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Analyzing the effect of heat stress on chlorophyll in flag leaves of different wheat varieties (*Triticum aestivum* L.) sown under different sowing conditions

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Abstract

Winter wheat is one of the most important cereal crops which occupy a key place in the cereal economy of the world particularly India as well. Its production and demand with the growing population has been continued to increase per year. Factors such as climate change, rise in temperature, inappropriate sowing timing causes declination in its production. Delay in sowing timing causes sudden elevation of air temperature during sensitive growth stages of wheat life cycle (anthesis and grain filling). Rise in air temperature causes negative effect on leaf photosynthetic pigments which are key variables in depicting photosynthetic response and gross primary production. These pigments play central role in light harvesting, photosystem protection and other growth functions. In this context, chlorophyll 'a', 'b' and a/b ratio in flag leaves of eight wheat varieties (UP2628, HD3086, UP2967, UP2784, UP2526, UP2565, UP2748 and HD3059) were evaluated at the time of anthesis and grain filling under different sowing conditions; sowing in month of November (normal) and December (heat stress) during year 2018-19. Due to an elevation of 3.7 °C at anthesis and 2.9 °C at grain filling in average air temperature, the photosynthetic pigments were significantly and negatively affected. Least reduction in photosynthetic pigment was observed in variety UP2748 while variety UP2784 was found maximum affected by elevated temperatures. UP2565 variety also performed well under heat stress condition with maximum chlorophyll a/b ratio at the time of anthesis. On basis of correlation analysis it was found that in comparison to grain filling anthesis was more sensitive for photosynthetic pigments to elevated temperature.

Keywords: Wheat, sowing timing, heat stress, chlorophylls, thermo-tolerant trait

Introduction

Wheat (*Triticum aestivum* L.) belongs to family *Poaceae* is a highly cultivated and most consumed cereal crop worldwide (Kimber *et al.*, 1987) ^[12]. Due to its prominent position in the international food market and population growth, the production and demand of wheat is also kept on increasing every year (Evenson *et al.*, 2003) ^[6]. As a cool season crop its cultivation is highly depend on various factors like abiotic stresses, climate change, global warming and temperature variations etc. which causes sudden decline in wheat production in past few years. Thus, increasing crop yield is the need of the hour to meet the feeding demand of the world in the 21st century. In 2050, the world's population is expected to reach 9 billion and the demand of wheat will also increase to >900 Tg (Tadess, *et al.*, 2019) ^[20].

For winter crops especially in wheat, sowing timing is another factor which can limit its productivity directly. Delay in sowing timing causes exposure of plants to elevated air temperature during anthesis and grain filling stage (stages of wheat life cycle which are sensitive to high temperatures) (Kanchan and Mahendra, 2000) ^[11]. A single degree elevation in air temperature during anthesis and grain filling stage can reduce the wheat yield upto 3 to 4% (Tripathi *et al.*, 2005). Temperatures above the optimal level (17.5 to 23.4°C for anthesis and grain filling) can cause irreversible damage to the crop and reduce yield potential by affecting physiological processes such as photosynthesis, flag leaf performances and yield related parameters (Akter and Islam, 2017) ^[1].

Heat stress during anthesis leads to disruption in photosynthetic process by disturbing the structure and function of chloroplast which causes reduction in chlorophyll pigments. Excess light responsible for oxidative damage (induced by lipid peroxidation), inactivates chloroplast enzyme and degrade proteins led to decreased rate of leaf photosynthesis (Sairam *et al.*, 2000) ^[14]. In many studies a significant reduction in chlorophyll pigments (chl a and b) was reported under elevated temperatures due to heat induced damage to thylakoids. An essential

enzyme which is required during chlorophyll biosynthesis known as protochlorophyllide oxidoreductase (POR), as it catalyzes the photoreduction of protochlorophyllide to chlorophyllide which ultimately converted to chlorophyll in developing leaves found greatly reduced due to heat stress in wheat cultivars as compared to control and shows a significant & positive correlation between loss of chlorophyll and heat stress (Ristic *et al.*, 2007) [13]. Six different wheat cultivars, namely; UP-2338, PBW-343, UP-2113, PBW-175, VL-616 and VL-421) when subjected to variable temperature due to a delay of 20 days in sowing, a significant reduction in chl_a+b and chl_a/b ratio were reported against normal sown cultivars. Highest total chlorophyll and chl a/b ratio during anthesis was estimated in wheat cultivar UP-2338 under normal sowing conditions while 63.41% and 57.89% decline in chl_a+b and chl_a/b ratio respectively was reported in late sown condition in comparison to normal (Srivastava *et al.*, 2012) [18]. Elevation in temperatures up to 37°C and 45°C for 8 hours in wheat seedlings (Karacadage and Firat) causes reduction in chlorophyll pigment accumulation (Iqbal *et al.*, 2017) [9].

Measurement of chlorophyll pigments could be useful for screening the heat tolerance trait in various crop species. Through various studies it was observed that that the heat tolerant wheat cultivar (Fang) possesses higher chlorophyll content (chlorophyll a, b, a/b ratio) in comparison to heat sensitive wheat cultivar (Siete Cerros) (Tahir *et al.*, 2009) [21]. According to previous studies, tolerant genotypes of wheat showed higher values of chlorophyll pigments or show least reduction in chlorophylls accumulation under elevated temperatures while the sensitive genotypes showed relatively lower values for chlorophyll pigments or show maximum reduction in chlorophyll content in comparison to non-stressed condition. For example, in an experiment, under normal growing condition, the wheat genotype BAW-1202 had highest flag leaf chlorophyll (1.63 mg g⁻¹ FW). On the other hand, BARI Gom-27 had the lowest chlorophyll (0.94 mg g⁻¹ FW) at 18 days after anthesis but under late sowing (heat stress conditions), the chlorophyll content in flag leaves was reduced significantly in BAW-1202 but it was remained unchanged in BARI Gom-27 wheat genotypes which was found tolerant towards elevated temperatures (Sharmin *et al.*, 2020) [17]. So overall under both sowing conditions, analyzing the flag leaves (topmost leaf which directly contributes upto 70% in total photosynthates production) of different wheat varieties on the basis of their chlorophyll pigments and their ratios, provides a better understanding of heat tolerant trait of the varieties, capability to delay the senescence or stay green trait and help in observing in which stage (anthesis or grain filling) the chlorophyll and the pigments ratio is most affected by elevated temperatures.

Plant material and Experimental details

To study the effect of heat stress on the photosynthetic pigments (chlorophyll a, b and a/b ratio) in flag leaves of eight different wheat varieties UP2628, HD3086, UP2967, UP2784, UP2526, UP2565, UP2748 and HD3059, a one-year field trial was conducted during Rabi season of 2018-19 at Dr. N.E. Borlaug Crop Research Center G.B.P.U.AT Pantnagar. The sowing was done on 20 Nov 2018 (normal) and 22 Dec 2018 (heat stress). The experiment was replicated thrice in randomized block design in 4 rows in 3m² per plot area. By using random sampling technique flag leaves were collected

at the time of anthesis and grain filling stage (15DAA).

Chlorophyll pigment analysis

Chlorophyll in flag leaves of wheat varieties was estimated by using the methodology given by (Hiscox and Isealesham 1979) [7]. 50 mg leaf sample were taken in a clear test tube containing 10ml of Dimethyl sulfoxide (DMSO) and incubated at 65 °C for 3 hours in oven. OD was taken at 649.1nm and 665.1nm by using a multi-wave length spectrophotometer against pure DMSO as blank. The chlorophyll content was calculated by using equation given by (Wellburn, 1994) [25].

Equations

$$\text{Chlorophyll 'a' (mg/g FW)} = \frac{(12.47 \times A_{665.1} - 3.62 \times A_{649.1}) \times \text{Volume of DMSO used (ml)}}{1000 \times \text{Wt of sample (g)}}$$

$$\text{Chlorophyll 'b' (mg/g FW)} = \frac{(25.06 \times A_{649.1} - 6.5 \times A_{665.1}) \times \text{Volume of DMSO used (ml)}}{1000 \times \text{Wt of sample (g)}}$$

$$\text{Chlorophyll a/b ratio} = \frac{\text{Value of Chlorophyll 'a'}}{\text{Value of Chlorophyll 'b'}}$$

Statistical Analysis

The statistical analysis of all the data collected were analysed by using the Two-way Analysis of Variance technique with STPR15 statistical tool and Least Significant Difference (LSD) test at 5% probability level was applied to compare the treatment's means. Data for correlation analysis was done in SPSS software version 26.

Results

Chlorophyll 'a' in flag leaves

Chlorophyll 'a' was found reduced from 7.33% to 30.06% at anthesis and 1.82% to 33.33% at grain filling (15DAA) stage, under heat stress in compared to normal conditions. Least reduction in values of chlorophyll 'a' during anthesis was observed in UP2967 while maximum reduction observed in UP2784. However, during grain filling, least reduction observed in UP2526 variety while maximum reduction due to heat stress observed for UP2628 variety. On comparing between two sensitive stages, values of chlorophyll 'a' was found higher at the time anthesis in all wheat varieties in comparison to grain filling (table 2). Statistically, during anthesis and grain filling, chlorophyll 'a' was found significantly and negatively affected by heat stress condition at 1% probability level. The values for chlorophyll 'a' between varieties also found significantly different with each other at 5% probability level while rest of the interactions were found insignificant at 5% probability level.

Chlorophyll 'b' in flag leaves

Chlorophyll 'b' in wheat varieties was found reduced from 8.54% to 34.61% at anthesis and 5.36% to 27.32% at grain filling (15DAA) stage respectively, under heat stress in compared to normal conditions. Least reduction in values of chlorophyll 'b' during anthesis was observed in HD3086 and UP2748 while maximum reduction observed in UP2967. However, during grain filling, least reduction observed in UP2748 variety while maximum reduction due to heat stress observed for UP2967 variety. On comparing between two sensitive stages, values of chlorophyll 'b' was found higher at the time anthesis in all wheat varieties in comparison to grain

filling. Statistically, during anthesis and grain filling, chlorophyll 'b' was found significantly and negatively affected by heat stress condition at 1% probability level. The values for chlorophyll 'b' between varieties also found significantly different with each other at 5% probability level while rest of the interactions were found insignificant at 5% probability level (table 2).

Chlorophyll a/b ratio in flag leaves

Chlorophyll a/b ratio was found reduced from 2.56% to 14.40% at anthesis and 0.60% to 13.29% at grain filling (15DAA) stage, under heat stress in compared to normal conditions. Least reduction in values of chlorophyll a/b ratio during anthesis was observed in UP2748 while maximum reduction observed in UP2784. However, during grain filling, least reduction observed in UP2748 variety while maximum reduction due to heat stress observed for UP2784 variety. On comparing between two sensitive stages, values of chlorophyll a/b ratio were found higher at the time anthesis in all wheat varieties in comparison to grain filling. Statistically, during anthesis and grain filling, the values of chlorophyll a/b ratio was found significantly and negatively affected by heat stress condition at 1% probability level. Between varieties, Chlorophyll a/b ratio also found significantly different with each other at 1% probability level while rest of the interactions were found insignificant at 5% probability level (table 2).

Correlation analysis between parameters

At anthesis, a positive but non-significant correlation was observed between values of chlorophyll 'a' and chlorophyll 'b', and chlorophyll a/b ratio, while a negative correlation was observed between chlorophyll 'b' and chlorophyll a/b ratio under November sown (normal) conditions. However, under December sown (heat stress) conditions, a positive and significant correlation was observed between chlorophyll 'a' and chlorophyll a/b ratio while the correlation between chlorophyll 'a' and chlorophyll 'b', chlorophyll 'b' and chlorophyll a/b ratio was found negative but non-significant with each other. From this it can be concluded that more negative correlations between photosynthetic pigments was observed under heat stress in comparison to normal. However, the correlations between chlorophyll 'a' and a/b ratio was enhanced and improved under heat stress condition and could be a reason for least reduction in photosynthetic pigments in some varieties under stress conditions (table 1a). At grain filling (15DAA), a positive and significant correlation was observed between values of chlorophyll 'a' and chlorophyll 'b', positive but non-significant correlations observed between chlorophyll 'a' and chlorophyll a/b ratio, while a negative correlation was observed between chlorophyll 'b' and chlorophyll a/b ratio under November sown (normal) conditions. However, under December sown (heat stress) conditions, a positive but non-significant correlation was observed between chlorophyll 'a' and chlorophyll 'b', chlorophyll 'a' and chlorophyll a/b ratio while the correlation between chlorophyll 'b' and chlorophyll a/b ratio was found negative but non-significant with each other. From this it can be concluded that positive and significant correlations between photosynthetic pigments was negatively affected by heat stress in comparison to normal. However, rest of the interactions under grain filling stage was not found much affected by sowing conditions except the correlation between

chlorophyll 'a' and chlorophyll 'b' (table 1b).

Discussion

In the present study, due to the delay of 30 days in sowing timing, the environmental temperature during sensitive growth stages (anthesis and grain filling) was found higher under December month in comparison November. An elevation of 3.75 °C during anthesis and 2.91 °C during grain filling period was reported. As a result of rise in temperature, chlorophyll 'a', 'b' and chlorophyll a/b ratio were found greatly reduced under stress conditions. From past researches, as a result of delay in sowing conditions in winter crops like wheat, elevated temperature causes heat stress during their growth period (especially anthesis and grain filling) which is responsible for disruption in photosynthetic processes due to altered structures and function of chloroplast as it causes reduction in chlorophyll content and amount of other pigments (Xu *et al.*, 1995). The excess light and oxidative stress causes inactivation of chloroplast enzyme, protein degradation and membrane rupture under elevated temperature and decreased total chlorophyll and chlorophyll pigments hence rate of leaf photosynthesis decreased (Sairam *et al.*, 2000) [14]. In a study 25 to 30% reduction in total chlorophyll content and chlorophyll a to b ratio was observed in different wheat varieties at the time of anthesis and grain filling when the varieties compared on the basics of different sowing conditions (November v/s December). Decline in chlorophyll pigments and their chlorophyll a/b ratios is mainly due to the loss of chlorophyll 'a' containing proteins under elevated temperature which are closely associated with PSII and PSI reaction (Srivastava, 2005) [19]. In many studies, reduction in the chlorophyll pigments under delay in sowing is also due to experience of heat stress by plant during anthesis and after anthesis. A sudden elevation in temperatures resulting onset of forced senescence and early yellowing of the flag leaves hence chlorophyll pigments and their ratios found reduced (Chandra, 2006) [4]. According to a study, a reduction up to 24.80% in chl 'a' at anthesis and 9.70% at grain filling (15DAA) was recorded along with a reduction upto 35.99% in chl 'b' at the time of anthesis and 64.97% at grain filling (15DAA) in wheat cultivars under heat stress in comparison to non-stressed conditions (Dhyani, 2010) [5]. Decline in chlorophyll pigments and total chlorophyll is one of the most important characteristic features of onset of senescence. Delay in sowing induces senescence quickly than the normal sown conditions, may be due to imposition of adverse environmental variables over late sown cultivars (Srivastava *et al.*, 2012) [18]. The photosynthetic machinery includes various processes and subunits. Any impairments in gaseous exchange process, photosynthetic pigments, photosystems, electron transport system, carbon reduction pathways, and enzyme systems leads to reduced photosynthetic activity, growth, and biomass production in plants (Ashraf *et al.*, 2013) [2]. Heat stress results in loss of leaf pigment and significantly damages photosynthetic activities. (Ashraf *et al.*, 2013) [2]. As chlorophyll pigments control the photosynthetic potential of the plant by capturing light energy directly from the sun, under heat stress the chlorophyll accumulation gets disturb which ultimately reduces biomass production (Houborg *et al.*, 2015) [15]. A reduction of 5%, 8% and 11% were observed in 12 different genotypes of soybean when treated with different temperatures range (an average daily temperature of 26, 29,

32 during their growth period, while maximum reduction of 21% was observed at 35°C in comparison to ambient temperature conditions, respectively (Jumrani *et al.*, 2017)^[10]. In another experiment, a treatment of 37 °C and 45 °C for 8 hours was given in seedlings of Karacadage and Firat (wheat cultivars). They showed reduced chlorophyll pigment accumulation under heat stress condition (Iqbal *et al.*, 2017)^[9]. Seven different wheat varieties (Joe, SY Monument, Larry, West Bred 4458, Zenda, West Bred Cedar and Everest) when investigated under higher temperatures 35/15 °C in comparison to normal 25/15 °C condition. Higher accumulation of chlorophyll pigment was observed under earlier growth stage and the values for pigments shows a steep declination as varieties moves towards senescence. However, the declination was fast under heat stress wheat varieties exposure (Sebal *et al.*, 2020). Some recent experiments also prove the fact that decrement in Chlorophyll a/b ratio in heat-stressed conditions may be related to the degradation of chlorophyll a by high temperature, which in turn may reduce the light-harvesting chlorophyll a/b-binding proteins (LHC) and the decrease in Chl a+b content by heat may be attributed to the reduction in biosynthesis and/or to the degradation of the pigments (Hassan *et al.*, 2021). More chlorophyll pigment accumulation may be considered as a thermo-tolerant trait. Higher leaf chlorophyll contents at anthesis and grain filling is considered as an indication of delayed senescence, high photosynthetic rate and remobilization of assimilates under terminal heat stress along with it also shows the stay green trait of particular plant variety. According to some previous reports, the thermo-tolerant genotypes of wheat possess higher chlorophyll pigment accumulation /lower reduction in chlorophyll content under heat stress condition as compared to ambient. In present study also, wheat variety UP2565 and UP2748 showed least reduction and maximum chlorophyll pigments at the time of anthesis under elevated temperatures. Similar trend was also followed by some other researches, in which two spring wheat cultivars (Millet-11, Punjab-11) and two advanced lines (V-07096, V-10110) when exposed to terminal heat stress by delay in sowing condition, the results showed 20% and 71–125% higher values of Chl a and b contents in the respective cultivars, under late sown compared to normal sown crop during anthesis so, estimation of chlorophyll content is considered as an indicator that reflects plant's resistance to stress. Higher leaf chlorophyll or least reduction in chlorophyll accumulation at the anthesis and grain filling indicates stay green trait of the variety, their thermo-tolerant nature which is responsible for high photosynthetic rate and good remobilization of assimilates

even elevated air temperatures (Tariq *et al.*, 2021)^[22].

Conclusion

The chlorophylls are the main photosynthetic pigment in chloroplast (thylakoid membrane) responsible for harvesting light energy and drive electron transfer during the process of photosynthesis. Degradation of chlorophylls leads to leaf senescence or chlorosis. Reduced chlorophylls during heat stress are mainly due to the increased activity of chlorophyllase and chlorophyll degrading peroxidase. So, least reduction or increased chlorophyll 'a', 'b' and a/b ratio (photosynthetic pigments and their ratios) under stress conditions is considered as thermo-tolerant trait. In this research, variety UP2748 and UP2565 were found tolerant towards terminal heat stress with least reduction in photosynthetic pigments during sensitive stages and so can be recommended for both sowing conditions while variety UP2784 found highly sensitive towards elevated air temperature in tarai region. More efforts are required in this area as the photosynthetic pigments analysis is a non-destructive investigation which is capable of finding the thermo-tolerant characteristic of plants under elevated temperatures, being a biochemical approach; they are stable, easy to measure, heritable and directly correlated to the photosynthetic potential of the crop.

Table 1a: Pearson's correlation between photosynthetic pigments during normal sowing (in November)

	chl a	chl b	chla/b		chl a	chl b	chla/b
chl a	1			chl a	1		
chl b	0.282	1		chl b	-0.111	1	
chl a/b	0.339	-0.595	1	chla/b	0.746*	-0.147	1

** Correlation is significant at the 0.01 level (2-tailed)*. Correlation is significant at the 0.05 level (2-tailed).

Note: Chlorophyll 'a' (chl a), Chlorophyll 'b' (chl b), and Chlorophyll 'a/b' ratio (chl a/b).

Table 1b: Pearson's correlation between photosynthetic pigments during late sowing (heat stressed) in December

	chl a	chl b	chla/b		chl a	chl b	chla/b
chl a	1			chl a	1		
chl b	0.768*	1		chl b	0.620	1	
chla/b	0.368	-0.116	1	chla/b	0.142	-0.603	1

** Correlation is significant at the 0.01 level (2-tailed)*. Correlation is significant at the 0.05 level (2-tailed).

Note; Chlorophyll 'a' (chl a), Chlorophyll 'b' (chl b), and Chlorophyll 'a/b' ratio (chl a/b).

Table 2: Effect of different sowing conditions on photosynthetic pigments and their ratios of different wheat varieties.

Sowing in December		Chlorophyll 'a'		Chlorophyll 'b'		Chlorophyll 'a/b'	
SNO.	Varieties	At anthesis	15DAA	At anthesis	15DAA	At anthesis	15DAA
1	UP2628	2.11 ± 0.07b	1.74 ± 0.07c	0.78 ± 0.01a	0.67 ± 0.02a	2.67 ± 0.08c	2.57 ± 0.06a
2	HD3086	1.78 ± 0.07c	1.65 ± 0.04c	0.72 ± 0.01a	0.62 ± 0.02a	2.59 ± 0.04c	2.38 ± 0.12b
3	UP2967	2.21 ± 0.10a	2.04 ± 0.08b	0.87 ± 0.06a	0.76 ± 0.07a	2.69 ± 0.04c	2.42 ± 0.01b
4	UP2784	2.23 ± 0.18a	1.56 ± 0.22d	0.86 ± 0.04a	0.72 ± 0.12a	2.59 ± 0.20c	2.49 ± 0.02b
5	UP2526	2.31 ± 0.06a	2.09 ± 0.02b	0.68 ± 0.02a	0.54 ± 0.02a	2.75 ± 0.03b	2.33 ± 0.02c
6	UP2565	2.15 ± 0.02b	2.01 ± 0.01b	0.56 ± 0.05b	0.47 ± 0.01b	3.33 ± 0.11a	2.50 ± 0.04b
7	UP2748	2.50 ± 0.15a	2.25 ± 0.01a	0.85 ± 0.01a	0.51 ± 0.07a	3.01 ± 0.20b	2.67 ± 0.03a
8	HD3059	2.11 ± 0.06b	1.95 ± 0.05b	0.81 ± 0.10a	0.71 ± 0.05a	2.76 ± 0.38b	2.54 ± 0.07a

*Values (means from replicates) in the same column under similar sowing conditions sharing similar alphabet are not significantly different (P>0.05). Values show mean ± SE (n=3).

Sowing in December		Chlorophyll 'a'		Chlorophyll 'b'		Chlorophyll 'a/b'	
SNO.	Varieties	At anthesis	15DAA	At anthesis	15DAA	At anthesis	15DAA
1	UP2628	1.74 ± 0.44b	1.16 ± 0.02b	0.58 ± 0.01a	0.52 ± 0.03a	2.30 ± 0.04c	2.26 ± 0.02c
2	HD3086	1.65 ± 0.40b	1.16 ± 0.02b	0.66 ± 0.01a	0.52 ± 0.04a	2.43 ± 0.04b	2.24 ± 0.14c
3	UP2967	2.04 ± 0.34a	1.36 ± 0.18ab	0.57 ± 0.02a	0.55 ± 0.03a	2.45 ± 0.03b	2.25 ± 0.13c
4	UP2784	1.56 ± 0.22b	1.40 ± 0.05a	0.70 ± 0.08a	0.65 ± 0.01a	2.22 ± 0.14c	2.16 ± 0.05c
5	UP2526	2.09 ± 0.21a	1.14 ± 0.02b	0.53 ± 0.03a	0.51 ± 0.01a	2.60 ± 0.14b	2.24 ± 0.07c
6	UP2565	2.01 ± 0.11a	1.12 ± 0.01b	0.49 ± 0.05b	0.42 ± 0.01a	3.07 ± 0.20a	2.43 ± 0.02b
7	UP2748	2.25 ± 0.11a	1.40 ± 0.01a	0.77 ± 0.05a	0.49 ± 0.01a	2.93 ± 0.03a	2.66 ± 0.01a
8	HD3059	1.95 ± 0.04a	1.35 ± 0.01a	0.70 ± 0.05a	0.64 ± 0.02a	2.59 ± 0.08b	2.26 ± 0.01c

*Values (means from replicates) in the same column under similar sowing conditions sharing similar alphabet are not significantly different ($P>0.05$). Values show mean \pm SE (n=3).

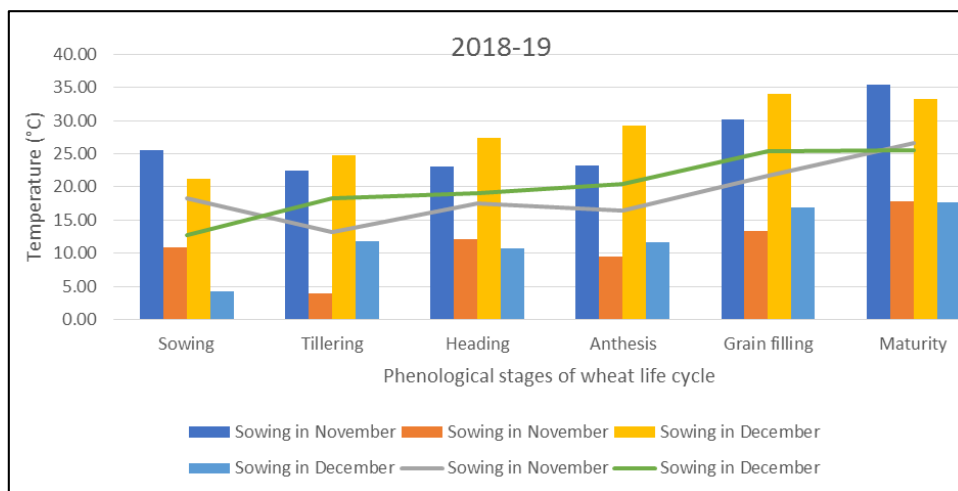


Fig 1: Maximum, minimum and average air temperature ($^{\circ}$ C) during different phenological stages of wheat in year 2018-19.
*(Max; Maximum temperature, Min; Minimum temperature; Avg; Average temperature)

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