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Effects of the Spatial Coupling of Water and Fertilizer on the Chlorophyll Fluorescence Parameters of Winter Wheat Leaves

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Abstract

Wheat is an important agricultural crop in the Loess region of China, where there is drought stress and low availability of soil nitrogen and phosphorus. Using a pulse modulation fluorometer, we studied the effects of water, nitrogen, and phosphorus on the kinetic parameters of chlorophyll fluorescence in winter wheat. The wheat was grown in layered columns of Eum-Orthic Anthrosol (Cinnamon soil), with the water content and nutrient composition of each layer controlled. The results showed that the kinetic parameters of chlorophyll fluorescence were sensitive to water stress. The basic fluorescence (F_0) of leaves was higher in the dry treatment (0-30 cm layer at 40-45% of field capacity, 30-90 cm at 75-80% of field capacity) compared to the wet treatment (entire soil column at 75-80% of field capacity). The maximal fluorescence (F_m), the variable fluorescence (F_v), the photochemical efficiency (F_v/F_m) and potential activites (F_v/F_0) of photosystem 2 (PS2) were significantly lower in the dry treatment. Although drought stress impaired PS2 function, this effect was significantly ameliorated by applying P or NP fertilizer, but not N alone. P application increased F_v/F_m, both in well-watered and water stressed plants, especially when fertilizer was applied throughout the column or within the top 30 cm of soil. A combined fertilizer improved photosynthesis in well watered plants, with F_m and F_v/F_m being the highest when fertilizer was applied throughout the columns. For drought stressed, plants F_v/F_m was significantly greater when combined fertilizer was added within the top 30 cm of soil. We concluded that, when growing winter wheat in both arid and semi-arid parts of the Loess region of China, it is important to guarantee the nutrient supply in the top 30 cm of the soil.

Key words: water stress, nutrient, spatial coupling, chlorophyll fluorescence, column experiment

INTRODUCTION

A great achievement in food production has been made through expanded crop production with the introduction of high yielding varieties, intensification of cropping, and increased use of irrigation and fertilizers. However, increased effect in crop yield due to the use of modern crop varieties has weaken at a certain extent. In fact, in order to meet still increasing demand for food, farmers in China have mainly focused on the application of N and P fertilizers. Fertilization could increase soil fertility, facilitate photosynthetic efficiency and consequently plant growth, and improve the ability of plant adaptation to dry conditions. However, the excessive accumulation of nutrient in farmlands can be a major environmental risk (Fang *et al*. 2006; Yang *et al*. 2006). Therefore, it is necessary to investigate the relation of environment factors, nutrient input, and plant growth.

Plants are frequently exposed to stresses such as

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drought, nutrient deficiency, salinity, and temperature extremes (Lima *et al*. 1999; Hassan 2006; Jiang *et al*. 2006; Zhou *et al*. 2009). The photosynthetic apparatus, especially photosystem 2 (PS2), may be temporarily affected by environmental stresses before irreversible morphological damage is observed. Non-invasive, remote sensing techniques, such as chlorophyll fluorescence, can provide insights into the ability of plants to tolerate stress, and the extent to which stress damages the photosynthetic apparatus (Degl'Innocenti *et al*. 2008). It can also identify the physiological condition of plants at larger spatial and temporal scales (Zarco-Tejada *et al*. 2002). Chlorophyll fluorescence could be an excellent tool for studying stress-induced changes in PS2 (Naumann *et al*. 2008), and predicting changes in the natural environment (Baker and Rosenqvist 2004; Dobrowski *et al*. 2005).

In many regions of the world, water stress in plants is the most common limitation to agricultural production. It may occur even where, overall, there is adequate irrigation because plants experience transient water stress during the middle of the day. Water deficit reduces leaf water potential and stomatal conductance, inhibits photosynthetic metabolism and eventually reduces plant productivity (Baker and Rosenqvist 2004; Hassan 2006; Rouhi *et al*. 2007; Degl'Innocenti *et al*. 2008). Under drought stress, disturbances of photosynthesis at the molecular level are connected with the restricted electron transport through PS2 and/or with structural injuries to PS2 (Flexas *et al*. 2004; Hura *et al*. 2007).

Mineral nutrients are important agricultural inputs that substantially affect crop growth. Nutrient deficiencies, which may in part be due to water deficit, can modify plant structure and adjust the growth pattern of crops. The extent to which photosynthetic capability is maintained may play an important role in plant adaptation to environmental stress. Dark-adapted values of photochemical efficiency (F_v/F_m) may reflect the potential quantum efficiency of PS2 and could be used as a sensitive indicator of plant photosynthetic activity under environmental stress (Roháček 2002).

Currently, there is no consensus about the effects of nitrogen (N) deficiency on PS2 functions. Some researchers have reported that N deficiency reduces F_v/F_m (Lima *et al*. 1999; DaMatta *et al*. 2002). Others have suggested that N deficiency has no significant effects on PS2 function (Ciompi *et al*. 1996; Kováčik and Bačkor 2007). Further research in this area is clearly needed. Although phosphorus (P) is known to be involved in the regulation of several photosynthetic processes, relatively little is known about the effect of P deficiency on PS2 functions. Significant declines in F_v/F_m have been observed in P deficient plants, suggesting that P deficit at least partly affects the chlorophyll fluorescence of plants (Zhou *et al*. 2009).

Winter wheat (*Triticum aestivum* L.) is a particularly important agricultural crop in northwestern China. However, wheat production there suffers from many environmental stresses, especially in the arid and semi-arid regions in the Loess region of China, where there are serious drought stress and limited availability of soil nitrogen and phosphorus. Despite the relationships between chlorophyll fluorescence and water or nutrient deficit being documented, there has been relatively little research into the simultaneous effects of both constraints, particularly the spatial coupling of water and fertilizer. Our study took into consideration of the characteristics of the Loess plateau soil, which is dry in the upper layer and moist lower down the profile (Shan and Chen 1993). Winter wheat was used in isolated layer soil column experiments. Several combinations of water and fertilizer were used to examine the effects of the spatial coupling of water, nitrogen, and phosphorus in different parts of the soil column on chlorophyll fluorescence parameters of the leaves of winter wheat. The aim was to extend knowledge on the use of fertilizers on dry land and to mitigate environmental risks in the arid and semi-arid region.

MATERIALS AND METHODS

Experimental materials and trial location

The experiments were performed at the State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences. Eum-Orthic Anthrosol soil (Cinnamon soil) was collected from farmland after the harvest of summer corn in Northwest A&F University, Shaanxi Province, China. Air dried soil samples were irrigated appropriately (see Experimental design and measurment) and packed into PVC columns with an inner diameter of 15 cm and a length of 90 cm, at a bulk soil density of 1.30 g cm^{-3}. The columns were divided into three 30 cm layers, each with soil from the strata corresponding to that recorded in the field. Each layer was separated by 2 cm of sand and stone, which controlled soil moisture and prevented water and fertilizer exchange between layers without constraining root growth. The soil of the top 0-30 cm layer was ground and sieved through a 2-mm sieve. It contained 8.78 g kg^{-1} organic C, 14.5 mg kg^{-1} available N, and 5.45 mg kg^{-1} available P, and had a of pH 7.6, measured with a soil/water ratio of 1:2.5 (w/v). There were two holes on opposite sides of the PVC columns in each of the top, middle, and substrate layers, one for watering (diameter 1.2 cm), and the other for monitoring soil water content (diameter 4.5 cm).

Winter wheat (*Triticum aestivum* L. *cv*. Xiaoyan 22) was planted in the columns which were then left in the open, but moved into the canopy during rainfall. After germination, the winter wheat was thinned and the upper layer drought treatment began at the regreening stage. Then, it was harvested at the ripening stage.

Experimental design and measurment

Two water treatments were used. In the first, the whole column was maintained at 75-80% of field capacity (referred to as W). In the other, the top (0-30 cm) layer was maintained under drought conditions (40-45% of field capacity), while the rest was at 75-80% of field capacity (referred to as D). There were four fertilizer treatments: no fertilizer (CK), N-fertilizer (using KNO_3 as the N source, referred to as N), P-fertilizer (using KH_2PO_4 as the P source, referred to as P), and N- and P-fertilizer together (using KNO_3 as N source and KH_2PO_4 as P source, referred to as NP). Fertilizer was mixed uniformly into the soil at a rate of 0.2 g N kg^{-1} of soil and 0.15 g P_2O_5 kg^{-1} of soil before packing column, according to the treatment. Fertilizer was applied in four spatial distributions: throughout the entire column (0-90 cm, referred to as A), in the top layer (0-30 cm, referred to as T), in the middle layer (30-60 cm, referred to as M), and in the bottom layer (60-90 cm, referred to as S). The total number of combinations was 26. There were three replicates of each combination, giving 78 columns, which were arranged completely at random.

Time domain reflectometer was used to determine water content before watering. Then, we added water drop by drop, on the basis of the existing water content and water treatment. This method prevented water and nutrient migration between different soil layers.

Chlorophyll fluorescence of winter wheat functional leaves was measured using a Pulse Modulation Fluorometer (PAM, Walz, Germany) at the jointing, booting, and early-filling stages. Leaves were dark-adapted for 20 min and then illuminated with a modulated measuring beam to obtain the initial fluorescence (F_0) and the maximum fluorescence (F_m). From these measurements the variable fluorescence (F_v), photochemical efficiency (F_v/F_m), and potential activities (F_v/F_0) of PS2 were calculated (Schreiber *et al*. 1994). ANOVA was used to test for statistical significance. Means were separated using least significant differences.

RESULTS

The effect of spatial coupling of water and fertilizer on the F_0 of winter wheat leaves

The basic fluorescence (F_0) is the fluorescence when the PS2 reaction center is completely open and in an active state. Its intensity is related to the excitation light and chlorophyll content. When all the activity centers of PS2 are active, F_0 is minimal. Increased F_0 indicates inactivation of reaction centers. Lower values of F_0 indicate a potentially greater resistance to the irreversible deactivation of the reaction centers. In our study, the F_0 of the leaves was higher for the dry treatment than for the wet treatment, showing drought stress was destructive to the PS2 reaction center. F_0 was not significantly different between different nutrient treatments where whole soil columns were at 75% of field capacity (Table 1). In the treatments where the upper soil was under water stress, F_0 was the greatest where no fertilizer was added, but was not significantly different from the F_0 for treatments with nitrogen only (Table 1). There were significant differences between F_0 for the treatment with no fertilizer and the P and NP treatments ($P<0.05$) (Table 1). For the N and P treatments, there was no significant difference between F_0 when the fertilizer was placed in different layers. For the NP treatment, F_0 was significantly higher when the fertilizer was applied to the 30-60 cm layer compared to when the fertilizer was applied throughout the column ($P<0.05$).

Table 1 Effects of different treatments on the F_0 values of winter wheat leaves

Treatment	CK	N	P	NP
W	0.0904 a	0.0884 a	0.0884 a	0.0881 a
D	0.0951 a	0.0900 ab	0.0890 b	0.0895 b

W, entire column was at 75-80% of field capacity; D, 0-30 cm layer at 40-45% of field capacity, 30-90 cm layer at 75-80% of field capacity. CK, no fertilizer; N, N-fertilizer; P, P-fertilizer; NP, N- and P-fertilizer. Different lowercase letters within rows indicate significant differences between different fertilizer treatments at the $P<0.05$. The same as below.

The effect of spatial coupling of water and fertilizer on F_m of winter wheat leaves

The maximal fluorescence (F_m) is the fluorescence value when the PS2 reaction center is completely shut down. Lower values of F_m indicate less PS2 activity, as occurs when PS2 becomes damaged. Table 2 shows that Fm was significantly lower in the dry treatment than in the wet treatment ($P<0.01$). For the wet treatment, the difference in F_m among CK, N, and P treatments was not significant. For the dry treatment, the F_m value for the NP treatment was higher compared to those for the CK, N, and P treatments, being significantly different from those for the CK and N treatments ($P<0.05$ in both cases). The difference in F_m between the N and the P treatments was not significant ($P>0.05$), but both were significantly higher compared to CK ($P<0.05$ in both cases).

Fig. 1 illustrates the effect of fertilizer placement on F_m. The ANOVA for the F_m values showed that there were no significant differences when N fertilizer was applied to different layers in both wet and dry treatments. The differences in F_m were highly significant when P fertilizer was applied to different layers in the dry treatment ($P<0.01$). The differences in Fm were highly significant when N and P fertilizer were applied to different layers in the wet treatment ($P<0.01$), but was insignificant in the dry treatment ($P>0.05$).

Table 2 F_m values for different water and fertilizer treatments

Treatment	CK	N	P	NP	Average
W	0.3914 b	0.3985 b	0.4022 b	0.4457 a	0.4095 A
D	0.3298 c	0.3784 b	0.3895 ab	0.4239 a	0.3804 B

The capital letters within last column indicate a significant difference between water treatments at the $P<0.01$. The same as in Table 4.

The effect of spatial coupling of water and fertilizer on F_v of winter wheat leaves

The variable fluorescence (F_v) is the difference between F_m and F_0, its intensity reflects the oxidation-reduction state of the PS2 initial electron acceptor and the maximum capacity for photochemical energy quenching by the sample. Table 3 shows that, as a whole, the F_v of leaves was significantly lower in the dry treatment than in the wet treatment ($P<0.01$). The F_v of the NP treatment was greater in the wet treatment and was significantly higher than for the CK, N, and P treatments ($P<0.01$ in all cases). The F_v of the NP treatment was not significantly different from that for the P treatment in the dry treatment, but was significantly higher than for the N treatment ($P<0.05$). F_v of CK was significantly lower compared to N and P treatments ($P<0.05$). It appears that fertilization, at least partially alleviated the decrease in F_v caused by drought. In both wet and dry treatments there was no significant difference among F_v values when N fertilizer was applied to different layers. The difference in F_v was highly significant when P fertilizer was applied to different layers, in both the wet and dry treatments ($P<0.01$ in both cases). For the NP fertilized plants which were not drought stressed, F_v was the greatest when fertilizer was applied to the 0-90 cm layer, with the difference being significant ($P<0.01$). For drought stressed, plants F_v was the greatest when the NP fertilizer was applied to the 0-30 cm layer, but there was no significant difference in F_v between treatments in which NP was applied to different levels.

The effect of spatial coupling of water and fertilizer on the PS2 photochemical efficiency of winter wheat leaves

F_v/F_m is proportional to the quantum yield of PS2 photochemistry and exhibits a high correlation with the

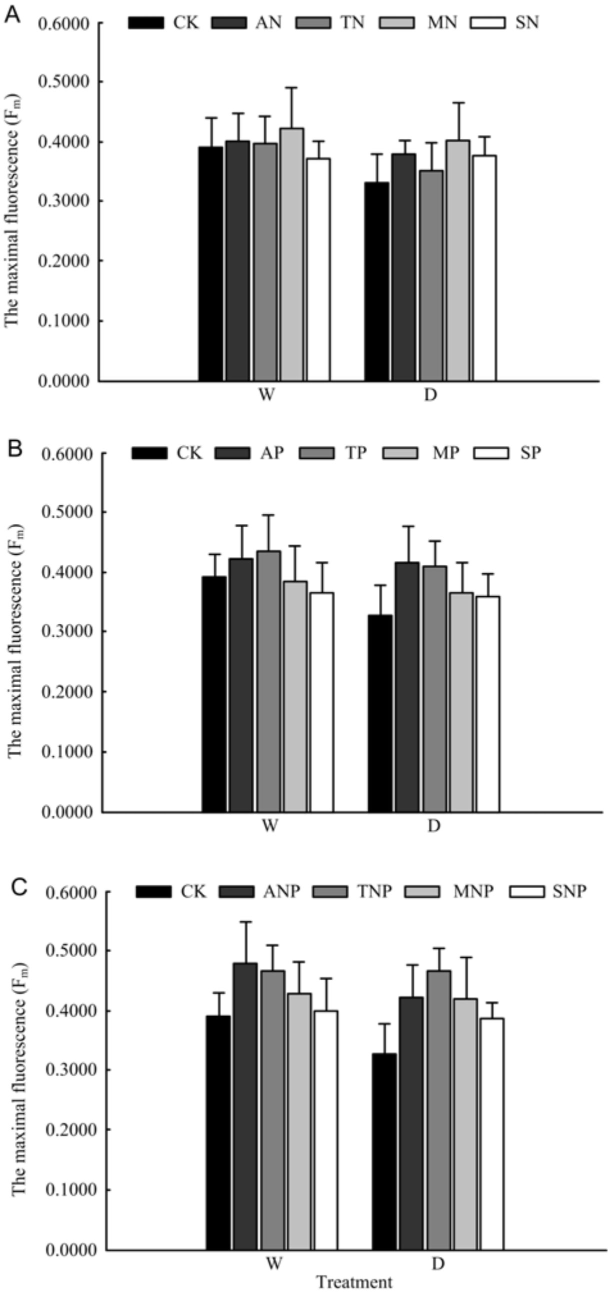

Fig. 1 The maximun fluorescence (F_m) for different placements of N, P, and NP fertilizer and water treatments of winter wheat leaves. W, entire column was at 75-80% of field capacity; D, 0-30 cm layer at 40-45% of field capacity, 30-90 cm layer at 75-80% of field capacity. CK, no fertilizer; AN, N-fertilizer applied to the 0-90 cm layer; TN, N-fertilizer applied to the 0-30 cm layer; MN, N-fertilizer applied to the 30-60 cm layer; SN, N-fertilizer applied to the 60-90 cm layer. AP, P-fertilizer applied to the 0-90 cm layer; TP, P-fertilizer applied to the 0-30 cm layer; MP, P-fertilizer applied to the 30-60 cm layer; SP, P-fertilizer applied to the 60-90 cm layer. ANP, N-, and P-fertilizer applied to the 0-90 cm layer; TNP, N-, and P-fertilizer applied to the 0-30 cm layer; MNP, N-, and P-fertilizer applied to the 30-60 cm layer; SNP, N and P-fertilizer applied to the 60-90 cm layer. The same as below.

Table 3 *F*-values from the ANOVA of F_v, comparing different water and fertilizer treatments

Treatment	N	P	NP
W	2.394	4.068^{*}	9.814^{**}
D	2.888	7.477^{*}	3.795

Values followed by * and ** are different significantly at $P<0.05$ and $P<0.01$, respectively.

quantum yield of net photosynthesis. It changes only slightly in non-stress environments, and is almost free from the influence of different species and growing environments, but it declines markedly under stress, making it a useful probe and indicator. Table 4 indicates that F_v/F_m was significantly lower in the dry treatment than in the wet treatment ($P<0.01$). The differences between F_v/F_m among N, P, and NP treatments were insignificant in both wet and dry treatments, but all were significantly greater than for the CK treatment ($P<0.05$), showing that fertilization helped to enhance wheat PS2 photochemical efficiency.

Table 4 Difference of F_v/F_m between different water and fertilizer treatments

Treatment	CK	N	P	NP	Average
W	0.7655 b	0.7745 ab	0.7761 ab	0.7984 a	0.7786 A
D	0.7046 b	0.7585 a	0.7680 a	0.7846 a	0.7539 B

Fig. 2 illustrates the effect of fertilizer placement on F_v/F_m. For the N treatment, F_v/F_m was higher when the fertilizer was applied to the 0-90 or 30-60 cm layers, compared to when it was applied to the 0-30 or 60-90 cm layers in both wet and dry treatments. For the P treatment, the order of F_v/F_m, from highest value to the lowest, was 0-90, 0-30, 30-60, and 60-90 cm, in both the wet and dry treatments. The same order was observed for the NP wet treatment. But, in the dry treatment, F_v/F_m was the largest when the fertilizer was applied to the 0-30 cm layer, followed by when the fertilizer was applied to the 0-90 cm layer. The effect of fertilizer placement on F_v/F_m was significant in both wet and dry treatments ($P<0.05$).

The effect of spatial coupling of water and fertilizer on the potential PS2 activity (F_v/F_0) of winter wheat leaves

The ratio of F_v to F_0 is an important indicator of poten-

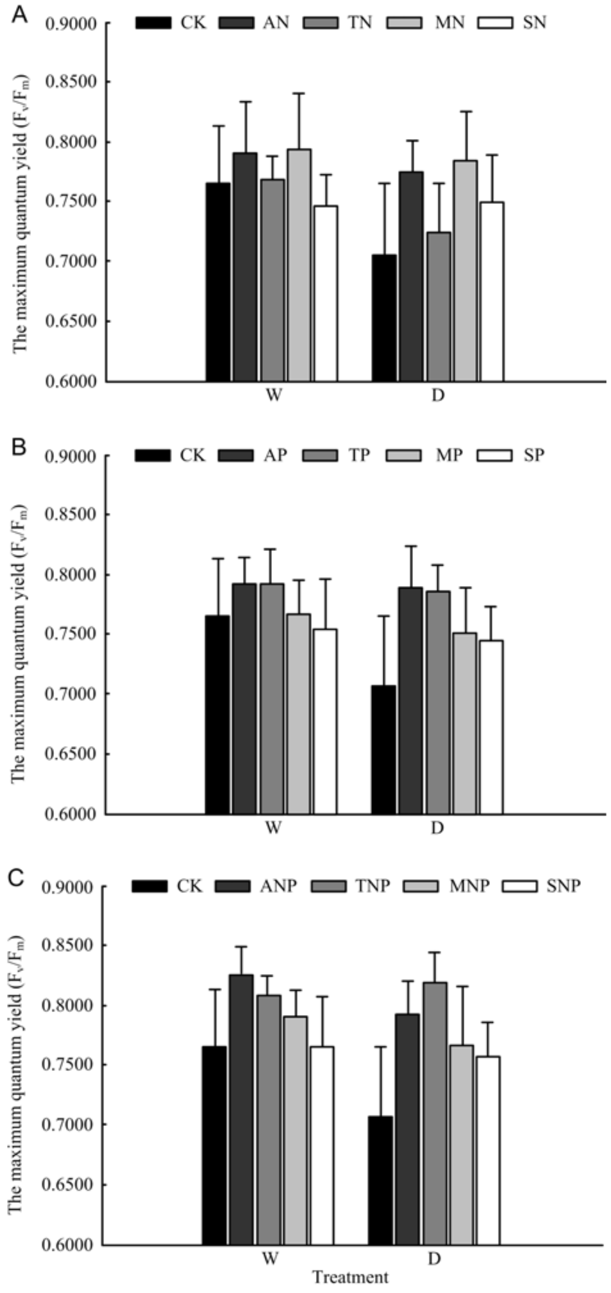

Fig. 2 The maximun quantum yield (F_v/F_m) for different placements of N, P, and NP fertilizer and water treatments of winter wheat.

tial PS2 activity. F_v/F_0 was generally lower in the dry treatment than in the wet treatment (Table 5). When the fertilizer was applied to the 0-90 cm layer, the differences in F_v/F_0 values among the CK, N, and P treatments were not significant. The values were smaller than those for the NP wet treatment ($P<0.05$). In the dry treatment, the F_v/F_0 values of NP, P, and N treatments were not significantly different. They were all significantly higher than that for the CK treatment ($P<0.05$). When the fertilizer was applied to the 0-30 cm layer, the F_v/F_0 of the NP wet treatment showed no significant difference from that for the P wet treatment, but was significantly different from that for the N and CK wet treatments ($P<0.05$). In the dry treatment, the F_v/F_0 of the CK treatment was close to that for the N treatment, there being no significant difference between them. They were significantly lower than that for the P treatment or NP treatments ($P<0.05$). The F_v/F_0 for the P treatment was significantly lower compared that for the NP treatment ($P<0.05$).

When the fertilizer was applied to the 30-60 and 60-90 cm layers, there was no significant difference in F_v/F_0 between different wet fertilizer treatments. The differences between the N, P, and NP dry treatments were not significant, but were all significantly higher than for the CK dry treatment ($P<0.05$). For the N treatment, F_v/F_0 was greatest when the fertilizer was applied to the 30-60 cm layer in both wet and dry treatments. For the P treatment, F_v/F_0 was the greatest when the fertilizer was applied throughout the column, in both wet and dry treatments. For the NP treatment, the order of F_v/F_0 was not consistent between plants that were well watered and those which were water stressed. In the wet treatment, the differences between F_v/F_0 values were highly significant ($P<0.01$), being the greatest when the fertilizer was applied throughout the column. For plants fertilized within a single 30 cm layer the F_v/F_0 values decreased significantly with increasing depth of the layer. In the dry treatment, F_v/F_0 was the highest when the fertilizer was applied to the 0-30 cm layer, followed by when the fertilizer was applied throughout the column. The difference was significant ($P<0.05$).

DISCUSSION

As expected, the photosynthetic characters of winter wheat, especially PS2, showed strong responses to environmental stress. In our study, all chlorophyll fluorescence parameters were affected, to some extent, by water stress, nutrient deficiency (N and P), and nutrient placement. These factors reliably produced differences in PS2 photochemistry. Our results indicate that water stress increased F_0, while F_m, F_v, F_v/F_m, and F_v/F_0 were significantly reduced.

Table 5 Effects of different treatments on F_v/F_0 for winter wheat

Treatment	F_v/F_0	Treatment	F_v/F_0	Treatment	F_v/F_0	Treatment	F_v/F_0
WCK	3.3944 b	WCK	3.3944 b	WCK	3.3944 a	WCK	3.3944 a
WAN	3.8979 b	WTN	3.3385 b	WMN	4.0744 a	WSN	2.9883 a
WAP	3.8957 b	WTP	3.8725 ab	WMP	3.3478 a	WSP	3.1362 a
WANP	4.8591 a	WTNP	4.2740 a	WMNP	3.8429 a	WSNP	3.3989 a
DCK	2.4952 b	DCK	2.4952 c	DCK	2.4952 b	DCK	2.4952 b
DAN	3.4801 a	DTN	2.7023 c	DMN	3.7740 a	DSN	3.0953 ab
DAP	3.8284 a	DTP	3.7392 b	DMP	3.1230 ab	DSP	2.9600 ab
DANP	3.9417 a	DTNP	4.6681 a	DMNP	3.4883 a	DSNP	3.1652 a

A, fertilizer placement in 0-90 cm; T, fertilizer placement in 0-30 cm; M, fertilizer placement in 30-60 cm; S, fertilizer placement in 60-90 cm.

In agreement with many previous studies, our results show that water stress seems to be the primary limitation to photosynthetic capacity (DaMatta *et al.* 2002; Hassan 2006; Wu *et al.* 2008). The increase in growth between well-watered unfertilized plants and water stressed fertilized plants was greater than between well-watered unfertilized and fertilized plants, especially for those fertilized with N or P only. This is consistent with the findings of Yin (2009).

Since, nutrient deficiency suppresses growth rate and leaf photosynthesis, we investigated the effect of fertilizer application on the chlorophyll fluorescence parameters. In sunflower (Ciompi *et al.* 1996) and sorghum (Cechin 1998), N deficiency did not significantly change F_v/F_m, indicating no reduction in PS2 efficiency. In contrast decreases in F_v/F_m were reported in maize (Khamis *et al.* 1990) and common bean (Lima *et al.* 1999) grown in sub-optimal conditions. Significant declines in F_v/F_m have been observed only in P deficient plants, suggesting that photoinhibition is at least partly responsible for the reduction in CO_2 assimilation by P deficient plants (Campbell and Sage 2006; Zhou *et al.* 2009). Our results indicate that although drought stress was destructive to the PS2 reaction center, the effects could be significantly ameliorated by the application of P fertilizer, but not by N alone. There was an increased F_v/F_m with P application, both in well-watered and water stressed plants. Overall the results indicated that applying a combination of the two fertilizers would be the most effective for reducing drought damage.

The indiscriminate application of fertilizer can waste resources and lead to environmental damage. For guiding the proper application of fertilizer to maximize production, while helping to preserve ecological integrity and ameliorating the effects of existing ecological degradation, the spatial coupling of water, nitrogen, and phosphorus was considered according to the characteristics of the Loess plateau soil in our study. The result showed that the effect of fertilizer placement varied among treatments. For plants, given just N fertilizer, there was no significant effect of varying the depth or extent of fertilizer application, and given P only, F_m and F_v/F_m were significantly higher when the fertilizer was applied either throughout the column or within the top soil layer. For well watered plants, given both fertilizers, the placement and extent of fertilizer application had a significant impact on fluorescence parameters, with F_m and F_v/F_m being the greatest when the fertilizer was applied throughout the column. This shows that adding the combined fertilizer can improve photosynthesis even in the absence of drought. For drought stressed plants, F_v/F_m was significantly greater when the combined fertilizer was placed within the top 30 cm of the soil.

Chlorophyll fluorescence has proven useful for quantifying the changes in the function of the photosynthetic apparatus. Intricate relationships between fluorescence kinetics and photosynthesis help our understanding of photosynthetic biophysical processes. Our results indicated that photosynthetic rate (P_n) was positively related to the mean values of F_v/F_m ($R^2=0.6978$, $P<0.05$) and F_v/F_0 ($R^2=0.6961$, $P<0.05$). In wheat, despite the extensive investigation of the relationship between photosynthetic rate and grain yield in recent years, the debate continues. In this analysis, positive relationships between grain yield and the values of P_n were found at different growth stages with R^2 values ranging from 0.63 ($P<0.01$) to 0.91 ($P<0.01$).

We conclude that in semi-arid areas, where the upper soil is dry and the lower soil is moist, applying a combined fertilizer to the top 30 cm of soil could improve photosynthesis and help to protect wheat plants from drought damage. So, in order to improve the efficiency of fertilizers and minimise the environmental impact, when growing winter wheat in the Loess region of China, it is important to guarantee the nutrient supply in the top 30 cm of soil, not only where the water distribution down the soil profile is similar to that simulated here, but also in arid and semi-arid areas.

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