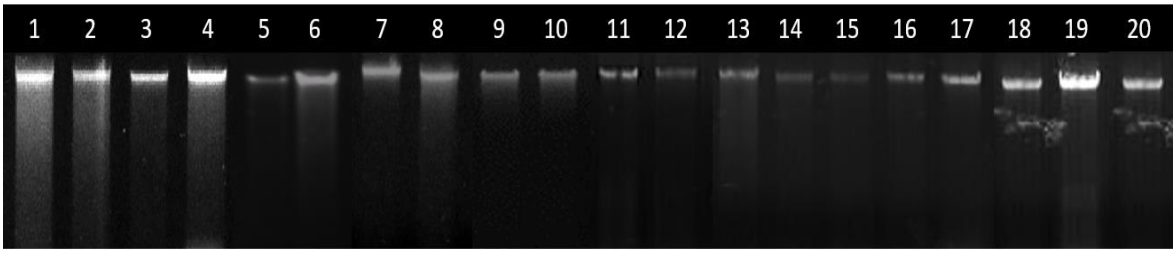
Based on the results of the phenotypic evaluation of the 144 F_2 generation plants, ten plants each that were extremely tolerant and extremely susceptible to heat stress were selected. The spikelet fertility percentage parameter was specifically used to characterize the plants as tolerant or susceptible. The F_2 generation line numbers 3, 6, 7, 56, 79, 82, 49, 98, 121, 143 were selected as tolerant and line numbers 25, 54, 72, 73, 92, 93, 103, 104, 126, 141 were selected as susceptible. Bulked segregant analysis (BSA) was carried out using 100 SSR markers to identify the polymorphic markers that were linked to high temperature tolerance through the amplification pattern of the DNA of the tolerant and susceptible bulks and that of the tolerant (NL-44) and susceptible (Uma) parents.

4.4.1 Quality of DNA isolated

The DNA from the selected ten tolerant and ten susceptible F_2 lines was isolated along with that of the two parental genotypes. The quality and quantification of the DNA isolated is presented in table 28. The quality of the DNA isolated, measured by the ratio (A260/A280) was in the range of 1.68 and 1.92 which is deemed to be of sufficient quality. The DNA of the F_1 hybrids was also isolated, and the quality and quantity determined in a similar manner, is presented in table 27. The gel profile of the bands isolated is shown in plate 13.



a) F_1 generation (1 to 10 – F_1 plants)



b) F_2 generation (1 to 20 – F_2 plants)

Plate 13. Gel profile of DNA derived from F_1 and F_2 generation plants

Table 27. DNA quantification of F_1 generation plants

S.no.	A_{260}	A_{280}	A_{260}/A_{280} value	DNA concentration (ng/µl)
1.	2.03	1.21	1.68	3045
2.	1.59	0.84	1.89	2385
3.	2.26	1.33	1.70	3390
4.	1.37	0.71	1.93	2055
5.	1.87	0.98	1.91	2805
6.	2.08	1.11	1.87	3120
7.	2.38	1.27	1.87	3570
8.	1.42	0.87	1.63	2130
9.	2.55	1.48	1.72	3825
10.	2.09	1.25	1.67	3135

Table 28. DNA quantification of F_2 generation plants

S.no.	A_{260}	A_{280}	A_{260}/A_{280} value	DNA concentration (ng/µl)
1.	1.14	0.66	1.75	1710
2.	1.73	0.92	1.88	2595
3.	2.21	1.22	1.81	3315
4.	2.46	1.28	1.92	3690
5.	3.02	1.64	1.84	4530
6.	1.38	0.79	1.75	2070
7.	2.07	1.13	1.82	3105
8.	1.98	1.14	1.74	2970
9.	2.82	1.65	1.71	4230
10.	1.74	0.92	1.89	3610
11.	3.06	1.65	1.85	4590
12.	1.71	0.87	1.97	2565
13.	1.38	0.75	1.84	2070
14.	3.06	1.77	1.73	4590
15.	2.07	1.23	1.68	3105
16.	1.23	0.66	1.86	1845
17.	2.97	1.62	1.83	4455
18.	2.61	1.46	1.78	3915
19.	1.38	0.78	1.77	2070
20.	2.37	1.38	1.72	3555

4.4.2 Quality of PCR products

The DNA was amplified through polymerase chain reaction (PCR) using the SSR markers and the amplification pattern obtained on the 3% agarose gel is presented in plate numbers 15 – 20. The size of the band was compared with a 100 bp ladder. The gel profile obtained indicates that they are deemed to be of sufficient quality. The F_1 lines were subjected to PCR amplification using the marker RM10793 to test whether the lines were true hybrids. The amplification pattern obtained confirms that they are indeed hybrids as they possessed heterozygous bands that had both bands corresponding to the bands obtained with the susceptible and tolerant parents. The amplification pattern has been shown in plate 14.

4.4.3 Polymorphism between the tolerant and susceptible bulks

To identify the potential polymorphic markers, PCR reactions with the 100 SSR markers were conducted using the two parents. Based on the amplification pattern obtained, 18 markers were determined to exhibit polymorphism between the parents. The list of the identified markers is given in table 29. The amplification pattern of the polymorphism between the parents is shown in plates 15 and 16. Through the technique of BSA, the identified polymorphic markers were tested for polymorphism between the tolerant and susceptible DNA bulks. In this technique, the DNA from the ten tolerant and ten susceptible lines were bulked by taking equal quantities from each of the respective tolerant or susceptible lines into the tolerant and susceptible bulks. Thereafter, PCR reactions were carried out using the polymorphic markers using the DNA from the two parents and the two bulks. The amplification pattern obtained between the bulks and the parents is shown in plate numbers 17, 18 and 19.

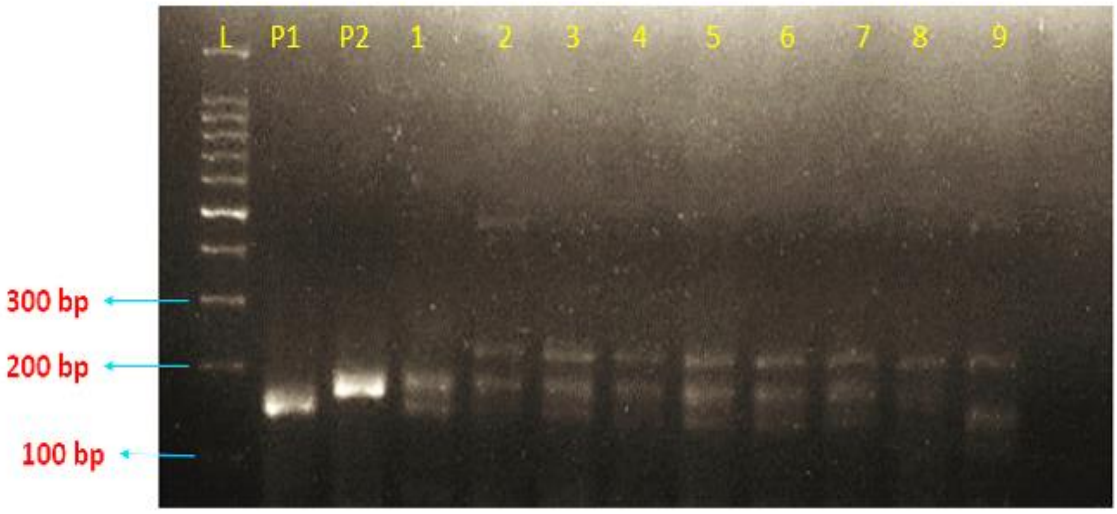


Plate 14. Amplification pattern of F_1 generation plants using SSR marker RM10793

(L- 100 bp ladder, P1 – Uma, P2 – NL-44, 1 to 8 – F_2 plants)

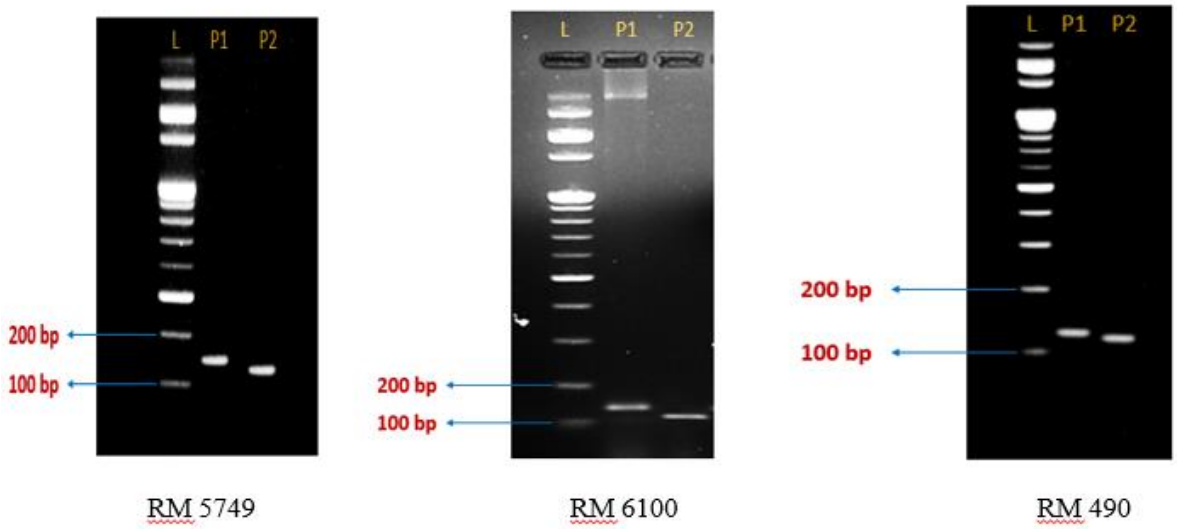


Plate 15. Amplification pattern of SSR markers RM5749, RM6100 and RM490 expressing polymorphism between parents (L-100 bp ladder, P1 – Uma, P2 – NL-44)

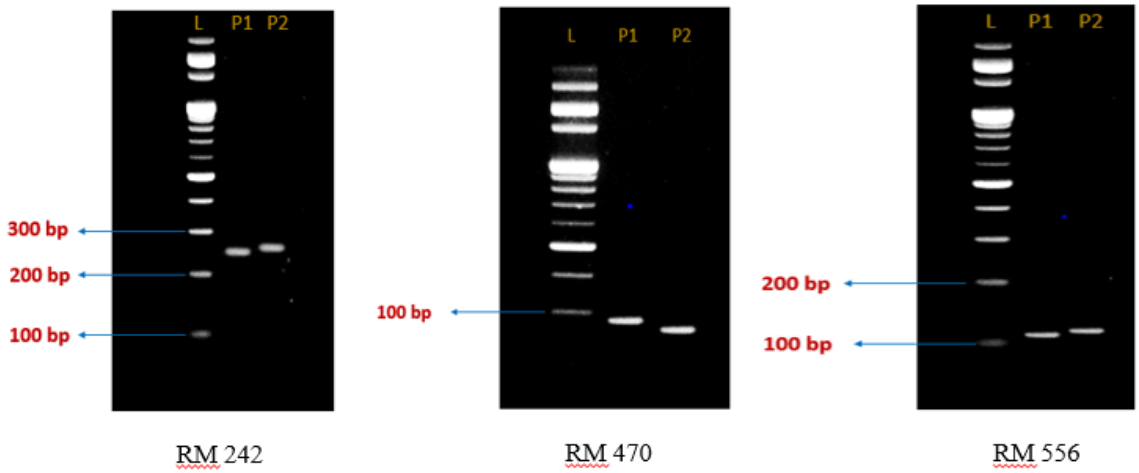


Plate 16. Amplification pattern of SSR markers RM242, RM470 and RM556 expressing polymorphism between parents (L-100 bp ladder, P1 – Uma, P2 – NL-44)

Table 29. List of SSR markers exhibiting polymorphism between parents

S. no.	Marker	Location
1	RM222	Chr. 10
2	RM237	Chr.1
3	RM242	Chr.9
4	RM310	Chr. 8
5	RM320	Chr. 7
6	RM337	Chr. 8
7	RM413	Chr. 5
8	RM470	Chr. 4
9	RM471	Chr. 4
10	RM473	Chr. 7
11	RM490	Chr. 1
12	RM554	Chr. 3
13	RM556	Chr. 8
14	RM3475	Chr. 1
15	RM3586	Chr. 3
16	RM5749	Chr. 4
17	RM6100	Chr. 10
18	RM10793	Chr. 1

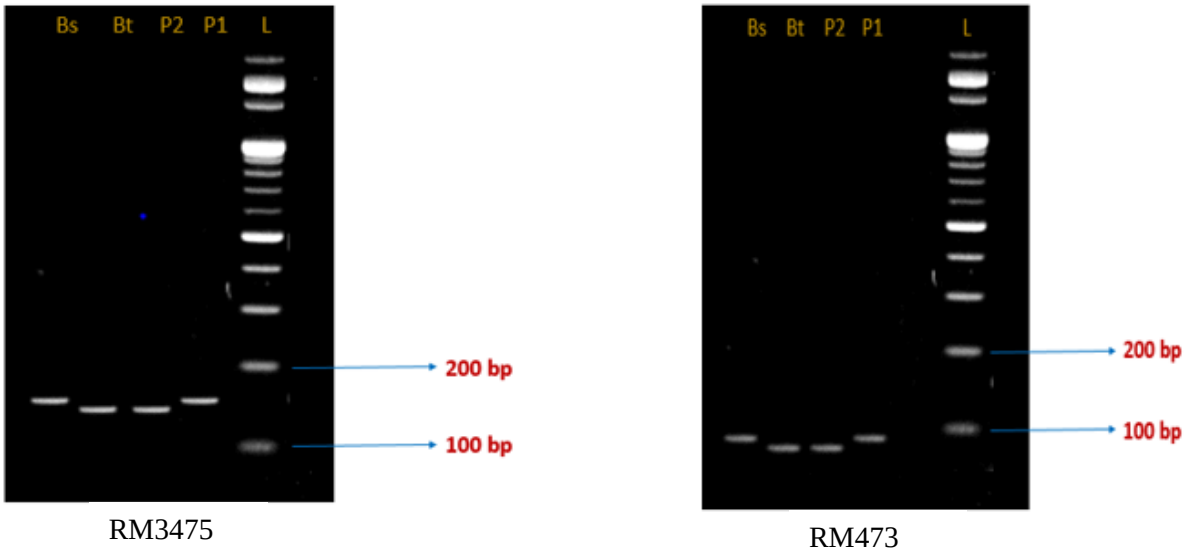


Plate 18. Amplification pattern of SSR markers RM3475 and RM473 expressing polymorphism between bulks (L-100 bp ladder, P1 – Uma, P2 – NL-44, Bt – tolerant bulk, Bs – susceptible bulk)

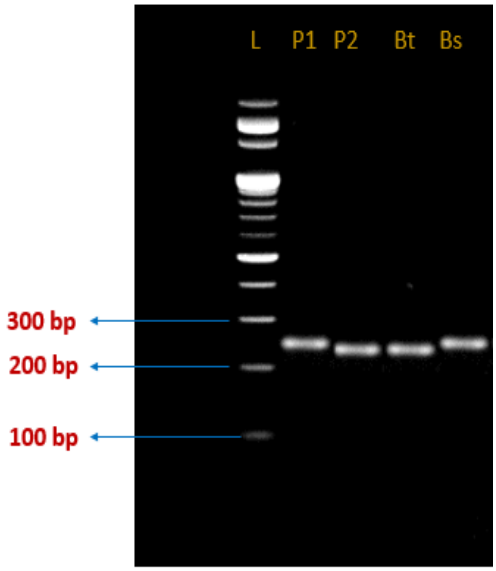


Plate 19. Amplification pattern of SSR marker RM222 expressing polymorphism between bulks (L-100 bp ladder, P1 – Uma, P2 – NL-44, Bt – tolerant bulk, Bs – susceptible bulk)

4.4.4 Segregation pattern of polymorphic markers among the selected lines

The identified F_2 lines were subjected to PCR reaction with SSR marker RM10793 to check whether they could segregate between the individual lines into tolerant or susceptible genotypes corresponding to the tolerant and susceptible parents. The amplification pattern obtained is shown in plate 20.

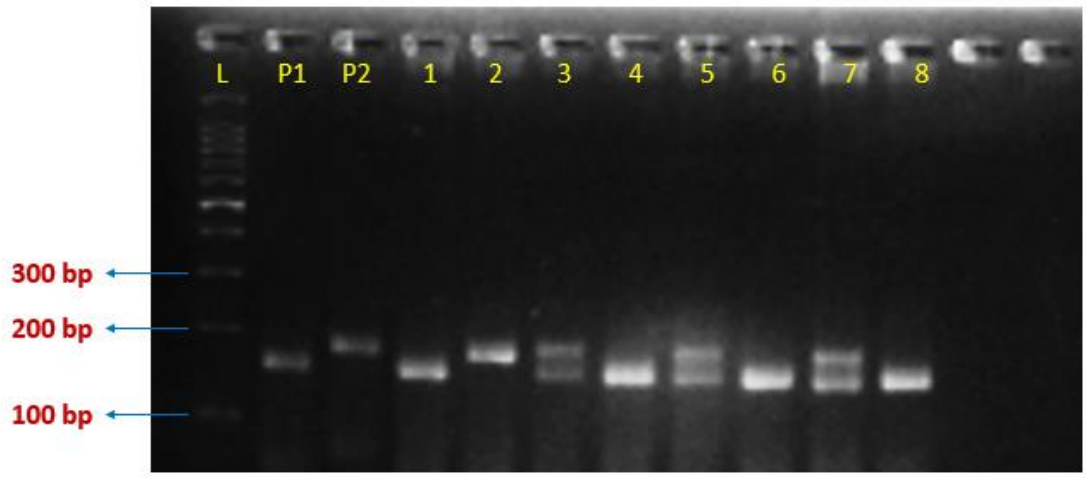


Plate 20. Amplification pattern of F_2 lines using SSR marker RM10793 (L- 100 bp ladder, P1 – Uma, P2 – NL-44, 1 to 8 – F_2 plants)

Discussion

5. Discussion

Rice, being the staple crop for more than half the world occupies major portion of the productive area in India (Mahajan *et al*., 2017; Rejeth *et al*., 2020). The effects of high temperature on crop performance has been well reviewed by several authors (Jagadish *et al*., 2015; Hassan *et al*., 2021) and its effects on the physiological and reproductive traits are well known for reducing the fertility leading to high losses in the yield of the plants (Wang *et al*., 2019a; Lawas *et al*., 2018).

The impending rise in temperature due to climate change (Pachauri and Meyer, 2014) poses a challenge to maintain the plant yield to meet the food demand of the increasing population. In this regard, it is imperative that we keep exploring the mechanisms that contribute to improving the performance of various accessions. In the current chapter, the focus has been to understand the effects of high temperature stress on the physiological and the sugar signaling mechanisms that are involved in contributing to the tolerance of the rice varieties, as well as to identify the microsattelite markers that are associated with the heat tolerance traits in rice.

5.1 ASSESSING THE IMPACT OF HEAT STRESS ON THE PHYSIOLOGICAL, YIELD AND SUGAR SIGNALING ASPECTS IN RICE

5.1.1 Effect of heat stress on growth related parameters

Both the varieties expressed significantly increased height under high temperature stress compared to control conditions (Figure 16). Unlike in drought, as the plants were subjected only to high temperature without any water limitation, the cell turgor seemed to be maintained negating the impact of heat stress on the reduction in height (Piveta *et al.*, 2020). Poli *et al*. (2013) also reported that plant height was increased in plants subjected to heat stress. The increase in plant height may also be attributed to increased panicle exertion. Jumiatun *et al*. (2016) in a study reported that certain rice varieties with better yield showed

a higher panicle exertion under high temperature conditions. Better panicle exertion is a contributing factor to more number of filled grains.

There was significant reduction in both the tiller number and the productive tiller number under stress which has a cumulative effect on the reduction in the yield of the crop (Figure 17, 18). The results correlate with Oh-e *et al.* (2007) who reported that the number of panicles was reduced when stress was given after the active tillering stage. The vegetative to reproductive transition of a tiller to produce a productive panicle is affected by stress as the transport of photo-assimilates from source to sink tissues is curtailed through modification in meristem identity genes. Fichtner *et al.* (2017) showed that trehalose-6-phosphate (T6P) triggers axillary bud outgrowth in garden pea by regulating sucrose levels. In their study, the increased T6P levels were correlated with increased amino acid synthesis. T6P is known to coordinate the carbon and nitrogen metabolic pathways to enhance growth. We can correlate this in our current investigation, where the down-regulation of *OsTPS1* under stress is associated with the decrease in the number of tillers.

The decrease in MSI of Vandana was significantly higher than that of NL-44 (Figure 19). Kumar *et al*. (2016) observed a decrease of 5-20% MSI in both sensitive and tolerant genotypes under elevated temperatures. The decrease in MSI was attributed to higher levels of lipid peroxidation (MDA content) resulting in disruption of cell membrane leading to electrolyte leakage. Cell membranes are the frontier structures that perceive and transmit heat stress and therefore are prime targets for disruption of the membrane integrity (Vivitha *et al*., 2017).

5.1.2 Effect of high temperature on the flowering characteristics

Under heat stressed conditions, the plants of both the varieties took more number of days to flower with regards to the plants under normal conditions (Figure 20). Early flowering is the resultant of better accumulation of starch and faster rates of growth. The delayed flowering habit in the heat stressed plants can be attributed to the requirement of sufficient biomass for the plant to perceive the developmental signals to transition to the

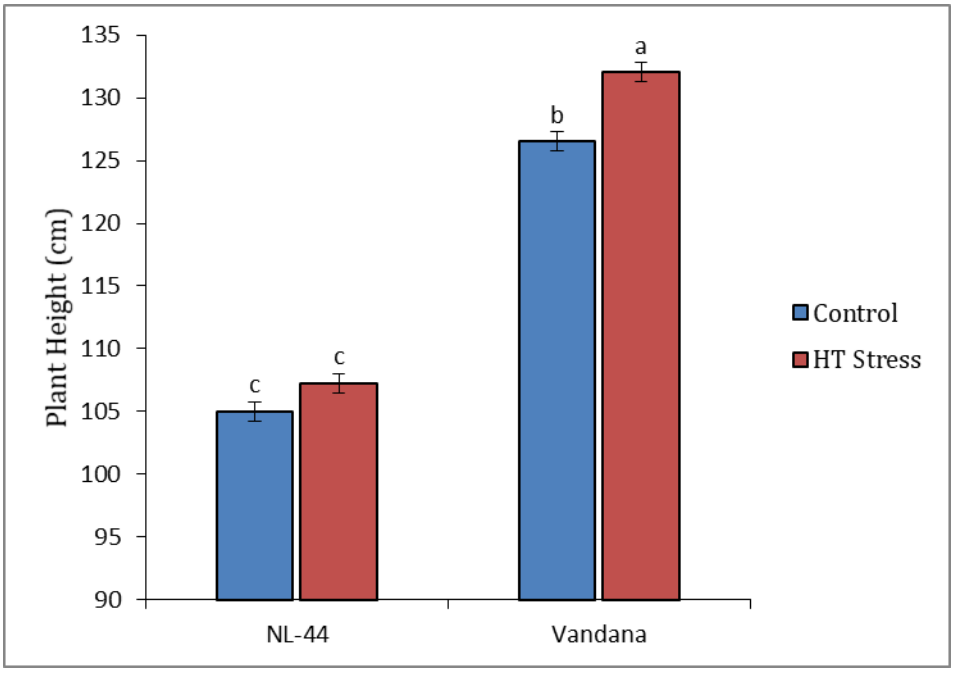


Figure 16. Difference in plant height (cm) of NL-44 and Vandana under the impact of high temperature (HT) stress

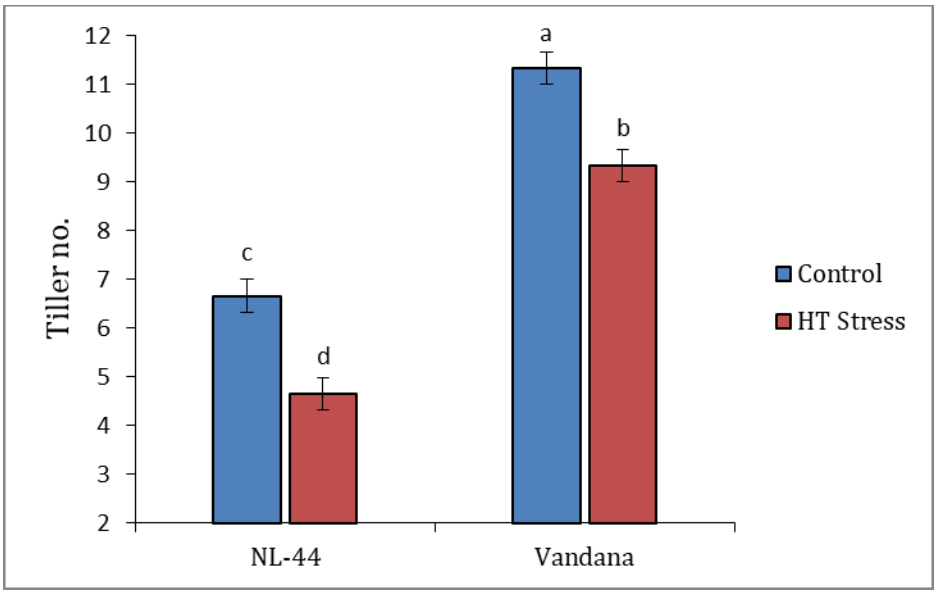


Figure 17. Difference in tiller number of NL-44 and Vandana under the impact of high temperature (HT) stress

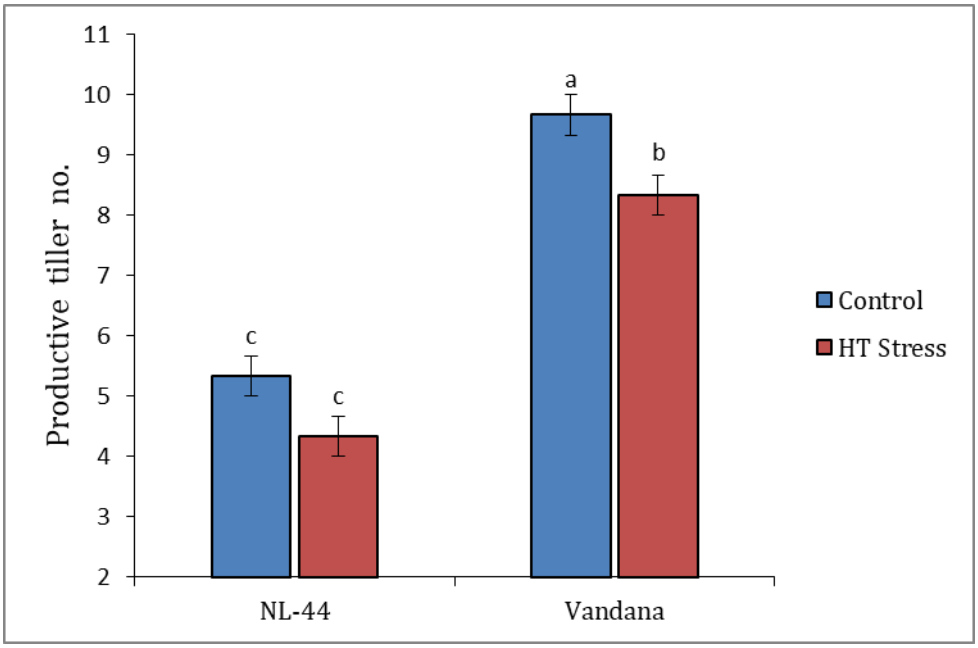


Figure 18. Difference in productive tiller number of NL-44 and Vandana under the impact of high temperature (HT) stress

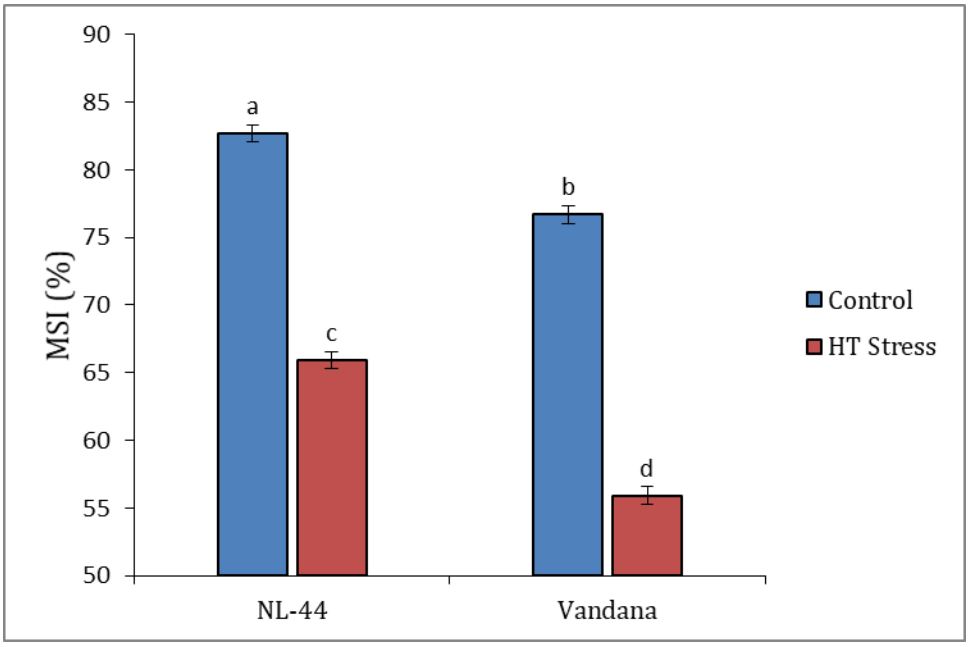


Figure 19. Difference in cell membrane stability index (MSI) of NL-44 and Vandana under the impact of high temperature (HT) stress

reproductive phase. In the vegetative-phase gene expression data, both the varieties showed down-regulation of *OsTPS1* indicating low sucrose content which could be the probable cause for the delayed flowering. According to Quilichini (2019), Target of Rapamycin (*TOR)* regulates cell growth by controlling the rates of protein synthesis. Moreau *et al.*, (2012) in their study, reported that over-expression of *TOR* resulted in early transition to flowering while mutants of *TORC1* exhibited late onset of flowering. In results on gene expression obtained in our study, we note that expression of *OsTOR* was down-regulated in NL-44 and low in Vandana indicating an inversely proportional relationship between the date of flowering and their expression levels.

Several studies have clearly established the fact that varieties that have shown an earlier time of anthesis have higher levels of tolerance against heat stress as they can escape the higher temperatures during the latter part of the day (Julia and Dingkuhn, 2012; Zhao *et al.*, 2016). In this regard, Vandana variety seemed to perform better with half hour advancement in its flowering time under stress (Figure 21). The NL-44 variety does not seem to follow the same mechanism as, under stress conditions, the flowering was delayed by half hour rather than being advanced. However, it is to be noted that the duration of active anthesis was reduced to one hour under heat stress compared to two hours under un-stressed conditions. This could also be a stress adaptive mechanism as the plant can escape the heat stress by reducing the duration for which the anthers are exposed to heat stress.

The pollen viability of NL-44 under heat stress was better maintained with just 7.79% reduction compared to that under ambient conditions (Figure 22). The failure of pollen to achieve viability is due to the impaired cell division of microspore mother cells. The anthers are highly sensitive to increased temperatures. The pollen of heat stressed anthers was found to be reduced in size with a wrinkled shape (Kumar *et al*., 2015). Accumulation of Reactive Oxygen Species (ROS) and decrease in the accumulation of carbohydrates are the leading causes for the decrease in pollen viability (Santiago and Sharkey, 2019). The decrease in pollen viability was greater in the sensitive variety, Vandana. The decrease in pollen fertility under heat stress in susceptible varieties has also been reported by Wang *et al*. (2019b). The inability to metabolize the incoming sucrose

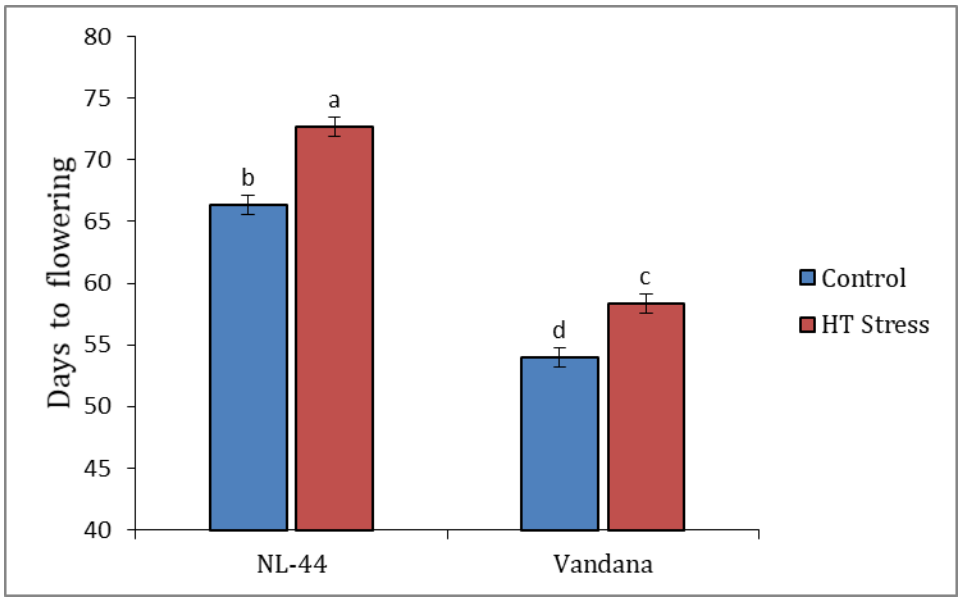


Figure 20. Difference in days to flowering of NL-44 and Vandana under the impact of high temperature (HT) stress

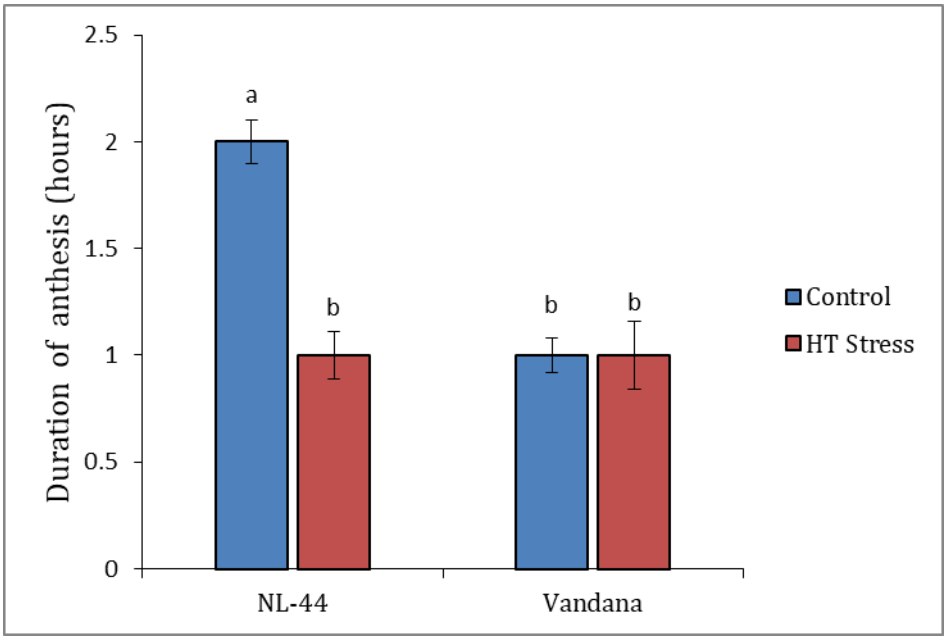


Figure 21. Difference in duration of anthesis (hours) of NL-44 and Vandana under the impact of high temperature (HT) stress

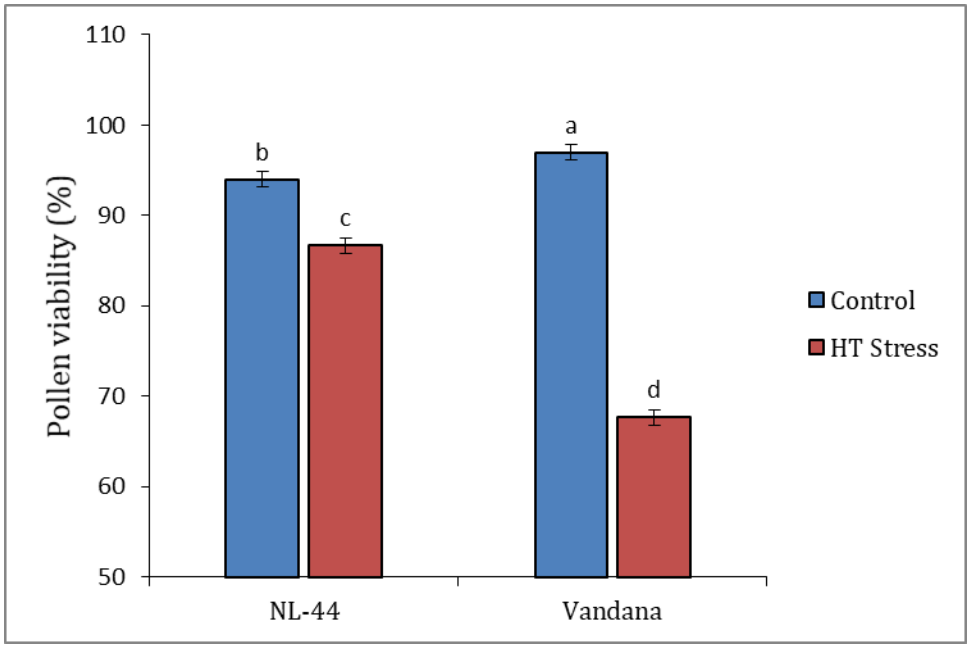


Figure 22. Difference in pollen viability (%) of NL-44 and Vandana under the impact of high temperature (HT) stress

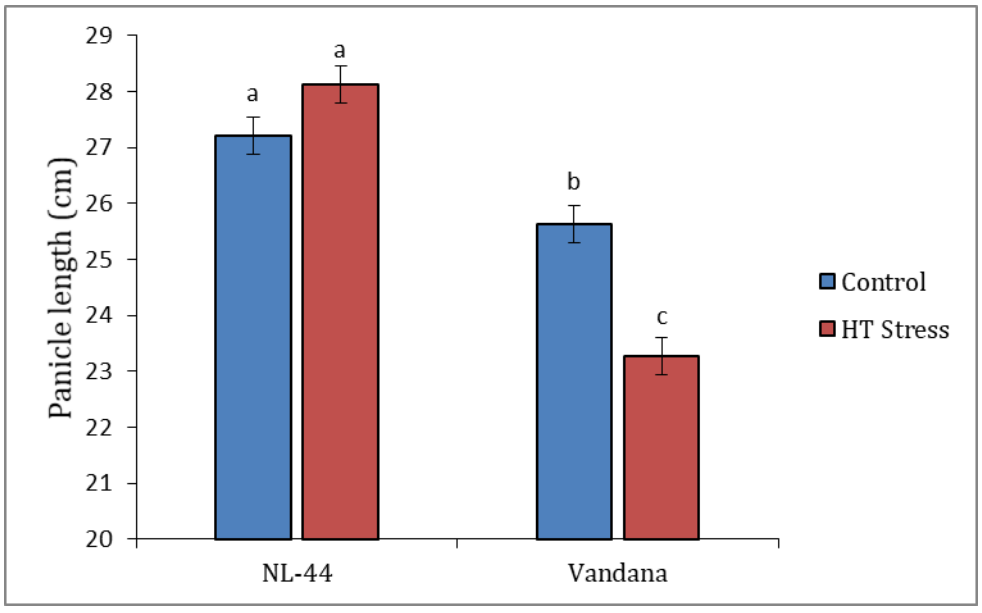


Figure 23. Difference in the panicle length (cm) of NL-44 and Vandana under the impact of high temperature (HT) stress

was noted to be the reason for poor pollen development in plants expressing anti-sense *SnRK1* (Zhang *et al.,* 2001). According to Halford and Dickson (2001), the expression of *SnRK1* is activated by low intracellular glucose. This is in line with our findings of high expression levels of *OsSnRK1* indicating low glucose content resulting in impaired growth.

The panicle length of NL-44 under both treatment conditions was on-par whereas a significant reduction in its length was observed in Vandana (Figure 23). Increased panicle length is a significant contributing character for the yield of the plant (Sun *et al*., 2016). Liu *et al*. (2016) reported an increase of 13.73% in yield correlated with an increase of 41.02% in the length of the panicle of NIL-LP1. The panicle length in combination with the number of spikelets and density modifies the panicle architecture determining the grain number per panicle. The reduction in panicle length in the susceptible variety Vandana can be related with higher expression of *OsSnRK1*. The low glucose status which activates *Snf1* related kinases that phosphorylate key metabolic enzymes which inhibit the growth and cell cycle related processes (Halford, 2003).

5.1.3 Effect of heat stress on Gas Exchange and Fluorescence related parameters

The lower percentage of reduction in the photosynthetic rate in NL-44 (14.19%) compared to Vandana (33.43%) (Figure 24) reveals the importance of the higher assimilation rate as a tolerance mechanism to withstand extreme temperature stress. In a study by (Gesch *et al*., 2003), the heat tolerant rice genotype (N22) could maintain photosynthetic activity for a longer time after heading leading to increased grain weights, compared to heat-sensitive genotypes (IR20, IR53, IR46). Bahuguna *et al*. (2015) described a 13% decrease in photosynthetic rate in NL-44 under elevated temperatures. They noted that NL-44 was able to maintain low hydrogen peroxide production and non-photochemical quenching (NPQ). Sharma *et al*. (2015) reported a positive correlation between Fv/Fm ratio and the photosynthetic rate as well as chlorophyll content resulting in increased dry matter.

Under higher temperature, the tolerant variety, NL-44 showed enhanced stomatal conductance (Gs) and transpiration (E) (Figure 25, 26) which is essential for the plant to

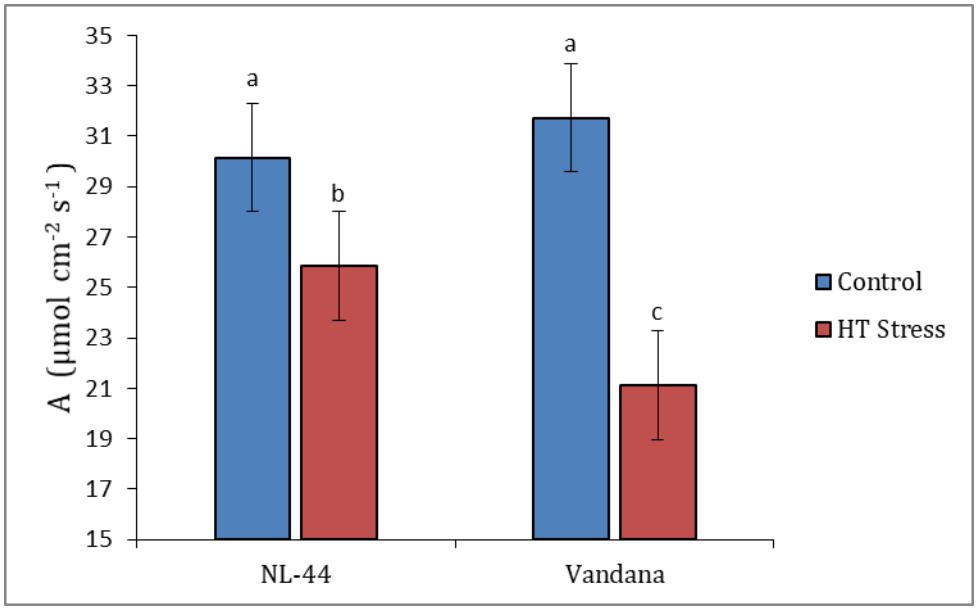


Figure 24. Difference in photosynthetic rate (A) of NL-44 and Vandana under the impact of high temperature (HT) stress

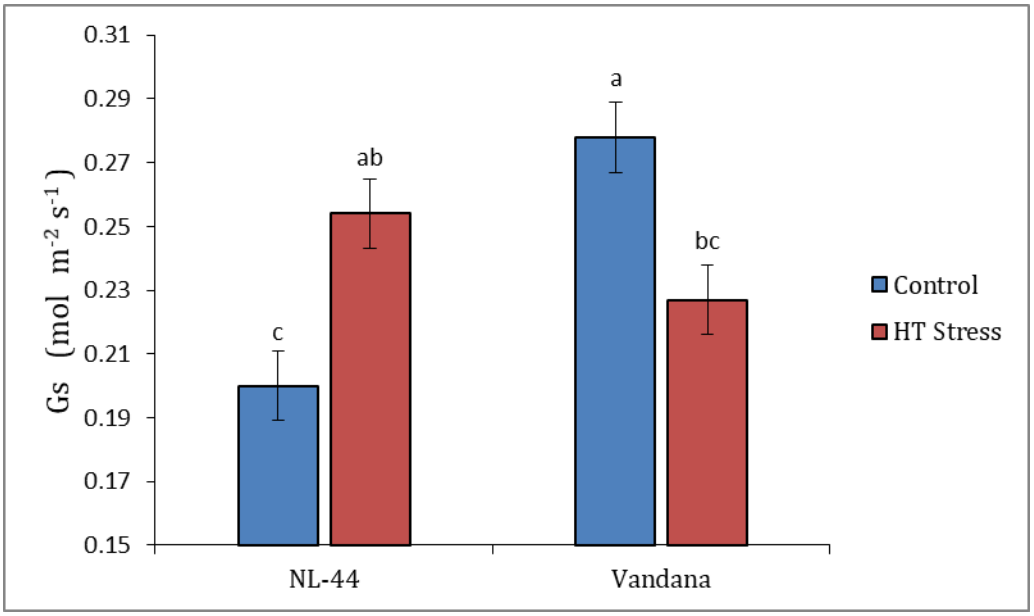


Figure 25. Difference in stomatal conductance (Gs) of NL-44 and Vandana under the impact of high temperature (HT) stress

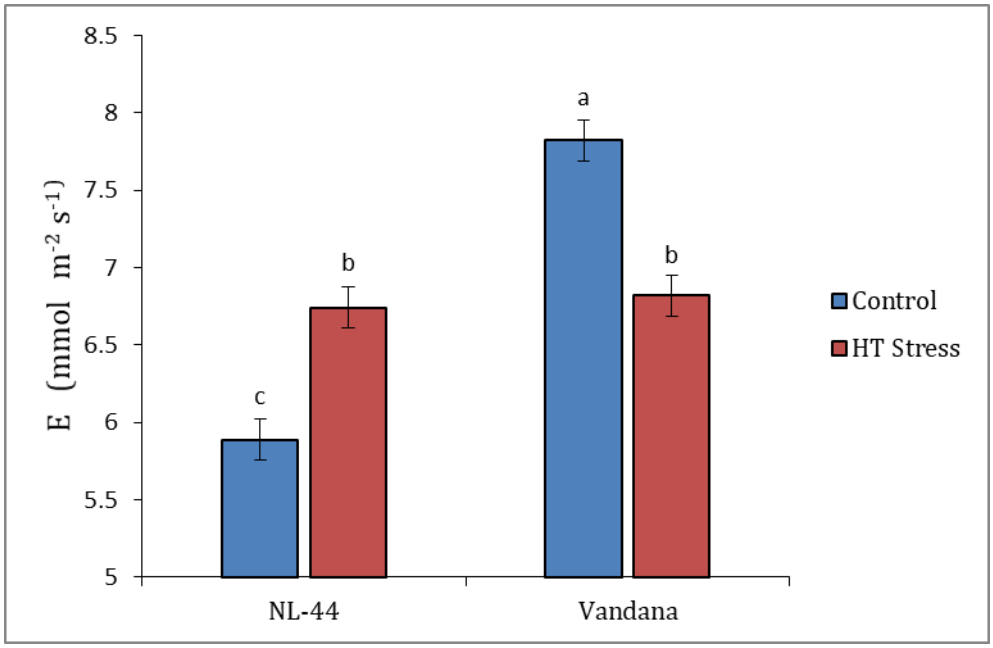


Figure 26. Difference in transpiration rate (E) of NL-44 and Vandana under the impact of high temperature (HT) stress

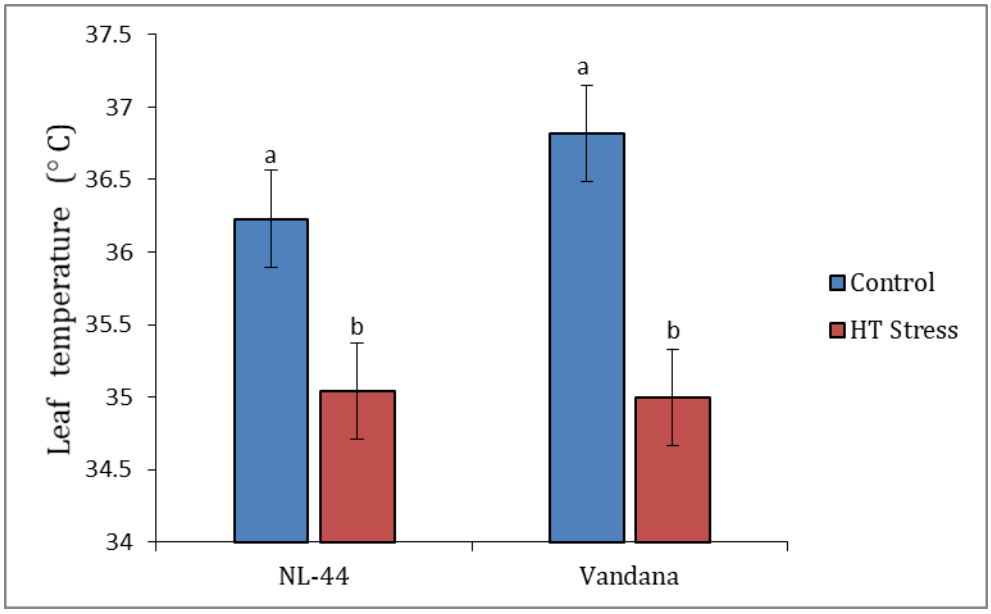


Figure 27. Difference in leaf temperature of NL-44 and Vandana under the impact of high temperature (HT) stress

lower its tissue temperature in order to adapt to the stress. By evaporating more water, plants can maintain cooler canopy temperature, thereby acclimatizing to higher temperatures (Crawford *et al*., 2012). The results are in agreement with Li *et al*. (2019) who also reported significant increase in the rate of transpiration and stomatal conductance under high temperature in rice. A study by Lugassi *et al.* (2015) reported that *HXK1* stimulates stomatal closure by regulating sucrose levels in the guard cells. In the current investigation, the stomatal conductance (Gs) of Vandana variety was decreased under stress. This is correlated with the gene expression results wherein, the higher expression of *OsHXK2* in Vandana during vegetative phase resulting in stomatal closure leading to reduced stomatal conductance. In another study, Van Houtte *et al*. (2013) reported that increased expression of trehalase (enzyme mediating hydrolysis of trehalose to glucose) reduces stomatal aperture. This is agreement with the results obtained in our study where Vandana variety, expressing higher relative fold change of *OsTPS1* under stress exhibited lower stomatal conductance. Heat stress causes damage to the chlorophyll molecules as well as the photosystem-II. There was reduction in leaf temperature in both the varieties under stress (Figure 27). The reduction in the leaf temperature helps to protect the photosynthetic apparatus from the effects of heat (Tang *et al*., 2018).

The water-use efficiency (WUE) of NL-44 was significantly higher under both the treatment conditions compared to Vandana (Figure 28). This is due to the reduced photosynthetic rate coupled with increased transpiration rate in the susceptible variety which results in reduced biomass accumulation per unit of water transpired. The water-use efficiency in the current study is physiological as it is based on the instantaneous physiological measurement of photosynthesis rate and transpiration rate. Photosynthetic rate and chlorophyll fluorescence are indirect indicators of abiotic stress. As the photosystem-II is the most heat labile structure (Vacha *et al*., 2007), a reduction in fluorescence due to high temperature stress is a clear indicator of its susceptibility. Therefore, the fluorescence parameters such as Fv/Fm ratio, ϕPSII and the electron transport rate gains prominence. The Fv/Fm ratio, ϕPSII and ETR were significantly higher in NL-44 compared to Vandana under the stress conditions although they were lower than

their corresponding controls (Figures 29, 30, 31). Shefazadeh *et al*. (2012) correlated higher Fv/Fm ratio in wheat lines to better grain yield under high temperature stress. In a study by Thussagunpanit *et al*. (2015), heat stress was found to reduce the stomatal conductance (Gs), Fv/Fm ratio, electron transport rate (ETR) and ϕPSII. Heat stress was reported to significantly reduce the Fv/Fm ratio of tolerant rice genotypes NL-44 and N-22 (Bahuguna *et al*., 2015). Sailaja *et al*. (2015) in their study on rice, reported that N-22 genotype which maintained high Fv/Fm (0.75) under heat stress was identified as heat-tolerant compared to Vandana variety which recorded a lower Fv/Fm (0.70). Higher expression levels of *TOR* gene reportedly increased the photosynthetic efficiency and biomass under water-limiting stress conditions (Bakshi *et al*., 2017). This is line with our current study wherein, the tolerant variety NL-44 whose Fv/Fm ratio was higher under stress compared to control conditions can be correlated with the expression levels of *OsTOR* which was recorded to be the highest in NL-44 in the grain filling stage.

5.1.4 Effect of heat stress on yield related parameters

Despite the extreme heat conditions, the ability of NL-44 to achieve higher spikelet fertility percentage compared to Vandana indicates its tolerance to heat stress (Figure 32). The decrease in spikelet fertility may be attributed to myriad factors such as anther indehiscence, pollen sterility, failure of pollen to germinate on stigma, impairment in the elongation of pollen tube in the pistil (Sunoj *et al*., 2017; Zhang *et al*., 2018). Spikelet sterility under elevated temperature was due to decrease in the duration of grain filling caused by reduced supply of assimilates such as starch and protein (Kumar *et al*., 2015; Beena *et al*., 2018). Sucrose is the major photo-assimilate that is transported into the grains (Pravallika *et al*., 2020). Therefore, a greater availability of sucrose in the leaves during higher temperatures indicates that the variety is more tolerant under stress contributing to increased grain yield (Snider *et al*., 2011). Prasad and Djanaguiraman (2011), in a study on sorghum reported that heat tolerant genotypes recorded higher sucrose contents compared to heat susceptible genotypes. The major enzymes involved in enhanced sucrose availability in sucrose synthase (*SuSy*) and sucrose phosphate synthase (SPS) (Kaushal *et al*., 2013).

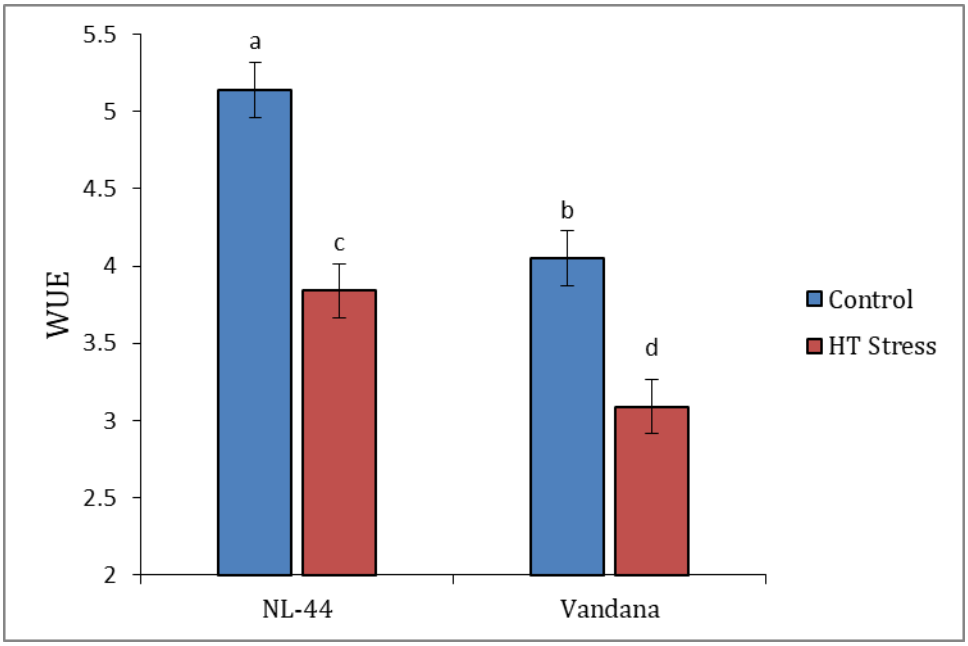


Figure 28. Difference in Water-Use Efficiency (WUE) of NL-44 and Vandana under the impact of high temperature (HT) stress

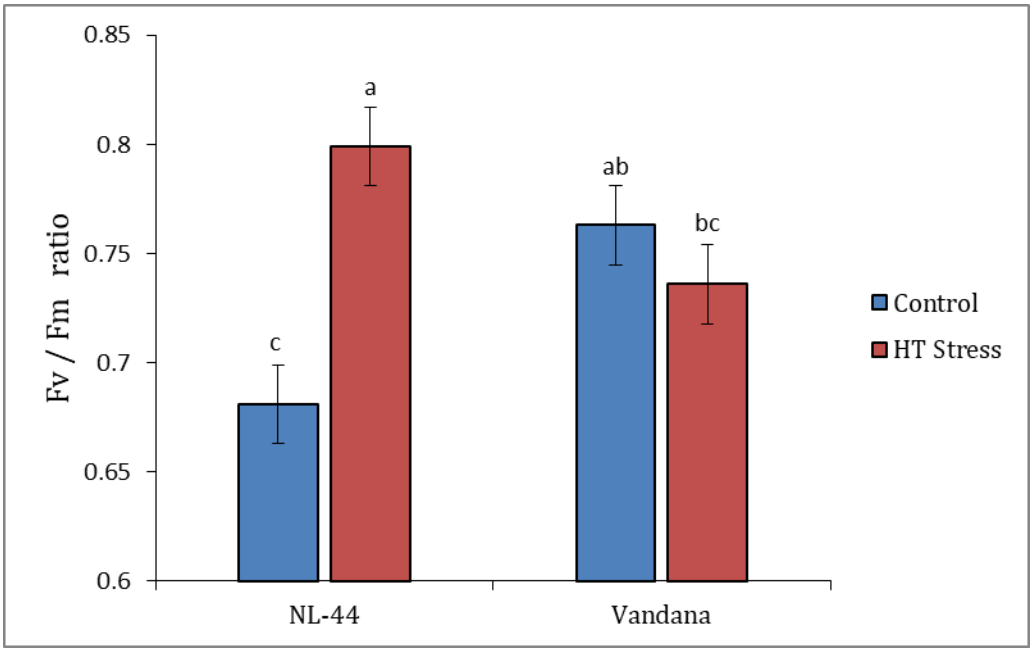


Figure 29. Difference in Fv/Fm ratio of NL-44 and Vandana under the impact of high temperature (HT) stress

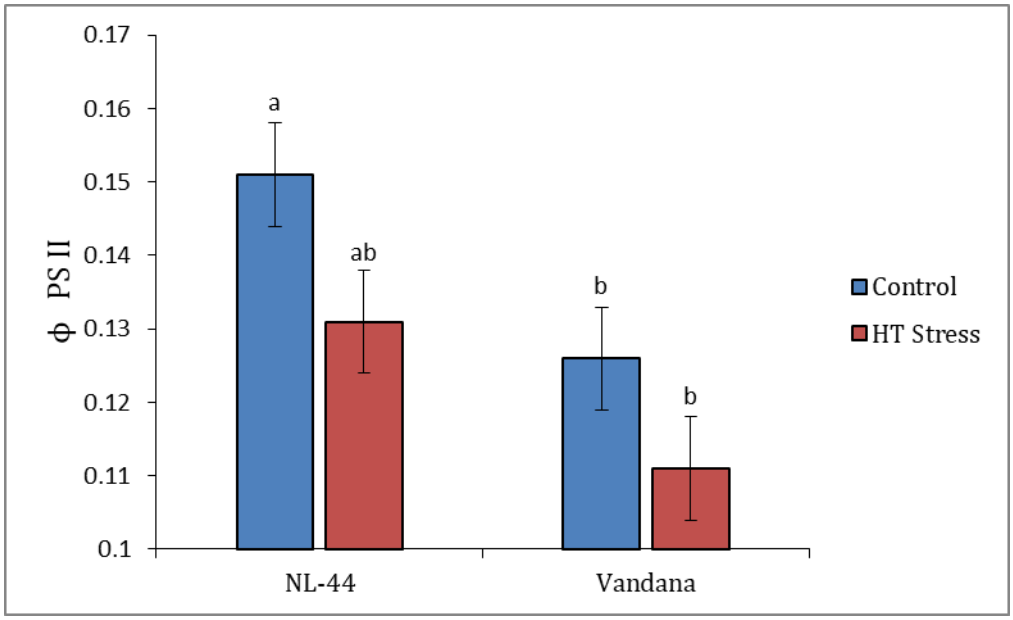


Figure 30. Difference in ϕPSII of NL-44 and Vandana under the impact of high temperature (HT) stress

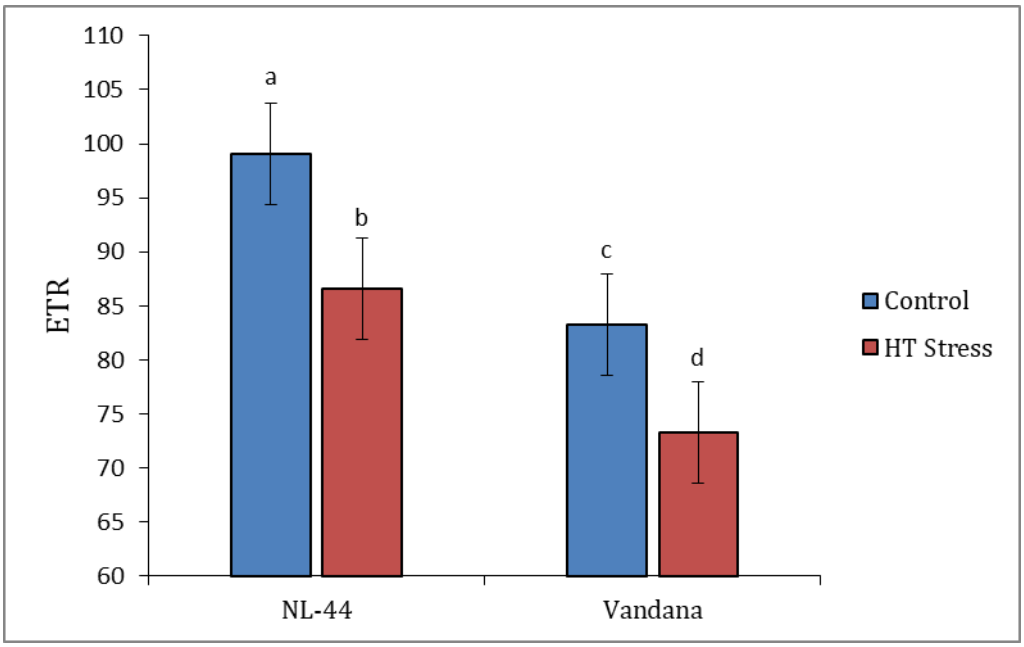


Figure 31. Difference in electron transport rate (ETR) of NL-44 and Vandana under the impact of high temperature (HT) stress

Crop yield is a result of combination of several factors such as tiller number, number of panicles, number of grains per panicle, spikelet fertility etc. (Wang *et al*., 2019b; Poli *et al*., 2013; Beena *et al*., 2021a). The 1000 grain weight is a component that signifies the accumulation and remobilization of assimilates indicating their partitioning efficiency (Beena *et al*., 2021b; Nithya *et al*., 2021). The ability of NL-44 to maintain the 1000 grain weight even under heat stress on par with the control treatment (Figure 33) is an essential trait to reduce the losses under stress. Target of Rapamycin (*TOR)* is implicated in sensing nutrient availability and coordinating growth and cell division. It induces genes involved in stress responses (Dobrenel *et al.,* 2011). This fact can be correlated with the higher expression of *OsTOR* in NL-44 during grain-filling under stress. According to Paul *et al*. (2018), the decreased trehalose-6-phosphate levels in the vegetative tissues result in resource re-allocation of sucrose to withstand abiotic stresses. Accordingly, we can see that in the current study, in the vegetative phase, the down-regulation of *OsTPS1* in NL-44 indicates better stress tolerance. This has resulted in the genotype NL-44 being able to maintain the 1000 grain weight even under stress conditions.

5.1.5 Correlation of factors influenced by heat stress

The negative correlation between the 1000 seed weight and tiller number is attributed to increased remobilization into the grain under stress which would otherwise be utilized for stress tolerance. The positive correlation of the days to flowering with the 1000 grain weight is significant as the prolonged vegetative phase accumulates greater amounts of starch in the stem which can be remobilized during grain-filling phase. The strong positive correlation between pollen viability and spikelet fertility indicates that higher number of pollen available for pollination ensures greater success in fertilizing greater number of spikelets. The association of panicle length with 1000 grain weight indicates that it is an important yield contributing factor. The significant negative correlation of the transpiration rate and stomatal conductance with the water-use efficiency (WUE) imply that their increase is detrimental to the plant biomass. The membrane stability index was also positively associated with pollen viability and ETR which is important for maintaining cellular homeostasis. The water use efficiency, pollen viability and efficiency of

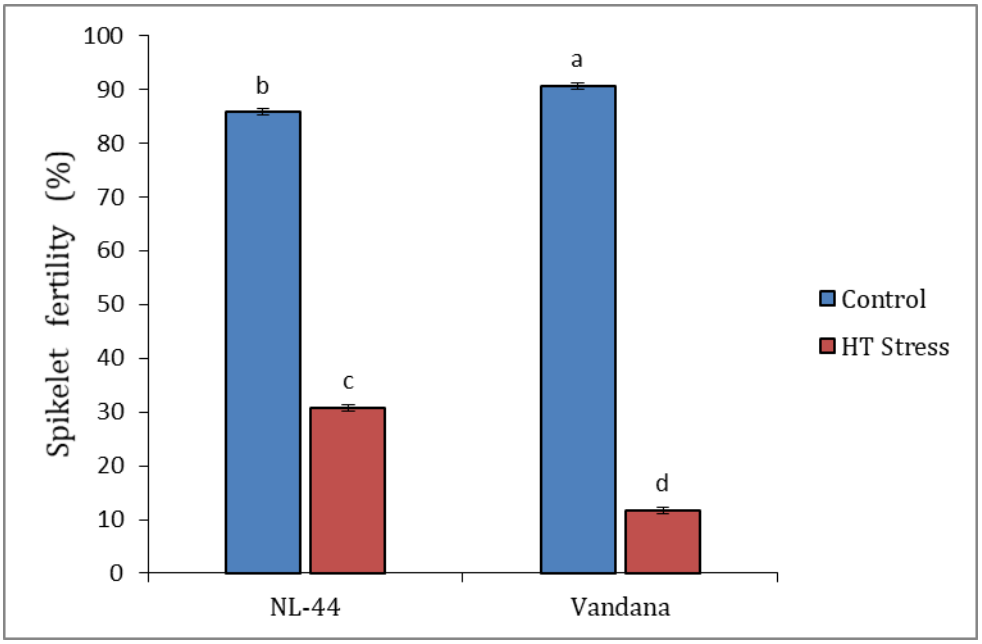


Figure 32. Difference in spikelet fertility (%) of NL-44 and Vandana under the impact of high temperature (HT) stress

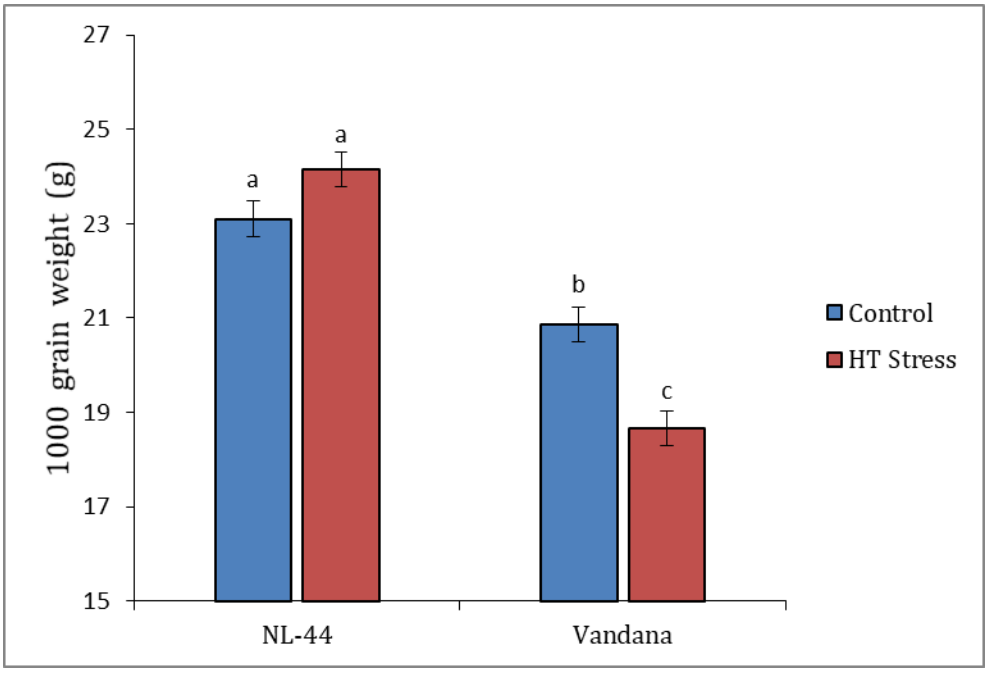


Figure 33. Difference in the 1000 grain weight (g) of NL-44 and Vandana under the impact of high temperature (HT) stress

photosystem-II (ϕPSII) which are major contributors to the thermo-tolerance of the plant were found to be positively correlated to the 1000 seed weight which is beneficial for reducing the yield loss under stress. The significant positive correlation between spikelet fertility with the leaf temperature, pollen viability, photosynthetic rate and membrane stability index is beneficial for improving the grain filling percentage which enhances the yield of the crop. Sailaja *et al*. (2015) also reported a positive correlation between spikelet fertility and grain yield as well as between photosystem-II efficiency and grain yield along with evapotranspiration rate.

5.1.6 Gene expression under heat stress

5.1.6.1 ***OsHXK2:*** Hexokinases (*HXKs)* primarily sense the glucose level which is the product of cleavage of sucrose by sucrose synthase (SuSy) and invertase (INV). Low glucose flux causes the down-regulation of *HXK*s. The glucose sensor gene, *OsHXK2* was expressed at a higher fold change in Vandana under vegetative phase (Figure 34) signifying higher glucose content. In the grain-filling phase (Figure 35), it was down-regulated indicating that in susceptible varieties, the glucose content was lower indicating decreased translocation or remobilization of photo-assimilates into the grains. The activity of hexokinase enzyme involved in glycolysis was reported to be significantly decreased in susceptible rice varieties under prolonged high temperature treatment (Yaliang *et al*., 2020). This results in the inhibition of transport of carbohydrates from the leaves to the panicles. This is correlated significantly with the decrease in 1000 grain weight and the photosynthetic rate in Vandana variety. Contrastingly, the *OsHXK2* was up-regulated in the tolerant variety NL-44 even in the grain-filling phase signifying its ability to transport photo-assimilates to the grains even under energy limiting conditions.

5.1.6.2 ***OsSnRK1:*** The energy-sensing protein kinase, *SnRK1* modulates the metabolic and transcriptional changes in plants as an adaptive mechanism to overcome stress under energy depleting conditions (Smeekens *et al*., 2010; Baena-Gonzalez *et al*., 2007; Cho *et al*., 2012). According to Nunes *et al*. (2013c), high sugar levels inhibit *SnRK1*. As the levels of expression of *OsSnRK1* during the vegetative phase were higher while significantly

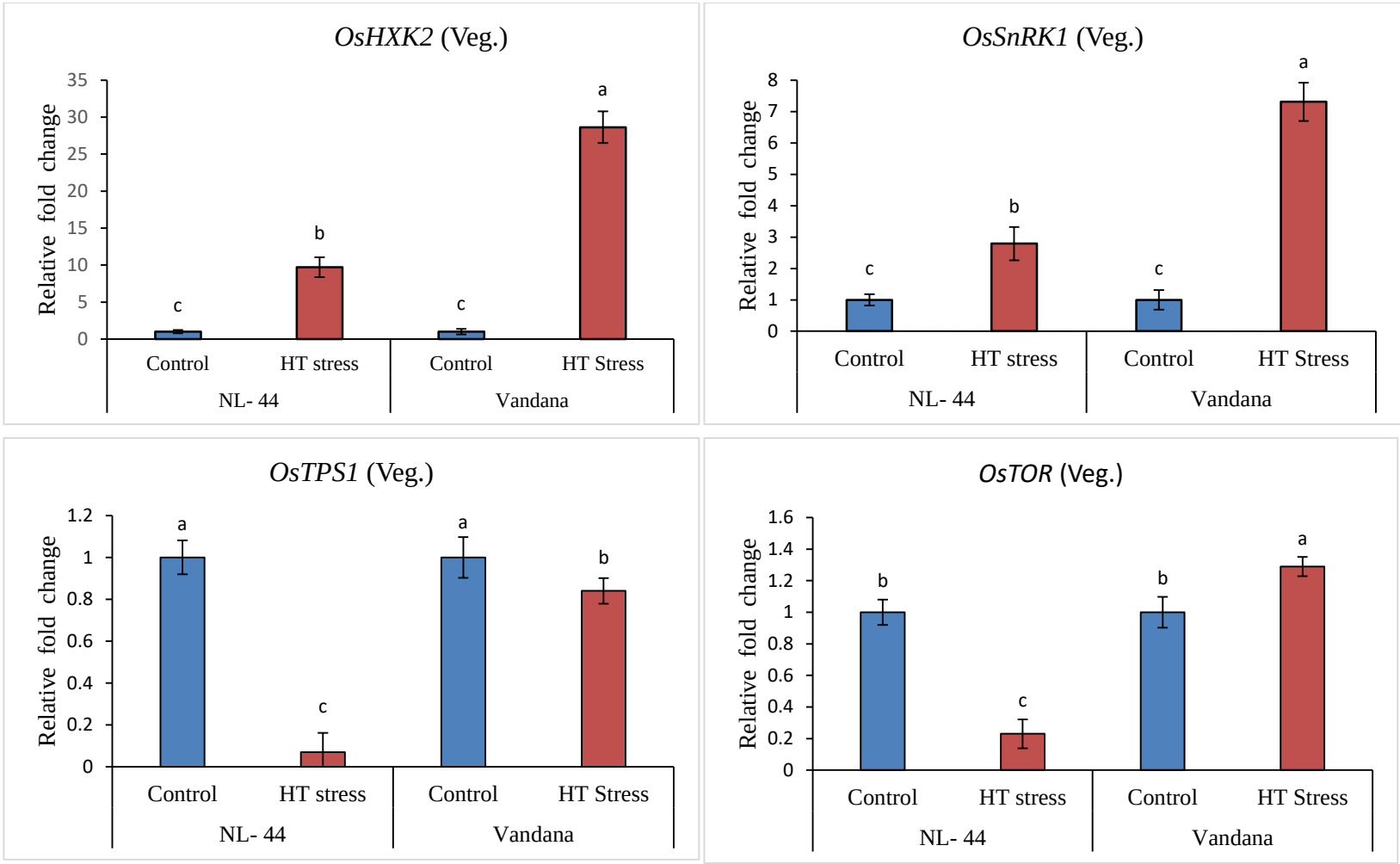


Figure 34. Expression levels of genes during vegetative (Veg.) phase

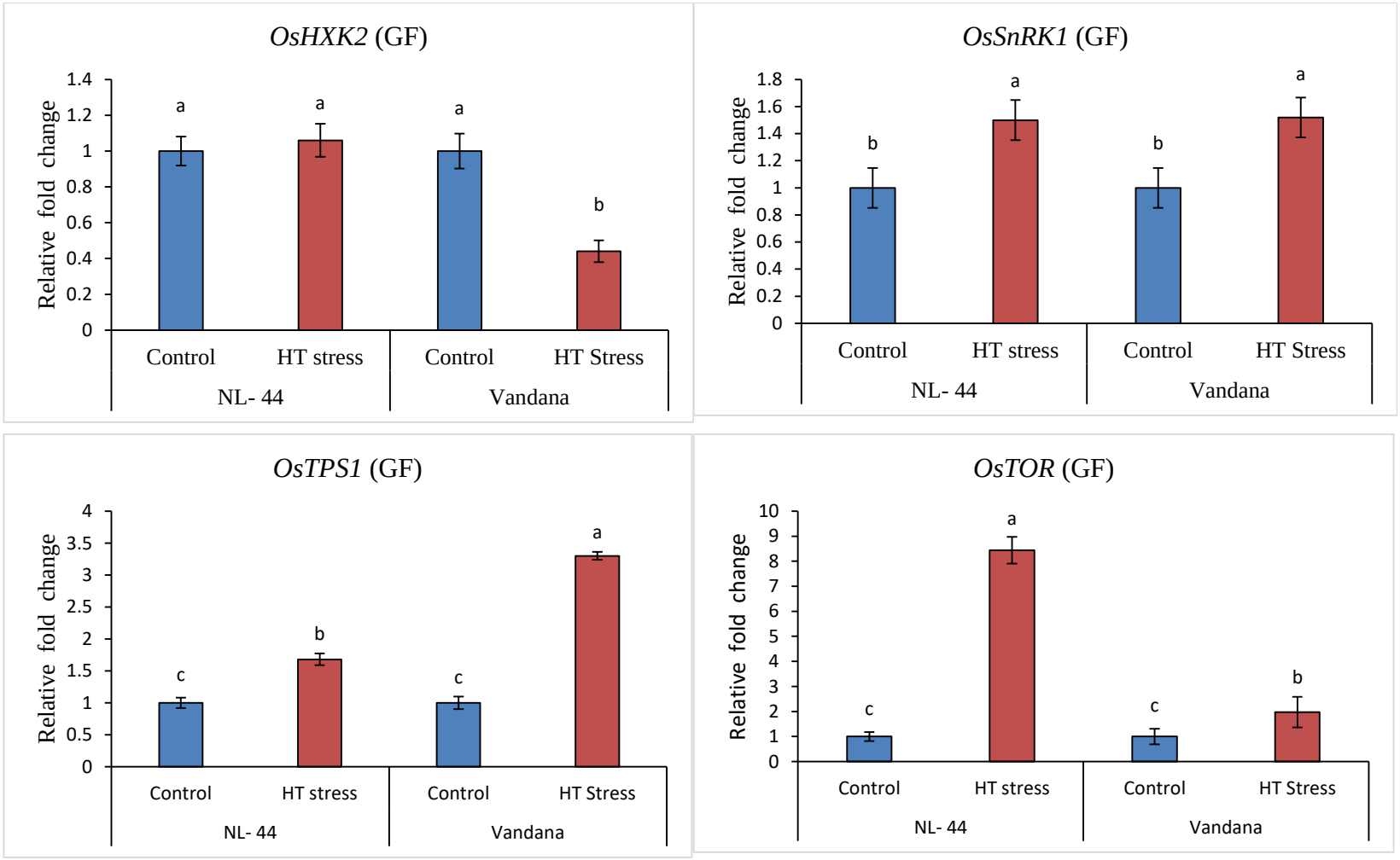


Figure 35. Expression levels of genes during grain-filling (GF) phase

lower during the grain filling stage, we can state that the sugar levels were relatively higher during the grain filling phase compared to the vegetative phase. This may be attributed to the requirement of sucrose for remobilization into the grain during the maturation phase. The non-significant difference between the two varieties points to the fact that change in *OsSnRK1* expression levels may not play a crucial role in mechanisms differentiating the tolerant and susceptible varieties. Both the varieties maintained an up-regulated status of *OsSnRK1* under stress in the grain filling stage indicating activation of starvation response which modulates the activity of genes directed towards storage of photo-assimilates rather than the growth activity. *TOR* kinase is repressed by *SnRKs* under conditions of starvation. It is derepressed under favorable conditions of nutrient availability promoting growth and division by down-regulation or suppression of *SnRKs*.

5.1.6.3 *OsTPS1:* The trehalose-6-phosphate (T6P) is a signal of sucrose availability (Yadav *et al*., 2014). *TPS* modifies the perception of glucose content sensed by hexokinase (*HXK)* (Eveland and Jackson, 2012). In the current investigation, *OsTPS1* was found to be down-regulated in the vegetative phase pointing towards diminished sucrose availability under heat stress. Contrastingly, we observe that the gene was up-regulated during the grain-filling phase indicating enhanced sucrose availability for translocation into the panicles. In maize kernels, Smeekens (2015) reported an increased sink activity with reduced levels of *T6P*. Based on these results, we can infer that in the grain-filling stage, although up-regulated, the NL-44 showing relatively lower expression levels might be implicated to have enhanced sink activity. In a study by Zhang *et al*. (2009a), the activity of At*SnRK1* was reportedly suppressed by the application of trehalose-6-phosphate. T6P is a direct negative regulator of *SnRK1* activity. The synchrony of these two genes indicates the sugar status in the source organs that can be transported to the sink organs i.e. panicles.

5.1.6.4 *OsTOR:* Target of Rapamycin (*TOR*) is a central regulatory hub that integrates varied signals related to plant growth and development including stress responses. Plants with mutations in the *TOR* gene were observed to express symptoms of stress even in the absence of a trigger (Fu *et al*., 2020). The study by Pererya *et al*. (2020) cautioned that the duration and intensity of stress plays an essential role in the level of gene expression. The

current study also imparts a similar opinion as the stress received in the vegetative phase was of shorter duration compared to that received during the grain filling phase. *TOR* inactivation is modulated by low carbon and nitrogen status by repression of *TORC1* and *RAPTOR* complexes (Dobrenel *et al*., 2016).

The data analysed from the current experiment reveals that during the vegetative phase, *OsTOR* was down-regulated in the tolerant variety, NL-44. Xiong *et al*. (2013) stated that low glucose status suppresses the activity of *TOR* kinase. Therefore, this can be correlated with the expression levels of *OsHXK2* wherein it was relatively lower although up-regulated in NL-44 implying relatively lower glucose content in the vegetative phase compared to the susceptible variety Vandana. The lower glucose content indicates a proportionally higher sucrose content that is available for translocation. During the grain-filling phase, *OsTOR* was up-regulated and significantly higher in the tolerant variety, NL-44 than the susceptible variety, Vandana. These results are in agreement with Sharma *et al*. (2019) who reported that over-expression of *TOR* increased the gene expression of heat shock factors in seedlings. *TOR* gene expression was also reported to be up-regulated in *Lolium perenne* under heat stress (Wang *et al*., 2017b) implying its expression as an indicator of heat tolerance.

5.1.6.5 *Gene Regulation Pathway*

In the vegetative phase, both varieties have shown commonality in the up-regulation of *OsHXK2*, *OsSnRK1* and down-regulation of *OsTPS1*, with the difference between the varieties in that *OsTOR* is up-regulated in the susceptible variety (Vandana), while it was down-regulated in the tolerant variety (NL-44). In the grain filling phase, the signaling pathway differs with regards to the one expressed in the vegetative phase. *OsSnRK1*, *OsTPS1* and *OsTOR* have been similarly expressed i.e. up-regulated in both the varieties under stress. However, the difference lay in the expression of *OsHXK2* which was down-regulated in the susceptible variety, whereas it was up-regulated in the tolerant variety. Several aspects regarding the functionality and expression of sugar-signaling genes

under various conditions have been reported (Lim *et al*., 2013; Min *et al*., 2014; Ljung *et al*., 2015; Smeekens, 2015; Wurzinger *et al*., 2018; Ryabova *et al*., 2019; Fu *et al*., 2020).

The data analyzed with regard to the four sugar signaling genes reveals unique interactions between them as well as the expression levels indicate the plant requirement in order to be relatively tolerant to high temperature stress by maintaining better physiological and yield parameters. Therefore, we can summarize that, in order to exhibit tolerance to heat stress, a variety should express up-regulation of *OsHXK2* in both the phases as it indicates enhanced glucose content. Also, down-regulation of *OsSnRK1* in the vegetative phase as growth processes need to be prioritized over starvation response while being relatively up-regulated in the grain-filling phase as starvation response in the grain filling phase enhances the translocation of photo-assimilates into the sink organs. A reduced expression of *OsTPS1* gene in the grain filling stage in the source organs is essential as the reduced levels of trehalose in the leaves allows for greater transport of sucrose into the sink tissues (Griffiths *et al*., 2016). The up-regulation of *OsTOR* in the grain filling phase would be a necessary pre-requisite for co-ordinating with abscisic acid (ABA) signaling pathway which is involved in stress adaptation (Fu *et al*., 2020).

5.2 PRODUCTION OF F_2 GENERATION SEEDS

The rice variety, NL-44 is a genotype that has traits that confer tolerance to heat stress but has a low yield per plant (Ravikiran *et al*., 2020). On the other hand, the variety Uma is a high yielding genotype grown in the southern-most part of the Indian peninsula in the State of Kerala. However, it is also a genotype that is susceptible to high temperature stress (Waghmare *et al*., 2021). The aim of the current experiment was to produce F_1 hybrids and their progeny that possess tolerance traits to withstand high temperature along with the ability to maintain high yielding capacity.

The two varieties were crossed by laying crossing blocks with staggered planting to synchronize the flowering time of both the varieties. In the crossing block I, about 106 F_1 hybrid seeds were produced. The NL-44 is an interspecific cross between *Oryza glabberima* and *Oryza sativa*, while Uma is an indica type (*Oryza sativa*). The large genetic

distance between the genotypes is a limiting factor for successful hybridization and successful seedling establishment. This was observed in our current investigation where, only ten of the F_1 seeds could be established successfully, with the majority of the seeds failing to germinate or the seeds which germinated failing to survive due to arrested development of the root growth. The factors that inhibited the germination of the seeds and growth of the seedlings need further investigation and elucidation using genetic and molecular tools.

In the crossing block II, the successfully established F_1 plants were raised and selfed to produce F_2 generation seeds. The Uma variety has a seed character that is short and bold, while the NL-44 has a grain character that is long and slender compared to Uma. The produced F_2 generation seeds were observed to have seed characteristics that was intermediate to both the parental genotypes with medium height and medium boldness.

5.3 PHENOTYPIC EVALUATION OF F_2 POPULATION OF NL-44 X UMA FOR HIGH TEMPERATURE TOLERANCE

The F_2 seeds produced in the crossing blocks was germinated and raised under ambient conditions according to standard cultivation practices. 144 F_2 plants along with the two parental genotypes, NL-44 and Uma were evaluated for their phenotypic characters under high temperature stress.

5.3.1 Influence of high temperature stress on the frequency of plant height in F_2 population

The F_2 population had a widely distributed range of 57 cm with regards to the plant height (Figure 36). However, the majority of the plants (1 standard deviation) were present between 90.08 cm and 108.1 cm which is a difference of 17.3 cm. The Uma variety recorded a lower height compared to NL-44 with the mean lying in between the two genotypes. The leptokurtic curve of the normal distribution indicates a heavy population of outliers while the negative skewness is indicative of greater number of plants with plant height below the mean value.

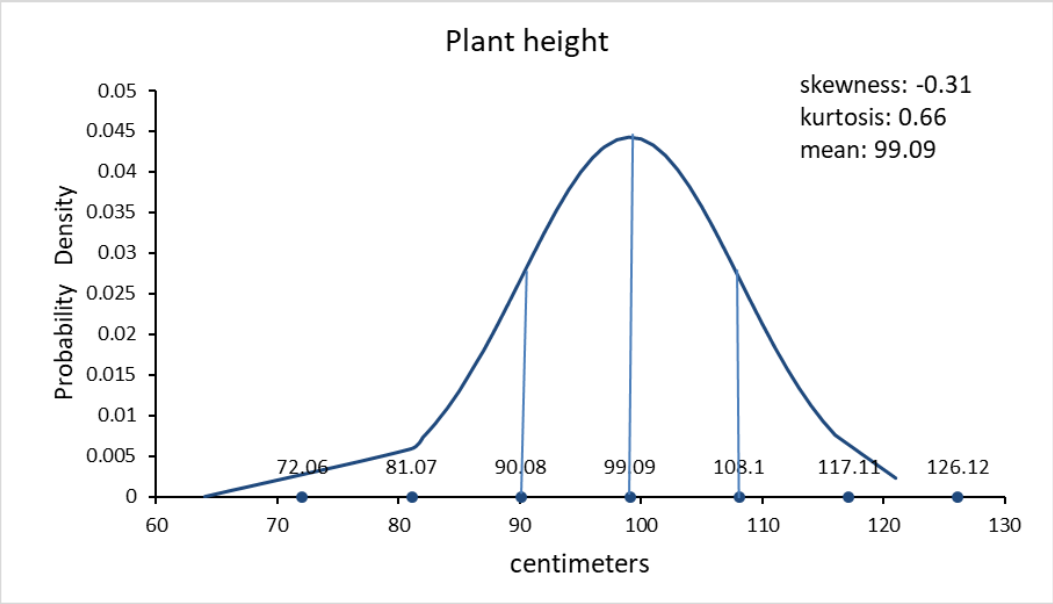


Figure 36. Normal distribution curve of F_2 population under the effect of high temperature stress on the plant height

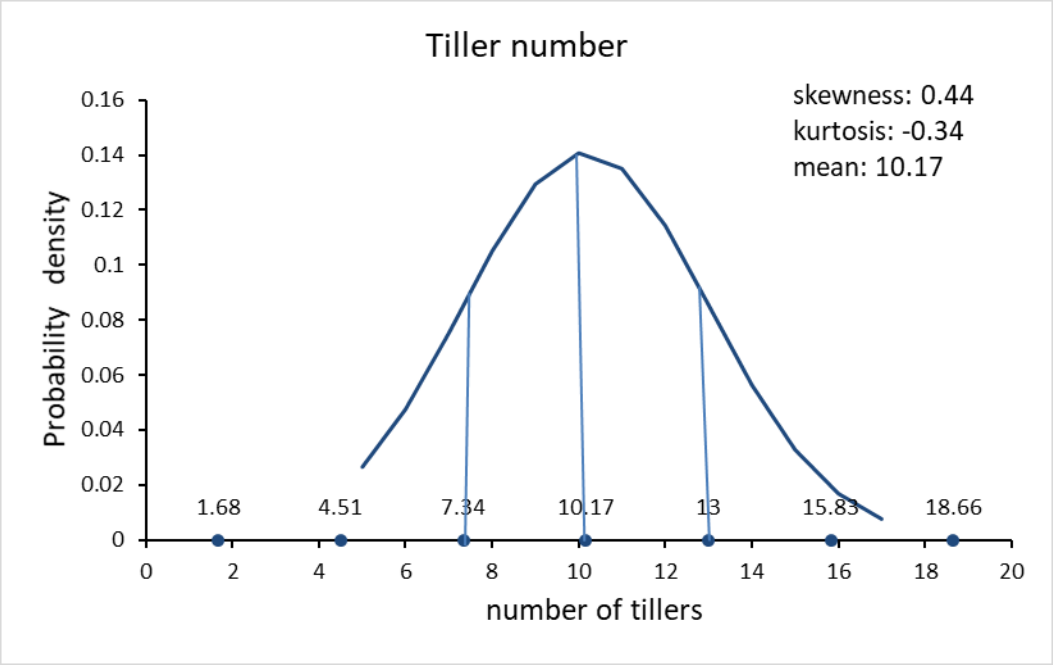


Figure 37. Normal distribution curve of F_2 population under the effect of high temperature stress on the tiller number

5.3.2 Influence of high temperature stress on the frequency of tiller number in F_2 population

The distribution of tiller number in the F_2 population ranged from 5 to 17 tillers with 68% (1 standard deviation) of population having tiller number between 7 and 13 (Figure 37). NL-44 recorded lower tiller number compared to Uma with the mean lying in between with 10 tillers. The mean was found to be greater than NL-44 variety and closer to Uma variety. The normal distribution curve was platykurtic which indicates that there are less number of outliers. The positive skewness of the curve shows that there are relatively greater number of plants towards the right of the mean.

5.3.3 Influence of high temperature stress on the frequency of productive tiller number in F_2 population

The number of productive tillers in the F_2 population ranged from 2 to 11 with 68% of the population producing productive tillers between 4 and 7 (Figure 38). The mean of the population was lower than both the genotypes with NL-44 producing lower number compared to that of Uma. The lower value of the mean shows that the trait in the F_2 population was influenced to a greater extent by NL-44 and was depressed compared to both the parents. The normal distribution curve was platykurtic with the least number of outlying plants. The positive kurtosis obtained for the population indicates that the majority of the population had produced productive tillers higher than that of the mean.

5.3.4 Influence of high temperature stress on the frequency of days to flowering in F_2 population

The range of the F_2 population was from 71 to 101 days to flowering (Figure 39). The normal distribution curve of 1 standard deviation ranged from 81 to 94 days to flowering. The genotype NL-44 took least number of days to flowering compared to that of Uma. Only the values of Uma and the mean were found to be within the confines of 1 SD. The mean value being greater than Uma shows that the trait in the majority of plants has been influenced by Uma. The skewness of 0.01 of the distribution curve was almost

normal. Both the genotypes had flowering dates that were lower than that of the mean. Thus it can be seen that the majority of the population had taken more number of days to flowering compared to both the parental genotypes. The platykurtic curve of the normal distribution shows that there are less number of outlying population.

5.3.5 Influence of high temperature stress on the frequency of time of anthesis in F_2 population

The F_2 population had a range spanning 3 hours and 45 minutes where the mean of the population was lower than that of both the parents. This shows that majority of the population had an earlier time of anthesis. Among the parents, Uma had earlier anthesis compared to NL-44. The earlier time of anthesis is a trait that is useful for escaping the high temperature stress.

5.3.6 Influence of high temperature stress on the frequency of membrane stability index in F_2 population

The membrane stability index of the F_2 population ranged from 56% to 91% with the values of 68% of the population lying between 68.54% and 83.14% (Figure 40). The mean of the population was greater than both the parental genotypes clearly indicating that the majority of the population has the potential for producing MSI greater than that of the parents. The negative skewness of the population distribution curve shows that the data leans towards the left of the mean value while negative kurtosis indicates low number of outlying population.

5.3.7 Influence of high temperature stress on the frequency of photosynthetic rate in F_2 population

The range of the F_2 population regarding the photosynthetic rate was 14.1 µmol $cm^{-2}s^{-1}$ with 1 SD ranging from 20.88 µmol $cm^{-2}s^{-1}$ to 27.82 µmol $cm^{-2}s^{-1}$ (Figure 41). The photosynthetic rates of Uma, NL-44 and the mean were within this range wherein Uma had lower photosynthetic rate compared to NL-44 with the mean lying in between the two genotypes. The mean of the population being greater than the susceptible Uma shows that

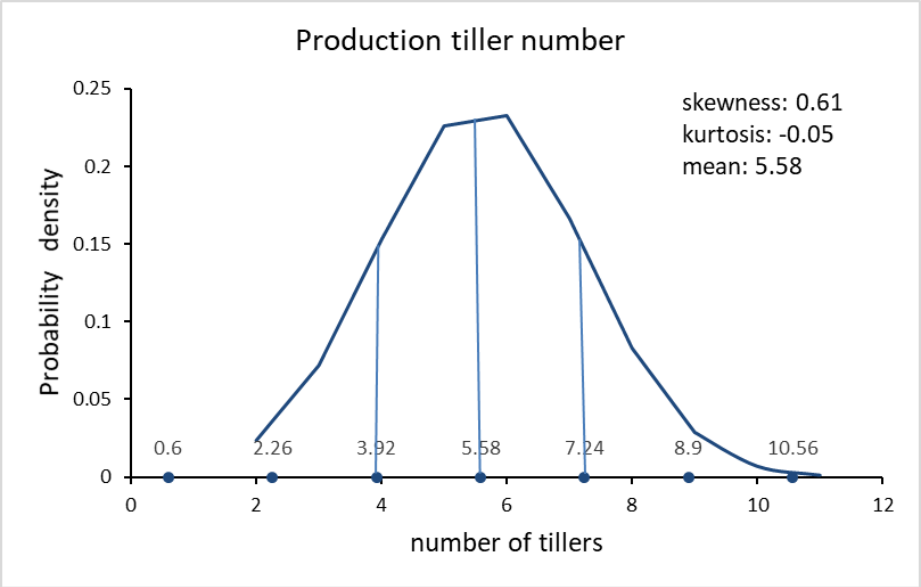


Figure 38. Normal distribution curve of F_2 population under the effect of high temperature stress on the productive tiller number

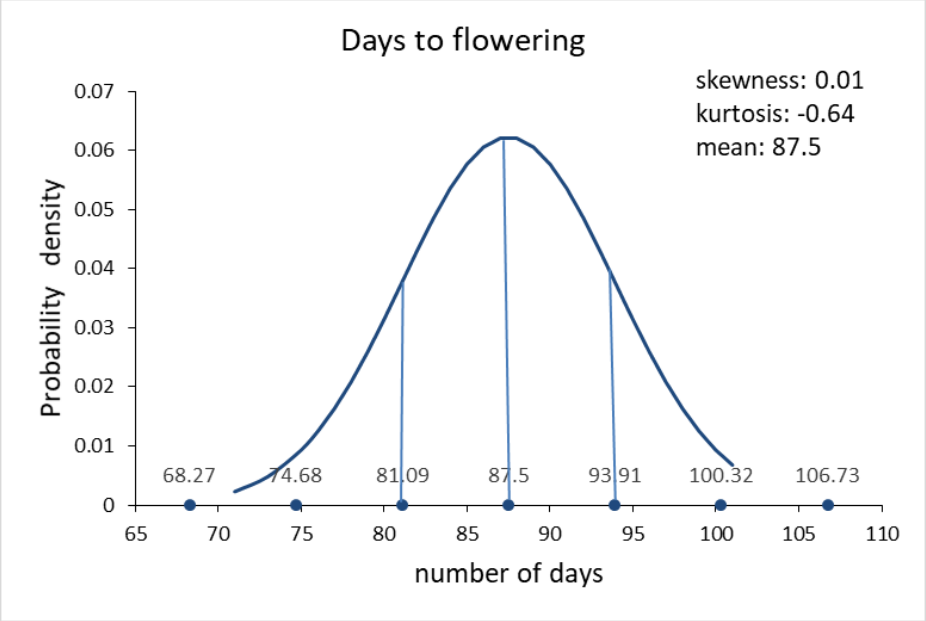


Figure 39. Normal distribution curve of F_2 population under the effect of high temperature stress on the days to flowering

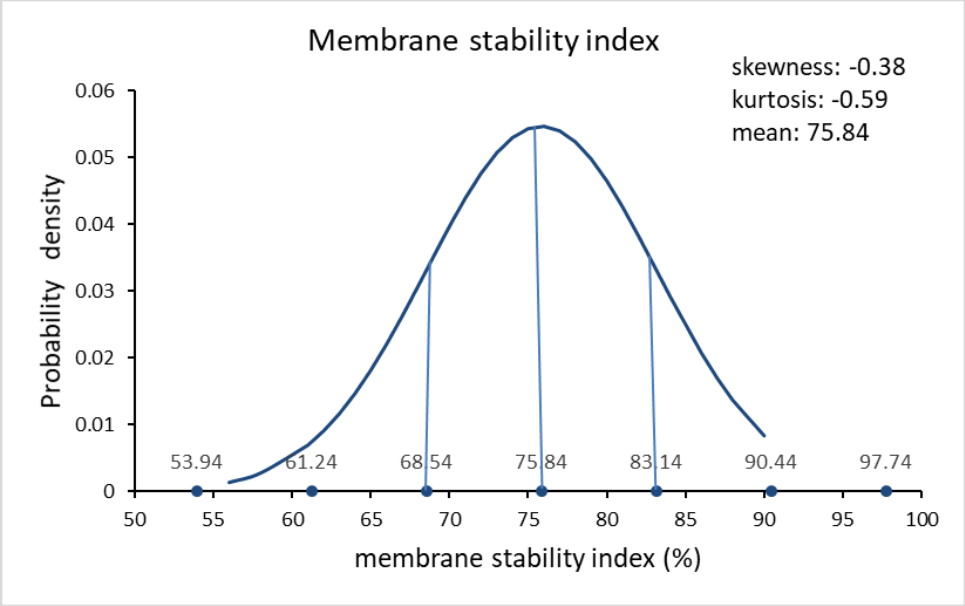


Figure 40. Normal distribution curve of F_2 population under the effect of high temperature stress on the membrane stability index

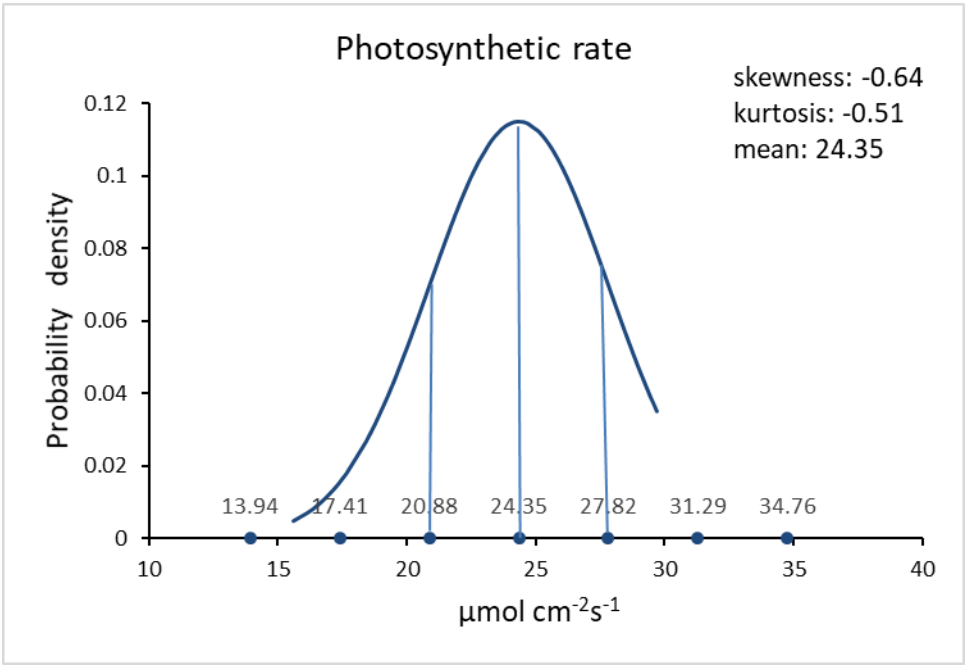


Figure 41. Normal distribution curve of F_2 population under the effect of high temperature stress on the photosynthetic rate

that the majority of the population had a higher photosynthetic rate which was influenced by the tolerant NL-44. The negative skewness in the normal distribution curve is representative of the fact that the majority of the population had photosynthetic rates lower than that of the mean.

5.3.8 Influence of high temperature stress on the frequency of rate of stomatal conductance in F_2 population

The confines of 1 standard deviation had a very narrow range of stomatal conductance rate, ranging from 0.2 mol $m^{-2}s^{-1}$ to 0.26 mol $m^{-2}s^{-1}$ in the F_2 population (Figure 42) with Uma variety recording a lower stomatal conductance compared to NL-44 with the mean lying in between the parents. The normal distribution curve was leptokurtic which is indicative of greater number of outlying population. The positive skewness of the curve is indicative of the curve leaning towards the right of the mean which shows that major number of plants have stomatal conductance higher than the mean.

5.3.9 Influence of high temperature stress on the frequency of transpiration rate in F_2 population

The transpiration rate of the F_2 population (Figure 43) ranged from 4.63 mmol $m^{-2}s^{-1}$ to 7.15 mmol $m^{-2}s^{-1}$. The majority of the plants (1 SD) had a transpiration rate between 5.29 mmol $m^{-2}s^{-1}$ and 6.53 mmol $m^{-2}s^{-1}$. The transpiration rate of the two parents and the mean lay within 1 SD. Uma variety recorded a lower transpiration rate compared to NL-44 with the mean lying in between the two. The mean value being greater than the susceptible Uma variety indicates that the majority of the population had a higher transpiration rate. The positive skewness of the normal distribution curve indicates that the data is leaning towards the right of the mean. The platykurtic curve shows that there are lesser number of outliers.

5.3.10 Influence of high temperature stress on the frequency of leaf temperature in F_2 population

The range of the F_2 population regarding the leaf temperature for 68% (1 SD) of the population was small, ranging from 33.8 ˚C to 35 ˚C (Figure 44). The variety NL-44 recorded a lower leaf temperature compared to Uma, with the mean lying in between them. The mean being closer to NL-44 and lower than Uma shows that the majority of the population has a lower leaf temperature.

5.3.11 Influence of high temperature stress on the frequency of pollen viability in F_2 population

The pollen viability of the F_2 population ranged from 59% and 91% with the majority of the population (within 1 SD) lying in between 70.94% and 84.58% (Figure 45). The variety NL-44 had a greater pollen viability percentage compared to Uma with the mean value greater than the susceptible variety Uma. This indicates that the majority of the F_2 population has a pollen viability percentage that has been influenced by the tolerant parent, NL-44. The negative skewness shows that more number of plants lie towards the left of the mean. The leptokurtic normal distribution curve is indicative of a large number of outlying plants.

5.3.12 Influence of high temperature stress on the frequency of panicle length in F_2 population

The panicle length of the tolerant NL-44 was extremely high while that of Uma was lower. The mean of the F_2 population was lower than both the parental genotypes and relatively closer to Uma. This clearly indicates that the panicle length of the majority of the population was influenced by the Uma variety. The majority of the lines (68%) had a panicle length between 20.8 cm and 25 cm (Figure 46). The negative kurtosis shows that the majority of the lines are concentrated in the centre with a very low number of outliers.

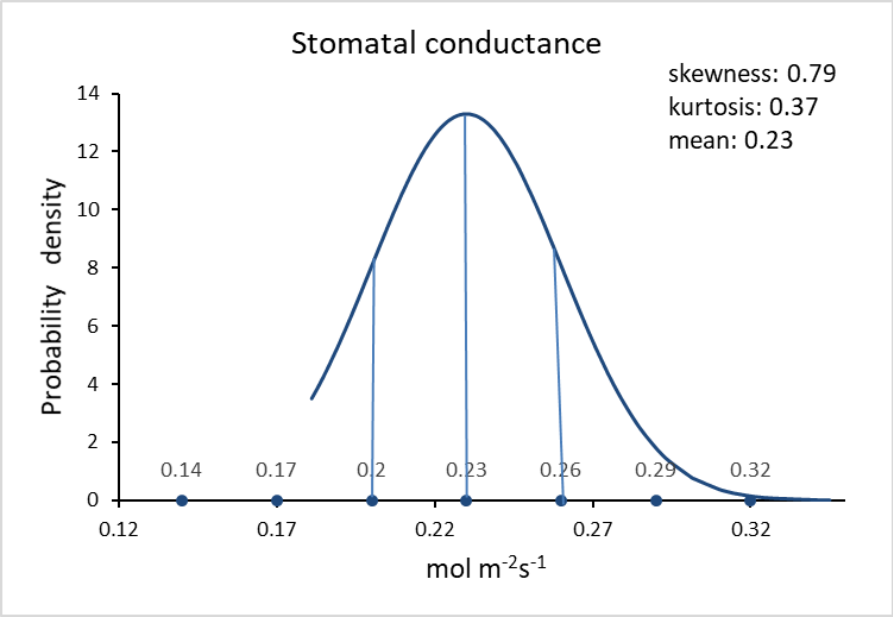


Figure 42. Normal distribution curve of F_2 population under the effect of high temperature stress on the stomatal conductance

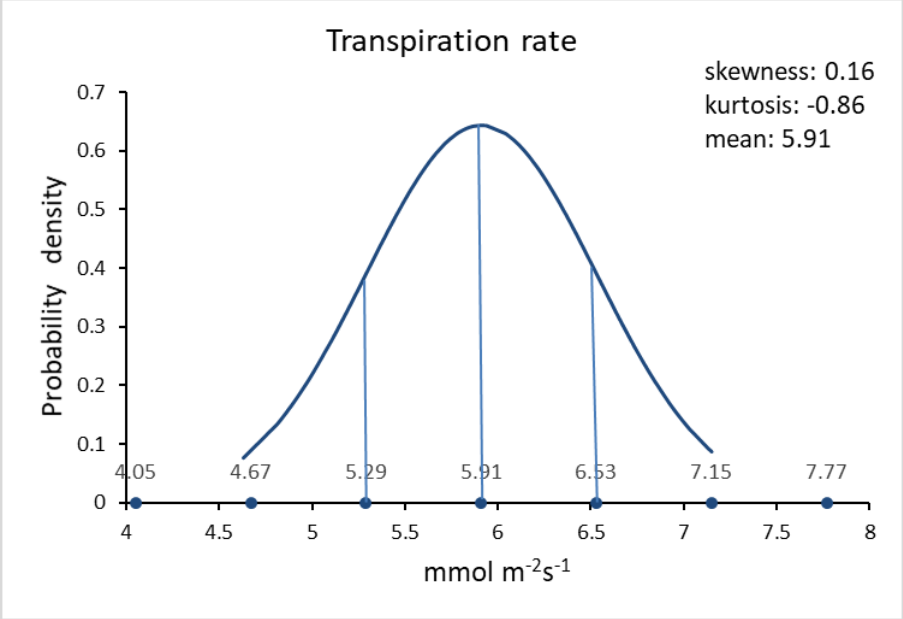


Figure 43. Normal distribution curve of F_2 population under the effect of high temperature stress on the transpiration rate

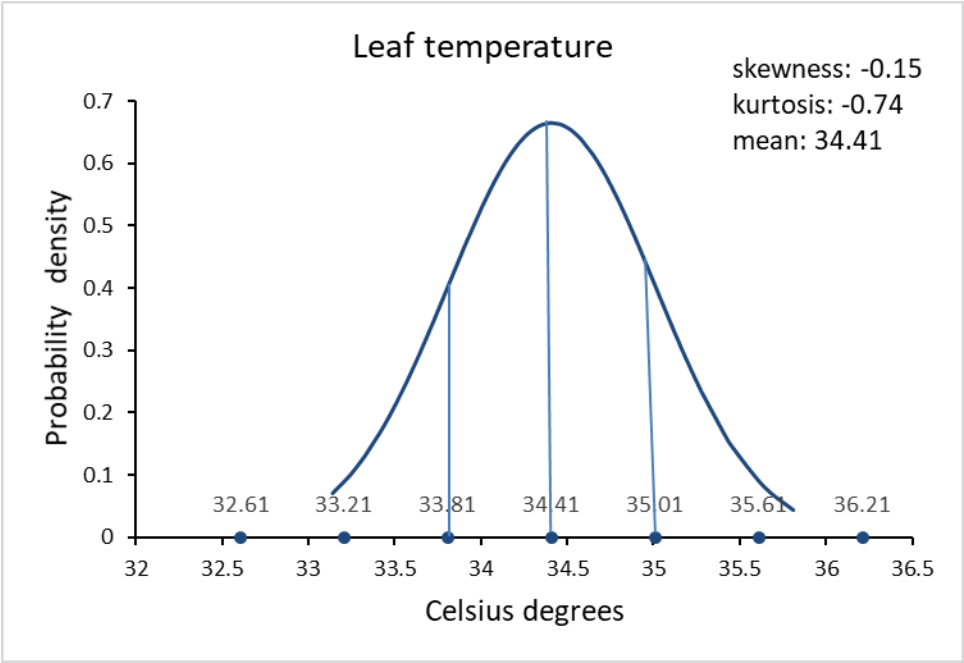


Figure 44. Normal distribution curve of F_2 population under the effect of high temperature stress on the leaf temperature

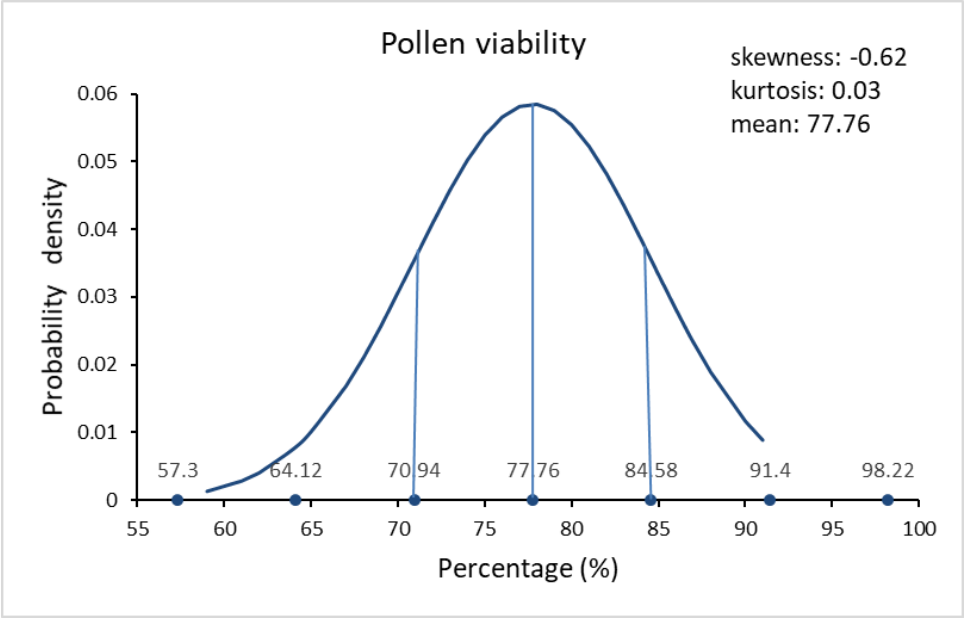


Figure 45. Normal distribution curve of F_2 population under the effect of high temperature stress on the pollen viability

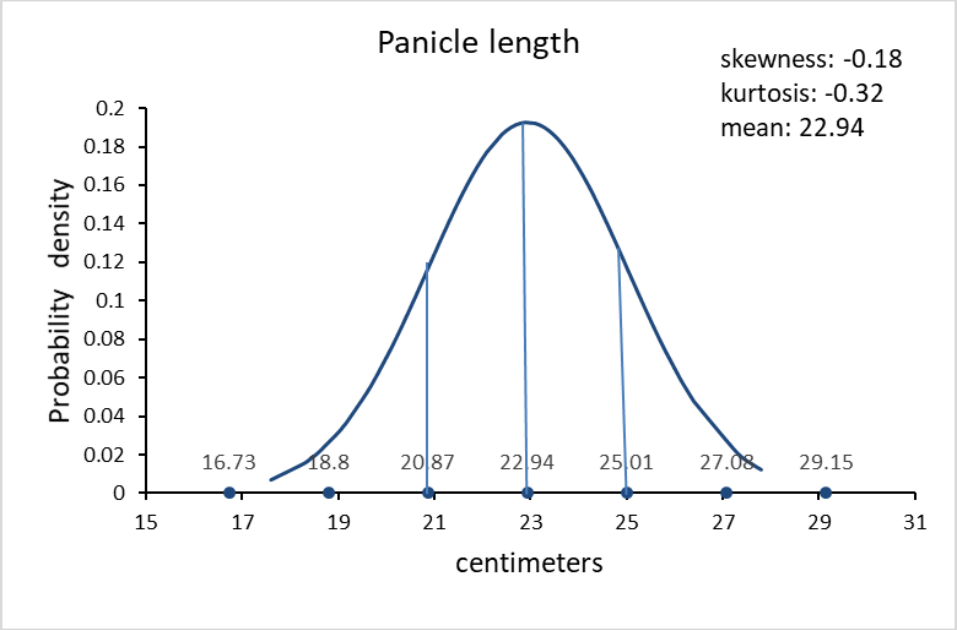


Figure 46. Normal distribution curve of F_2 population under the effect of high temperature stress on the panicle length

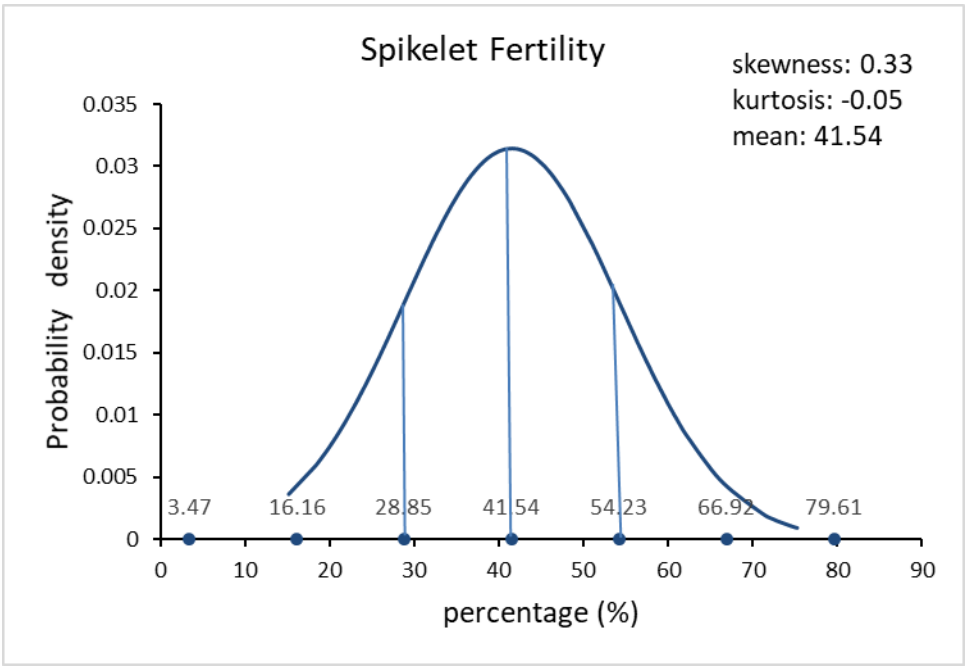


Figure 47. Normal distribution curve of F_2 population under the effect of high temperature stress on the spikelet fertility

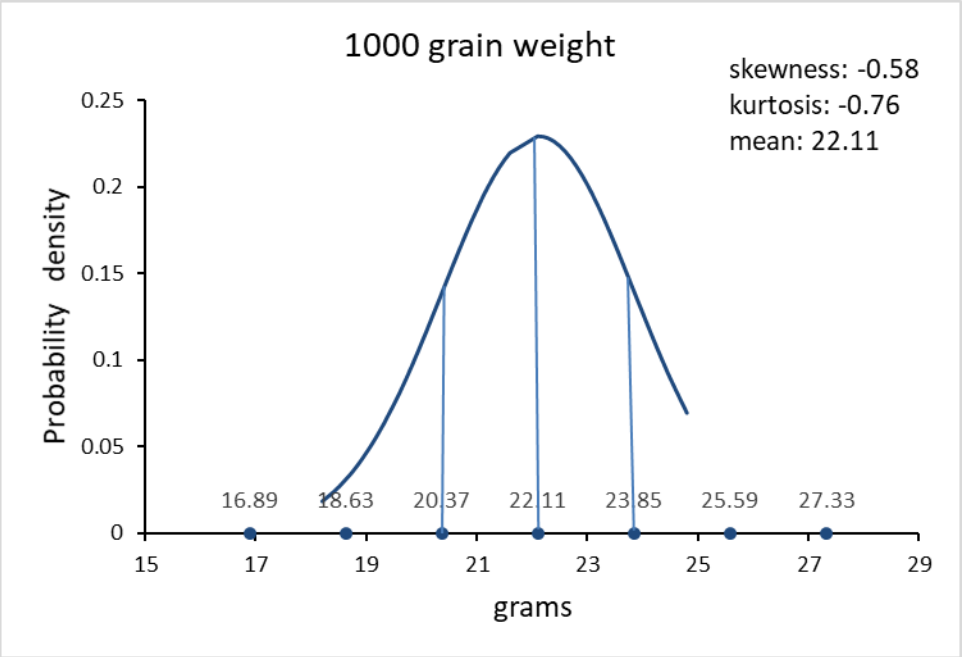


Figure 48. Normal distribution curve of F_2 population under the effect of high temperature stress on the 1000 grain weight

5.3.13 Influence of high temperature stress on the frequency of spikelet fertility percentage in F_2 population

The total range of the F_2 lines with regards to the spikelet fertility was in between 15% and 75%. However, the range of 1 standard deviation was within the confines of 28.8% and 54.2% spikelet fertility (Figure 47). The tolerant variety NL-44 had a much higher spikelet fertility compared to the susceptible parent. The mean of the population lay in between the two parental genotypes. The greater value of the mean compared to Uma indicates that this important trait for heat tolerance has been beneficially transferred from the parent NL-44 to the majority of the population.

5.3.14 Influence of high temperature stress on the frequency of 1000 grain weight percentage in F_2 population

The 1000 grain weight of the F_2 population ranged from 18.2 g to 24.8 g with the confines of 1 standard deviation ranging from 20.31 g to 23.8 g (Figure 48). The tolerant parent NL-44 recorded a higher 1000 grain weight compared to the susceptible parent while the mean was greater than Uma variety and in between the two parental genotypes. The mean being closer to NL-44 clearly indicates that the trait has been greatly contributed by the tolerant parent. The negative kurtosis of the normal curve shows that the majority of the population is concentrated in the centre with lesser number of outliers. The negative skewness indicates that the majority of the population is slightly towards the left of the mean.

5.3.15 Analysis of correlation between the parameters

The 1000 grain weight was significantly correlated with the tolerance contributing traits such as membrane stability index, pollen viability and spikelet fertility which explains the positive impact on the yield characteristic. The positive correlation of the membrane stability index on the pollen viability and spikelet fertility is a major contribution to the heat tolerance of the plants. The negative correlation of the time of anthesis with the spikelet fertility and 1000 grian weight indicates than an earlier time of anthesis is a trait that is beneficial in avoiding the effects of high temperature stress leading to higher grain yield. The positive correlation of the stomatal conductance with the photosynthetic rate is important as higher rate of gaseous exchange significantly improves the photosynthetic rate due to greater influx of carbondioxide, whereas, the positive correlation between the photosynthetic rate and the evapo-transpiration points to the beneficial effect of transpirational cooling under high temperature stress. The negative correlation of leaf temperature on the pollen viability shows that lower temperature contributes to higher pollen viability percentage. As the correlation matrix is a result of mathematical inference, all parameters cannot be assumed to be correlated correctly as the physiological functions of the parameters also need to be considered. In this regard, the negative correlation

obtained between certain parameters cannot be explained as the physiological basis of their relationship is not possible.

5.3.15 Inference of the evaluation of the F_2 population

Based on the results of the phenotypic evaluation of the F_2 population and their parents, we can characterize the NL-44 variety as a tolerant parent as it had performed better with regards to traits such as 1000 grain weight, spikelet fertility, pollen viability, transpiration rate, photosynthetic rate, membrane stability index and days to flowering compared to the variety Uma under high temperature stress conditions. The poor performance of Uma variety under the stress conditions makes it a suitable candidate for characterization as a susceptible variety to heat stress. The mean of the population was closer to NL-44 with regards to the traits of plant height, membrane stability index, photosynthetic rate, stomatal conductance, transpiration rate, pollen viability, spikelet fertility and 1000 grain weight. However, for the traits such as tiller number, days to flowering, time of anthesis, leaf temperature and panicle length, the mean of the population was influenced by the susceptible parent, Uma. The yield contributing character such as productive tiller number has been depressed in the F_2 population.

5.4 IDENTIFICATION OF POLYMORPHIC MARKERS RELATED TO HIGH TEMPERATURE TOLERANCE USING BSA

Simple sequence repeats (SSR) are molecular markers that are relatively more efficient, cheaper and easier to use in marker assisted selection for crop improvement, as there is a probability of higher polymorphism rate (Gao *et al*., 2016). Identifying SSR markers that are associated with QTLs contributing to heat tolerance has huge potential in breeding programmes through gene pyramiding (Ye *et al*., 2015). In this regard, the bulk segregant analysis is a technique that can rapidly identify markers that are tightly linked to the genes for a given phenotype (Zou *et al*., 2016).

In the current study, out of the 100 SSR markers tested on the parental genotypes and the bulks, 18 were found to be polymorphic indicating a polymorphism of 18%. BSA was used to link the markers to heat tolerance on the basis of spikelet fertility percentage recorded under stress conditions. Waghmare *et al*. (2018) had demonstrated the efficiency of BSA, through which they had identified 41 SSR markers that were found to be polymorphic between parents and associated with QTLs for heat tolerance. Varying levels of polymorphism between the tolerant and susceptible parents were obtained, ranging from 8.07 - 27.99 %, in studies that had utilised SSR markers in order to link them with heat tolerance (Vikram *et al*., 2011, Wei *et al*., 2013, Salunkhe *et al*., 2011, Kanaraj *et al*., 2010).

Although a good number of markers linked to different traits for tolerance to heat stress have been reported, each segregating population is unique due to genetic differences between the parents and therefore, the markers validated in a particular cross of parental genotypes may not be applicable to other populations. In the present study, inspite of testing a significant proportion of previously reported validated markers for heat tolerance, only a few of them could be confirmed with the results we have obtained. Out of the 18 polymorphic markers identified, two of them, i.e. RM320 and RM470 are expected to be unique for the current investigation as significant findings about them have not been reported in the literature surveyed. The markers RM222, RM237, RM556 and RM3475 have also been linked to drought tolerance traits (Freeg *et al*., 2016, Yue *et al*., 2006).

The rest of the identified polymorphic markers have been reported to be linked to various aspects of heat tolerance. RM554 was reported to be associated with QTLs that controlled unfilled grain percentage (Buu *et al*., 2014). RM3586 was validated to be associated with QTLs responsible for phenotypic variation for heat tolerance at the flowering stage (Zhang *et al*., 2009b, Buu *et al*., 2014). Xiao *et al*. (2011) associated RM471 with QTLs influencing seed set percentage. RM242 was reported to be affect the erect panicle trait (Kong *et al*., 2007) and was also associated with plant height, panicle length and spiklet fertility under heat stress by Pradhan *et al*. (2016) and Wei *et al*. (2013). Sunohara *et al*., 2006 reported that RM3475 and RM237 were associated with controlling the panicle characters. Liao *et al*. (2011) identified RM473 as a polymorphic marker that

was strongly associated with heat tolerance. Pradhan *et al*. (2016) had associated RM3586 with days to 50% flowering and spikelet fertility percentage under high temperature stress. Bharathkumar *et al*. (2014) associated RM6100 with a major quantitative trait locus affecting tolerance to heat stress at the flowering stage. Waghmare *et al*. (2021) identified RM5749 as a polymorphic marker that could differentiate between the tolerant and susceptible bulks under heat stress in a cross of the parental genotypes, N22 and Uma.

The identified polymorphic markers can be linked to traits known to contribute to heat tolerance through QTL analysis. The polymorphic markers are useful in identifying marker loci associated with various phenotypic traits. In the studied F_2 population, the plants were segregated into tolerant or susceptible genotypes based on the spikelet fertility percentage alone. Therefore, the markers may or may not be linked to other phenotypic traits that were used for evaluation. However, an inference can be made on the correlation analysis between the traits wherein, the spikelet fertility percentage was significantly and positively associated with the tolerance and yield contributing traits such as pollen viability, MSI and 1000 grain weight. The markers can be utilised for marker assisted selection to incorporate tolerance traits. As some of the markers have been reported to be associated with drought, further studies are needed to identify markers that are specific to heat tolerance and also the traits which may be useful for such characterization.

Summary

6. SUMMARY

Rice is an important crop and consumed widely across the globe as staple food. It is the dominant crop of India and contributes 43% of the total food. For every 1° C increase, there will be 10% decrease in grain yield. At present the temperature may go up to 39° C during second/third crop at Palakkad, Thrissur and Kuttanad tract of Alappuzha which are the main rice growing areas of Kerala. It was observed that the second and third crop rice faces failure of seed setting (spikelet sterility). Hot summers in many agricultural regions can negatively affect the vegetative and reproductive growth phases of such crops and can result in up to 80 percent losses in rice yield. One of the important mechanisms to impart tolerance to heat stress is by regulating the sugar metabolism. Sugars have a dual role as they can function as both a metabolite and a signaling molecule in a manner similar to hormones. The sugar signaling mechanism is key to regulating the allocation, partitioning and assimilation of the photo-assimilates in the source and sink organs of the plant. As heat stress is a serious impediment for optimum crop growth and development, it is crucial to identify genomic regions with Quantitative Trait Loci (QTL) that are associated with tolerance traits.

The present investigation was carried out to understand the effect of high temperature stress on the changes in the sugar signaling pathway and to identify the molecular markers associated with heat tolerance in rice. The investigation was carried out as four experiments, the first being the study of heat stress effects on the sugar signaling pathway, and the remaining three experiments being interconnected. The second experiment was the laying of crossing blocks to produce F_1 and F_2 generation seeds, after which the third experiment was conducted to phenotypically evaluate the F_2 population derived from the second experiment. The fourth experiment was conducted to identify polymorphic micro-satellite markers that were associated with heat tolerance in the F_2 population evaluated in the previous experiment.

In the first experiment, the genotypes NERICA L-44 (NL-44) and Vandana were evaluated under two different temperature conditions viz. high temperature stress (38-42 ˚C) and ambient (26-34 ˚C) which was taken as control. The expression of four genes viz. *OsHXK2, OsSnRK1, OsTOR* and *OsTPS1* was studied using quantitative real-time polymerase chain reaction (qRT-PCR) at the vegetative phase just before panicle initiation and at the grain filling stage. The results of the experiment showed that under high temperature stress conditions, the performance of the variety NL-44 was superior to the variety Vandana, as measured by parameters such as cell membrane stability index (+10%), pollen viability (+19%), panicle length (+4.8 cm), photosynthetic rate (+4.75 µmol $cm^{-2}s^{-1}$), stomatal conductance (+0.027 mol $m^{-2}s^{-1}$), spikelet fertility (+19.1%), 1000 grain weight (+5.5 g) as well as greater photochemical efficiency (Fv/Fm ratio), maximal quantum yield (ΦPSII), electron transport rate and higher water-use efficiency. The correlation analysis of the various parameters revealed that the water use efficiency, pollen viability and efficiency of photosystem-II (ϕPSII) which are major contributors to the thermo-tolerance of the plant were found to be positively correlated to the 1000 seed weight which is beneficial for reducing the yield loss under stress. The significant positive correlation between spikelet fertility with the leaf temperature, pollen viability, photosynthetic rate and membrane stability index is beneficial for improving the grain filling percentage which enhances the yield of the crop. The results of the experiment clearly reinforce the tolerance characteristics of NL-44 and establish the susceptibility of Vandana to high temperature stress.

In the vegetative phase, both varieties have shown commonality in the up-regulation of *OsHXK2*, *OsSnRK1* and down-regulation of *OsTPS1*, with the difference between the varieties in that *OsTOR* is up-regulated in the susceptible variety (Vandana), while it was down-regulated in the tolerant variety (NL-44). In the grain filling phase, *OsSnRK1, OsTPS1* and *OsTOR* have been similarly expressed i.e. up-regulated in both the varieties under stress, but differing in the expression of *OsHXK2* which was down-regulated in the susceptible variety, whereas it was up-regulated in the tolerant variety. The expression of each gene was correlated with multiple traits that explained the tolerance or

susceptibility of the genotypes under heat stress. The down-regulation of *OsHXK2* in the susceptible Vandana genotype in the grain-filling phase indicated lower glucose content compared to the up-regulation in the tolerant NL-44. Relatively higher expression of *OsTOR* in NL-44 in the grain filling phase indicating high nutrient status was correlated with the resulting higher 1000 grain weight. The down-regulation of *OsTPS1* in the vegetative phase in both the genotypes resulted in delayed flowering as *OsTPS1* is an indicator of sucrose content. Based on such correlations, the proposed pathway of sugar signaling in tolerant rice genotypes should necessarily cause upregulation of *OsHXK2*, as it indicates high glucose content; down-regulation of *OsSnRK1*, as it would prevent the induction of catabolic processes; up-regulation of *OsTOR*, as it indicates higher nutrient status as well as being involved in inducing stress responses; and down-regulation of *OsTPS1* as the low T6P content would signal remobilization of nutrients towards stress response.

In the second experiment, the two varieties NL-44 (heat-tolerant) and the high yielding Uma (heat-susceptible) were crossed to produce F_1 generation hybrids. The F_1 seeds were then selfed to produce F_2 generation seeds. The F_2 seeds produced had morphological characters that were intermediate to the parents with medium length and medium boldness unlike NL-44 which was long and slender grain whereas Uma has characteristic short and bold grain characteristics.

The F_2 seeds were raised and phenotypically evaluated under high temperature stress (36-40 ˚C) in the third experiment. 144 F_2 plants along with the parents, NL-44 and Uma were assessed using various physiological and yield-related parameters after stress induction from maximum tillering stage till the grain-filling stage in a high temperature polyhouse. The mean of the F_2 population was calculated and the frequency distribution of the lines for each trait was determined along with the normal distribution curve which represented the skewness and kurtosis. The mean of the population was closer to NL-44 with regards to$_1$ the traits of plant height, membrane stability index, photosynthetic rate, stomatal conductance, transpiration rate, pollen viability, spikelet fertility and 1000 grain weight. However, for the traits such as tiller number, days to flowering, time of anthesis,

leaf temperature and panicle length, the mean of the population was influenced by the susceptible parent, Uma. The yield contributing character such as productive tiller number have been found to be depressed in the F_2 population.

Based upon the spikelet fertility percentage, the ten most tolerant and ten most susceptible plants were selected from the F_2 plants phenotypically evaluated in the third experiment. The Bulked Segregant Analysis (BSA) technique was used, wherein the DNA from the tolerant and susceptible lines are pooled into the two contrasting bulks, in order to identify polymorphic SSR markers that are linked to high temperature tolerance. The results of the study have identified 18 SSR markers that exhibited polymorphism between the parents out of the 100 SSR markers used. The identified polymorphic markers could distinguish between the tolerant and susceptible bulks as seen in their differential banding pattern. The identified polymorphic markers were also found to segregate between the individual lines and characterize them into tolerant or susceptible lines based on their similarity to the banding pattern of the tolerant parent, NL-44 or the susceptible parent, Uma.

In the present study, the expression levels of the sugar signaling genes and their association with phenotypic characters has elucidated their role in imparting heat tolerance in rice. The phenotyping of the F_2 generation indicated that the tolerance traits in the population were majorly contributed by the tolerant parent i.e. NL-44. The identified polymorphic markers were able to segregate the individual lines of F_2 population into tolerant and susceptible genotypes. The elucidation of the sugar signaling mechanism in tolerant genotypes of rice and the association of molecular markers linked to the heat tolerance trait in the segregating second generation filial populations is validated to be beneficial in undertaking crop improvement studies for enhanced sugar metabolism as well as to introgress the tolerance traits into high-yielding regional varieties.

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HIGH TEMPERATURE MEDIATED CHANGES IN SUGAR SIGNALING PATHWAY AND IDENTIFICATION OF ASSOCIATED MICROSATELLITE MARKERS IN RICE (*Oryza sativa* L.)

By

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Abstract

The research work entitled 'High temperature mediated changes in sugar signaling pathway and identification of associated microsatellite markers in rice (*Oryza sativa* L.)' was undertaken at the College of Agriculture, Vellayani during 2018-2022. The aim of the study was to understand the effect of high temperature stress on the changes in the sugar signaling pathway and to identify the molecular markers associated with heat tolerance in rice. The investigation was carried out as four experiments, the first being the study of heat stress effects on the sugar signaling pathway, and the remaining three experiments being interconnected. The second experiment was the laying of crossing blocks to produce F_1 and F_2 generation seeds, after which the third experiment was conducted to phenotypically evaluate the F_2 population derived from the second experiment. The fourth experiment was conducted to identify polymorphic micro-satellite markers that were associated with heat tolerance in the F_2 population evaluated in the previous experiment.

In the first experiment, the genotypes NERICA L-44 (NL-44) and Vandana were evaluated under two different temperature conditions viz. high temperature stress (38-42 ˚C) and ambient (26-34 ˚C) which was taken as control. The expression of four genes viz. *OsHXK2, OsSnRK1, OsTOR* and *OsTPS1* was studied using quantitative real-time polymerase chain reaction (qRT-PCR) at the vegetative phase just before panicle initiation and at the grain filling stage. The results of the experiment showed that under high temperature stress conditions, the performance of the variety NL-44 was superior to the variety Vandana, as measured by parameters such as cell membrane stability index (+10%), pollen viability (+19%), panicle length (+4.8 cm), photosynthetic rate (+4.75 µmol $cm^{-2}s^{-1}$), stomatal conductance (+0.027 mol $m^{-2}s^{-1}$), spikelet fertility (+19.1%), 1000 grain weight (+5.5 g) as well as greater photochemical efficiency (Fv/Fm ratio), maximal quantum yield (ΦPSII), electron transport rate and higher water-use efficiency. The results of the experiment clearly reinforce the tolerance characteristics of NL-44 and establish the susceptibility of Vandana to high temperature stress. The expression of each gene was correlated with multiple traits that explained the tolerance or susceptibility of the genotypes

under heat stress. Based on such correlations, the proposed pathway of sugar signaling in tolerant rice genotypes should necessarily cause upregulation of *OsHXK2*, as it indicates high glucose content; down-regulation of *OsSnRK1*, as it would prevent the induction of catabolic processes; up-regulation of *OsTOR*, as it indicates higher nutrient status as well as being involved in inducing stress responses; and down-regulation of *OsTPS1* as the low T6P content would signal remobilization of nutrients towards stress response.

In the second experiment, the two varieties NL-44 (heat-tolerant) and the high yielding Uma (heat-susceptible) were crossed to produce F_1 generation hybrids. The F_1 seeds were then selfed to produce F_2 generation seeds. The F_2 seeds produced had morphological characters that were intermediate to the parents with medium length and medium boldness unlike NL-44 which was long and slender grain whereas Uma has characteristic short and bold grain characteristics.

144 F_2 plants along with the parents, NL-44 and Uma were phenotypically evaluated under high temperature stress (36-40 ˚C) in the third experiment. . The mean of the population was closer to NL-44 with regards to the traits of plant height, membrane stability index, photosynthetic rate, stomatal conductance, transpiration rate, pollen viability, spikelet fertility and 1000 grain weight. However, for the traits such as tiller number, days to flowering, time of anthesis, leaf temperature and panicle length, the mean of the population was influenced by the susceptible parent, Uma.

Based upon the spikelet fertility percentage, the ten most tolerant and ten most susceptible plants were selected from the F_2 plants and Bulked Segregant Analysis (BSA) technique was used to identify polymorphic SSR markers that are linked to high temperature tolerance. The results of the study have identified 18 SSR markers that exhibited polymorphism between the parents out of the 100 SSR markers used. The identified polymorphic markers could distinguish between the tolerant and susceptible bulks as seen in their differential banding pattern. The identified polymorphic markers were also found to segregate between the individual lines and characterize them into tolerant or

susceptible lines based on their similarity to the banding pattern of the tolerant parent, NL-44 or the susceptible parent, Uma.

In the present study, the expression levels of the sugar signaling genes and their association with phenotypic characters has elucidated their role in imparting heat tolerance in rice. The phenotyping of the F_2 generation indicated that the tolerance traits in the population were majorly contributed by the tolerant parent i.e. NL-44. The identified polymorphic markers were able to segregate the individual lines of F_2 population into tolerant and susceptible genotypes. The elucidation of the sugar signaling mechanism in tolerant genotypes of rice and the association of molecular markers linked to the heat tolerance trait in the segregating second generation filial populations is validated to be beneficial in undertaking crop improvement studies for enhanced sugar metabolism as well as to introgress the tolerance traits into high-yielding regional varieties.

സംഗ്രഹം

നെല്ലിൽ (*Oryza sativa* L.) ഉയർന്ന താപനിലയുടെ മധ്യസ്ഥതയിൽ പഞ്ചസാര സന്ദേശം പകരുന്ന പാതയിലെ മാറ്റങ്ങളും നെല്ലിലെ അനുബന്ധ മൈക്രോസാറ്റലൈറ്റ് മാർക്കറുകൾ തിരിച്ചറിയലും" എന്ന തലക്കെട്ടിൽ 2018-2022 കാലയളവിൽ വെള്ളായണിയിലെ കാർഷിക കോളേജിൽ, പ്ലാന്റ് ഫിസിയോളജി വിഭാഗത്തിൽ ഗവേഷണ പ്രവർത്തനങ്ങൾ നടത്തി. ഉയർന്ന താപനിലയുടെ സമ്മർദ്ദത്തിൽ പഞ്ചസാര സന്ദേശം പകരുന്ന പാതയിലെ മാറ്റങ്ങൾ മനസ്സിലാക്കുക, നെല്ലിലെ ചൂട് സഹിഷ്ണുതയുമായി ബന്ധപ്പെട്ട തന്മാത്രാ മാർക്കറുകൾ തിരിച്ചറിയുക എന്നിവയായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം. നാല് പരീക്ഷണങ്ങളായാണ് അന്വേഷണം നടത്തിയത്, ആദ്യത്തേത് പഞ്ചസാര സന്ദേശം പകരുന്ന പാതയിലെ താപ സമ്മർദ്ദ ഫലങ്ങളെക്കുറിച്ചുള്ള പഠനമാണ്, ശേഷിക്കുന്ന മൂന്ന് പരീക്ഷണങ്ങൾ പരസ്പരം ബന്ധപ്പെട്ടിരിക്കുന്നു. F_1, F_2 തലമുറ വിത്തുകൾ ഉൽപ്പാദിപ്പിക്കുന്നതിനായി ക്രോസിംഗ് ബ്ലോക്കുകൾ രണ്ടാമത്തെ പരീക്ഷണത്തിൽസ്ഥാപിച്ചു. അതിനുശേഷം രണ്ടാമത്തെ പരീക്ഷണത്തിൽ നിന്ന് ഉരുത്തിരിഞ്ഞ F_2 ജനസംഖ്യ സ്ഥൂലരൂപം ആയി വിലയിരുത്തുന്നതിനായി മൂന്നാമത്തെ പരീക്ഷണം നടത്തി. മൂന്നാമത്തെ പരീക്ഷണത്തിൽ വിലയിരുത്തിയ F_2 ജനസംഖ്യയിൽ ചൂട് സഹിഷ്ണുതയുമായി ബന്ധപ്പെട്ട ബഹുരൂപം മൈക്രോ-സാറ്റലൈറ്റ് മാർക്കറുകൾ തിരിച്ചറിയുന്നതിനാണ് നാലാമത്തെ പരീക്ഷണം നടത്തിയത്.

ആദ്യ പരീക്ഷണത്തിൽ, NERICA L-44 (NL-44), വന്ദന എന്നീ ജനിതകരൂപങ്ങളെ ഉയർന്ന താപനില (38-42˚C) സമ്മർദ്ദത്തിലും തുറസായ താപനില (26-34 ˚C) സമ്മർദ്ദത്തിലും വളർത്തിയെടുത്തു. ആംബിയന്റ് നിയന്ത്രണമായി എടുത്തു. കപാണ്ടിറ്റേറ്റീവ് തൽസമയം പോളിമറേസ് ചെയിൻ പ്രതികരണം (qRT-PCR) ഉപയോഗിച്ച് നാല് ജീനുകളുടെ (*OsHXK2, OsSnRK1, OsTOR, OsTPS1*) ആവിഷ്കാരം കതിർ വരുന്നതിനുമുന്പുള്ള ഘട്ടത്തിലും, ധാന്യം നിറയുന്നതിനു മുന്പുള്ള ഘട്ടത്തിലും പഠനം നടത്തുക ഉണ്ടായി . ഉയർന്ന താപനില സമ്മർദ്ദ സാഹചര്യത്തിൽ കോശ സ്തര സൂചിക (+10 %), പൂമ്പൊടിയുടെ പ്രവർത്തന ക്ഷമത (+19 %), കാതിരിന്റെ നീളം (+4

.8 സി. എം), പ്രകാശ സംശ്ലേഷണ നിരക്ക് (+4.75 µmol $m^{-2}s^{-1}$), ആസ്യരന്ധ്ര വിനിമയ നിരക്ക് (+0.027 mmol $m^{-2}s^{-1}$), ചെറു കതിരിന്റെ ഫെർട്ടിലിറ്റി (+19.1%),), ആയിരം ധാന്യത്തിൻറെ ഭാരം (+5.5g), പ്രകാശത്തിന്റെ രാസപ്രവർത്തനവു മായി ബദ്ധപ്പെട്ടകാര്യക്ഷമത (Fv/Fm) അനുപാതം), പരമാവധി ഊർജതന്മാത്ര വിളവ് (ΦPSII), എലെക്ട്രോണ് ഗതാഗത നിരക്ക് , ഉയർന്ന ജല ഉപയോഗ ക്ഷമത എന്നീ സ്വഭാവങ്ങൾ വന്ദന എന്ന നെല്ലിനത്തെ അപേക്ഷിച്ചു NL-44 എന്ന നെല്ലിൽ കൂടുതൽ ആണെന്ന് കണ്ടെത്തി. പരീക്ഷണത്തിന്റെ ഫലങ്ങൾ വന്ദന എന്ന നെല്ലിനത്തിന്റ താപ സഹിഷ്ണുത ഇല്ലായ്മയും ഉയർന്ന താപനില സമ്മർദ്ദത്തിന് NL-44 ന്റെ താപ സഹിഷ്ണുത സ്ഥാപിക്കുകയും ചെയ്യുന്നു. ഓരോ ജീനിന്റെയും പ്രകടനവും താപ സമ്മർദ്ദത്തിൻ കീഴിലുള്ള ജനിതകരൂപങ്ങളുടെ സഹിഷ്ണുതയോ സംവേദനക്ഷമതയോ വിശദീകരിക്കുന്ന ഒന്നിലധികം സ്വഭാവസവിശേഷതകളുമായി ബന്ധപ്പെട്ടിരിക്കുന്നു. അത്തരം പരസ്പര ബന്ധങ്ങളെ അടിസ്ഥാനമാക്കി, താപസഹിഷ്ണുത ക്ഷമതയുള്ള നെല്ലിൻറെ ജനിതകരൂപങ്ങളിൽ പഞ്ചസാര സന്ദേശം നിർദ്ദേശിക്കപ്പെടുന്ന പാത നിർബന്ധമായും *OsHXK2* എന്ന ജീനിൻറെ അപ്പ് റെഗുലേഷന് കാരണം ആകണം, കാരണം ഇത് ഉയർന്ന ഗ്ളൂക്കോസ് ഉള്ളടക്കത്തെ സൂചിപ്പിക്കുന്നു; OsSnRK1 എന്ന ജീനിൻറെ ഡൗൺ-റെഗുലേഷൻ, കാറ്റബോളിക് പ്രക്രിയകളുടെ ഉത്തേജകത്തെ തടസ്സപ്പെടുത്തുന്നു; *OsTOR*-ന്റെ അപ്പ്-റെഗുലേഷൻ, ഉയർന്ന പോഷക നിലയെ സൂചിപ്പിക്കുന്നതോടൊപ്പം സമ്മർദ്ദ പ്രതികരണങ്ങൾ ഉണ്ടാക്കുന്നതിൽ പ്രധാന പങ്കു വഹിക്കുന്നു; കൂടാതെ *OsTPS1* ന്റെ താഴ്ന്ന നിയന്ത്രണം T6P ഉള്ളടക്കത്തെ കുറക്കുകയും സമ്മർദ്ദ പ്രതികരണത്തിലേക്കുള്ള പോഷകങ്ങളുടെ പുനർനിർമ്മാണത്തെ സഹായിക്കുകയും ചെയ്യുന്നു .

രണ്ടാമത്തെ പരീക്ഷണത്തിൽ, F_1 തലമുറ സങ്കരയിനം ഉത്പാദിപ്പിക്കാൻ താപസഹിഷ്ണുതയുള്ള ഇനമായ NL-44 ഉം ഉയർന്ന വിളവ് നൽകുന്ന ഉമ (താപ സംവേദന ക്ഷമതയുള്ള) എണ്ണവും തമ്മിൽ പരാഗണം നടത്തി F_1 തെലമുറ ഉൽപ്പാദിപ്പിച്ചെടുത്തു . F_1 തലമുറയുടെ സ്വപരാഗണത്തിലൂടെ F_2 തെലമുറ ഉൽപ്പാദിപ്പിച്ചെടുത്തു. NL-44 ഇനത്തിൽ നീളമുള്ളതും മെലിഞ്ഞതുമായ ധാന്യങ്ങളും ഉമാ എന്ന ഇനത്തിൽ ചെറുതും

ഉരുണ്ടതുമായ ധാന്യങ്ങളുമാണ് ഉണ്ടാകുന്നത് എന്നാൽ F_2 വിത്തുകൾ ഇടത്തരം നീളത്തിലും വന്നതിലും കാണപ്പെട്ടു.

മൂന്നാമത്തെ പരീക്ഷണത്തിൽ ഉയർന്ന താപനില സമ്മർദ്ദത്തിൽ (36-40 ˚C) മാതാപിതാക്കളായ NL-44, ഉമ എന്നി ഇനങ്ങളോടൊപ്പം 144 F_2 സസ്യങ്ങളും വളർത്തി അതിന്റെ സ്വഭാവ സവിശേഷതകൾ വിലയിരുത്തി. സസ്യങ്ങളുടെ ഉയരം, സ്മര സ്ഥിരത സൂചിക, പ്രകാശസംശ്ലേഷണ നിരക്ക്, സ്റ്റോമറ്റൽ കണ്ടക് ടൻസ്, ട്രാൻസ്പിറേഷൻ നിരക്ക്, പൂമ്പൊടിയുടെ പ്രവർത്തനക്ഷമത, ചെറു കതിരിന്റെ ഫെർട്ടിലിറ്റി, 1000 ധാന്യങ്ങളുടെ ഭാരം എന്നിവ NL-44 ന്റെ സവിശേഷതകളോട് സാമ്യം കാണിക്കുകയും ടില്ലർ നമ്പർ, പൂവിടുന്ന ദിവസങ്ങൾ, പുഷ്പവികാസതിന്റെ സമയം, ഇലയുടെ താപനില, പാനിക്കിൾ നീളം എന്നിവ ഉമയുടെ സവിശേഷതകളോട് സാമ്യം കാണിക്കുകയുംചെയ്തു.

ചെറു കതിരിന്റെ ഫെർട്ടിലിറ്റി, ശതമാനത്തിന്റെ അടിസ്ഥാനത്തിൽ, F_2 സസ്യങ്ങളിൽ നിന്ന് ഏറ്റവും സഹിഷ്ണുതയുള്ളതും ഏറ്റവും സംവേദന ക്ഷമതയുള്ളതുമായ പത്ത് സസ്യങ്ങളെ തിരഞ്ഞെടുത്തു, അതിനുശേഷം ഉയർന്ന താപനില സഹിഷ്ണുതയുമായി ബന്ധപ്പെട്ടിരിക്കുന്ന ബഹുരൂപ SSR മാർക്കറുകൾ തിരിച്ചറിയുന്നതിനായി ബൾക്ക് സിഗ്രിഗന്റ അനാലിസിസ് (BSA) സാങ്കേതിക വിദ്യ ഉപയോഗപ്പെടുത്തി. ഉപയോഗിച്ച 100 എസ്എസ്ആർ മാർക്കറുകളിൽ രക്ഷിതാക്കൾക്കിടയിൽ ബഹുരൂപത പ്രകടിപ്പിക്കുന്ന 18 എസ്എസ്ആർ മാർക്കറുകൾ ഉണ്ടെന്ന് പഠന ഫലങ്ങൾ കണ്ടെത്തി. ക്രമത്തിൽ കാണുന്ന വ്യതിയാനത്തിലൂടെ താപവ്യാപന സഹിഷ്ണുത ഉള്ളതും താപ സംവേദന ക്ഷമത ഉള്ളതുമായ ഇനങ്ങളെ തിരിച്ചറിയാൻ കഴിയും. താപനില സഹിഷ്ണുതയുള്ള NL-44 ഇനത്തിന്റെയും താപനില സംവേദന ക്ഷമത ഉള്ള ഉമ ഇനത്തിന്റെയും ബാന്ഡിങ് സമാനതയുടെ അടിസ്ഥാനത്തിൽ തിരിച്ചറിഞ്ഞ പോളിമോർഫിക് മാർക്കറുകൾ വ്യക്തിഗത F_2 ചെടികളെ വേർതിരിച്ചു അവയെ താപസഹിഷ്ണുത ഉള്ളത്, താപസംവേദന ക്ഷമത ഉള്ളത് എന്നിങ്ങനെ തരംതിരിച്ചു

നിലവിലെ പഠനത്തിൽ പഞ്ചസാര സന്ദേശം പകരുന്ന ജീനുകൾക്ക്, നെല്ലിലെ ഫീനോടൈപിങ് പ്രതീകങ്ങളുമായും

താപസഹിഷ്ണുതയുമായുള്ള പങ്കു വ്യക്തമാക്കുന്നുണ്ട്. F_2 തലമുറയിലെ സ്വഭാവ സവിശേഷതകൾ വിലയിരുത്തുമ്പോൾ താപനിലസഹിഷ്ണുതയുടെ സ്വഭാവസവിശേഷതകൾ കൂടുതലായി സംഭാവന ചെയ്തിരിക്കുന്നത് NL-44 നെല്ലിനത്തിൽ നിന്നാണ്. ഉയർന്ന താപനിലയിൽ നെല്ലിലെ പഞ്ചസാര സന്ദേശം നൽകുന്ന പ്രക്രിയയും താപസഹിഷ്ണുതസ്വഭാവവുമായി ബന്ധപ്പെട്ട തന്മാത്ര മാർക്കറുകളുടെ കണ്ടുപിടിത്തവും നെല്ലിലെ താപ സഹിഷ്ണുത ഉയർത്താൻ സഹായിക്കുമെന്നു ഈ പഠനത്തിലൂടെ കണ്ടെത്തി.