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Evaluation of heat tolerant traits using morpho-physiological and biochemical markers at three different sowing dates in wheat (*Triticum aestivum* L.)

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Abstract
Heat stress poses a major constraint to wheat production by impairing grain yield and quality, particularly at the reproductive and grain-filling stages. The screening and detection of heat-tolerant genotypes plays a vital role in minimizing yield losses under heat stress. The experiment involved the assessment of ten wheat genotypes (Trimbak, NIAW-34, MACS-6222, MACS-6478, MACS-2496, RAJ-3765, Phule Samadhan, Sardar, HI-1633, HI-1605) grown during the 2021-22 season under three irrigated environments, timely sown (non-stress environment (S1), late sown (S2) and very late sown (heat stress environment, S3) at Post Graduate Instructional Farm and Botany laboratory, PGI, Mahatma Phule Krishi Vidyapeeth, Rahuri. Delayed sowing was used to impose heat stress as 20-20 days delayed from normal sowing date. Wheat genotypes were evaluated for heat tolerance on the basis of multiple morpho-physiological and biochemical traits such as days to first flowering, days to maturity, plant height, tillers per plant, length of spike, number of grains per spike, weight of grains per plant, 1000-grain weight, CTD, chlorophyll content, RLWC, SOD, TSI, and NDVI. Varieties HI-1605 and NIAW-34 showed superior traits, such as higher productive tillers, larger spikes, and improved grain weight even under late sowing conditions. Genotype HI-1605 exhibited higher chlorophyll retention, superior CTD, and better RLWC. HI-1605 and Phule Samadhan showed high SOD activity. Genotypes HI-1633, and NIAW-34 consistently demonstrated superior heat tolerance with low TSI values. Phule Samadhan and HI1605 consistently showed high NDVI values. Thus, these traits may serve as effective criteria for screening heat-tolerant traits in wheat.

Keywords: S1-timely sowing, S2- late sowing, S3- very late sowing

Introduction
Wheat (*Triticum aestivum* L.) is one of the most important cereal crops, playing a crucial role in global food security. However, the increasing prevalence of high temperatures during critical growth stages poses a significant threat to wheat productivity. Rising temperatures, altered rainfall patterns, and shortened winter seasons have intensified abiotic stresses, particularly drought and heat stress, which severely affect crop growth, yield potential, and food quality. Among these, heat stress is a major limiting factor for staple crops like wheat, and its impact is expected to worsen under ongoing climatic shifts. Projections suggest that by the end of the 21st century, global temperatures will rise by 1-4 °C, leading to an estimated decline in wheat yields of 4-6% (Zheng *et al*., 2025) [28]. Elevated temperatures disrupt plant growth and development by impairing physiological processes, grain formation, protein synthesis, enzyme activity, and cellular stability, while also causing membrane damage and reducing cell division. In wheat, the optimal temperature during anthesis to grain maturity is 22-25 °C; exposure to higher temperatures causes irreversible damage (Farooq *et al*., 2011) [11]. Under late sowing conditions, when crops encounter 25-33 °C during anthesis and grain filling, early maturation occurs, resulting in significant yield losses. Grain filling duration is considered an important parameter for identifying heat-tolerant wheat genotypes (Ali *et al*., 2018) [2]. Thus, developing heat-tolerant wheat genotypes is imperative to mitigate the adverse effects of climate change on crop yield. Consequently, identifying heat-tolerant genotypes is essential for wheat improvement (Sarkar *et al*., 2025) [22].

Stress-tolerant lines can be selected by evaluating advanced wheat genotypes under both optimal and stress conditions. Harnessing the existing genetic variability among wheat genotypes provides breeders with opportunities to enhance tolerance to heat stress. However, developing an efficient and reliable selection strategy remains one of the greatest challenges in identifying heat-tolerant cultivars.

Although numerous stress tolerance indices have been developed for screening cultivars, certain indices prove more reliable in identifying heat tolerance in wheat. Chlorophyll Content Index, Chlorophyll is essential for photosynthesis and its reduction under stress conditions directly impacts plant productivity (Fahad *et al*., 2017) [10]. Canopy temperature depression (CTD) is a measured to evaluate a plant's ability to cool itself under stress. The relative leaf water content (RLWC) is a physiological trait that helps assess a plant's water retention capacity under stress conditions. RLWC is a useful indicator of a plant's ability to tolerate water stress (Shaukat *et al*., 2021) [23, 24]. Thermal Stress Index (TSI) is commonly used as a physiological indicator to assess the extent of stress-induced damage to cell membranes. NDVI is a widely used remote sensing index that measures vegetation health and density by utilizing the difference between near-infrared (NIR) and red (RED) light reflectance (Rouse *et al*., 1974) [20]. Biochemical indices such as Superoxide dismutase (SOD) acts as a multi-functional protector against heat stress, safeguarding plants through Osmo-protection (Almeselmani *et al*., 2009) [3]. Together with certain phenological characteristics, offer better criteria for the selection of stable and tolerant cultivars.

In the present investigations, 10 wheat genotypes were evaluated under three environmental conditions (normal, late sowing and very late sowing). It was observed that under late-sown conditions, grain yield declined due to exposure to elevated temperatures (~3-5 °C above normal sowing) during the anthesis and grain-filling stages. Indices such as Chlorophyll Content Index, CTD, RLWC, TSI and NDVI and other biochemical marker (SOD) have been adopted for the identification of heat-tolerant genotypes, with their selection carried out on the basis of stress indices assessment.

Materials and Method

The experiment involved the evaluation of ten wheat genotypes (Trimbak, NIAW-34, MACS-6222, MACS-6478, MACS-2496, RAJ-3765, Phule Samadhan, Sardar, HI-1633, HI-1605) grown during the 2021-22 season under three irrigated environments, timely sown (non-stress environment-November last week (S1), late sown- December second week (S2) and very late sown - second week of January (heat stress environment, S3) at Post Graduate Instructional Farm and Botany laboratory, PGI, Mahatma Phule Krishi Vidyapeeth, Rahuri. Thus, the reproductive stage of wheat was exposed to intense heat stress, while standard agronomic practices were implemented periodically according to the crop's requirements. The days required from sowing to first flowering of the plants showing emergence of spikes was considered as days to first flowering. The days required from sowing to the stage at which earhead turned yellow was considered as days to maturity. The height of plant from ground level to the tip of ear head excluding awn length in respect of main tillers were recorded at maturity. The productive tillers bearing spike in a single plant were counted at physiological maturity. The total number of grains in spikelet on the main spike of selected plant were manually counted. Randomly selected plants from each generation of replication were harvested in single bag. All the ears belonging to single plant were threshed, cleaned and stored in single packet and the weight of grains from each individual plant was measured in grams. The weight of 1000 randomly selected grains were counted using a digital counter and then weighted in grams using electronic balance average value considered as individual observation. Canopy temperature depression measurement was made by using hand held infrared thermometer (Model 0S 530 HR, Omega Engineering Inc Stamford CT USA). SPAD (Soil plant analysis development) index was estimated non-destructively using SPAD-502 chlorophyll meter (Minolta Corp., Ramsey, NJ, USA) at 50% flowering grain filling stages from flag leaf only SPAD value is directly associated with the chlorophyll content of the leaf. The procedure for relative leaf water content given by Barrs and Weatherley (1962) [5] was used for the estimations. Thermal stress index provided the degree of cell membrane injury against the heat stress (C_1/C_2). It was calculated at 50% flowering by the following procedure given by Blum and Adelina (1981) [8]. Spectral reflectance measurements (For NDVI) were recorded using the SVC HR1024i spectroradiometer, which operates within the wavelength range of 350-2500 nm and is equipped with a 4° Field of View (FOV) lens (Rouse *et. al.* 1973) [19]. Superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium using the method described by Dhindsa *et al*. (1981) [9].

CTD = Ambient or air temperature - Canopy temperature (Rosyara *et al*. 2008)

NDVI (Normalized difference Vegetation Index) (Rouse *et. al.* 1973) [19]

$$NDVI = (NIR-R)/(NIR+R)$$

RLWC (%) = Wf - Wd / Wt-Wd *100

Where,

Wf = Fresh weight, Wd = Dry weight and Wt = Turgid weight

Results and Discussion

Days to First Flowering

Days to First Flowering showed significant variations among the varieties and different sowing conditions; it revealed substantial amount of variability present in the varieties (Fig.1). In timely sowing, the genotype MACS-2496 exhibited the highest days to first flowering (67.8 days). Conversely, HI-1633 showed the shortest time to flowering (54.2 days), demonstrating its suitability for timely sowing and its potential for early maturity.

In late and very late sowing, Varieties such as HI-1633 (53.3, 50.4) and MACS-6222 (54.9 and 52.2), with consistently shorter flowering durations, could serve as promising candidates for late-sowing conditions, ensuring timely maturity and yield stability. In contrast, a similar trend was observed, with MACS-2496 requiring 65.7 and 58.7 days to first flowering, the highest under this condition, but significantly reduced compared to S_1, S_2 and S_3. Across the three sowing conditions.

Days to Maturity

The trait reflects highly significant differences among varieties and interaction of sowing conditions (timely, late and very late) (Fig.2). Varieties MACS-2496 took the longest to reach maturity in all conditions (127, 125.7 and 112.4 days), whereas MACS-6222 had shorter maturity days in all conditions (112.4, 108.8 and 97.4 days) followed by MACS- 6478 (110.6, 106.8 and 97.9). HI-1633 maturing earlier at 112.3 days under S1, while 112.3 days and 109.1days required under late and very late sowing. The days to maturity trait showed notable variation across varieties and conditions. Varieties such as MACS-6222 and MACS- 6478 followed by HI-1633 demonstrated resilience under heat stress. Early maturity as inherent trait indicated better thermal tolerance (Bhagat *et al*., 2023) [7].

Plant Height

Under all sowings and for all the varieties for Plant height showed significant variations (Fig.3). HI-1605 exhibiting the tallest average height across conditions (91.95, 91.0 and 87.6 cm), indicating superior genetic traits for growth under stress (Kartseva *et al*., 2024) [14], while MACS-2496 displayed shorter stature (76.3, 76.9 and 74.3 cm). This reduction under late and very late sowing highlights the impact of heat stress. NIAW-34 showed reduced heights (81.1 cm in S1, 81.9 in S2 and 78.7 in S3). Varieties Phule Samadhan (86.0, 84.5 and 81.8 cm; S1, S2 and S3) and Trimbak (88.6, 85.9 and 82.2 cm; S1, S2 and S3) maintained comparatively stable plant heights across all sowing times, suggesting moderate adaptability.

Productive tillers per plant

Wheat genotypes exhibited genetic variation in the Productive tillers per plant under both control and stress conditions (Fig.4). As temperature increased tillers number decreased and the performance of all varieties is tabulated in Table 4.8. HI-1605 exhibited the highest tillering capacity, with averages of 10.1 in S1 (timely), 8.8 in S2 (late) and 7.7 (very late) in S3 followed by Sardar 6.7 in S3. NIAW-34 had lower productive tillers, with averages of 6.5, 6.7 and 5.8 in S1, S2 and S3, respectively. MACS- 6478 had average tillers (9.6, 8.9, 6.2; S1, S2, S3) followed by Sardar (9.1, 8.5, 6.7; S1, S2, S3) showed better tolerance. Abro *et al*., (2019) [1] concluded that cultivar showed minimal tiller reduction under heat stress, indicating tolerance and potential for future breeding. While, MACS-6222 showed a significant decrease under S3 (4.8 tillers). Reduction in productive tillers during late sowing caused due to terminal heat stress (Abro *et al*., 2019) [1].

Length of spike

Genetic variation in Length of Spike was observed among the wheat varieties (Fig.5). MACS-6222 demonstrated the longest spikes with average lengths of 12.01 cm in S1 (timely), 11.87 cm in S2 (late) and 9.97 cm in S3 (very late), suggesting a high yield potential. On the other hand, Trimbak had the shortest spike lengths, with values of 9.69 cm (S1), 8.77 cm (S2) and 7.81 cm (S3). Under very late condition (S3), varieties HI1633 followed HI1605 had spike length of 10.14 cm, 10.02 cm respectively, while genotype Phule Samadhan showed 11.06 cm 10.68 cm and 9.97 cm spike length under S1, S2 and S3, respectively. Varieties showed minimal reduction under heat stress, indicating potential for breeding heat-tolerant varieties (Abro *et al*., 2019) [1].

Grains per Spike

The genetic variability in Grains per Spike was observed among the wheat varieties (Fig.6). The number of grains per spike varied, HI-1605 produced the highest number of grains per spike averaged (54.2 in S1, 50.2 in S2, 45.9 in S3) and MACS-6222 (54 grains in S_1), whereas Trimbak had the lowest, especially in S3 (39.8 grains), highlighting the negative impact of late sowing on yield. The mean grains per spike across environments were 50.33 (S1timely), 48 (S2 late) and 41.76 (S3 very late), the overall decline in grains per spike under late and very late sowing conditions indicates the adverse effects of heat stress on pollination and grain formation.

Under late sowing MACS-6222 (51.1) and HI-1605 performed best, while HI-1605 continued to excel in very late conditions, followed by NIAW-34 (43.6) and Phule Samadhan (43.1) achieved the best performance under heat stress. Asseng *et al.* (2011) [4] observed that maintained the highest grain count across stressed environment, highlighting its tolerance to temperature stress.

Yield per plant (g)

Significant variations in Yield per plant were observed among the wheat genotypes under both timely and heat stress conditions (Fig. 7). The genotype Phule Samadhan recorded the highest weight (22.44 g in S1, 20.97 g in S2 and 16.65 g in S3) showcasing its adaptability and resilience. Followed by MACS-6222 (21.87 g in S1, S2 21.02 g), while MACS-2496 had the lowest (17.39 g in S1, 16.38 g in S2, 11.01 g in S3), indicating its lower potential for productivity. Naveen *et al.* (2024) [17] noted that varieties with superior yield under heat stress exhibits flexibility and lesser yield indicating its lower potential for productivity.

The mean grain weight per plant across environments was 19.69 g (S1), 18.73 g (S2) and 14.03 g (S3). Phule Samadhan (16.65 g) consistently outperformed followed by HI-1605 (15.96 g) maintaining a higher grain weight even under very late sowing.

Test weight (g)

Significant variations in Test weight were observed among the wheat genotypes under both timely and heat stress conditions (Fig.8). The 1000-grain weight varied, with MACS-6222 producing the substantial grains on average (42.99 g in S1, 42.13 g in S2 and 35.20 g in S3). HI-1633 recorded lower values, particularly in S3-very late sowing (34.77 g). In timely sowing, MACS-6222 (42.99 g) performed best among the varieties. Under late condition, MACS-6222 (42.13 g), followed closely by HI-1605 (40.58 g) had TW. The genotype such as HI-1605 (37.90 g) followed by Phule Samadhan (37.83) weighted highest under S3 (very late). MACS-6222 exhibited superior grain size and uniformity under S1 and S2 conditions, while HI-1605 maintained relatively high grain weights under very late sowing. Pandey *et al*. (2014) [18] evaluated the use of 1000-grain weight (dTGW) as an indirect selection criterion has proven effective, with certain markers correlating with dTGW under varying sowing conditions.

Canopy Temperature Depression (CTD)

There was significant variation in CTD recorded in wheat varieties under timely and late sowings (Fig.9). During timely sowing (S1), MACS-6478 exhibited lowest CTD value (6.01), with a mean of 8.40, indicating differences in heat stress tolerance. CTD values generally decreased from S1 (timely) to S3 (very late) across varieties, with early-stage values (S1) as high as 11.01 in Sardar, followed by HI-1633 at 10.95, indicating good water status and evaporative cooling Gautam *et al.* (2015) [13].

Under late sowing (S2), genotype (HI1605) 8.02 followed by (Phule Samadhan) 7.79 showed better performance under elevated temperature. Trimbak (4.23) and MACS 6478 (4.3) reflect minimum CTD values under very late sown conditions. HI1605 exhibited the highest CTD in S3 (very late) at 6.81, followed by Phule Samadhan at 5.9, indicating these varieties' superior ability to maintain cooler canopies, which may correlate with enhanced water uptake and transpiration rates Khan *et al.* (2020) [15].

Chlorophyll Content Index (SPAD)

Chlorophyll Content significantly varied in wheat genotypes under late sown conditions (Fig. 10). Varieties Trimbak, NIAW-

34 and HI-1605 showed higher chlorophyll content in the early stage (S1), with values around 55-57, indicating higher photosynthetic efficiency in initial growth phases. Under late sowing HI 1605 followed by RAJ 3765 depicted peak values 54.71 and 54.31, respectively maintained high chlorophyll content and photosynthetic efficiency.

By S3, all varieties exhibited a decrease, with values ranging from 47 to 51, but the performance of Sardar gradually decreased (from 54.22 to 51.54) as compared to other varieties suggesting the gradual reduction in chlorophyll content over stages can be attributed to the plant's growth cycle. Retaining chlorophyll during heat stress is crucial; higher chlorophyll levels correlate with better grain yield. For instance, the cultivar Otis maintained 79.2% chlorophyll content under severe heat stress, leading to a 55% increase in yield compared to other lines (Fu *et al*., 2022) [12].

Relative Leaf Water Content (RLWC, %)

There was significant variation in RLWC recorded in wheat varieties under timely and late sowings (Fig.11). The RLWC was relatively high in early stages (S1), with values of 87.19% for MACS-6478 and 86.45% for MACS-6222, highlighting their ability to maintain adequate hydration, while RAJ-3765 had the lowest at 82.83%. This is important for sustaining physiological processes and biomass accumulation. By S3, RLWC dropped significantly across all the varieties.

Under late sowing (S2) 78.85%, RAJ 3765 showed lowest value, whereas with 84.35%, MACS 6478 followed by 84.31% for Trimbak exhibited higher values, imparting better tolerance. For instance, MACS-6222's RLWC reduced from 86.45% in S1 to 54.43% in S3. This decrease suggests that as plants matured and environmental demands increased, water retention capability declined, likely impacting physiological functions and potential yield. Genotype MACS-6478, which had higher RLWC in early stages, NIAW-34 had highest RLWC (56.50%) over S3 may be better suited for stress conditions, as they show better hydration retention. These results are in accordance with Vedi *et al.* (2024) [27].

Superoxide Dismutase (SOD)

SOD significantly varied in wheat genotypes under late sown conditions (Fig. 12). Trimbak exhibited the lowest SOD (18.39 mg^{-1} protein in S3), (11.47 mg^{-1} protein in S2) and (6.18 mg^{-1} protein in S1 for HI1633). SOD is an important antioxidant enzyme that scavenges harmful superoxide radicals produced under stress conditions, helping to maintain cellular health. SOD activity increased significantly from S1 (timely) to S3 (very late), with the highest activity observed in HI-1605 (33.43mg^{-1} protein in S1 and 69.26 mg^{-1} protein in S3) and Phule Samadhan (24.92 mg^{-1} protein in S1 and 56.28 mg^{-1} protein in S3). This suggests that these varieties possess strong antioxidant responses, crucial for protecting cellular structures during prolonged stress.

The mean SOD activity increased from 16.58 mg^{-1} protein in S1 to 39.23 mg^{-1} protein in S3, indicating that all varieties adapted by enhancing their antioxidant capacity as stress intensified. For example, NIAW-34 exhibited a significant increase from 10.20 mg^{-1} protein in S1 (timely) to 31.21 mg^{-1} protein in S3 (very late). Under late sowing HI-1605 (48.57 mg^{-1} protein) followed by Sardar (38.87 mg^{-1} protein) exhibited best performance. Varieties with high SOD activity in later stages, such as HI-1605 (69.26 mg^{-1} protein in S3), indicate a strong capacity to neutralize oxidative stress. Sairam *et al.* (2000) [21] suggested that ability to counteract oxidative stress is essential for maintaining plant health and ensuring resilience under adverse conditions.

Thermal Stress Index (TSI)

TSI significantly varied in wheat genotypes under late sown conditions (Fig. 13). The genotype with the highest mean TSI across all stages was Trimbak, followed closely by MACS-2496 with a mean TSI of 0.56. The mean TSI for all varieties across stages was calculated at 0.49 for S1, 0.42 for S2 and 0.58 for S3, showing an increase over time. The genotype RAJ-3765 showed the lowest TSI values among the entries (S1: 0.36, S2: 0.47, S3: 0.56) with a mean of 0.46. Similarly, varieties HI-1633 (0.51) followed by NIAW-34 (0.52) reflects lowest values under very late sown condition, suggesting good adaptability and consistent trait expression across different environments. Identifying varieties with lower TSI values may help in developing varieties that are more resilient to heat stress, crucial for food security under climate change scenarios.

Normalized Difference Vegetation Index (NDVI)

Wheat genotypes showed genetic variability in NDVI (Fig. 14). Phule Samadhan exhibited high NDVI values (S1: 0.95, S2: 0.94, S3: 0.83) followed by HI1605 (S1: 0.91, S2: 0.94, S3: 0.82), varieties Sardar (0.82, S3) also showed better performance under very late sowing, reflecting robust photosynthetic activity, which may contribute to favourable biomass production. Trimbak also showed relatively high NDVI values in the earlier stages (S1: 0.90, S2: 0.93), although it declined in S3 (0.75). On the contrary, MACS-6222 had the lowest NDVI values in S1 (0.81) but showed an increase by S2 (0.90) before slightly declining in S3 (0.76). This pattern could suggest a delayed but moderate vegetative growth compared to other varieties. Lemma *et al.* (2022) [16] indicated that varieties with fluctuating NDVI values can be indicative of stress tolerance, as they may possess stay-green traits that allow for sustained growth under stress.

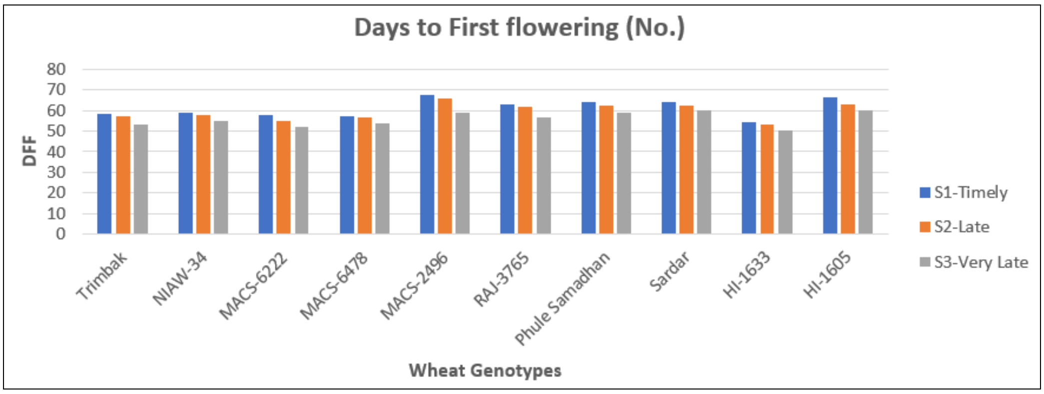

Fig 1: Days to First flowering and its reduction of wheat genotypes under timely and heat stress condition

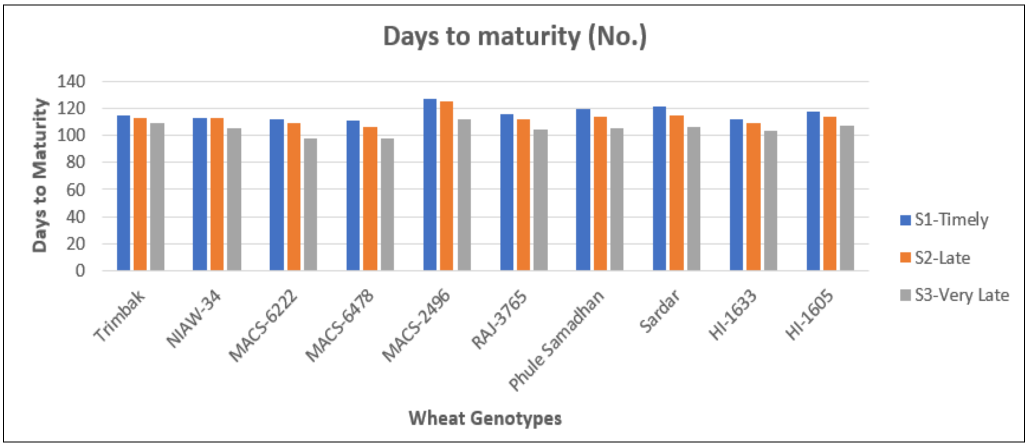

Fig 2: Days to Maturity and its reduction of wheat genotypes under timely and heat stress condition

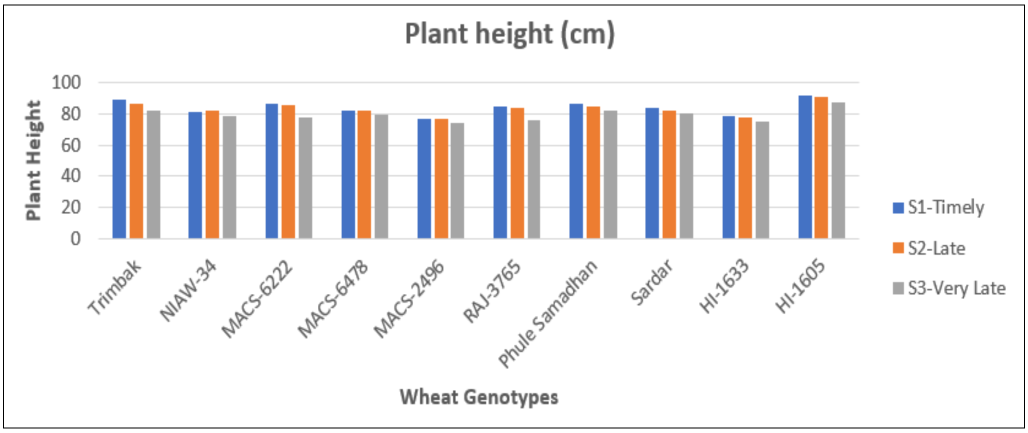

Fig 3: Plant Height and its reduction of wheat genotypes under timely and heat stress condition

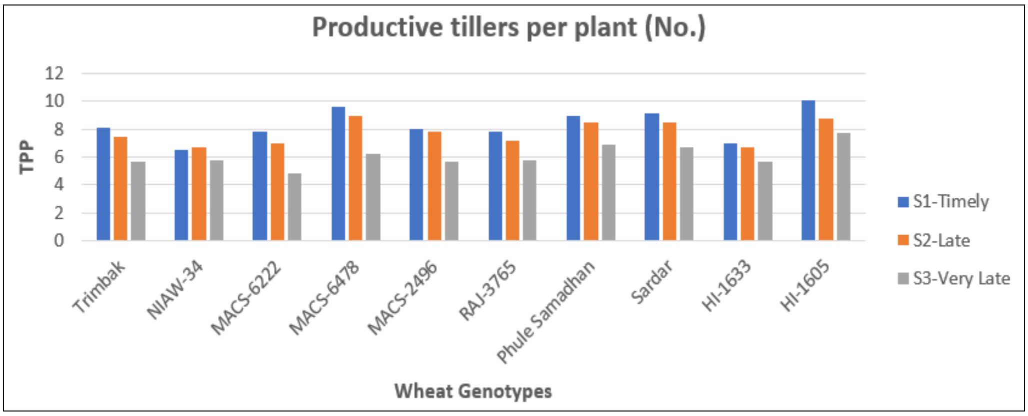

Fig 4: Productive tillers per plant and its reduction of wheat genotypes under timely and heat stress condition

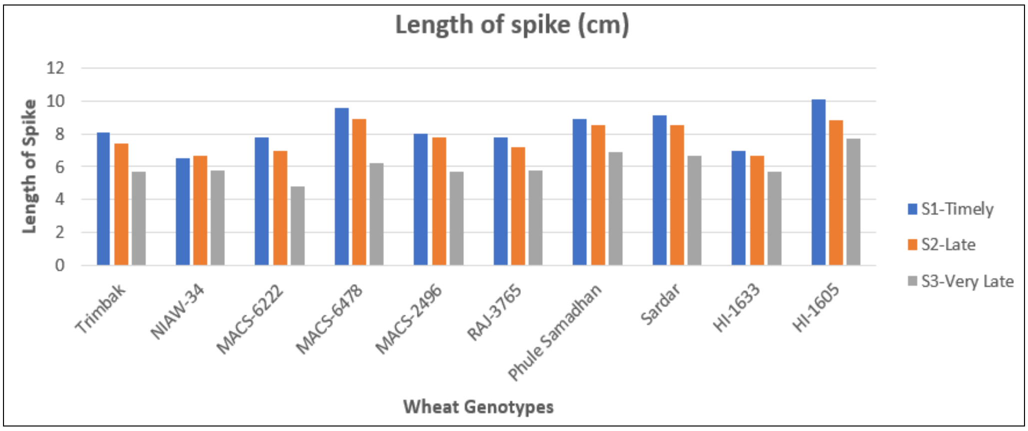

Fig 5: Length of spike and its reduction of wheat genotypes under timely and heat stress condition

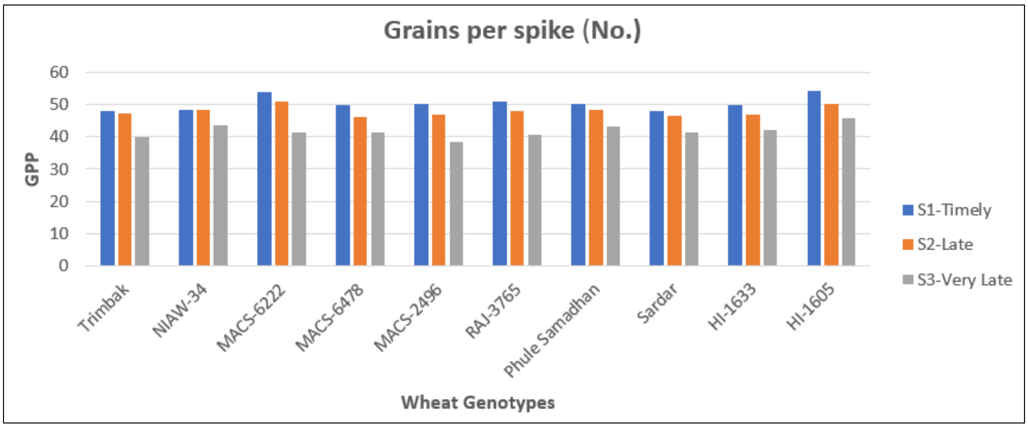

Fig 6: Grains per spike and its reduction of wheat genotypes under timely and heat stress condition

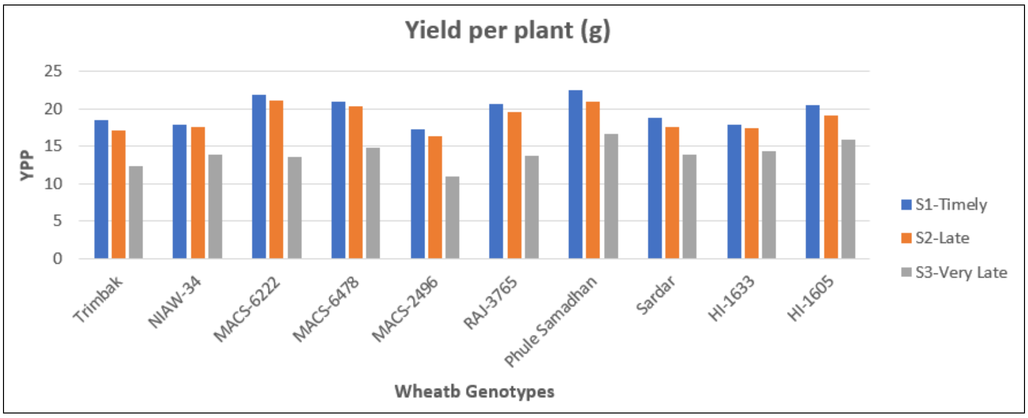

Fig 7: Yield per plant and its reduction of wheat genotypes under timely and heat stress condition

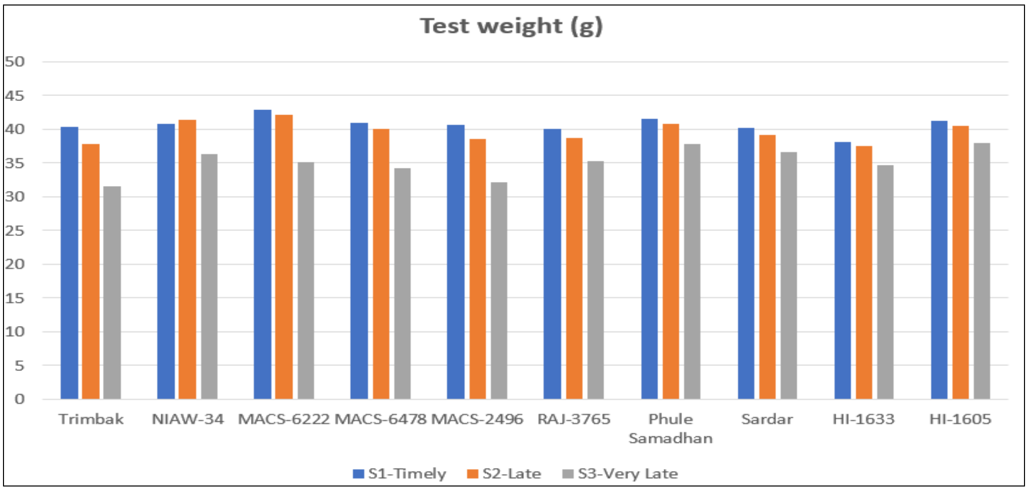

Fig 8: Test weight and its reduction of wheat genotypes under timely and heat stress condition

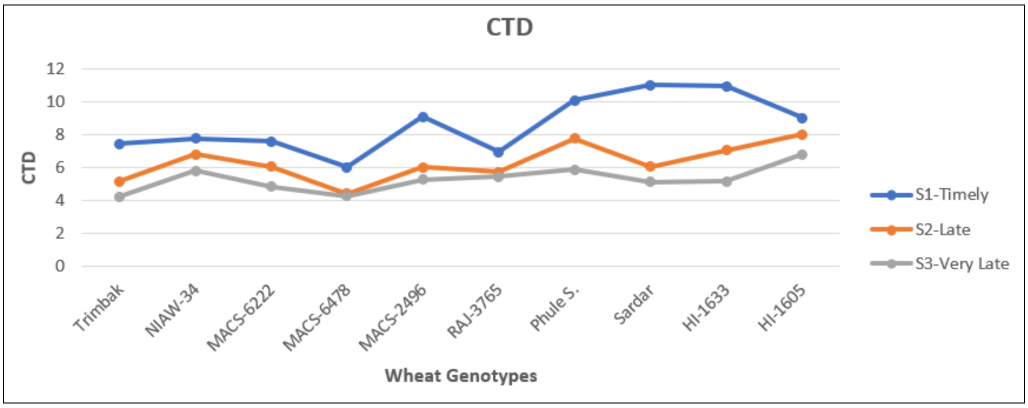

Fig 9: CTD and its reduction of wheat genotypes under timely and heat stress condition

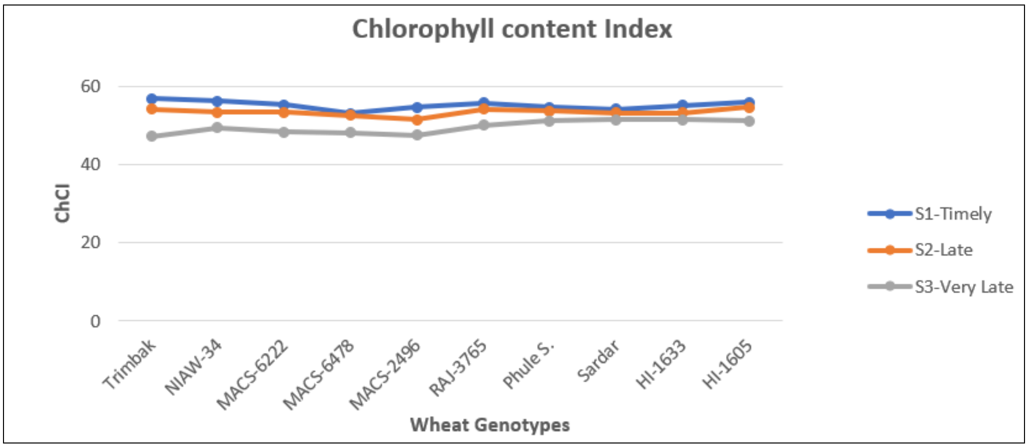

Fig 10: Chlorophyll Content Index and its reduction of wheat genotypes under timely and heat stress condition

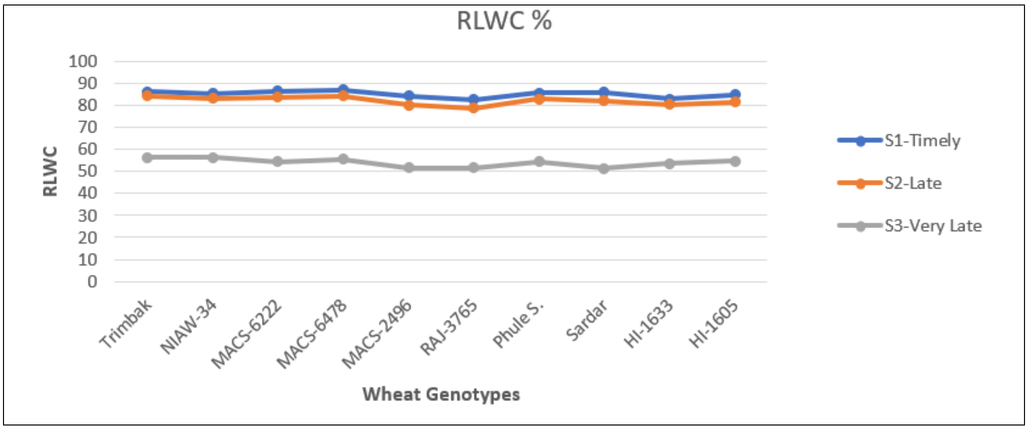

Fig 11: RLWC and its reduction of wheat genotypes under timely and heat stress condition

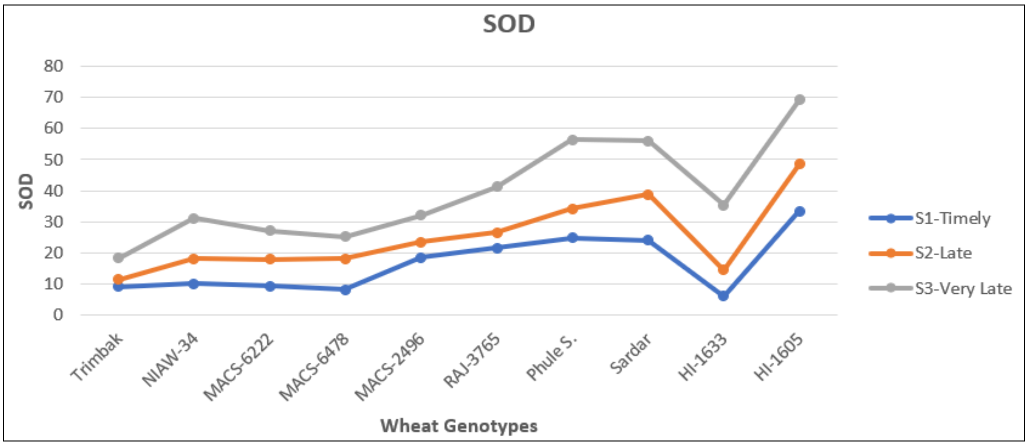

Fig 12: Productive tillers per plant and its reduction of wheat genotypes under timely and heat stress condition

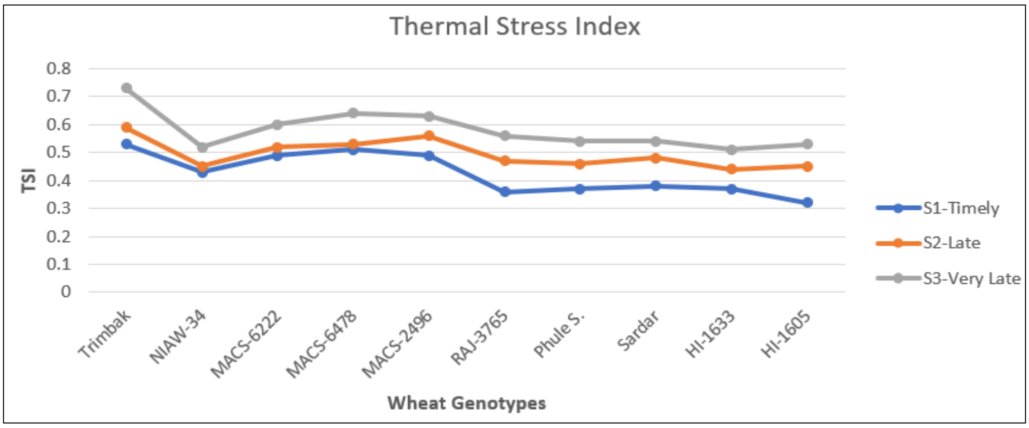

Fig 13: Thermal Stress Index and its reduction of wheat genotypes under timely and heat stress condition (Lower values of TSI are desirable)

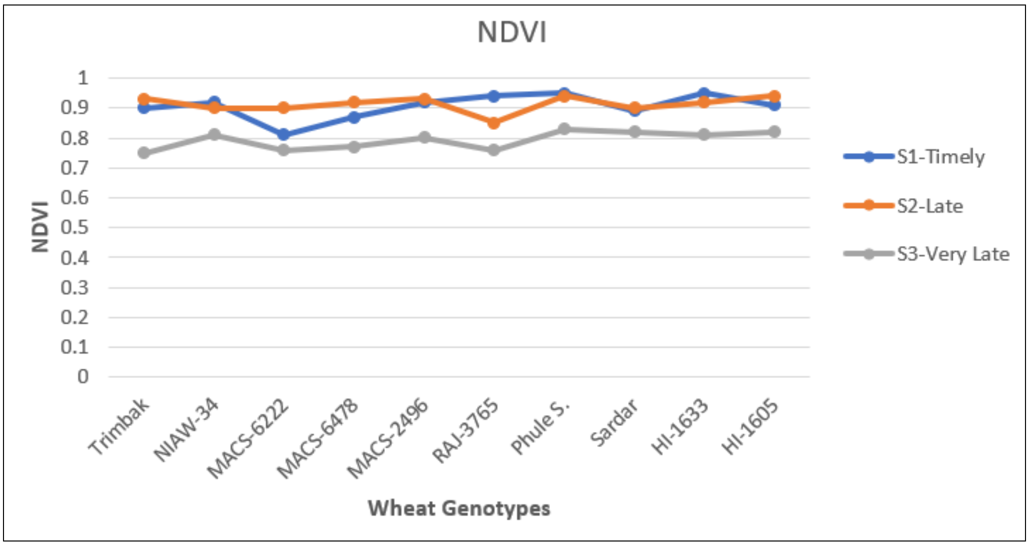

Fig 14: NDVI and its reduction of wheat genotypes under timely and heat stress condition

Conclusions

Field evaluations of ten diverse genotypes revealed HI-1605 as superior, with high performance in phenological traits, indicating adaptability to diverse sowing conditions. MACS-6222 displayed high yield potential with longer spikes and heavier grains. The study demonstrated the genotype-specific responses to heat stress, highlighting significant variations in physiological and biochemical traits. HI-1605, NIAW- 34, Phule Samadhan and HI-1633 emerged as best performing genotypes with superior adaptability across multiple traits, making them suitable for cultivation in heat-stressed environments.

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