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In-field assessment of foliar cytokinin timing and dosage effects on morphological traits, photosynthesis, yield and quality in common wheat

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ABSTRACT

The increasing frequency of heat waves and drought during wheat ripening undermines yield potential and grain quality by accelerating plant senescence. This study investigated the effects of late foliar cytokinin (6-benzyladenine, 6-BA) application timing and dosage on leaf greenness, photosynthesis, yield, and grain quality in open-field wheat cultivation in NE Italy. Four application timings were evaluated: at flowering (T1), and at 10 (T2), 20 (T3), and 30 (T4) days after flowering, and two double applications (T1+T3; T2+T3), at three 6-BA doses (25, 50 and 150 g ha^{-1}) compared with untreated controls (C). T1 application significantly prolonged canopy greenness ($p \leq 0.05$), while increases in CO_2 assimilation (+15 %) and yield (+6 %) were non-significant. Grain protein content improved significantly, and gluten composition shifted toward a higher glutenins/gliadins ratio. Later applications, especially T3, significantly increased leaf chlorophyll content, the glutenins/gliadins ratio, and low-molecular-weight glutenins, whereas double applications mainly enhanced root development in the upper soil profile (+20 to +33 %) without yield benefits. T2 and T4 were largely ineffective for canopy greenness, but showed selective positive effects on protein or root traits. Overall, yield responses remained non-significant, but cytokinin effects were stage-dependent, with treatments at flowering providing the most consistent benefits at doses ≥50 g ha^{-1}. Taken together, these findings support a dual-role model of cytokinin action: early 6-BA sprays delay senescence and enhance N remobilization, while later sprays alter gluten composition, highlighting the potential of cytokinin treatments to improve wheat resilience and quality under field conditions; validation across environments and genotypes is still required.

1. Introduction

Global demand for wheat grain is steadily increasing in parallel with rapid population growth and stricter food technology standards. Meanwhile, wheat yield and quality are increasingly threatened by several constraints, most notably spring/summer heat stress and drought—factors that are likely expected to increase in frequency and intensity due to climate change (Farhad et al., 2023; Moriondo and Bindi, 2007; Lamaoui et al., 2018). Drought stress rapidly reduces leaf expansion and stomatal conductance, leading to a decline in both the activity and duration of photosynthesis (Sedaghat et al., 2017). Meta-analyses of global wheat production have reported average yield losses ranging from 17 % to 70 % associated with drought events, significantly undermining the global stability of wheat supplies (Wan et al., 2022; Nouri-Ganbalani et al., 2009). The duration and severity of drought stress are critical, with the reproductive and grain-filling periods showing the higher susceptibility to water deficits (Farooq et al., 2014).

Heat stress similarly triggers multiple adverse biochemical and physiological changes at both the cellular and whole-plant levels, resulting in yield and quality impairments. At the physiological level, high temperatures disrupt the photosynthetic system by altering the ultrastructure of organelles and lowering Rubisco activity, ultimately leading to decreased carbon assimilation and accelerated leaf senescence (Scafaro et al., 2023; Mathur et al., 2014). Regarding crop phenology, high temperatures during the final developmental stages shorten the growing cycle by accelerating the onset of leaf senescence and decreasing the grain-filling period. Consequently, both the

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interception of photosynthetically active radiation by green tissues and the remobilization of nutrients to developing kernels are significantly reduced (Kumar et al., 2023; Criado et al., 2009; Gooding et al., 2000; Prasad et al., 2017). Banowetz et al. (1999) found that a 7-day high-temperature treatment applied within one day of anthesis significantly reduced kernel cytokinin content by ~80 %, leading to a ~27 % reduction of mature kernel weight. This highlights a direct hormonal pathway that links heat stress to impaired sink activity and decreased kernel weight.

Heat stress adversely affects reproductive organs and kernel growth processes. During grain filling, high temperatures have been associated with substantial alterations in carbon metabolism, including decreased activity of key enzymes involved in starch synthesis, which leads to a reduced starch accumulation in kernels (Zahra et al., 2023; Zhao et al., 2008). Furthermore, the incidence of pathogens and diseases on plant tissues can increase, reducing green leaf area and potentially compromising the sanitary quality of grains due to mycotoxin contamination (Juroszek et al., 2020).

Enhancements in both yield and technological quality of winter wheat may be achieved through strategies that preserve photosynthetic capacity by delaying leaf senescence during grain filling. Several studies have demonstrated that extending the duration of the photosynthetically active leaf area–where the role of the flag leaf is crucial–can positively affect grain yield as well as kernel size and quality parameters (Augšpole et al., 2024; Zhang et al., 2010; Luo et al., 2006; Marinaccio et al., 2015; Entz and Fowler, 1990; Chen et al., 2010). The flag leaf, in particular, strongly contributes to grain filling by supplying photosynthates to the developing grain and serving as an essential source of remobilized nutrients after senescence (Jeong et al., 2017).

Various strategies to delay leaf senescence have been investigated in the recent literature, including the use of prolonged stay-green cultivars and the application of late-season nitrogen and sulfur foliar fertilizers (Blandino et al., 2020; Thomas and Howarth, 2000; Chen et al., 2010; Marinaccio et al., 2015; Bly and Woodard, 2003). While these approaches have shown promise, additional strategies that can be readily integrated into current wheat management systems are still needed, especially given the increasingly variable climatic conditions.

Cytokinins are adenine- or phenylurea-based compounds that regulate various plant growth and developmental processes and play a key role in modulating plant responses to abiotic stresses (Cortleven et al., 2019; Ha et al., 2012). At the molecular level, CKs alter the gene expression linked to photosynthesis, nutrient mobilization, and antioxidant systems, and they also interact with other hormonal signalling pathways to control responses to stress (Hudeček et al., 2023).

Under a short-term or mild stress, a transient elevation in CKs level is observed, which is associated with the promotion of adaptation strategies such as enhanced cell division, increased antioxidant activities, and greater sink capacity (Havlova et al., 2008; Yang et al., 2002, 2016; Panozzo et al., 2025). More recently, Liu et al. (2025) found that moderate water limitation improved source-to-sink N remobilization in wheat by moving trans-zeatin and trans-zeatin riboside from stems and leaves to spikes, enhancing grain N accumulation and nitrogen-use efficiency.

In contrast, under severe and prolonged stresses, adaptive responses–such as the maintenance of cell membrane integrity and stability–are instead associated with the down regulation of CKs, which can act as negative regulators of stress signalling. In light of their dual role, cytokinins are promising for their exogenous use in crop management in versatile environmental settings (Li et al., 2022b; Kumari et al., 2018). A decline in cytokinin levels in senescing leaves acts as a conserved molecular trigger for senescence across species, influencing chlorophyll retention, photosynthetic ability, and nutrient remobilization. While acting as a crucial signal for the onset of the senescence process, CKs delay senescence by a variety of mechanisms, including inhibiting chlorophyll breakdown enzymes, upregulating antioxidant defence systems and maintaining photosystem II efficiency. Therefore, the exogenous application of cytokinins on wheat could delay the onset of leaf senescence and prolong the photosynthetic competence—particularly in the flag leaf—potentially benefiting grain filling and enhancing abiotic stress tolerance (Jibran et al., 2013; Ciura and Kruk, 2018; Hönig et al., 2018).

Cytokinins have been also shown to alter root system architecture by enhancing root density and lateral branching, hence increasing water and nutrient absorption capacity—characteristics that are especially important under drought stress circumstances (Mao et al., 2020; Ramireddy et al., 2018). Genetic modification of CK metabolism enzymes, such as cytokinin oxidase/dehydrogenase, has shown that higher CK levels increase root development and give stronger stress tolerance in cereals (Jameson and Song, 2016).

In parallel, CKs can indirectly increase grain protein formation and grain filling by stabilizing chlorophyll concentration, delaying leaf senescence, and preserving photosynthetic efficiency throughout the key grain-filling phase. These physiological effects work collectively to promote assimilation and nitrogen remobilization in growing kernels, potentially increasing grain production and quality (Cortleven et al., 2019; Jameson and Song, 2016; Yang et al., 2016). Although direct evidence that CKs modulate the glutenin-to-gliadin protein ratio in wheat is limited, the balance between these two protein classes is a well-established driver and major determinant of dough strength and bread-making quality (Shewry and Halford, 2002; Wieser, 2007). The ability of CKs to impact this equilibrium needs further exploration, particularly in field conditions.

Despite their widespread use in fruit tree management, the exogenous application of phytohormones has received little attention in field crop agriculture. To date, very few experimental studies investigated the effects of exogenous application of cytokinins in wheat crop; most have been carried out in vitro (Xie et al., 2004) or in pots under controlled conditions (Criado et al., 2009), and have focused on the plant's responses related to adaptation to abiotic stresses (Gupta et al., 2012; Kumari et al., 2018; Yang et al., 2016).

Notably, Chipilski et al. (2021) found that field application of 6-benzyladenine (6-BA) or kinetin increased seed viability, flag-leaf chlorophyll index, and productivity in two winter wheat cultivars, indicating the effectiveness of foliar cytokinin interventions in the field.

However, knowledge of the effects of exogenous cytokinins application under open field-conditions on post-anthesis canopy photosynthetic dynamics, root traits, yield and grain quality remains scant. Moreover, information concerning the optimal timing and dosage of CKs application is very limited, underscoring the need for further research. To our knowledge, no peer-reviewed field study has systematically tested multiple post-anthesis application timings combined with varying doses of 6-BA to evaluate their effects on canopy photosynthesis, root development, yield components, and grain protein partitioning (including gliadins and glutenins, and low- and high-molecular weight glutenin fractions), underscoring the novelty of the present study.

Within this framework, the present study investigated the effectiveness of the exogenous cytokinins application via foliar spraying under open-field conditions to delay leaf senescence, prolong the leaf photosynthetic competence, and enhance grain yield and quality. A product containing the synthetic cytokinin 6-BA was used. Innovatively, four different timings of CKs application after flowering and three doses of the active ingredient 6-BA for each timing were investigated, with the following objectives: (***i***) to assess the impact of cytokinin application on the duration of leaf greenness and photosynthetic efficiency during grain filling; (***ii***) to assess the impact of exogenous cytokinins on root growth parameters; and (***iii***) to identify the most effective timing and dosage of CK application that best translate canopy and root effects into yield and quality of grains at harvest, with particular emphasis on gluten composition. Overall, this study aimed to determine whether cytokinin treatments might mitigate abiotic stress during wheat's grain-filling, presenting a holistic approach for improving production stability in a changing climate.

2. Materials and methods

2.1. Experimental site

A field trial was conducted during the 2019–2020 growing season at the "Lucio Toniolo" experimental farm of the University of Padua, Italy (Legnaro, Padua, NE Italy). The site had a silty-loam soil (fulvi-calcaric-cambisol, USDA classification), with 19 % clay, 65 % silt, 16 % sand, 1.65 % organic matter, 0.1 % total nitrogen content, a cation exchange capacity (CEC) of 11.4 cmol (+) kg^{-1}, and a pH of 7.75. Climatic conditions were recorded locally by a meteorological station, which revealed deviations from historical averages during the trial period. Mean monthly temperatures were lower than the 10-year historical average in December (−1 °C), February, and March (~−2 °C), but were consistently higher from April until June (+1.5 °C) (Figure SI1). From October 2019 to June 2020, the site received approximately 550 mm of precipitation (~60 mm per month), with significant heterogeneity in the rainfall patterns. February and March experienced abundant rainfall, whereas the other months were drier than the 10-year historical mean–particularly in January (17 vs. 42 mm), and during spring, April (30 vs.63 mm) and May (70 vs. 87 mm) (Figure SI1).

2.2. Field preparation and crop management

Soil was prepared by ploughing to a depth of 0.3 m and harrowing to 0.15 m. Prior to sowing, the experimental field was fertilized with 32 kg ha^{-1} N, 96 kg ha^{-1} P_2O_5, and 96 kg ha^{-1} K_2O, which were incorporated into the soil by harrowing. The previous crop was sugar beet.

Common wheat (*Triticum aestivum* L.) var. Bologna (SIS, Bologna, Italy) was chosen for this experiment due to its widespread cultivation in the region and its high-quality bread-making characteristics. Seeds, treated with Prothioconazole and Tebuconazole, were sown on October 27, 2019, at a sowing density of 650 seeds m^{-2} (equivalent to 221 kg ha^{-1}) in rows 0.14 m apart. Nitrogen was supplied in three applications: 32 kg N ha^{-1} before sowing, followed by two applications of ammonium nitrate (26.5 % N) during the growing season, and one foliar N fertilizer application at anthesis, totalling 160 kg N ha^{-1}. This fertilization regime follows standard agronomic practices for high-quality bread wheat in the region. The crop was protected against fungal pathogens by spraying Azoxystrobin and Cyproconazole twice—at the beginning of April and in early May 2020—following local agronomic recommendations. Mechanical harvest was conducted on June 20, 2020.

2.3. Experimental design and treatments

A completely randomized design was employed with three replicates per treatment. Each experimental plot measured 3-m in length and 1-m in width (3 m^2). Cytokinins (CKs) were applied via foliar spraying using the plant growth regulator MaxCel (Sumitomo Chemical, Milano, Italy), which contains 6-benzyladenine (6-BA) as the active ingredient (a.i.) at a concentration of 20 g L^{-1} and is commercially registered for use in apple orchards, given the lack of commercial products registered for wheat crops. Four timings of CKs application were tested: at flowering (T1), 10 days after flowering (DAF) (T2), 20 DAF (T3) and 30 DAF (T4). Additionally, two double treatments were also investigated: T1+T3 (applications at flowering and 20 DAF) and T2+T3 (applications at 10 and 20 DAF), resulting in six timing treatments (Table 1).

For each application timing, three concentrations of 6-BA, and amounts of the a. i., were tested:

- 50 ppm (D1): 25 g ha^{-1} of 6-BA (1.25 L $ha^{-1}$of MaxCel)
- 100 ppm (D2): 50 g $ha^{-1}$of 6-BA (2.5 L $ha^{-1}$of MaxCel)
- 300 ppm (D3): 150 g $ha^{-1}$of 6-BA (7.5 L $ha^{-1}$of MaxCel)

These concentrations were selected based on preliminary trials and literature on fruit tree applications, adapted for field crop conditions. In total, 18 'time × dose' treatments were included and compared with an untreated control (C). MaxCel was applied using a calibrated shoulder pump sprayer at a consistent spraying water volume of 500 L ha^{-1} to ensure uniform coverage.

2.4. Canopy and leaf measurements

2.4.1. Normalized Difference Vegetation Index (NDVI)

Normalized Difference Vegetation Index (NDVI) was measured from the last week of April until maturity twice a week on wheat canopy of each plot, using an active handheld Greenseeker spectrometer (Ntech Industries, Ukiah, CA, USA). The sensor measures canopy reflectance at wavelengths 590 nm (red) and 880 nm (near-infrared) and calculates NDVI as follows:

$$NDVI = \frac{refNIR - refRED}{refNIR + refRED}$$

This index, which values range from 0 to +1, provides an accurate indication of the canopy greenness, which correlates with plant health and soil coverage by green vegetation.

To account for baseline variability, NDVI values were standardized using the measurement taken on 6 May (before cytokinin application) as the baseline; subsequent values were expressed relative to this reference, allowing to take into account for possible treatment differences existing before CKs spraying (Figure SI2).

2.4.2. Chlorophyll content

The leaf chlorophyll content was measured twice per week from mid-May to the first week of June, using a Soil Plant Analysis Development (SPAD)-502 chlorophyll meter (Konica-Minolta, Hong Kong) (Hoel and Solhaug, 1998; Chang and Robison, 2003). Measurements were taken on the flag leaf of six randomly selected tagged plants in each plot/replicate. To account for potential within-leaf variation, two measurements were taken on each plant, one at one-third and one at two-thirds of the leaf length, then averaged. Similarly to NDVI, SPAD values were also standardized to the 6 May measurement, with later values expressed relative to this baseline (Figure SI3).

2.4.3. Chlorophyll fluorescence and gas exchange

Leaf chlorophyll fluorescence and gas exchange measurements were conducted after all CKs treatments were completed, on June 2, 2020, at roughly early dough maturity, between 8:30 and 11:30 a.m. using an

Table 1
List of treatments with the timing of cytokinin applications, corresponding application dates, and the associated wheat phenological stage with reference to days after flowering (DAF).

List of treatments Time of CKs application	Date	Phenological stage/Time
T1	May 7, 2020	Flowering
T2	May 17, 2020	10 DAF
T3	May 27, 2020	20 DAF
T4	June 6, 2020	30 DAF
T1+T3	May 7, 2020 + May 27, 2020	Flowering +20 DAF
T2+T3	May 17, 2020 + May 27, 2020	10 DAF +20 DAF

infrared gas analyzer Li-Cor-6800 (Li-Cor Inc., Lincoln, NE, USA). Measurements were performed on the flag leaf of three randomly selected wheat plants per plot/replicate per treatment. The instrument was calibrated according to the manufacturer's recommendations. In the measurement chamber, the photosynthetic photon flux density (PPFD) was set at 1000 μmol m^{-2} s^{-1}, the temperature was maintained at 27 °C, and the relative humidity controlled at 55–65 %. The reference CO_2 concentration was set at 400 μmol mol^{-1}, reflecting ambient atmospheric conditions.

After leaves were allowed for dark adaptation, a weak measuring light was switched on to elicit a minimum value for chlorophyll fluorescence (F_o) with all PSII reaction centers open. Actinic light of 1000 μmol m^{-2} s^{-1} (10 % blue, 90 % red) was then applied to the same leaves for 15–20 min, followed by a saturating light pulse applied for 0.8 s to record maximum fluorescence (F_m). PSII photosynthetic efficiency, as a ratio between variable and maximum fluorescence (Fv'/Fm'), and CO_2 Net Assimilation (A) were determined following the methodology described by Murchie and Lawson (2013).

2.5. Grain yield and quality assessment

Grain yield was determined at maturity by manually harvesting all plants from a 1 m^2 area per plot (n = 3) and threshing them with a stationery thresher.

The harvest Index (HI, grain-to-total shoot weight ratio) was determined from a sampling area of 0.5 m^2 in each plot. Plants from this area were harvested, and grains and straw separated and weighed after oven-drying at 105 °C for 36 h. The testing weight of wheat grains was determined using the GAC 500XT (Dickey-John, Auburn, IL-USA). Thousand kernel weight (TKW) was calculated by weighing three replicate samples of 1000 grains from each treatment.

Nitrogen concentration in the grains and straw was determined according to the Kjeldahl method (Sáez-Plaza et al., 2013). Gluten proteins analysis was performed on 30-g seed samples (n = 3) gently milled using six pulses of 10 s each with a Knifetec 1095 (Foss, Hillerod, Denmark). Gliadins, high-molecular-weight glutenin subunits (HMW-GS), and low-molecular-weight glutenin subunits (LWM-GS) were sequentially extracted from 30-mg subsamples, following the protocol described by Visioli et al. (2018).

Relative quantification of HMW-GS, LMW-GS, and gliadins was conducted spectrophotometrically using the Bradford colorimetric assay (Bio-Rad, Hercules, CA, USA) at the 595 nm wavelength, with three technical replicates for each sample. Linear regression between absorbance and protein concentration was established through calibration with Bovine Serum Albumin (BSA) standards, and the results were expressed as mg g^{-1} of wheat flour.

2.6. Root growth analysis

Root growth parameters were assessed by collecting 70-mm diameter, 1-m deep soil cores on June 11, 2020, at end-dough maturity. One core per plot was sampled from the inter-row space between two wheat rows at the centre of the plot to minimize edge effects. This timing was selected to capture potential treatment effects during the late grain-filling period. Each soil core was divided into 0.1 m-deep subsamples to analyse the vertical distribution of root profile. The roots were separated from the soil by flotation using a hydraulic centrifugation device and collected in a 500-μm mesh sieve according to the methodology of do Rosário et al. (2000). The separated roots were stored at 4 °C in ethanol solution (12 % v/v) until analysis.

TIFF-format images (1-bit at 400 DPI resolution) of the root samples were acquired using a flatbed scanner (EPSON Expression 11000XL, Canada). WinRHIZO ver. 2007b image analysis software (Regent Instruments Inc., Canada) was used to measure root length, area, and diameter, with a threshold area >30 pixels (0.0015 cm^2) applied to eliminate background noise. Root length and area were measured per unit soil volume for each depth interval to calculate root length density (RLD, cm cm^{-3}) and root surface density (RSD, cm^2 cm^{-3}).

2.7. Statistical analysis

The data of the leaf characteristics, yield and its components, root parameters, and grain quality parameters were subjected to analysis of variance (ANOVA) using R studio software ver. 1.4 (RStudio Public Benefit Corporation (PBC), Boston, MA, USA). For treatments with significant effects ($p \leq 0.05$), means were separated using Tukey's Honest Significant Difference (HSD) test.

To holistically assess the overall effects of cytokinin timing and dose, multivariate analyses were performed, including Multigroup Discriminant Analysis (MDA) with Wilks' lambda and Pillai's trace tests, and principal component analysis (PCA). Analyses included the following significant key parameters: yield, harvest Index (HI), thousand kernel weight (TKW), grain protein content, HMW- and LMW-GS, glutenins-to-gliadins ratio, straw N content, SPAD, NDVI, CO_2 net assimilation (A), PSII efficiency (Fv'/Fm'), and root diameter. For NDVI and SPAD, the averages of the last two measurement dates were used, as these values better reflect cytokinin activity during the onset of senescence. Multivariate data normality was first verified by the Shapiro test. Before analysis, data were standardized by subtracting the mean and dividing the result by the standard deviation within each variable. All analyses were performed in MS Excel XLSTAT (Addinsoft, Paris, France).

3. Results

3.1. Normalized Difference Vegetation Index (NDVI)

The foliar application of cytokinins (CKs) significantly enhanced the vegetation indexes in wheat, with the timing of application emerging as the most critical factor determining efficacy.

Applications at flowering (T1) demonstrated the most pronounced and sustained effects on canopy greenness (Fig. 1). Following CKs application at flowering on 7 May, T1-treated plants maintained significantly higher NDVI values compared to untreated controls across all measurement dates until the final measurement date on 7 June, regardless of dosage concentration. The NDVI improvement under T1 was particularly evident during the critical grain-filling period in late May and early June, when control plants were already exhibiting accelerated senescence.

Similarly, CK application on 20 DAF (T3) sustained higher canopy greenness than controls from late May onwards, though the variation was smaller and not statistically significant relative to that observed with T1 treatment. Surprisingly, applications at 10 DAF (T2) and 30 DAF (T4) had no influence on canopy greenness, with NDVI values remaining equivalent to controls or even decreasing slightly, particularly for T2 treatments from early June onward.

In contrast, the double treatment T1+T3 did not reproduce the early-stage NDVI gains observed with T1 alone. Specifically, NDVI values in T1+T3 plots prior to the second application date (27 May) did not differ significantly from controls ($p > 0.05$), despite including the same initial spray at flowering.

The double treatments (T1+T3 and T2+T3) showed intermediary effects overall, with NDVI values numerically higher than controls from late May onward. A statistically significant difference was observed only on 20 May ($p \leq 0.05$), with NDVI values of 0.67 and 0.70 for T1+T3 and T2+T3, respectively, compared to 0.60 in the control. On all other dates, these differences remained non-significant.

The CK dose effects were generally small across all application schedules, with only minimal differences between the three doses investigated. The intermediate dosage D2 (50 g 6-BA ha^{-1}) produced the most significant increases in NDVI values within T1 treatments, whereas the highest dose D3 (150 g 6-BA ha^{-1}) was slightly superior in T3, T1+T3, and T2+T3 treatments (Fig. 1). The visible effect of T1-D2

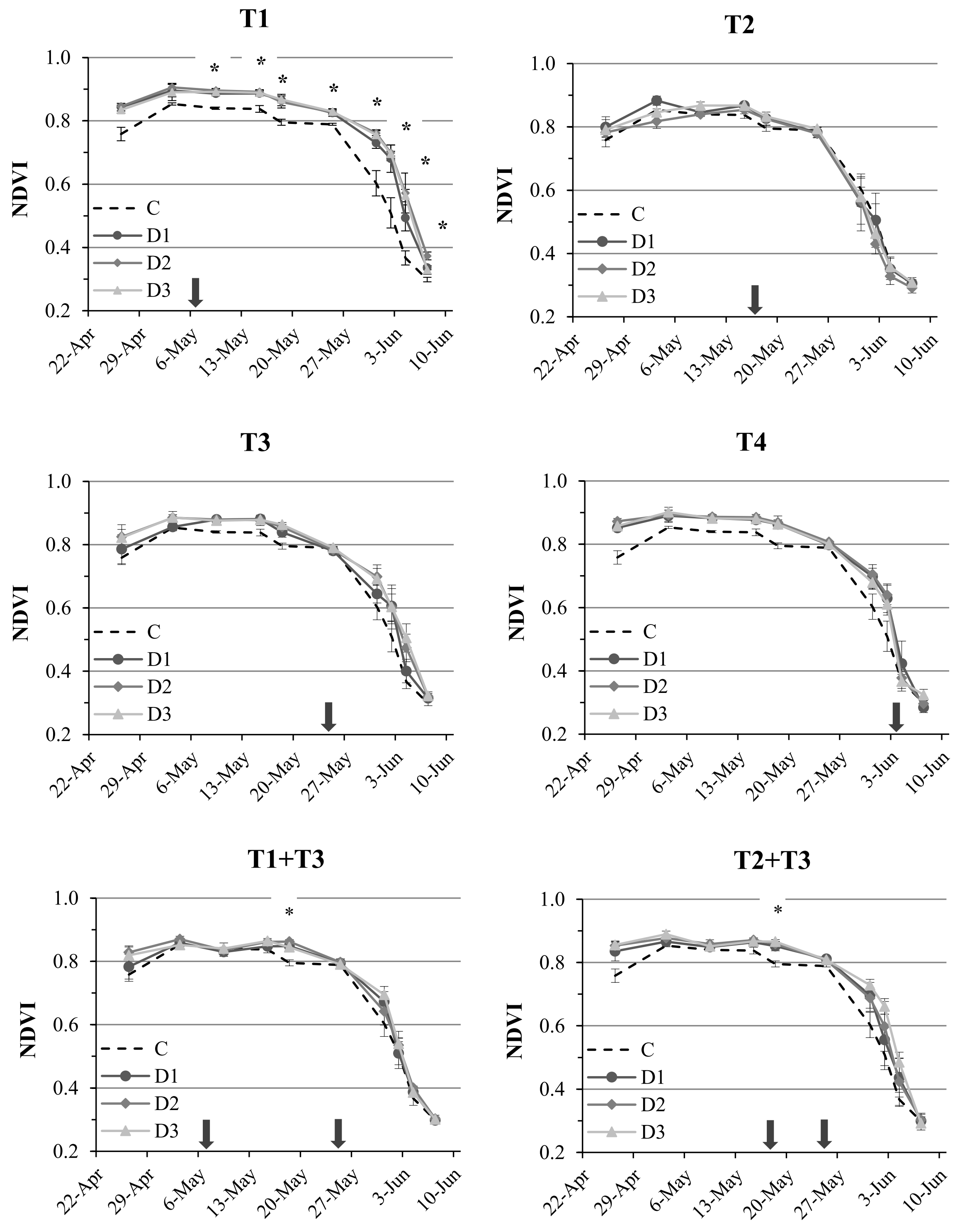


Fig. 1. Dynamics of Normalized Difference Vegetation Index (NDVI) from heading to maturity (mean ± S.E.; n = 3) in wheat plants treated with 6-benzyladenine (6-BA) at different timings of application (T) and doses (D), compared to untreated control (C). Asterisks denote significant differences among treatments for each date of measurement (Tukey's HSD test; $p \leq 0.05$). Arrows indicate cytokinin application dates for each treatment.

combination on wheat canopy greenness was particularly noticeable when compared to untreated plants, as shown in pictures (Fig. 2). The same evidence regarding both CKs application timing and dose effects was observed by analyzing the seasonal dynamics of standardized (on pre-treatment values) NDVI, which highlighted consistently better canopy greenness late in the season for T1 treatment, occasional improvements in T1+T3 and T2+T3, and greater efficiency with intermediate (D2) and high doses (D3) (Figure SI2).

3.2. Chlorophyll content of the flag leaf

The chlorophyll content in the flag leaf, as indicated by SPAD values, closely reflected the patterns seen in canopy NDVI (Fig. 3). T1 treatment yielded the most significant enhancements in leaf chlorophyll content,

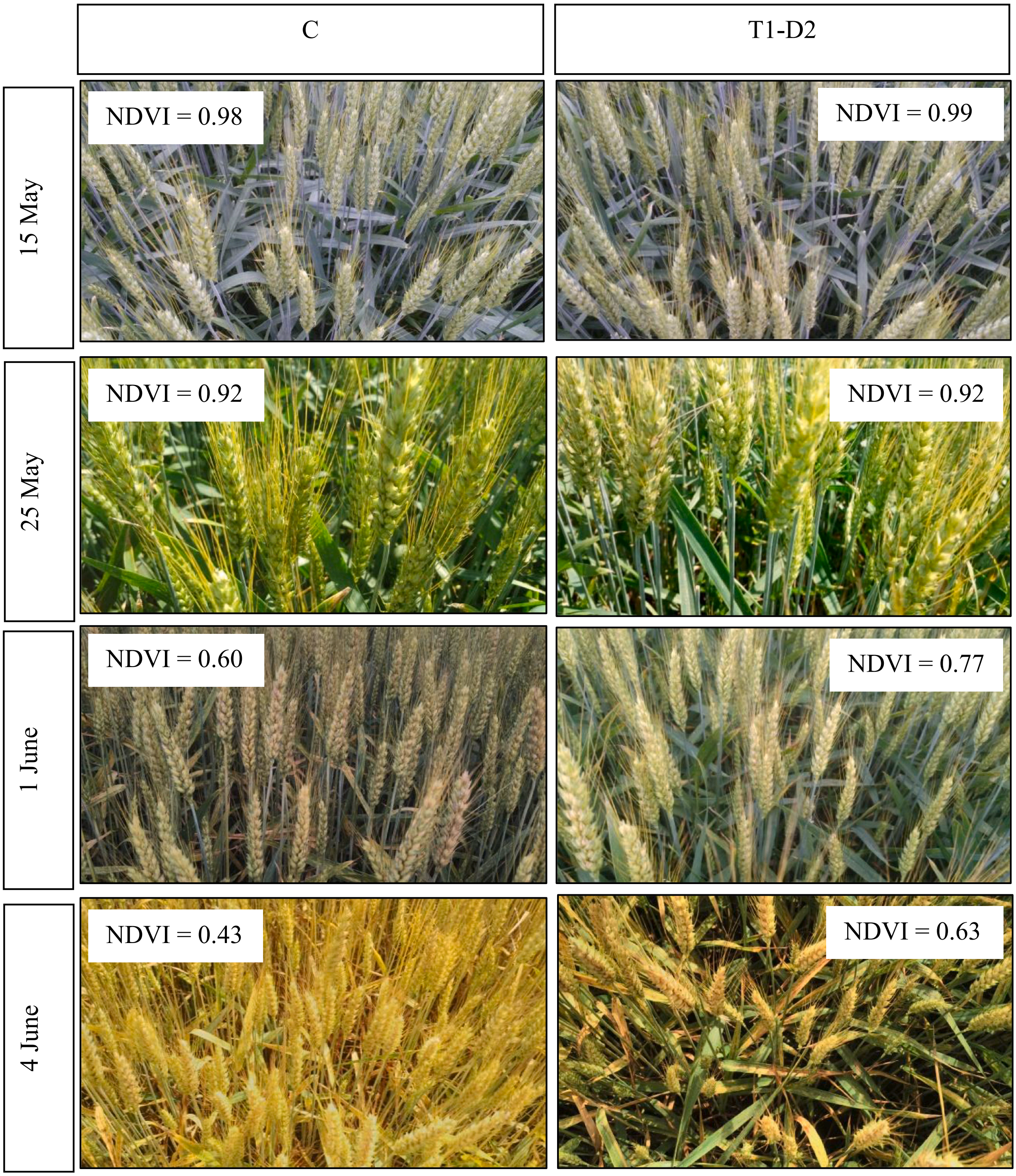


Fig. 2. Visual comparison of wheat plot canopies illustrating differences in leaf senescence from mid-May until early June by comparing untreated control (C; left) and plants treated at flowering (time T1) with 50 g of 6-BA ha^{-1} (dose D2).

sustaining markedly elevated SPAD values relative to controls until the final assessment in early June. Specifically, on 4 June significantly higher SPAD values were recorded under T1 treatment regardless of the dosage, notably with D2 and D3 concentrations (Fig. 3). A similar trend was observed in T3 treatment, with considerably higher chlorophyll retention in the flag leaf following CK application than controls, until the first week of June—again with more pronounced effects at D2 and D3 doses. In contrast with NDVI measurements, for the T1+T3 treatment, only D2 dose maintained slightly higher SPAD values than the untreated control from late May, although these differences were not statistically significant. Conversely, the impact of the double treatment T2+T3 was in line with NDVI, improving SPAD values until the last date of measurement, with significant differences observed at the highest dose (D3). Applications at T2 and T4 had no discernible effect on SPAD, regardless of the CK dosage (Fig. 3).

Standardized SPAD (on pre-treatment values) showed similar results as non-standardized trends, although slight, not significant decreases were observed under T2 and T1+T3 treatments (Figure SI3).

3.3. CO_2 net assimilation (A) and PSII photosynthetic efficiency

Beyond sustaining greenness, cytokinin treatments influenced the functional photosynthetic capability of wheat flag leaf late in the season (2 June). CO_2 net assimilation (A) increased non-significantly only after CKs application at flowering (T1), showing a mean improvement of 12 % across the three investigated doses (2.7 μmol m^{-2} s^{-1} vs. 2.4 in controls;

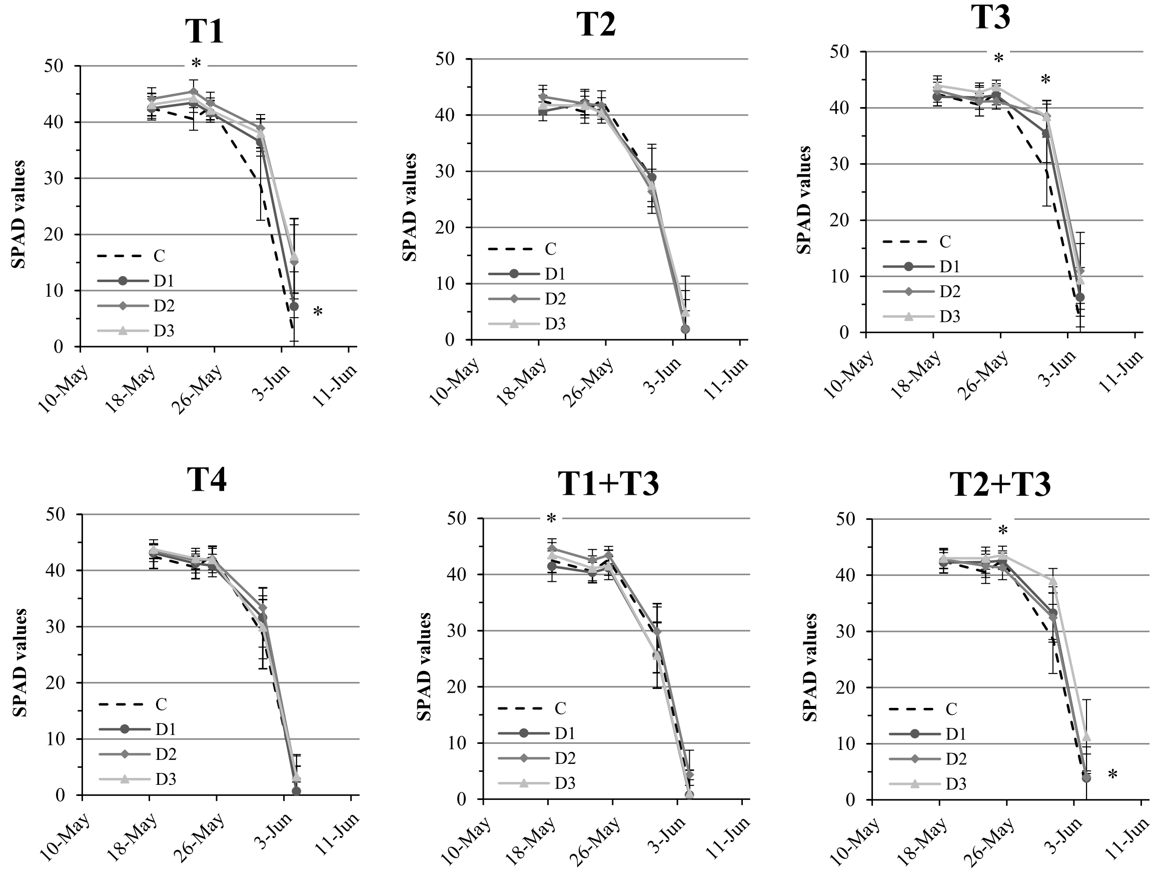


Fig. 3. Dynamics of the flag leaf chlorophyll content, measured as Soil Plant Analysis Development (SPAD) units (mean ± S.E.; n = 3), in wheat plants treated with 6-benzyladenine (6-BA) at different timings of application (T) and doses (D), compared to untreated control (C). Asterisks denote significant differences among treatments for each date of measurement (Tukey's HSD test; $p \leq 0.05$).

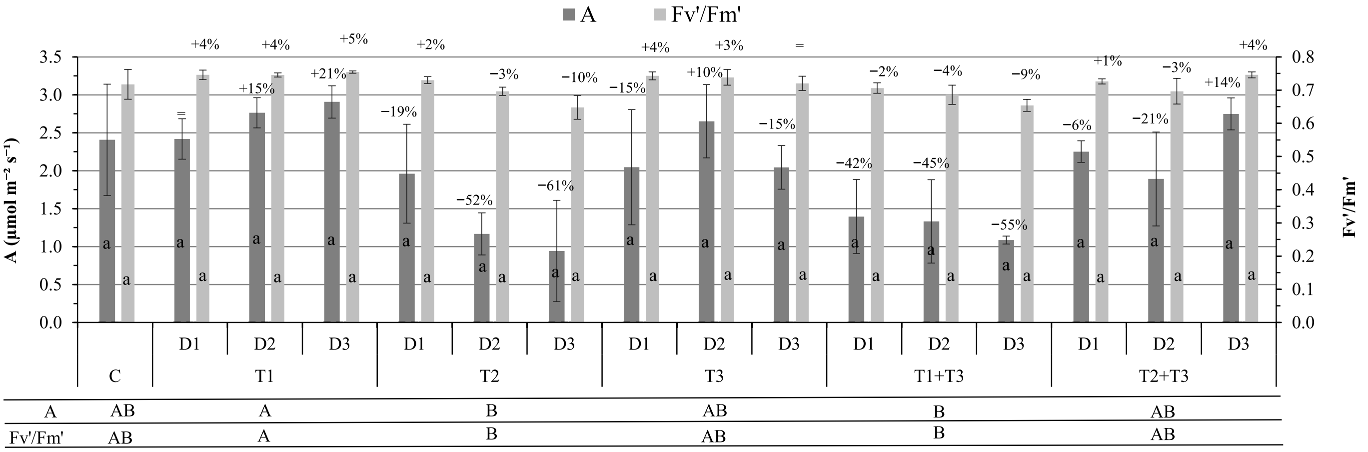


Fig. 4. Flag leaf CO_2 net assimilation (A) and PSII photosynthetic efficiency (Fv'/Fm') (means ± S.E.; n = 3), in wheat plants treated with 6-benzyladenine (6-BA) at different timings of application (T) and doses (D), compared to untreated control (C), measured on June 2, at early dough maturity. Numbers above histograms indicate percentage variation from controls (C). Within each parameter, lowercase letters indicate significant differences for multiple comparisons 'Time × Dose' interaction, and uppercase letters differences for the main effect Application Timing T (Tukey's HSD test, $p \leq 0.05$). Values above histograms represent percentage variation from C. Note: T4 treatment was excluded from the measurement.

$p \geq 0.05$); although the highest dose D3 exhibited a 21 % increase, this difference also did not reach statistical significance (Fig. 4).

Among the other 'time × dose' treatments, only T3-D2 and (T2+T3)-D3 exhibited slight improvements in CO_2 net assimilation, by +10 % and +14 % respectively, though not statistically significant. In contrast, both T2 and T1+T3 treatments resulted in a reduction of CO_2 assimilation following all the CK doses applied, on average 45 % decrease compared to controls ($p \leq 0.05$), possibly related to the weak negative trend on leaf chlorophyll reduction (standardized SPAD) in these treatments.

The photosystem II (PSII) efficiency (Fv'/Fm') showed even slighter variations among treatments than the net assimilation of CO_2. Considering the average of the three CKs doses, a slight increase of Fv'/Fm' was measured with T1 (+4 %) and T3 (+2 %) treatments compared to untreated controls (Fv'/Fm' = 0.71), while small reductions were noted again with treatments T2 (−4 %) and T1+T3 (−5 %) (Fig. 4). No significant dosage effects were appreciable, regardless of the time of application.

3.4. Yield and yield components

The effects of cytokinin treatments on leaf physiology translated into considerable influences on grain yield and quality parameters. Grain yield under T1, T4 and T2+T3 showed numerical increases of 4 %, 3 % and 2 %, respectively, compared to controls (811 g DW m^{-2}), but these differences did not reach statistical significance ($p > 0.05$). T1 showed the most considerable enhancement, particularly at D2 (+6 %) and D3 (+5 %) doses, whereas no positive effects were observed with the lowest dose (D1) (Table 2). Conversely, T2 and T3 applications resulted in modest yield reductions (−5 % and −2 % respectively), and T1+T3 double treatment showed no significant effect on yield when compared to the control group.

Grain testing weight remained unaffected by CKs treatment, showing only slight and not significant reductions relative to controls (~83 kg L^{-1}, $p \geq 0.05$), up to −2 % at maximum depending on the time of application.

Thousand Kernel Weight (TKW) showed a slight increase following applications at T2, T3 and T1+T3 treatments (+2 %, +3 % and +1 % respectively) compared to controls (34.5 g; $p \geq 0.05$), while a negligeable reduction was observed for T1 and T4 treatments (−1 % and −2 % vs. controls; $p \geq 0.05$).

Harvest Index (HI) was affected by CKs application, ranging from 0.45 to 0.47 depending on the application timing but not the dose—with a decrease up to 3 % observed in (T2+T3)-D1, while T3 showed an increase (+1 % vs. C, $p \geq 0.05$).

Statistical analysis revealed that application timing of CKs had a significant impact on grain yield, TKW, and HI ($p < 0.05$), while their dosage had no significant influence on any yield parameters.

3.5. Grain protein content and composition

Cytokinin treatment substantially affected both grain protein content and gluten composition. Grain protein increased following nearly all the application timings, except for the T1+T3 double treatment, which exhibited a slight decrease (−0.3 as absolute variation vs. controls; $p \geq 0.05$). T1 showed the most significant enhancement in protein content, reaching 13.5 % on DW basis compared to 12.5 % in controls ($p \leq 0.05$), particularly with doses D2 and D3 (about +1.2 compared to controls) (Table 3). Applications at T3, T4 and T2+T3 were also associated with higher grain protein content (+0.7, +0.3 and + 0.3, respectively) compared to controls, whereas no appreciable variations were observed following T2 treatment.

The effects on gluten protein composition exhibited complex patterns of response. T3 treatments resulted in a small increase in total gluten proteins (+2 %), reaching 37.6 mg g^{-1} of wheat flour compared

Table 2
Grain yield and yield components (mean ± S.E.; n = 3) in wheat plants after cytokinin treatments at different timings of application (T) and doses (D), compared to untreated controls (C). TKW = thousand kernel weight, HI = harvest index. Within each parameter, lowercase letters indicate significant differences for multiple comparisons 'Time × Dose' interaction, and uppercase letters differences for the main effect Application Timing T (Tukey's HSD test, $p \leq 0.05$). Values in parentheses represent percentage variation from C.

Treatments		Yield (g DW m^{-2})		Testing weight (kg L^{-1})		TKW (g)		HI	
C		811 ± 38 a [AB]	Ref.	82.8 ± 0.7 a [A]	Ref.	34.5 ± 0.3 a [ABC]	Ref.	0.466 ± 0.001 a [AB]	Ref.
T1	D1	809 ± 23 a	(=)	81.6 ± 0.4 a	(−1)	34.4 ± 0.6 a	(=)	0.455 ± 0.010 a	(−2)
	D2	862 ± 22 a	(+6)	83.1 ± 1.1 a	(=)	33.7 ± 0.5 a	(−2)	0.456 ± 0.003 a	(−2)
	D3	853 ± 11 a	(+5)	82.2 ± 0.3 a	(−1)	34.1 ± 0.6 a	(−1)	0.458 ± 0.006 a	(−2)
	Average	841 ± 13 [A]	(+4)	82.2 ± 0.4 [A]	(−1)	34.1 ± 0.3 [BC]	(−1)	0.456 ± 0.003 [B]	(−2)
T2	D1	788 ± 39 a	(−3)	81.8 ± 0.8 a	(−1)	35.0 ± 0.9 a	(+1)	0.469 ± 0.005 a	(+1)
	D2	759 ± 32 a	(−7)	80.6 ± 1.2 a	(−3)	35.3 ± 0.4 a	(+2)	0.469 ± 0.006 a	(+1)
	D3	767 ± 15 a	(−5)	81.2 ± 0.4 a	(−2)	35.6 ± 0.3 a	(+3)	0.460 ± 0.003 a	(−1)
	Average	771 ± 16 [B]	(−5)	81.2 ± 0.5 [A]	(−2)	35.3 ± 0.3 [AB]	(+2)	0.466 ± 0.003 [AB]	(=)
T3	D1	788 ± 16 a	(−3)	81.3 ± 0.7 a	(−2)	35.6 ± 0.7 a	(+3)	0.473 ± 0.001 a	(+2)
	D2	795 ± 7 a	(−2)	81.5 ± 0.8 a	(−2)	35.7 ± 0.5 a	(+4)	0.470 ± 0.004 a	(+1)
	D3	810 ± 15 a	(=)	81.3 ± 0.9 a	(−2)	35.1 ± 0.3 a	(+2)	0.469 ± 0.001 a	(+1)
	Average	798 ± 7 [AB]	(−2)	81.4 ± 0.4 [A]	(−2)	35.5 ± 0.3 [A]	(+3)	0.471 ± 0.001 [A]	(+1)
T4	D1	843 ± 13 a	(+4)	80.4 ± 0.7 a	(−3)	33.5 ± 0.6 a	(−3)	0.461 ± 0.005 a	(−1)
	D2	825 ± 28 a	(+2)	81.3 ± 0.7 a	(−2)	33.7 ± 0.3 a	(−2)	0.460 ± 0.007 a	(−1)
	D3	842 ± 9 a	(+4)	81.0 ± 0.6 a	(−2)	34.1 ± 0.8 a	(−1)	0.463 ± 0.005 a	(−1)
	Average	836 ± 10 [A]	(+3)	80.9 ± 0.4 [A]	(−2)	33.8 ± 0.3 [C]	(−2)	0.461 ± 0.003 [AB]	(−1)
T1+T3	D1	795 ± 6 a	(−2)	82.2 ± 0.5 a	(−1)	35.2 ± 0.6 a	(+2)	0.458 ± 0.002 a	(−2)
	D2	831 ± 41 a	(+2)	81.5 ± 0.2 a	(−2)	34.6 ± 0.2 a	(=)	0.461 ± 0.006 a	(−1)
	D3	816 ± 16 a	(+1)	81.9 ± 0.8 a	(−1)	34.6 ± 0.4 a	(=)	0.458 ± 0.004 a	(−2)
	Average	814 ± 14 [AB]	(=)	81.8 ± 0.3 [A]	(−1)	34.8 ± 0.2 [ABC]	(+1)	0.459 ± 0.002 [AB]	(−1)
T2+T3	D1	776 ± 14 a	(−4)	80.9 ± 1.1 a	(−2)	34.6 ± 0.4 a	(=)	0.450 ± 0.015 a	(−3)
	D2	862 ± 14 a	(+6)	82.2 ± 0.7 a	(−1)	34.8 ± 0.2 a	(+1)	0.464 ± 0.004 a	(=)
	D3	853 ± 20 a	(+5)	79.8 ± 0.7 a	(−4)	34.2 ± 0.7 a	(−1)	0.459 ± 0.003 a	(−2)
	Average	831 ± 16 [A]	(+2)	81.0 ± 0.6 [A]	(−2)	34.5 ± 0.3 [ABC]	(=)	0.458 ± 0.005 [AB]	(−2)
Time (T)		**		ns		**		*	
Dose (D)		ns		ns		ns		ns	
T × D		ns		ns		ns		ns	

ns: not significant; *: significant at $p \leq 0.05$; **: significant at $p \leq 0.01$.

Table 3

Grain protein content and gluten fractions (mean ± S.E.; n = 3) in wheat after cytokinin treatments at different timings of application (T) and doses (D), compared to untreated controls (C). DW = dry weight, N = nitrogen, HMW-GS = high molecular weight glutenin subunits, LMW-GS = low molecular weight glutenin subunits. Within each parameter, lowercase letters indicate significant differences for multiple comparisons 'Time × Dose' interaction, and uppercase letters differences for the main effect Application Timing T (Tukey's HSD test, $p \leq 0.05$). Values in parentheses represent percentage variation from C (absolute variation for grain protein content).

Treatment		Grain proteins % DW		Gliadins mg g^{-1}		HMW-GS mg g^{-1}		LMW-GS mg g^{-1}		Glutenins/Gliadins		Straw N content % DW	
C		12.5 ± 0.2 a [BC]	Ref.	25.7 ± 1.1 a [A]	Ref.	6.1 ± 0.2 a [AB]	Ref.	5.1 ± 0.3 bc [B]	Ref.	0.44 ± 0.01 b [C]	Ref.	0.51 ± 0.03 a [BC]	Ref.
T1	D1	13.2 ± 0.2 a	(+0.7)	24.0 ± 0.4 a	(–7)	6.2 ± 0.2 a	(+1)	5.0 ± 0.1 bc	(–3)	0.47 ± 0.02 ab	(+7)	0.58 ± 0.02 a	(+13)
	D2	13.6 ± 0.2 a	(+1.1)	23.4 ± 0.8 a	(–9)	6.2 ± 0.1 a	(+2)	4.8 ± 0.1 bc	(–6)	0.47 ± 0.02 ab	(+8)	0.66 ± 0.01 a	(+29)
	D3	13.7 ± 0.2 a	(+1.2)	24.2 ± 0.7 a	(–6)	6.3 ± 0.1 a	(+4)	5.2 ± 0.1 bc	(+2)	0.48 ± 0.01 ab	(+10)	0.65 ± 0.01 a	(+27)
	Average	13.5 ± 0.1 a [A]	(+1.0)	23.9 ± 0.3 a [A]	(–7)	6.2 ± 0.1 a [A]	(+2)	5.0 ± 0.1 [B]	(–2)	0.47 ± 0.01 [BC]	(+8)	0.63 ± 0.01 a [A]	(+23)
T2	D1	12.8 ± 0.9 a	(+0.3)	24.3 ± 0.4 a	(–5)	5.2 ± 0.2 a	(–15)	8.0 ± 0.7 a	(+56)	0.54 ± 0.02 ab	(+24)	0.50 ± 0.05 a	(–1)
	D2	12.5 ± 0.5 a	(=)	23.8 ± 0.9 a	(–7)	5.5 ± 0.5 a	(–10)	6.4 ± 0.1 abc	(+25)	0.50 ± 0.01 ab	(+14)	0.49 ± 0.06 a	(–4)
	D3	12.4 ± 0.4 a	(–0.1)	25.3 ± 0.2 a	(–1)	5.4 ± 0.1 a	(–11)	7.0 ± 0.3 abc	(+36)	0.49 ± 0.01 ab	(+12)	0.48 ± 0.02 a	(–5)
	Average	12.6 ± 0.3 a [BC]	(+0.1)	24.5 ± 0.4 a [A]	(–5)	5.4 ± 0.2 a [B]	(–12)	7.1 ± 0.3 [A]	(+39)	0.51 ± 0.01 [ABC]	(+17)	0.49 ± 0.02 a [C]	(–4)
T3	D1	13.2 ± 0.1 a	(+0.7)	24.1 ± 0.3 a	(–6)	5.2 ± 0.1 a	(–14)	6.3 ± 0.5 abc	(+23)	0.48 ± 0.03 ab	(+9)	0.53 ± 0.01 a	(+4)
	D2	13.0 ± 0.4 a	(+0.5)	24.4 ± 0.6 a	(–5)	5.8 ± 0.1 a	(–5)	7.4 ± 0.6 abc	(+44)	0.54 ± 0.04 ab	(+24)	0.55 ± 0.01 a	(+7)
	D3	13.2 ± 0.2 a	(+0.7)	25.2 ± 0.8 a	(–2)	5.7 ± 0.3 a	(–6)	8.8 ± 0.5 a	(+72)	0.58 ± 0.02 a	(+32)	0.64 ± 0.05 a	(+25)
	Average	13.2 ± 0.1 a [AB]	(+0.7)	24.6 ± 0.3 a [A]	(–4)	5.6 ± 0.1 a [AB]	(–8)	7.5 ± 0.5 [A]	(+46)	0.53 ± 0.02 [AB]	(+22)	0.57 ± 0.02 a [AB]	(+12)
T4	D1	13.2 ± 0.3 a	(+0.7)	23.7 ± 1.3 a	(–8)	6.1 ± 0.2 a	(+1)	4.9 ± 0.1 bc	(–4)	0.47 ± 0.01 ab	(+7)	0.63 ± 0.0.3 a	(+22)
	D2	12.8 ± 0.1 a	(+0.3)	23.6 ± 0.5 a	(–8)	6.0 ± 0.1 a	(–1)	4.7 ± 0.1 c	(–8)	0.46 ± 0.01 ab	(+4)	0.54 ± 0.03 a	(+6)
	D3	12.6 ± 0.3 a	(+0.1)	24.7 ± 0.3 a	(–4)	6.4 ± 0.4 a	(+6)	5.2 ± 0.3 bc	(+1)	0.47 ± 0.01 ab	(+8)	0.57 ± 0.04 a	(+12)
	Average	12.8 ± 0.2 a [ABC]	(+0.3)	24.0 ± 0.6 a [A]	(–7)	6.2 ± 0.1 a [A]	(+2)	4.9 ± 0.1 [B]	(–4)	0.46 ± 0.01 [C]	(+6)	0.58 ± 0.02 a [AB]	(+14)
T1+T3	D1	12.6 ± 0.2 a	(+0.1)	23.9 ± 0.6 a	(–7)	6.0 ± 0.8 a	(–1)	8.1 ± 1.4 a	(+58)	0.59 ± 0.06 a	(+35)	0.50 ± 0.02 a	(–2)
	D2	12.0 ± 0.2 a	(–0.5)	22.8 ± 0.3 a	(–11)	5.4 ± 0.2 a	(–11)	8.0 ± 0.7 a	(+56)	0.59 ± 0.03 a	(+35)	0.47 ± 0.01 a	(–9)
	D3	12.0 ± 0.3 a	(–0.5)	24.2 ± 0.5 a	(–6)	5.3 ± 0.1 a	(–12)	7.3 ± 0.3 abc	(+43)	0.52 ± 0.01 ab	(+20)	0.48 ± 0.02 a	(–6)
	Average	12.2 ± 0.1 a [C]	(–0.3)	23.6 ± 0.3 a [A]	(–8)	5.6 ± 0.3 a [AB]	(–8)	7.8 ± 0.5 [A]	(+52)	0.57 ± 0.02 [A]	(+30)	0.48 ± 0.01 a [C]	(–5)
T2+T3	D1	12.7 ± 0.3 a	(+0.2)	23.5 ± 1.3 a	(–8)	5.0 ± 0.1 a	(–17)	7.0 ± 0.5 abc	(+37)	0.52 ± 0.05 ab	(+19)	0.51 ± 0.02 a	(–1)
	D2	12.9 ± 0.3 a	(+0.3)	24.8 ± 0.5 a	(–3)	5.7 ± 0.1 a	(–7)	7.3 ± 0.4 abc	(+43)	0.52 ± 0.01 ab	(+20)	0.54 ± 0.01 a	(+6)
	D3	12.7 ± 0.4 a	(+0.2)	24.4 ± 0.8 a	(–5)	5.5 ± 0.2 a	(–9)	7.4 ± 0.3 ab	(+45)	0.53 ± 0.02 ab	(+22)	0.57 ± 0.02 a	(+11)
	Average	12.8 ± 0.2 a [ABC]	(+0.3)	24.2 ± 0.5 a [A]	(–6)	5.4 ± 0.1 a [B]	(–11)	7.3 ± 0.2 [A]	(+42)	0.52 ± 0.02 [ABC]	(+20)	0.54 ± 0.01 a [BC]	(+5)
Time		**		ns		***		***		***		***	
Dose		ns		ns		ns		ns		ns		ns	
T × D		ns		ns		ns		ns		ns		ns	

ns: not significant; *: significant at p ≤ 0.05; **: significant at p ≤ 0.01.

to 36.9 mg g^{-1} in controls ($p < 0.05$) (data not shown). In contrast, T1 and T4 showed a decrease of ~5 %, while no effects were observed after T2 and the double treatments. Variations among treatments were associated to gluten protein composition, as the glutenins-to-gliadins ratio increased across all the timings of CKs application (from +4 % to +35 %), with significant increases in T3 and T1+T3 treatments (+22 % and +30 % respectively compared to controls) (Table 3).

This shift in gluten composition resulted from a slight not significant decrease in gliadin content (from −4 % to −8 % across treatments) depending on the timing of CKs application, while glutenins variations were mainly associated to substantially increased low-molecular-weight glutenin subunits (LMW-GS: from +23 % to +72 % in T3, depending on the dose). In contrast, high-molecular-weight glutenin subunits (HMW-GS) were generally slightly decreased after cytokinin treatment (from −8 % to −12 % vs. controls), except for T1 and T4 which showed a slight increase (+2 %).

Notable variations among treatments were observed for the nitrogen content in the straw at harvest. The T1 treatments provided a significant rise in straw N (+23 % on average), particularly with D2 and D3 (+28 % on average). Most other treatments also increased straw N content (from +5 % to +14 %), while T2 and T1+T3 applications resulted in decreases (−4 % and −5 % vs. C; $p \geq 0.05$).

Statistical analysis revealed significant effect of the application timing on both grain protein content ($p \leq 0.1$) and gluten composition ($p \leq 0.05$) (except for gliadins). The effects of dose and "Time × Dose" interaction remained non-significant for all quality parameters (Table 3).

3.6. Root growth parameters

Cytokinin treatments also altered root architecture and distribution across the soil profile, with some treatments showing statistically significant ($p \leq 0.05$) changes. Considering the 0–1 m on average, root length density (RLD) increased in most of the treatments, with the greatest improvements in T1 (+14 %) and T1+T3 (+19 %), except for T2 and T3 which reduced RLD by approximately 9 % across the entire profile (Table SI1).

All the timings of CKs application showed higher RLD values in the 0–0.5 m soil profile, especially within the arable soil layer (0–30 cm) (Fig. 5); the double treatments T1+T3 and T2+T3 were associated with the highest percentage increases (+33 % and +20 % respectively vs. controls, $p \geq 0.05$) (Table SI1). Nevertheless, most treatments substantially decreased root proliferation in the deeper layers of soil (50–100 cm depth: 29 % to −74 % compared to controls), with the exception of T1 and T4 treatments, which sustained or slightly enhanced (+2 and + 8 %) deep root growth.

Root surface area density (RSD) exhibited patterns similar to RLD, with higher (n.s.) values in top layers (particularly after double treatments), but strongly reduced in deeper soil profile beneath 50 cm of depth (from −33 % to −72 %).

Root thickness measurements showed that T2 and T3 applications enhanced mean root diameter by around 5 % ($p \geq 0.05$), especially in the top profile of the soil (0–50 cm: +9 % as average of T2 and T3). Conversely, all other treatments decreased the mean root diameter by 3 %–8 %, but the changes were not statistically significant.

3.7. Multivariate analysis

Multivariate analysis successfully summarized the substantial variability present in the whole dataset, condensing the information from measured traits into two main synthetic variables (F1 and F2) (Fig. 6). By retaining only significant traits, these two variables collectively explained 97.63 % of the total variability (F1: 95 %; F2: 2.63 %). Traits with absolute loadings greater than |0.4| were considered primary contributors to interpretation, thereby avoiding over-weighting weak signals. The most important variable F1 was primarily defined by PSII efficiency (Fv'/Fm'), CO_2 assimilation (A), and LMW-GS, indicating that the dominant axis of variation reflected differences in photosynthetic performance and specific protein fractions. Variable F2, although accounting for a smaller proportion of variance, further separated traits related to nitrogen allocation and compositional quality, including traits with positive loadings (LMW-GS, TKW, glutenins/gliadins ratio) and

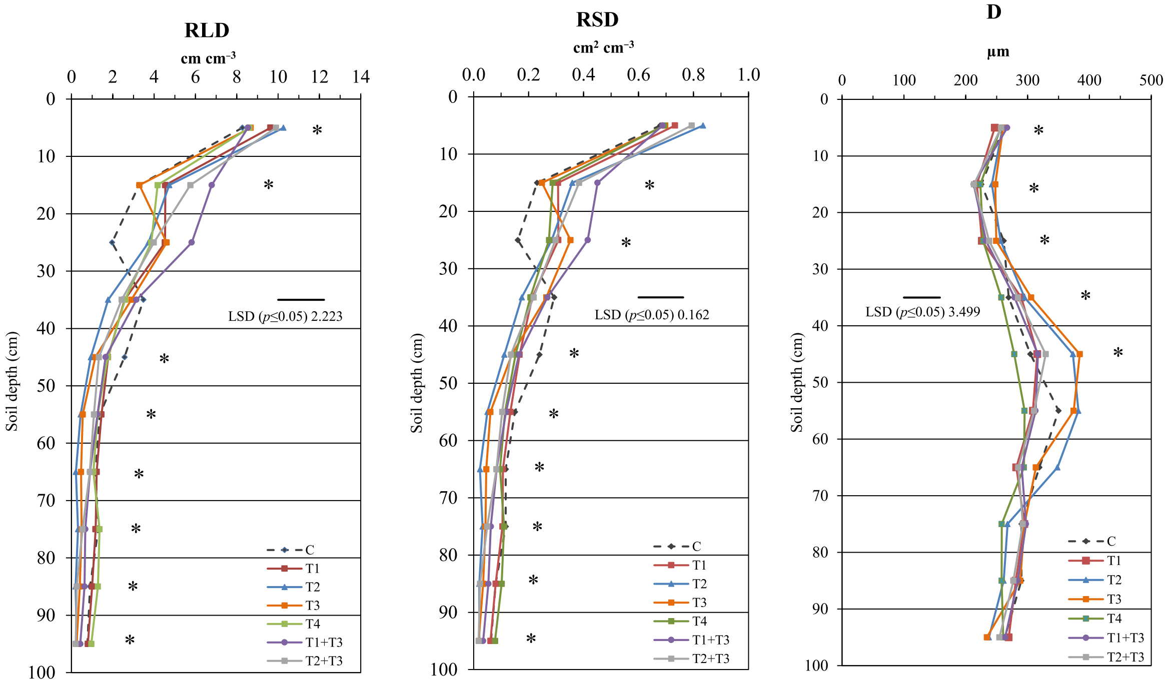
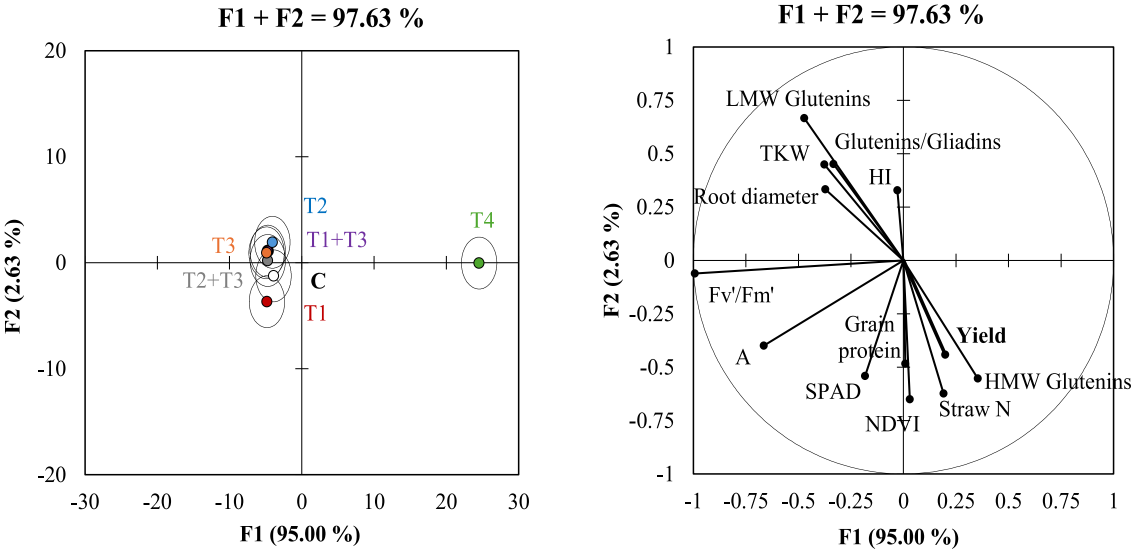


Fig. 5. Root length density (RLD), root surface area density (RSD), and root diameter (D) (mean; n = 3) across the 0–1 m soil profile at end dough maturity (11 June) in wheat after cytokinin treatments at different Application Timing (T) as main effect (doses averaged). Asterisks indicate significant differences among treatments (Tukey's HSD test, $p \leq 0.05$). LSD: Least Significant Difference for multiple 'Treatment × Depth' comparisons at $p \leq 0.05$.



Variables	F1	F2
Yield	0.199	**–0.442**
HI	–0.029	0.329
TKW	–0.376	**0.449**
Grain protein	0.008	**–0.484**
HMW Glutenins	0.353	**–0.552**
LMW Glutenins	**–0.472**	**0.667**
Glutenins/Gliadins	–0.333	**0.451**
Straw N	0.190	**–0.623**
SPAD	–0.182	**–0.542**
NDVI	0.030	**–0.651**
A	**–0.665**	–0.399
Fv'/Fm'	**–0.993**	–0.061
Root diameter	–0.372	0.334

Fig. 6. Principal Component Analysis (PCA; right) with variable loadings (bold values > |0.4|; bottom) and Discriminant Analysis (DA; left) for cytokinin application timings (T1, T2, T3, T4, T1+T3, T2+T3) and control (C). The two synthetic variables (F1 and F2) explained 97.63 % of total variance. DA showed distinct treatment clusters, with circles comprising 70 % of cases.

negative loadings (straw N percentage, HMW-GS, SPAD, NDVI, and grain protein). Along F2, canopy indicators (SPAD, NDVI, straw N) and grain protein loaded strongly on the negative side, whereas kernel filling and glutenin composition (TKW, LMW-glutenins, glutenins/gliadins ratio) loaded on the positive side, indicating a nitrogen allocation vs. grain-filling gradient. Discriminant Analysis (DA) further validated these patterns, demonstrating that treatment groups were statistically distinct, as observed by the position of their centroids. Among treatments, T1 emerged as improving vegetational and photosynthetic traits, with positive effects of grain yield, while T2 and T1+T3 treatments enhancing root diameter and glutenins, underscoring the role of cytokinin application timing in shaping both physiological performance and grain quality outcomes.

4. Discussion

The findings of this study present extensive evidence that foliar application of cytokinins (CKs) could effectively enhance wheat resilience and quality in open-field conditions, with the timing of CKs application serving as crucial determinant influencing efficacy. Overall, this experimental evaluation of diverse application times and dosages provides important insights into enhancing this agronomic strategy for practical use.

4.1. Leaf greenness and photosynthetic competence

The most substantial and persistent increases in leaf greenness were observed following CKs treatment at flowering (T1). Wheat plants treated at T1 exhibited significantly longer canopy greenness, with persistently higher NDVI values and greater flag leaf chlorophyll content throughout grain filling into early June. These corresponded to higher net CO_2 assimilation rates and improved PSII efficiency in early June as compared to the yellowing leaves of untreated controls. These effects suggest enhanced photosynthetic capacity of the flag leaf (the most important leaf from flowering onward), when subjected to CKs application at flowering, and are consistent with previous studies that demonstrated increased photosynthetic activity and delayed chlorophyll degradation after CKs treatment, especially under drought or heat stress conditions (Wang et al., 2019; Yang et al., 2018; Rivero et al., 2010; Criado et al., 2009; Koprna et al., 2016; Islam et al., 2022). However, the current study's assessment of senescence is limited to physiological indicators (NDVI, chlorophyll content, PSII efficiency), and molecular analysis of senescence markers, such as SAG12, may provide more detailed insight into senescence onset and progression. Therefore, future research using RT-PCR or other molecular techniques could clarify the understanding of CK-mediated anti-senescence effects in wheat (Fuentes et al., 2025; Guo and Gan, 2005; Vatov et al., 2021; Sýkorová et al., 2008).

Despite the evident visual changes in greenness seen across treatments (Fig. 2), NDVI and SPAD readings may not consistently capture this canopy-level variation. This restriction is likely due to NDVI saturation at high levels and possible insensitivity of single-point SPAD measurements to spatial heterogeneity, as previously described by other authors (Zarco-Tejada et al., 2005; Gao et al., 2024). Future research could examine using alternate chlorophyll assessment methods, such as chlorophyll extraction, multispectral indices, or sun-induced fluorescence, to give a more nuanced definition of canopy greenness (Aasen et al., 2019; Li et al., 2022a).

Treatments applied after flowering produced progressively smaller and often non-significant increases in canopy greenness, suggesting that the window for effective exogenous foliar cytokinin action probably narrows once senescence processes are initiated. Interestingly, the T1+T3 treatment did not reproduce the early NDVI increase observed with T1 alone, although the two treatments did not differ until the second CKs application when standardized (on pre-treatment) NDVI values were considered. This raises the question of why T1 sustained a better stay-green effect later in the season compared with T1+T3. This trend suggests that there are no additive hormonal effects, although receptor desensitization or feedback, as well as lower uptake of subsequent sprays due to leaf aging or wax deposition can be expected (Fernández and Eichert, 2009). Late additional CKs applications may fail to enhance—or may suppress the benefits of early treatments, possibly due to an inverse dose–response effect. Elevated CKs levels can also trigger feedback regulation that suppresses biosynthesis and enhance degradation, while desensitizing signalling components, thereby reducing cytokinin responsiveness and disrupting crosstalk with ethylene and auxin, which may impair source-sink coordination (Hönig et al., 2018; Zwack and Rashotte, 2015). Overproduction of cytokinins has also been shown to inhibit growth and reduce stress resistance in model plants (Wang et al., 2015), highlighting the importance of optimal CKs levels (further than timing) for maximal physiological benefit. It should also be acknowledged that spatial separation and micro-environmental heterogeneity among field plots may have contributed to treatment variability, potentially influencing the apparent lack of additive effects (Hoefler et al., 2020). Cytokinin interactions with nitrogen metabolism and remobilization processes, even though root growth stimulation, can influence grain protein and straw N, indicating that a single, well-timed spray at flowering is the most dependable technique for improving wheat canopy greenness and grain quality parameters (Abiola et al., 2025).

Previous research has predominantly focused on controlled environments, with limited information available on wheat crops in open field conditions. Our results support those of Xie et al. (2004), who reported higher photosynthetic rates and chlorophyll content in wheat flag leaves after continuous CK treatment during in vitro ear culture. Similarly, delayed chlorophyll degradation and enhanced photosynthetic rates were observed in wheat grown in pots by Criado et al. (2009) and Gupta et al. (2012) when CKs were applied 17 days after sowing and at anthesis. Furthermore, it has been documented that under controlled combined drought and high temperature stress conditions, the exogenous application of 6-BA on 10-day old wheat seedlings induces multiple stress adaptation responses—including reduction of lipid peroxidation, enhanced membrane stability, improved water status, and leaf chlorophyll content (Kumari et al., 2018).

In our study, treatments applied after flowering (T2, T3, T4) exhibited gradually declining impacts on leaf greenness metrics, with lower and frequently non-significant enhancements. Despite identical starting NDVI values to T1, there was no lasting CK effect on canopy greenness and CO_2 assimilation with T2, possibly due to physiological and environmental restrictions. At the time of T2 application (early boot), natural cytokinin receptor density and signaling component expression may be temporarily diminished, reducing response to exogenous CK (Hönig et al., 2018; Jameson and Song, 2016). Plants at this stage may have already begun irreversible senescence processes, or field stressors may have inhibited metabolic activity, reducing CK potency (Zwack and Rashotte, 2015). The modest, but non-significant, increases in SPAD and Fv'/Fm' following T3 treatment imply, however, that CKs may still provide some physiological advantages in advanced senescence. This supports findings that CK-induced stay-green responses are stage- and context-dependent (Sýkorová et al., 2008; Hönig et al., 2018). Future research incorporating transcriptome or proteomic indicators of CK signaling and senescence (e.g., SAG12 expression) might aid in identifying precisely appropriate developmental windows for CK modulation.

Overall, once the senescence processes initiate via endogenous hormone regulation, susceptibility to exogenous CK applications likely declines, as suggested by Mantilla et al. (2021). This is consistent with earlier studies showing that CK serves as an anti-senescence regulator, delaying chlorophyll degradation by downregulating senescence-associated genes (SAGs) and maintaining Rubisco activity (Hönig et al., 2018; Jiang et al., 2019). Furthermore, CKs affect nitrogen metabolism by altering nitrate absorption and protein remobilization, which might explain the observed discrepancies in grain protein content and straw nitrogen buildup (Gu et al., 2018; Criado et al., 2009). However, the minimal impact of subsequent administrations (T2, T3, T4) implies that the window of CK efficacy narrows as ABA and ethylene levels rise, supporting earlier results that CKs applied early after anthesis optimize leaf longevity and photosynthetic performance (Yang et al., 2016; Luo et al., 2006).

4.2. *Grain yield and performance*

Crucially, among all the timings of CKs application tested, only the enhanced leaf greenness observed in T1 treatment translated into a yield increase (+4 %: 841 vs. 811 g DW m^{-2}) across the three doses compared to controls—although this difference was modest and did not reach statistical significance ($p > 0.05$). This reflects a broader trend in wheat and other cereals, where prolonged canopy longevity does not necessarily translate into higher grain output. While delayed senescence preserves photosynthetic tissue, it may not enhance assimilate remobilization or sink activity, particularly if feedback regulation limits source strength or if environmental constraints during grain filling outweigh potential gains (Sýkorová et al., 2008; Kučeřová et al., 2020). Yield responses to CKs are therefore strongly contingent on genotype and environment, with both positive and neutral yield responses reported under stress and non-stress conditions (Joshi et al., 2019). Mechanistically, variability in yield response likely reflects constraints on assimilate export and remobilization from "stayed-green" leaves, feedback regulation in source-sink signaling, and overriding environmental limitations during grain filling—factors that can modulate or negate the benefits of prolonged photosynthesis and help explain trade-offs observed across genotypes (Lekhana et al., 2025). These findings further emphasize that the effectiveness of CK-induced stay-green traits depends not only on maintaining photosynthetic capacity, but also on synchronized source-sink coordination under favorable post-anthesis conditions (Koprna et al., 2016).

Endogenous phytohormones play an important role in determining sink strength and grain filling rate (Slafer et al., 2023; Yang et al., 2003; Ha et al., 2012; Chen et al., 2010), and previous studies have linked enhanced endosperm cell division and capacity of kernel sink to exogenous cytokinins application in cereals (Jameson, 2023). Such enhancement leads to an improved and prolonged grain filling period, when a great part of gluten proteins is accumulated, ultimately resulting in higher grain yield and proteins content. Empirical data supports this view, since studies reveal increased yields in maize (Gao et al., 2017; Ren et al., 2016) and rice (Yang et al., 2000) following CKs application. Similarly, our study indicates that applying CKs at flowering significantly improves these processes. Consistent with our T1 treatment, an average yield improvement of +4 % has been observed when CKs are applied immediately after flowering (Luo et al., 2018; Yang et al., 2016). Conversely, treatments applied later in development often produced negative effects, as seen in both our T2 treatment (−5 % vs. controls) and a prior study by Yang et al. (2003), who reported significant yield impairments (−6 %) following CK application roughly 10 days after anthesis.

Beyond the time of application, the environmental conditions are also relevant in determining the potential of this agronomic technique to promote yield increases. The environmental conditions during our study, featuring above-average temperatures (+1.5 °C) and limited precipitation during flowering and grain filling, may have increased the effectiveness of CK treatments. Previous research suggests that CK treatments offer better yield gains under rising temperatures and moderate nitrogen fertilization than in ambient circumstances or high nitrogen inputs (Slafer and Savin, 2018; Yang et al., 2016; Luo et al., 2018). This implies that CK-based agronomic strategy may be particularly useful for mitigating crop losses under increasingly changing climatic conditions.

4.3. Grain protein content and composition

In addition to phytohormones, N is another key factor in regulating leaf senescence, and numerous studies have reported prolonged canopy longevity and increased grain yield and protein content following the application of a foliar N fertilizer at anthesis (Nehe et al., 2020; Marinaccio et al., 2015; Bly and Woodard, 2003). In this study, CKs application significantly increased grain protein content across most treatments except for T1+T3. The most substantial enhancements were again observed with the treatment at flowering T1 (13.5 % DW vs. 12.5 % of untreated controls; $p \leq 0.05$). This reinforces the role of cytokinins in mediating absorption, transport, assimilation and metabolism of nitrogen (Gu et al., 2018; Shah et al., 2023; Sakakibara et al., 2006; Takei et al., 2004). In wheat, 60–95 % of the grain nitrogen is remobilized from reserves stored in roots and shoots prior to anthesis, which significantly affects grain protein concentration and baking quality of flour (Sharma et al., 2023; Hirel et al., 2007).

The extended leaf greenness and photosynthetic capacity, as well as stimulation of root growth following CKs application may partially explain these protein content changes (Criado et al., 2009). Previous research suggests that CKs stimulate assimilate accumulation in chloroplasts, maintaining sink activity in older leaves (Criado et al., 2009; Cowan et al., 2005). This was mirrored in our study by greater nitrogen content in straw at harvest, notably in T1-treated plants (+23 % vs. controls), suggesting enhanced nitrogen assimilation. Although some of this nitrogen was effectively remobilized into grains, resulting in greater protein concentrations, a considerable portion remained in vegetative tissues. This incomplete remobilization might be due to cytokinin (CK)-mediated decreases in amino acid and sugar export to the phloem, coupled with inhibition of protein degradation in leaves (Barneix, 2007; Criado et al., 2009; Cowan et al., 2005). These variables may partially counterbalance the increased kernel sink capacity, although in our study both grains and straw had increased N accumulation with CKs treatment at flowering.

This study also investigated the impact of CKs on flour quality of wheat and implications on bakery characteristics, revealing complex but promising impacts of CK treatments. Interestingly, while the overall gluten content increased marginally in late CKs applications, like in T3 (application at 20 DAF), a slight reduction of gluten in the most interesting treatment T1 suggests that there probably was a major accumulation of enzymatic proteins (albumins) or globulins. Regardless of application time, CKs increased the glutenins-to-gliadins ratio with a clearly time-dependent effect, that was lighter for early (+8 % of T1 vs. controls) and heavier for late applications (+22 % of T3 vs. controls).

These findings are consistent with previous research which found that accumulation of gluten proteins is a complex process driven by spatial and temporal regulation, and significant variations in gluten composition can be observed under abiotic constraints such as heat and drought stress or N limitations (Flagella et al., 2010; Visioli et al., 2018; Ferrari et al., 2025). However, limited information is available regarding possible changes in gluten protein composition in response to CKs application. For instance, Pan et al. (2023) reported that exogenous CK treatments not only alter the balance of gliadins and glutenins, but also increase the fraction of high-quality glutenin subunits, which may enhance dough elasticity and overall baking performance.

4.4. Root responses

Variations in root parameters must be carefully evaluated, as they may play an important indirect role in contributing to enhanced abiotic stress tolerance. This study offers unique insights into how foliar CK treatments impact below-ground growth in the field. Root characteristics exhibited complicated responses to CK treatments, with changes depending on both application timing and soil depth. A general rise in root length and surface area across the arable layer (0–30 cm depth) across all treatment timings (average + 30 % compared with controls) was obtained, while this increase was accompanied by a reduction in root thickness. Conversely, most treatments significantly reduced root development in deeper soil profiles (0.5–1 m depth: −29 % to −74 % vs. control), with considerable exceptions for T1 and T4.

These contrasting effects may be related to the well-known role of cytokinins as antagonists to abscisic acid (ABA) (Kurepa and Smalle, 2022; Ha et al., 2012). ABA and CKs are related to act synergically to communicate with auxin signalling pathway, to modulate root development in response to water deficit (Shkolnik-Inbar and Bar-Zvi, 2010; Iqbal et al., 2006; Chen et al., 2006). The exogenous application of cytokinin likely altered these hormonal signalling pathways triggering the observed changes in root architecture. While the molecular components and pathways mediating hormone response have been increasingly elucidated in recent years, and the intricate mechanisms underlying hormone interaction remain poorly understood (Verma et al., 2016; Santner et al., 2009; Wang et al., 2018), the positive root enhancements we generally found under CKs treatments is an essential, but still under-considered, effect.

4.5. Single versus double applications, timing and dose optimization of cytokinins

Besides the timing of application, this study also investigated the potential benefits of double CKs applications, with the second application scheduled 20 or 10 days after the first (T1+T3, T2+T3). Assessment of double CKs applications revealed that, despite modest improvements in the glutenins/gliadins ratio and root length density, these treatments produced fewer notable improvements in leaf photosynthetic capacity than single applications, particularly at flowering (T1). More importantly, these small benefits were not translated into relevant improvements of grain yield and protein content.

Previous studies in the literature investigated the impact of cytokinin applications in wheat, including daily applications for 3–5 days after anthesis; however, the variations in grain yield were inconsistent and never exceeded the percentages of increase obtained after a single treatment at flowering in this trial (Yang et al., 2003, 2016). This suggests that double applications provide limited additional benefits while substantially increasing application costs, reducing the economic and environmental sustainability of this agronomic technique.

Statistical analysis confirmed that application timing had a substantial impact on most measured parameters, including root morphology, but dose effects were largely non-significant, including the ‘Time × Dose’ interaction. However, the effect of CKs application at flowering (T1) were consistently stronger at intermediate (D2: 50 g ha^{-1} of a. i.) and high (D3: 150 g ha^{-1} of a. i.) dosages of 6-BA than at the lowest concentration investigated (D1: 25 g ha^{-1} of a. i.).

Since the intermediate and maximum studied dosages had similar impacts across most variables, our findings are consistent with prior studies advocating 6-BA treatments of 30–50 g ha^{-1} of a. i. (meaning 30–100 ppm concentration in water solution) for cereals. This suggests that exceeding 50 g ha^{-1} of a. i. offers no additional advantages while increasing expenses, thereby lowering the economic efficiency of this agronomic approach.

Beyond T1, later applications also had a beneficial impact on selected attributes. T3 significantly increased SPAD values, leaf N content, glutenins/gliadins ratio, and grain protein percentage ($p < 0.05$). Double treatments increased root growth in the top soil profile without detrimental yield effects, although more resources are addressed to the root system. These findings indicate again that CK activity is stage-dependent: T1 provides the most consistent canopy and protein advantages, whereas later or combination treatments supplement these effects by improving quality and root characteristics. Importantly, no adverse effects of subsequent CK applications were observed, highlighting CKs’ wider potential across the developmental timeline (Li et al., 2019).

However, as discussed earlier, the lack of additive benefits in T1+T3 suggests multiple late sprays may exceed the optimal CK exposure window, consistent with evidence that prolonged or excessive CK exposure can delay senescence without yield gain and is constrained by feedback and hormonal crosstalk (Sýkorová et al., 2008; Hönig et al., 2018; Zwack and Rashotte, 2015).

Multivariate analyses reinforced these interpretations by revealing clear trait groupings linked to cytokinin application timing. In PCA, T1 separated along mainly the second component (F2), and secondly along the first one (F1), fostering vegetational indexes (leaf photosynthetic parameters, SPAD, NDVI, straw N), and agronomic traits (yield, grain protein, HMW glutenins). This indicates that flowering-stage sprays primarily enhanced nitrogen assimilation and protein partitioning, indirectly also sustaining photosynthetic capacity, consistent with reports that cytokinins delay senescence and modulate nitrogen remobilization (Criado et al., 2009; Jameson and Song, 2016). Later and double CKs applications aligned with F2, linking with yield traits (TKW, HI) and gluten composition (LMW glutenins, glutenins-to-gliadins ratio), underscoring their role in grain quality. The untreated control clustered in between T1 and the other application timings, with lower photosynthetic efficiency. Discriminant analysis confirmed these separations, with photosynthetic efficiency and CO_2 assimilation rate, kernel weight, and glutenin fractions emerging as the main discriminators, in line with prior evidence that cytokinins shift source-sink balance and protein composition (Yang et al., 2016; Li et al., 2019). Overall, application timing—rather than dosage or repetition—was the key driver of phenotypic divergence, from canopy retention at T1 to grain development and protein synthesis at later stages.

It should be acknowledged, however, that PCA and discriminant axes represent statistical abstractions, and the associations described here are based on variable loadings and biplot geometry. Future physiological validation—such as ^{15}N tracer studies, enzyme activity profiling, or temporal expression analyses of N assimilation and photosynthetic genes—would be valuable to substantiate these mechanistic interpretations.

Holistically, these results support a dual role model of CK action, with (i) delaying senescence and preserving photosynthetic potential during vegetative and early reproductive growth, and (ii) facilitating nitrogen remobilization and quality trait enhancement during later reproductive stages (Nguyen et al., 2020; Atanasov et al., 2025). This stage dependent framework highlights CKs as a flexible agronomic tool, with T1 as one of the most reliable entry point, and later applications offering complementary improvements in quality and resilience.

4.6. *Formulation effects on foliar penetration and future directions*

The efficacy of foliar-applied cytokinins is heavily impacted by formulation properties which impact plant cuticle penetration. The cuticular layer is the major barrier to the penetration of exogenously applied compounds, composed of crystallized branched hydrocarbon chains, alkanes, alkenes, fatty acids, and fatty alcohols on the exterior layer of plant tissues (Staiger et al., 2019). Active ingredients like 6-BA enter plant tissues primarily via diffusion through the cuticle, a complex barrier with varying permeability based on molecule size and electrolytic state, and lipophilicity determining penetration rate, vertical penetration depth, and lateral diffusion (Buchholz, 2006).

The type of cytokinin utilized in formulations has a major impact on both penetration kinetics and subsequent physiological activity. Isoprenoid cytokinins, such as trans-zeatin are absorbed and metabolized at different rates than aromatic cytokinins, such as kinetin or p-topolin. This variation is due to changes in molecular structure, which influence charge-transfer complex formation and enzyme interactions within plant tissues (Popelková et al., 2006).

Environmental conditions, such as air humidity and temperature also modulate cuticle permeability and drying rates (Gao et al., 2015). Additionally, adjuvants can improve formulation efficacy: surfactants reduce surface tension, penetrants increase cuticle permeability, and adhesive agents prolong contact duration (Castro et al., 2014).

Future research should prioritize the development of alternative formulations with penetration-enhancing adjuvants to increase consistency in diverse field settings. Comparative research on the penetration kinetics among various cytokinin types with different adjuvant systems might help discover the best formulations for certain crops and settings. Moreover, developing standardized methods for evaluating cytokinin penetration and translocation inside plant tissues would enhance our understanding of formulation effects, allowing for more focused advances in foliar spray technologies (Fernández and Eichert, 2009).

5. Conclusions

This study demonstrates that exogenous cytokinin application via foliar spraying can effectively delay leaf senescence and sustain photosynthetic activity in wheat flag leaves during grain filling under field conditions. Among the tested timings, applications at flowering consistently produced the most favorable outcomes across morpho-physiological, yield, and quality parameters. Notably, the significant increase in protein content and altered gluten composition following CK application at flowering, underscores its potential to improve wheat quality for premium markets, even at a minimal dose of 50 g ha^{-1} of the a. i. 6-BA. Because this intervention can be combined with standard post-heading fungicide sprays, it can integrate readily into conventional farming systems without incurring additional application costs and aligning with existing farming practices.

The benefits of CKs application at flowering extend beyond delayed senescence, supporting nutrient assimilation, root development, and kernel sink capacity. While yield responses were not statistically significant, the observed positive trends suggest that CKs may contribute to improved crop performance. However, these findings should be interpreted with caution. Later applications also contributed positively by enhancing SPAD values and grain quality without adverse effects, highlighting the stage-dependent nature of CKs action and offering flexibility for different production goals.

These findings taken together provide a preliminary framework for incorporating cytokinins into wheat cultivation, with a single application strategy at flowering recommended for optimal results. Future research should focus on linking canopy traits to source-sink dynamics, particularly by optimizing assimilate translocation and remobilization to grains, potentially in combination with late-season nitrogen management. Integrating molecular markers of senescence and CK signaling, alongside standardized canopy measurement protocols to overcome NDVI saturation and spatial heterogeneity, will further clarify when CK-mediated stay-green can be translated into yield gains. Finally, the role of CKs in shaping root architecture and their complex hormonal interactions with other phytohormones deserve deeper investigation, especially in relation to drought resilience under increasingly variable climate conditions.

CRediT authorship contribution statement

Anna Panozzo: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Giuseppe Barion:** Methodology, Investigation. **Pranay Kumar Bolla:** Writing – review & editing. **Giovanna Visioli:** Methodology, Investigation. **Teofilo Vamerali:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Statements and declarations

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Teofilo Vamerali reports financial support was provided by Bimbo QSR. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.110739.

Data availability

Data will be made available on request.

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