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Impact of Nitrogen Addition on Spring Wheat Physiological and Agronomic Traits Under Contrasting Environmental Conditions

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Abstract: Optimizing the nitrogen (N) timings and rate can improve nutrient uptake, and nutrient-efficiencies, especially of N in wheat under the changing climate scenario. Climatic stress in the form of high temperature and drought resulted in the decreased crop physiology and, ultimately, grain yield. Taking the example of rainfed wheat, we quantified the impact of N application rates as full and split-dose at three variable sites of rainfed Pothwar, Pakistan by conducting field experiments for two years (2013-14 and 201-15). Treatments include, T_1 = Control (No fertilizer applied), full dose of N applied at the time of crop sowing, i.e. T_2 = 50 kg N ha^{-1}, T_3 = 100 kg N ha^{-1} and T_4 = 150 kg N ha^{-1} and split application of N at different timings during different stages of the crop called as split application of N, i.e. T_5: Application of 50 kg N ha^{-1} (15 kg N ha^{-1} (Sowing) : 20 kg N ha^{-1} (Tillering) :15 kg N ha^{-1} (Anthesis), T_6: Application of 100 kg N ha^{-1} (30 kg N ha^{-1} (Sowing): 40 kg N ha^{-1} (Tillering) : 30 kg N ha^{-1} (Anthesis) and T_7: Application of 150 kg N ha^{-1}(45 kg N ha^{-1} (Sowing) : 60 kg N ha^{-1} (Tillering) : 45 kg N ha^{-1} (Anthesis). Three study sites include viz. Islamabad (High rainfall with optimum temperature), University Research Farm (URF)-Koont (Medium rainfall with moderate temperature), and Talagang (low rainfall with high temperature). Results showed that the highest stomatal conductance (0.80 mole m^{-2} sec^{-1}), net photosynthetic rate (20.07 µmole $m^{-2}s^{-1}$), transpiration rate (9.58 mmole $m^{-2}s^{-1}$), intercellular CO_2 concentration (329.25 µmole CO_2 mol^{-1} air), SPAD values (58.86 %) and proline contents (35.42 µg g^{-1}) were obtained for split application of N (T_6 = Split N_{100}) compared to control and full dose of N treatments. Among sites, these physiological traits remained highest at Islamabad and lowest at Talagang, while among years, maximum values of the measured parameters were obtained in 2013-14. A similar trend was observed for crop total N, N efficiencies, and agronomic traits of the crop. Our results suggest that optimum N application rate and its suitable timings can help to harvest real benefits of N as in our findings, split dose resulted in the maximum performance of the crop from physiological parameters to the agronomic traits of the rainfed wheat crop.

Keywords: climate; rainfed wheat; N fertilization; split and full N application; photosynthetic rate; agronomic traits

1. Introduction

Rainfed regions occupy 45% of the earth and are home to the 38% of global production [1]. Climate change in the form of rising temperatures, rainfall variability, and elevated CO_2 showing significant impacts on the productivity of this region. Since elevated CO_2 [eCO_2] is one of the documented global changes thus, it is showing its effects on the plants in the form of reduction in stomatal conductance (g_s), transpiration rate (E), and increased light use efficiency [2,3]. However, this [eCO_2] could help to increase the photosynthesis of crops by increasing the Ribulose-1,5-bisphosphate carboxylase (Rubisco) carboxylation rate and inhibition of its oxygenation. It has been reported that [eCO_2] resulted in a 31% increase in the light-saturated leaf photosynthetic rate and 28% daily photosynthetic carbon assimilation. However, stress full conditions like low N and drought resulted in the decreased g_s and reduced net photosynthetic rate [4]. A similar trend of decreased g_s (20%) was observed for C_3 and C_4 species. The decrease in the photosynthesis is more pronounced under N-limited conditions as less N supply might limit the development of new sinks and disturbs the source-sink balance in plant growth under [eCO_2] [5,6].

Dry matter production in plants depends upon the process of photosynthesis. It is the process in which plants convert light energy into the photoassimilates through the action of CO_2 and water. However, the supply of N is also one of the determinant factors for the process of photosynthesis. During the light reaction of photosynthesis, an electron generated is passed to NADP and helps to create NADPH by accepting hydrogen from the photolysis of water. This NADPH is then used in the CO_2 carboxylation process to generate photosynthate [7]. However, water and nutrient deficiency, mainly N, could lead to a decreased in the g_s, and intercellular CO_2 concentration (Ci) due to the reduces transpiration rate (E) resulting in the reduced photosynthetic CO_2 carboxylation rate [8-10]. This reduction in crop physiological traits could be improved by the application of N as it affects plant adaptation to abiotic stress [11-13]. The physiological process in plants is significantly affected by N deficiency, and it is well documented that N deficiency has an impact on the photosynthetic CO_2 assimilation rate. It has been reported that lower levels of N lead to the deceased CO_2 assimilation rate by the leaves leading to the less photosynthetic yield [14-19]. Furthermore, it has been confirmed that this decrease in the photosynthetic CO_2 assimilation capacity is related to the supply of the N, which resulted in the decreased Rubisco content and activity of an enzyme (RuBPcase) in the dark reaction of photosynthesis [20].

A positive correlation between leaves photosynthetic capacity and N contents have been shown in earlier work [14, 21-23]. Application of N in wheat plants resulted in the improved properties of photosynthetic pigments and increased net photosynthetic rate (An) [24-25]. N deficiency could decrease the yield of PSII (Photosystem II) and CO_2 assimilation of photosynthesis, resulting in the lower crop yield [26-28]. However, some earlier work depicted that N deficiency has no effect on the PSII [29] but has an impact on the return of CO_2 assimilation and light-saturated rate of photosynthesis [26]. Lu and Zhang, (2000) [30] studied the effect of N deficiency on photosynthetic CO_2 assimilation, PSII photochemistry, and photoinhibition in maize under field conditions. Their results showed that N deficiency resulted in the lesser CO_2 assimilation capacity and increased susceptibility to photoinhibition. N fertilization positive effects on the PSII was documented by Zhang et al.[31] in their work and reported a higher net photosynthetic rate in the flag leaf of wheat. Cai et al. [32] concluded that the photosynthetic response to the N application rate is variable among wheat cultivars. Similarly, an increase in the total chlorophyll contents of leaves was positively correlated with the N supply [33-34]. Moran et al. [34] showed that foliar concentration of photosynthetic pigments has a relationship with N supply, and it then ultimately leads to the increased physiological traits of plants. Similar results were depicted by the Shrestha et al.[35] in their studies. They used SPAD meter, a handheld device for the measurements of chlorophyll, and reported that N-supply had significant effects on SPAD chlorophyll contents and photochemical reflectance index (PRI).

Zhong et al. [36] studied the effect of N addition on the sensitivity of photosynthesis among C_3 versus C_4 grass species under extreme drought and re-watering conditions. Their results concluded that N addition resulted in an increase in biomass, but photosynthesis resilience was lower under the drought conditions. However, faster recovery of photosynthesis was observed due to the N addition. They further indicated that during drought, the N addition effect on photosynthesis was asymmetric, and it is more specific for the plants which have different photosynthetic nitrogen use efficiency (PNUE). Their studies further confirm that N could be used to mitigate the abiotic stress like drought and could help plants to build resilience to the climate change. Similarly, N could help to improve the carbon fixation in crops as it is one of the essential components of amino acids. Additionally, it has been well documented that N addition improves the proline, sugars, and antioxidant enzymes in the plants. Sánchez et al. [37] reported an increased proline level under adequate N supply. Similarly, the accumulation of N-containing compounds could lead to the higher survival of plant species under stress [38]. Burns [39], in their findings, showed that N manipulation could help to improve plant growth and development. It has been reported that 90% of the biomass produced by the crops are derived from photosynthesis; thus, Makino et al. [40] emphasized the design of strategies that could help to improve crop photosynthesis and grain yield under given N supply. N is the main component of the plant body and part of biological molecules like protein and enzymes, which are involved in different metabolic processes in the plants. Thus, deficiency of N could lead to the overall decline in the physiological traits of crops, e.g., net photosynthesis (An), stomatal conductance (g_s), biomass, and finally, crop yield. Hence in this study, we want to see the relationship of different N application rates and methods on wheat crop physiological traits, biomass ad grain yield under field conditions as this will be useful for wheat crop management from the perspective of crop physiological processes. The objectives of the study were (i) to investigate the wheat crop physiological traits (SPAD chlorophyll contents, stomatal conductance (gs), stomatal resistance (rs), transpiration rate (E), net photosynthetic rate (An)), crop total N, N efficiencies and agronomic traits in response to N application rates and methods under field conditions and (ii) to see the relationship of physiological characteristics with wheat crop yield only.

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2. Results

2.1. Crop physiological traits

A significant difference was found for all physiological characteristics against all treatments. Stomatal conductance (g_s) results showed that it remains significantly higher during 2013-14 as compared to 2014-15. Among sites maximum, gs was observed for Islamabad followed by URF-koont while it was minimum at Talagang, i.e., low rainfall and high-temperature location. N treatment's effects on stomatal conductance revealed that it was maximum at T_4 while the minimum was observed for control treatment among full application of N at sowing. However, among split N application highest g_s were recorded for T_6, which was at par with T_7 (Table 1). The interactive effect of YxL was significant on g_s while all other interactions were non-significant. The results for stomatal resistance (R_s) was inverse to g_s. Among years the highest R_s (1.05 m^2 s $mole^{-1}$) was observed during 2014-15, while at sites, it remains high at Talagang, followed by URF-Koont and Islamabad. N treatment's impacts on R_s revealed that with the application of more N, it decreases significantly. Lowest stomatal resistance was found for T_5 and T_6, while the highest stomatal resistance was observed for control treatment T_1. Only YxL interactions were found significant for stomatal resistance, while all other interactions were non-significant. Net Photosynthesis (A_n) results showed that it remains highest for the first year compared to 2014-15. Among sites, the maximum A_n was recorded for Islamabad while it remained minimum at Talagang. N treatments have shown significant impacts on A_n and the highest A_n was observed for split treatment T_6, which was at par with T_7. However, among the full dose application of N maximum, A_n was found for T_4 while it was minimum for T_1. Transpiration rate (E) remained highest during 2013-14, while the lowest E was recorded for 2014-15. Among sites, the highest E was recorded for Islamabad, followed by URF-Koont and Talagang. The impact of N treatments on transpiration rate revealed that it remained highest for split application of N i.e.T_6, which was at par with T_7 as well as with T_4. However, the lowest E was observed for the control treatment (T_1). A similar trend was observed for Ci (µmole CO_2 mol^{-1} air) and SPAD chlorophyll contents. However, proline contents remained maximum for the second year compared to the first year. Among sites, the highest proline contents were observed at Talagang while it remained lowest at Islamabad. N addition resulted in the positive effects on proline contents, and the highest proline was observed for split treatment T_6.

2.2.Crop Total Nitrogen

Significant variation for crop/biomass total nitrogen (TN) was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments. Both years differed significantly for crop N at the tillering stage. Maximum TN (2.81 kg ha^{-1}) was observed during 2013-14, while minimum TN (2.68

kg ha^{-1}) recorded during 2014-15 (Table 2). During 2013-14, 5% higher TN was recorded than 2014-15. At tillering among locations, maximum TN (3.61 kg ha^{-1}) was recorded at Islamabad, while minimum TN (1.99 kg ha^{-1}) was observed at Talagang. There was a 44 % variation among study sites for TN at the tillering stage. Meanwhile, the maximum TN (4.16 kg ha^{-1}) was recorded under T_4, while minimum TN at tillering was recorded under T_5 N treatment (1.84 kg ha^{-1}). Under N treatment T_4, 42 % higher TN was registered than T_5 at the tillering stage. The interactive effects of Years x Locations (YxL) and Locations x Treatments (LxT) were highly significant, while YxT and YxLxT remained non-significant. For interactive effect, YxL maximum crop TN was accumulated during 2014-15 (3.77 kg ha^{-1}) at Islamabad while minimum crop TN (1.82 kg ha^{-1}) was recorded during 2014-15 at Talagang (Figure 1). Maximum TN was recorded at Islamabad under T_4 (5.46 kg ha^{-1}), while minimum TN at tillering was recorded (1.34 kg ha^{-1}) at Talagang under T_5 (Figure 1). Similarly, a significant difference was noted during both years at the anthesis stage for TN. Maximum crop TN (33.53 kg ha^{-1}) was recorded during 2013-14, while minimum crop TN (31.51 kg ha^{-1}) was observed during 2014-15. During 2013-14, 6 % higher N was noted than 2014-15. A significant difference in Crop TN was recorded at three climatic locations. Higher TN (39.93 kg ha^{-1}) was observed at Islamabad, while lower TN (23.39 kg ha^{-1}) was recorded at Talagang. The difference in TN among Islamabad and Talagang was 41 %. Total N also differed under different N treatments. Maximum crop TN (44.23 kg ha^{-1}) was recorded under T_7, while minimum TN (17.64 kg ha^{-1}) was observed under T_1. There was 88 % higher TN for T_7 compared to T_1. The interactive effects YxL and LxT were highly significant while YxT and YxLxT remained non-significant. For interactive effect, LxT maximum crop TN was accumulated during 2013-14 (42.71 kg ha^{-1}) at Islamabad while minimum crop TN was recorded (22.91 kg ha^{-1}) during 2013-14 at Talagang (Figure 1). For interactive effect, YxL highest crop TN (55.59 kg ha^{-1}) was recorded during 2013-14 under T_7 while lowest TN (12.39 kg ha^{-1}) was recorded at Talagang under control N treatment (Figure 1).

Meanwhile, significant variation for crop TN was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments at maturity. Both years differed significantly for crop TN at the maturity stage. Maximum TN (66.42 kg ha^{-1}) was observed during 2013-14, while minimum TN (62.95 kg ha^{-1}) recorded during 2014-15 (Table 2). During 2013-14, 5 % higher TN was recorded than 2014-15. At maturity among locations, maximum TN (87.22 kg ha^{-1}) was recorded at Islamabad, while minimum crop TN (43.22 kg ha^{-1}) was observed at Talagang. There was a 47 % variation among study sites for TN at the maturity stage. Meanwhile, maximum TN (89.20 kg ha^{-1}) was recorded under T_7, while minimum TN at maturity was recorded under control N treatment (32.91 kg ha^{-1}). Under N treatment T_7, 46 % higher TN was registered than T_1 at the maturity stage. The interactive effects YxL and LxT were highly significant while YxT and YxLxT remained non-significant. For interactive effect, YxL maximum crop TN was accumulated during 2014-15 (89.63 kg ha^{-1}) at Islamabad followed by 2013-14 at Islamabad (84.81 kg ha^{-1}) while minimum crop TN (37.53 kg ha^{-1}) was recorded during 2014-15 at Talagang (Figure 1). Maximum TN was recorded at Islamabad under T_7 (123.19 kg ha^{-1}), while minimum TN at maturity was recorded (25.55 kg ha^{-1}) at Talagang under T_1 (Figure 1).

2.3. Nitrogen efficiencies

2.3.1. Nitrogen Use Efficiency (kg kg^{-1})

A significant variation for NUE was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments. Both years differed significantly for NUE. Maximum NUE (18.35 kg kg^{-1}) observed during 2013-14, while minimum NUE (16.63 kg kg^{-1}) recorded during 2014-15 (Table 3). During 2013-14, 9 % higher NUE was recorded than 2014-15. Among locations, maximum NUE (22.96 kg kg^{-1}) was recorded at Islamabad, while minimum NUE (11.31 kg kg^{-1}) was observed at Talagang. There was a 51 % variation among study sites for NUE. Meanwhile, maximum NUE (21.72 kg kg^{-1}) was recorded under the T_1 while the minimum NUE was recorded under T_7 N treatment (13.395 kg kg^{-1}). Under N treatment T_1, 38 % higher NUE was registered than T_7. The interactive effects viz. YxL, YxT, LxT, and YxLxT remained statistically non-significant for NUE.

2.3.2. Nitrogen Uptake Efficiency (NUpE)

A significant variation for NUpE was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments. Both years differed significantly for NUpE. Maximum NUpE (0.41) observed during 2013-14 while minimum NUpE (0.39) recorded during 2014-15 (Table 3). During 2013-14, 5 % higher, NUpE was recorded than 2014-15. Among locations, maximum NUpE (0.53) was recorded at Islamabad, while minimum NUpE (0.27) was observed at Talagang. There was a 49 % variation among study sites for NUpE. Meanwhile, maximum NUpE (0.49) was recorded under T_5, while minimum NUpE was recorded under T_4 N treatment (0.32). Under N treatment T_5, 35 % higher NUpE was recorded than T_4.

2.3.3. Nitrogen Utilization Efficiency (NUtE) (kg kg^{-1})

A significant variation for (NUtE) was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments. Both years differed significantly for N utilization efficiency at the maturity stage. Maximum NUtE (44.36 kg kg^{-1}) observed during 2013-14 while minimum NUtE (44.19 kg kg^{-1}) recorded during 2014-15 (Table 3). During 2013-14, 0.3 % higher NUtE was recorded than 2014-15. At maturity, among locations, maximum N utilization efficiency (47.09 kg kg^{-1}) was recorded at URF-Koont, while minimum N utilization efficiency (42.2 kg kg^{-1}) was observed at Talagang. There was a 10 % variation among study sites for N utilization efficiency at the maturity stage. Similarly, maximum NUtE (53.99 kg kg^{-1}) was recorded under T_2, while minimum NUtE at the maturity was recorded under T_7 (35.16 kg kg^{-1}). Under N treatment T_2, 34 % higher N utilization efficiency was recorded than T_7 at the maturity stage. The interactive effects YxL, LxT, YxT, and YxLxT for NUtE were highly significant (Table 3)

2.4.Agronomic traits

Results showed that the number of tillers remained non-significant during both years (2013-14 and 2014-15), while a significant difference was observed at the three different climatic sites under different N treatments (Table 4). Similarly, thousand-grain weight (TGW) remained non-significant during both study year. However, it was significantly different at sites and under different N treatments. Under N treatment T_6, 32 % higher TGW was recorded than T_1. The interactive effects YxL was significant while LxT, YxT, and YxLxT were non-significant. Among locations, maximum TGW (34.3 g) was recorded at Islamabad, while minimum TGW (24.7 g) was observed at Talagang. There was a 28 % variation among study sites for TGW. Meanwhile, maximum TGW (34.5 g) was recorded under the treatment T_6 while the minimum TGW was recorded under control treatment. Significant variation for the grain yield was observed during both years (2013-14 and 2014-15) at three varying climatic locations under different N treatments. The highest biological yield (9380.9 kg ha^{-1}) was recorded for the first year (2013-14) while it was lowest (8704.7 kg ha^{-1}) for the second year (2014-15). Among locations, biological yield remained maximum at Islamabad while the addition of N resulted in the maximum biomass under split application of N, i.e., T_6. Maximum grain yield (3001.9 kg ha^{-1}) was observed during 2013-14, while minimum grain yield (2611.40 kg ha^{-1}) was during 2014-15 (Table 4). During 2013-14, a 10 % higher grain yield was recorded than 2014-15. Among locations, the highest grain yield (3957.5 kg ha^{-1}) was recorded at Islamabad, while the lowest grain yield (1760.6 kg ha^{-1}) was detected at Talagang. There was a 52 % variation among the study sites for the grain yield. Meanwhile, the highest grain yield (3517.2 kg ha^{-1}) was recorded under T_6, while the lowest grain yield was recorded under T_1 N treatment (1737.8 kg ha^{-1}). For N treatment T_6, a 44 % higher grain yield was recorded than T_1. The interactive effects of LxT were highly significant, while YxL, YxT, and YxLxT were non-significant. The considerable difference for harvest index was observed during both years (2013-14 and 2014-15). During 2013- 14, a 6 % higher harvest index was recorded than 2014-15. Among locations, the maximum harvest index (0.35) was recorded at Islamabad, while the minimum harvest index (0.25) was observed at Talagang. There was a 29 % variation among study sites for the harvest index. Meanwhile, the maximum harvest index (0.32) was recorded under T_2, T_3, T_4, and T_7, while the minimum harvest index was recorded under T_1 and T_5 N treatment (0.29).

2.5.Relationship of physiological traits with grain yield

Linear regression analysis was performed to see the relationship between grain yield and physiological characteristics combined over the years, locations, and N treatments. The results showed that physiological traits (e.g., g_s, R_s, A_n, E, and SPAD chlorophyll contents) have a significant relationship with grain yield of wheat (Figure 2). The regression equation for grain yield with stomatal conductance showed a positive trend with R^2 = 0.98. The equation obtained for grain yield and g_s is presented below:

$$Grain\ yield = -1476.28 + 6518.68g_s$$

The inverse relationship between grain yield and stomatal resistance was observed with R^2 = 0.98. The equation obtained was:

$$Grain\ yield = 5353.31 - 3591.18R_s$$

A positive, strong association was obtained for net photosynthesis and grain yield (R^2 = 0.99). The regression equation for this relationship is:

$$Grain\ yield = -5614.79 + 433.27A_n$$

Transpiration rate outcomes revealed that with the increase in transpiration rate, grain yield of wheat crop increases significantly (R^2=0.98). The equation obtained for this trend is:

$$Grain\ yield = -3490.54 + 833.24E$$

A similar pattern was observed for Intercellular carbon dioxide concentration and SPAD chlorophyll contents with R^2 values of 0.98 and 0.97, respectively. The model equations were:

$$Grain\ yield = -6828.66 + 32.06C_i$$

$$Grain\ yield = -1501.84 + 74.45\ \mathrm{SPAD}_{chlorphyll\ contents}$$

3. Discussions

Climate extremes in the form of rainfall variability, drought, and rise in temperature are the primary abiotic stressor affecting wheat crop physiological traits, crop total N, N efficiencies, and agronomic characteristics. Consistent with previous findings, our results showed that the physiology of wheat crop decreases under stress (water, temperature, or nutrient); however, this stress could be managed by the application of N[8]. Stomatal conductance (g_s) could be the critical determinant of crop yield and productivity as it balances cop CO_2 uptake and water loss. It impacts on the total rate of photosynthesis and water use during the crop growing period. Ahmed et al. [46], in their works, concluded that crop physiological traits have a strong association with prevailing climatic conditions. Higher temperatures and lower availability of water resulted in the decline in the physiological characteristics like g_s, A_n, and E while an increase in the stomatal resistance. However, they suggested that change in the sowing date could be an option to mitigate the effect of climatic variables on crop physiological traits while we recommend here the addition of N as a split application could build resilience in the crop physiological traits. Since N helps in the process of photosynthesis, thus, its addition could be beneficial for the crop. Higher photosynthetic rates lead to the higher biomass production and grain yield. Yu et al. [47] reported that photosynthesis and transpiration are interdependent upon each other, and improvements of one are linked with the development of others. We also got the same trend for both photosynthesis and transpiration rate, and higher values for both parameters were obtained under split application of N. Therefore, the management of N by applying at the proper time with proper amount could help to improve the physiology of wheat crop. Kimball et al. [48] reported reduced stomatal conductance (33-50%) and transpiration rate (20-27%) with the change in the microclimate of the crop. Our results of N addition and its relationship with photosynthetic capacity was at par with the earlier findings in which they concluded that N addition could help to replenish the negative effect of stress by building resilience in the physiological traits of the crop [14, 21-23]. Like our work, the increased net photosynthetic rate (An) was observed due to the application of N [34]. Gyuga et al. [49] reported that N greatly influences photosynthetic processes, and deficiency of N leads to the declined photosynthesis. Similarly, Abid et al. [50] in their findings concluded that management of N nutrition could build drought tolerance in wheat by maintaining higher photosynthetic activities and antioxidative defense system during vegetative growth periods. A primary driving force for dry matter production in photosynthesis; thus, its management through the optimization of N could help to improve dry matter production in the plant. A positive correlation between leaves photosynthetic capacity and N contents have been shown in earlier work [14, 21-23]. Application of N in wheat plants resulted in the improved properties of photosynthetic pigments and increased net photosynthetic rate (An) [24-25]. Similarly, the fitness of plants could be determined by the indicators like chlorophyll contents and photosynthetic rate. Thus, moderate stress (drought or nutrients) could decrease the photosynthesis, mainly due to the stomatal limitations [51-53]. Hence, we suggest here that the application of N in split dosage tackle this limitation. Variability in the SPAD chlorophyll contents due to the temperature and water stress were reported in the earlier work, and they suggested the use of suitable genotypes and optimum sowing time to provide suitable environmental conditions to the crop [54]. CO_2 is an important ecological factor for plant matter, being directly involved in photosynthesis; however, its optimum fixation is linked with stomatal traits and chlorophyll contents in the leaves [55]. Proline is a vital stress defender, and, in our findings, it has been revealed that N addition resulted in the increased N compound, i.e., proline (Table 1). Improved tolerance in plants was observed due to the accumulation of proline under water and temperature stress [56-60], which resulted in the improved crop physiological processes. The role of antioxidants like proline in plant drought tolerance was well-reviewed by Laxa et al. [61]. Higher survival of plant under stress have a strong link with the accumulation of N-containing compounds, and their concentration has been increased due to the addition of N as reported in our studies [37-38].

Lu et al. [30] reported that reduced N supply could lead to the severe plant growth and lower grain yield. The addition of N could help to increase total N in a plant as elaborated in our findings (Table 2). A similar conclusion was made by Ladha et al. [13] in their work, and they emphasized the management of N to increase its uptake efficiency. Nitrogen use efficiency (NUE) and agronomic efficiency (AEN) were evaluated by Srivastava *et al*. [62] under different N rates (0 (N_0), 75 (N_{75}), 100 (N_{100}) and 125 (N_{125}) kg ha^{-1} and 0 (N_0), 60 (N_{60}), 80 (N_{80}) and 100 (N_{100}) kg ha^{-1})) and sowing scenarios in maize. They concluded that the under rainfed N rate from N_0 to N_{100} resulted in decreased NUE and AEN. Today wheat cultivars require higher input of N, but it resulted in a risk of environmental pollution. Therefore, the management of N is very important. Guarda et al. [63] investigated the impacts of different N rates (N_0, N_{80}, N_{160}, kg ha^{-1}) on wheat yield quality and NUE. The results showed that the management of N resulted in improved plant N uptake, NUE, and grain quality. Nitrogen uptake efficiency is the measure of how much N is taken up by the wheat crop. Raun and Johnson [64] reported that to increase NUE, its uptake must be enhanced. Raun et al. [65] concluded that N fertilization helps to improve NUE in wheat. The results of the current study depicted that NUpE is affected by N treatments during both the years at three study sites. Rahimizadeh et al. [66] concluded decreased N uptake efficiency under increased N rates, which might be due to more N losses.

Agronomic traits were significantly changed in our findings for all treatments, and it has been reported that dry matter and grain yield of the plants could be improved by N fertilization [67]. However, N fertilization should be matched with the crop demand as, in our case, higher agronomic traits were observed for split treatments compared to a full application at the time of sowing. Generally, farmers apply N at the time of sowing or at the earlier growth stages of the crop, which resulted in the maximum loss of N and lowered crop dry matter and yield. Thus, an integrated soil-crop system management strategy could help to improve grain yield and NUE as proposed by Meng et al. [67]. Hawkesford et al. [68] reported that 33% of applied N fertilizer is recovered in the harvested grain. Thus, 67% of N is lost, which could be a major source of pollutants and should be a major target for crop improvement. Therefore, agronomic management and crop breeding traits could help to improve NUE. Furthermore, optimizing the N application rate could be a good option to minimize N losses and increase crop yield [69]. The relationship of grain yield with crop physiological traits showed that stomatal conductance could be the major determinant of the grain yield (Figure 2). Since global temperature and frequent occurrence of drought could maximize N losses, thus new avenues for improving crop productivity must be exploited [70-71].

4. **Materials and Methods**

4.1. Study sites, treatments, plant material, and experimental design

Field experiment was carried out during wheat growing season of 2013-2014 and 2014-2015 at three variable climatic locations of Pothwar i.e. low rainfall area, Talagang (32.55°′N, 72.25°′E), medium rainfall area, URF-Koont (32.93°′N, 72.86°′E) and high rainfall area, Islamabad (33.40°′N, 73.10°′E) [41] (Figure 3). Urea $((NH_2)_2CO)$ (46% N) fertilizer was applied as full at the time of sowing and split doses (sowing, tillering, and anthesis). Treatments include, T_1= Control (No fertilizer applied), full dose of N applied at the time of crop sowing, i.e. T_2 = 50 kg N ha^{-1}, T_3= 100 kg N ha^{-1} and T_4= 150 kg N ha^{-1} and split application of N at different timings during different stages of the crop called as split application of N, i.e. T_5: Application of 50 kg N ha^{-1} (15 kg N ha^{-1} (Sowing) : 20 kg N ha^{-1} (Tillering) :15 Kg N ha^{-1} (Anthesis), T_6: Application of 100 kg N ha^{-1} (30 kg N ha^{-1} (Sowing): 40 kg N ha^{-1} (Tillering) : 30 kg N ha^{-1} (Anthesis) and T_7: Application of 150 kg N ha^{-1}(45 kg N ha^{-1} (Sowing) : 60 kg N ha^{-1} (Tillering) : 45 kg N ha^{-1} (Anthesis). Each treatment was replicated three times Weed control was done manually. A field experiment was laid out using a randomized complete block design (RCBD). The plot size was 5 x 6 m^2 in which one wheat cultivar (Pakistan-13) was sown on 15th November for two years with a row to row distance of 25 cm. The one-meter path was maintained to isolate treatments. Pedigree/parentage of sown cultivar is PTSS02B00132T-0TOPY-0B-0Y-0B-38Y-0M-0SYMEX94.27.1.20/3/SOKOLL//ATTILA/3*BCN, and it was released in the year 2013 for the rainfed conditions of Punjab Pakistan.

4.2.Physiological traits measurements

Wheat physiological traits which includes g_s (Stomatal Conductance), R_s (Stomatal resistance) (m^2 s $mole^{-1}$), Net Photosynthetic rate (A_n) (µ mole/m^2/s) , E (Transpiration rate) (mmole m^{-2} s^{-1}) and Ci (Intercellular CO_2) (µmole CO_2 / mol air) was measured with Infra-red gas analyser (IRGA) at anthesis (Zadok 60) stage of wheat by putting leaf into chamber and adjusting its leaf area and when value became constant it was recorded. The averages of five samples were taken as measured by Long and Bernacchi [42]. Similarly, chlorophyll contents were measured by using a SPAD chlorophyll meter. The chlorophyll contents were taken from the top, middle, and base of leaves, and then the average value was used to represent SPAD chlorophyll contents. Proline Content (μg g^{-1}) was measured by taking fresh leaves (0.5 g) from plants at the flag leaf stage from each plot. The Samples were normalized in ten ml of three percent sulfosalicyclic acid ($C_7H_6O_6S$) and then filtered. Stress amino acid, Proline (C_5H_9NO), estimated spectrophotometrically following the ninhydrin method [43].

4.3.Crop Total Nitrogen

The amount of N in the plant was determined at tillering, anthesis, and maturity stages. The one-meter square area was used to take plant samples. Nitrogen contents from plant samples were determined after oven drying at 65°C for 48 hours. After drying, samples were ground by using Wiley Mill, and samples were placed in plastic bottles to determined N contents. The sample of 2 g in 30 to 50 mL of acid and approximately 100 mL sodium hydroxide (NaOH) solutions were used. The TN was measured after wet digestion using the Kjeldahl procedure [43].

4.4.Nitrogen efficiencies

Rahimizadeh et al. [44] procedure was used to determined Nitrogen Uptake Efficiency (NUpE), Nitrogen Utilization Efficiency (NUtE), and Nitrogen Use Efficiency (NUE).

$$\text{NUpE} = \frac{N_T}{N_{supply}}$$

Where

$$\text{NUtE} = \frac{G_Y}{N_T}$$

$$\text{NUE} = \frac{G_Y}{N_{supply}}$$

Where N_T = Total plant N uptake, G_Y = Grain yield, and S_{upply} = Sum of soil N content at sowing and N fertilizer.

4.5. Agronomic traits

At physiological maturity, total numbers of fertile tillers were counted from an area of one m² from each plot. Furthermore, a sub-sample of thousand grains from each treatment was weighed using a digital weighing balance. The biological yield was measured by harvesting one m² area per plot, and it will be converted to get the final yield in kg ha^{-1}. Grain yield was calculated by harvesting a one m² area per plot, and it was converted to get final yield in kg ha^{-1}. Finally, harvest index (HI) was measured using the following formula.

$$Harvest\ index\ (HI) = {}^{Grain\ yield}/_{Biological\ Yield} \times 100$$

4.6.Statistical Analysis

The significance of the effects of years, locations, and N treatments at the 0.05 level was determined by a three-way analysis of variance (ANOVA) using SPSS 19.0 software (SPSS, Inc., Chicago, IL, United States). Means obtained were compared by LSD at 0.05% level of significance.

5. Conclusions

Crop physiological traits, total N, and N efficiencies have a significant strong relationship with crop dry matter and grain yield. Since crop yield is the most commonly used indicator; thus, it needs to be improved by considering the interaction of N addition with crop physiology such as stomatal conductance, net photosynthetic rate, and transpiration rate. Optimum N application rate and its timings can help to harvest real benefits of N as in our findings split dose resulted to the optimum physiological traits, i.e., g_s, A_n and E. Improvement in the stomatal conductance through the management of N under stress could be the key determinants of crop yield as it balances the cop CO_2 uptake and water loss. Similarly, a split application of N resulted in the higher N efficiencies, i.e., NUE, nitrogen uptake efficiency, and nitrogen utilization efficiency at all sites for two years. Thus, the idea of Hawkesford et al.[68] to recover maximum applied N fertilizer is possible through its split application during different stages of the crop. Furthermore, a split application of N resulted in the maximum agronomic traits, and a significant combined strong relationship was obtained between grain yield and crop physiological parameters. The results showed that T_6 = Split N_{100} could be used to get optimal returns from N. However, in the future, we will be further using quadratic plateau model approach to build the relationship between N methods and rates. This will ultimately help us to optimize crop physiological traits and grain yield under different sets of N scenarios at these variable field sites.

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Table 1. Physiological traits of wheat for two years, at three variable sites and under different nitrogen treatments; * p≤0.05; ** p ≤ 0.01; *** p≤ 0.001; NS = Non-significant.

	gs (mole m^{-2} sec^{-1})	Rs (m^2 s $mole^{-1}$)	An µmole $m^{-2}s^{-1}$	E (mmole $m^{-2}s^{-1}$)	Ci (µmole CO_2 mol^{-1} air)	Chlorophyll contents (SPAD)	Proline Content (µg g^{-1})
Years (Y)							
2013-14	0.65a	0.73b	18.70a	7.52a	298.41a	54.672a	30.77b
2014-15	0.50b	1.05a	16.24b	6.32b	272.60b	45.56b	38.25a
Study Sites/Locations (L)							
Islamabad	0.67a	0.66c	18.88a	7.95a	317.79a	51.80a	27.85c
URF-Koont	0.56b	0.87b	16.77b	6.89b	290.12b	46.79b	33.86b
Talagang	0.46c	1.07a	15.55c	6.13c	252.60c	40.29c	40.13a
Nitrogen Treatments (T)							
T_1=N_0	0.60c	0.71a	15.25c	6.82c	290.79d	44.51d	27.97d
T_2=N_{50}	0.73b	0.56b	17.50b	8.15b	310.18c	48.39c	29.52c
T_3=N_{100}	0.78a	0.49c	18.10b	8.75b	318.33b	54.55b	32.15b
T_4=N_{150}	0.79a	0.48c	19.49a	9.22a	320.23b	57.28a	33.82b
T_5=Split N_{50}	0.73b	0.56b	17.52b	8.15b	310.19c	54.39b	30.74c
T_6=Split N_{100}	0.80a	0.48c	20.07a	9.58a	329.25a	58.86a	35.42a
T_7=Split N_{150}	0.80a	0.48c	19.79a	9.31a	325.27a	58.88a	35.42a
Interactions							
YxL	***	***	***	***	NS	NS	***
YxT	NS	NS	NS	NS	NS	NS	NS
LxT	NS	NS	NS	NS	NS	***	NS
YxLxT	NS	NS	NS	NS	NS	NS	NS

Table 2.

Crop total N (kg N ha^{-1}) at tillering, anthesis and maturity stages of wheat crop during two years at three study sites under seven nitrogen treatments with significance of their interactions; * p≤0.05; ** p ≤ 0.01; *** p≤ 0.001; NS = Non-significant.

	Crop N at Tillering (kg N ha^{-1})	Crop N at Anthesis (kg N ha^{-1})	Crop N at Maturity (kg N ha^{-1})
Years (Y)			
2013-14	2.81^{a}	33.53^{a}	66.417^{a}
2014-15	2.68^{b}	31.51^{b}	62.95^{b}
Study Sites/Locations (L)			
Islamabad	3.61^{a}	39.93^{a}	87.22^{a}
URF-Koont	2.64^{b}	34.25^{b}	63.60^{b}
Talagang	1.99^{c}	23.38^{c}	43.22^{c}
Nitrogen Treatments (T)			
$T_1=N_0$	2.11^{e}	17.64^{f}	32.91^{e}
$T_2=N_{50}$	2.87^{c}	25.64^{e}	48.60^{d}
$T_3=N_{100}$	3.54^{b}	31.61^{d}	64.17^{c}
$T_4=N_{150}$	4.16^{a}	36.62^{b}	75.32^{b}
T_5=Split N_{50}	1.84^{f}	33.70^{c}	63.81^{c}
T_6=Split N_{100}	2.17^{e}	38.18^{b}	78.74^{b}
T_7=Split N_{150}	2.52^{d}	44.23^{a}	89.20^{a}
Interactions			
YxL	***	***	***
YxT	NS	NS	***
LxT	***	***	***
YxLxT	NS	NS	***

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Table 3. Wheat nitrogen use efficiencies (Nitrogen use (kg kg^{-1}), Utilization and uptake efficiencies) crop during two years at three study sites under seven nitrogen treatments with significance of their interactions; * p≤0.05; ** p ≤ 0.01; *** p≤ 0.001; NS = Non-significant.

Years (Y)	NUE	NUtE	NUpE
2013-14	18.35^{a}	44.18^{b}	0.41a
2014-15	16.63^{b}	44.35^{a}	0.39b
Study Sites/Locations (L)			
ISLAMABAD	22.96^{a}	43.53^{b}	0.53a
URF-Koont	18.22^{b}	47.08^{a}	0.39b
Talagang	11.31^{c}	42.20^{c}	0.27c
Nitrogen Treatments (T)			
T_1=N_0	21.72^{a}	52.78^{b}	0.41^{bc}
T_2=N_{50}	20.03^{b}	53.98^{a}	0.37^{d}
T_3=N_{100}	16.49^{c}	46.33^{c}	0.35^{de}
T_4=N_{150}	13.48^{d}	41.38^{d}	0.32^{e}
T_5=Split N_{50}	20.03^{b}	40.84^{e}	0.49^{a}
T_6=Split N_{100}	17.31^{c}	39.42^{f}	0.43^{b}
T_7=Split N_{150}	13.39^{d}	35.15^{g}	0.38^{cd}
Interactions			
YxL	NS	***	***
YxT	NS	NS	***
LxT	NS	***	***
YxLxT	NS	NS	***

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Table 4.

Agronomic traits of wheat crop during two years at three study sites under seven nitrogen treatments with significance of their interactions; * p≤0.05; ** p ≤ 0.01; *** p≤ 0.001.

	No of tillers m^{-2}	1000 grain weight (gm)	Biological yield (kg ha^{-1})	Grain yield (kg ha^{-1})	Harvest index
Years (Y)					
2013-14	222.4^{NS}	31.3^{NS}	9380.9^{a}	3001.9^{a}	0.32^{a}
2014-15	212.7	30.7	8704.7^{b}	2611.4^{b}	0.3^{b}
Study Sites/Locations (L)					
Islamabad	236.5^{a}	34.3^{a}	10450.1^{a}	3957.5^{a}	0.35^{a}
URF-Koont	226.9^{a}	33.9^{a}	8578.2^{b}	2830.8^{b}	0.33^{b}
Talagang	189.2^{b}	24.7^{b}	7042.4^{c}	1760.6^{c}	0.25^{c}
Nitrogen Treatments (T)					
T_1=N_0	184.5^{c}	23.5^{c}	5992.4^{c}	1737.8^{c}	0.29^{c}
T_2=N_{50}	205.8^{b}	28.1^{b}	8140.6^{b}	2605^{b}	0.32^{a}
T_3=N_{100}	218.8^{ab}	31.7^{a}	9275.9^{a}	2968.3^{a}	0.32^{a}
T_4=N_{150}	229.3^{a}	33.1^{a}	10942.8^{a}	3501.7^{a}	0.32^{a}
T_5=Split N_{50}	221.1^{ab}	31.6^{a}	8982.7^{b}	2605^{b}	0.29^{c}
T_6=Split N_{100}	234.4a	34.5a	11345.5^{a}	3517.2^{a}	0.31^{b}
T_7=Split N_{150}	229.1a	34.3a	10976.3^{a}	3512.4^{a}	0.32^{a}
Interactions					
YxL	***	NS	***	NS	***
YxT	NS	NS	NS	NS	***
LxT	NS	***	NS	***	***
YxLxT	NS	NS	NS	NS	***

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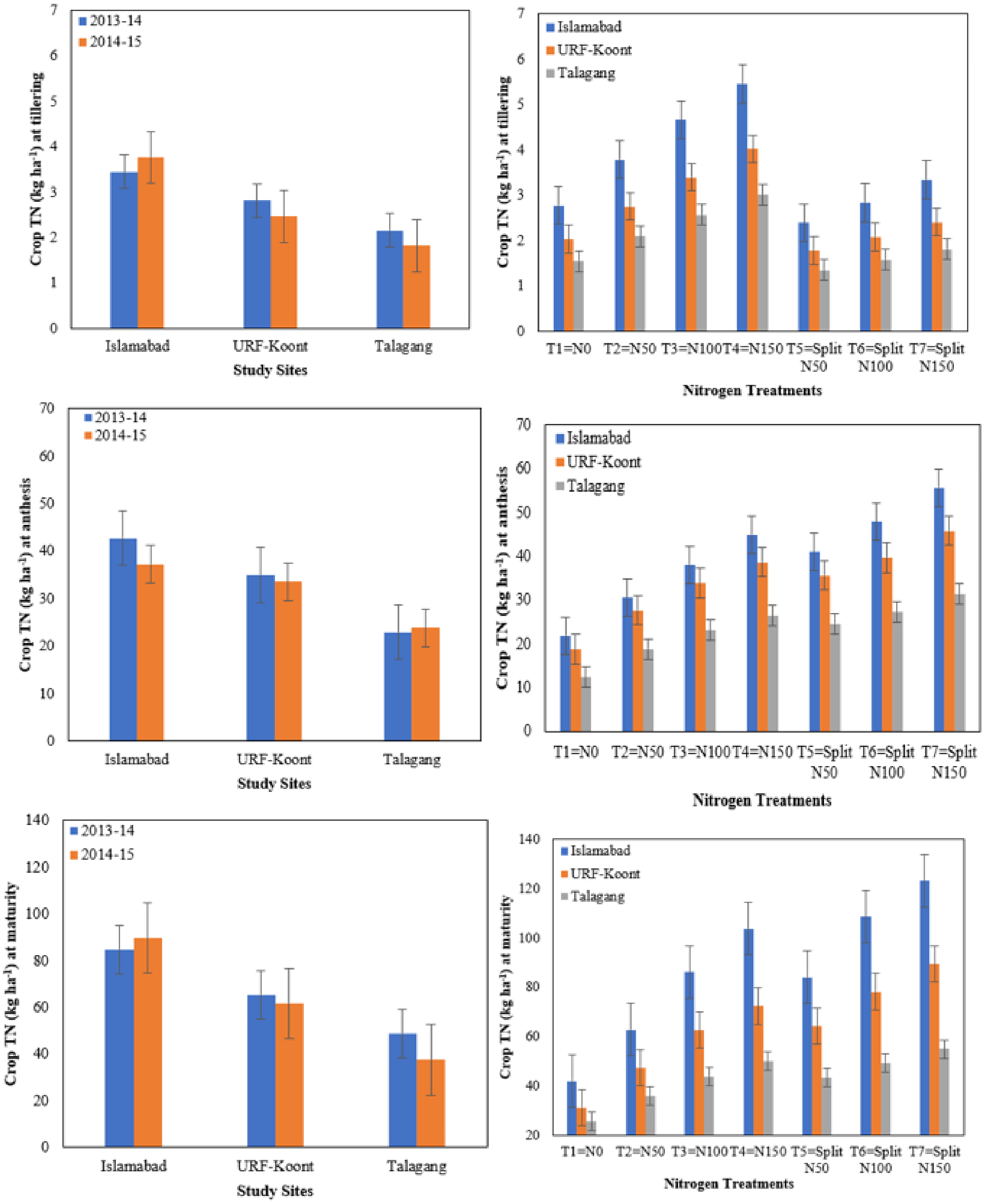

Figure 1. Crop total nitrogen (TN) Kg N ha^{-1} at tillering, anthesis and at maturity stages for years x locations (YxL) and Locations x Treatments (LxT) interactions

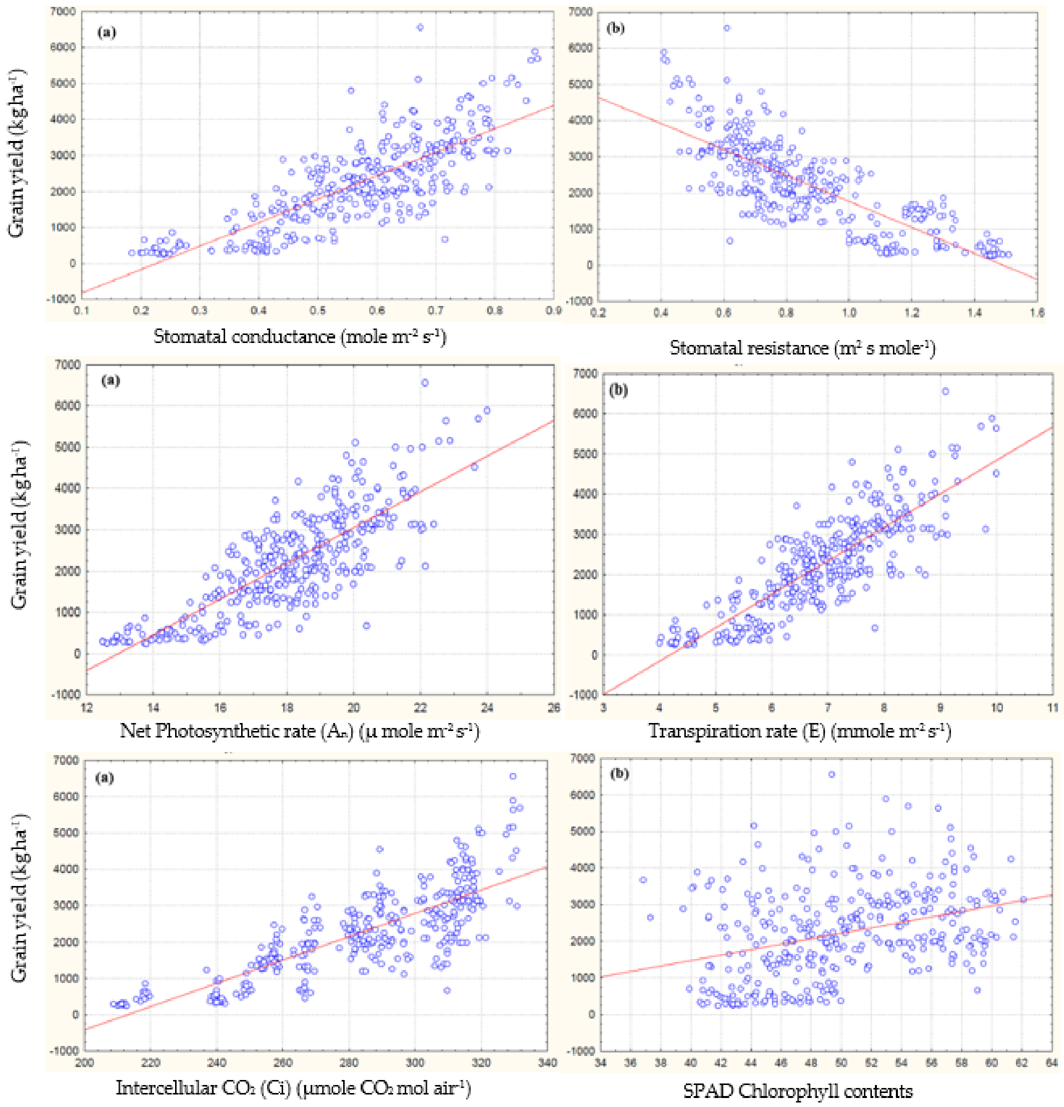

Figure 2. Relationship between physiological traits of wheat with grain yield combined over years, locations and Nitrogen treatments.

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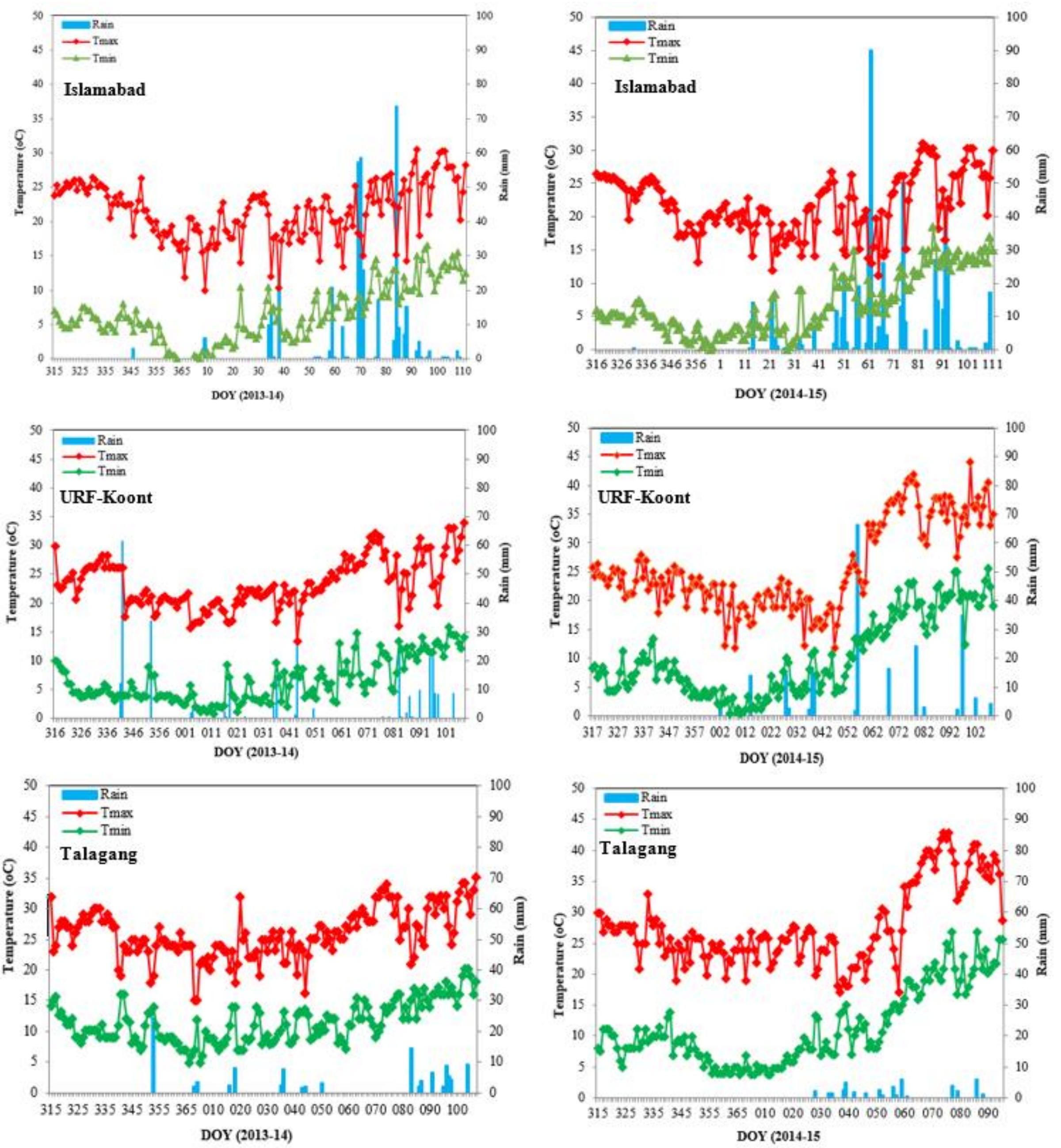

Figure 3. Climatic variables (Temperature (Tmax and Tmin) and Rainfall) during wheat crop growing season for two years at three study sites (DOY=Days of year)