



Quantification of spatial heterogeneity of different chlorophyll fluorescence parameters over tomato leaf areas affected by Cd²⁺ stress

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Abstract

Cadmium impairs plant growth by inducing oxidative damage through excessive ROS production. Sulfur and nitrogen supplementation can affect Cd mobility, uptake, and toxicity. This study quantifies the spatial heterogeneity of Cd-induced photochemical limitation in *Solanum lycopersicum* leaves using chlorophyll fluorescence imaging, and examines Cd²⁺ distribution in roots, stems, and leaves of hydroponic plants treated with ammonium sulfate. We assessed four treatments: control, ammonium sulfate (5 mM), cadmium (100 µM CdCl₂), and their combination, using fluorescence imaging, the JIP-test, and polarography. Cd stress was intrinsically heterogeneous within single leaves, with the strongest PSII inhibition along vascular tissue and leaf tips. Contrary to expectations, ammonium sulfate failed to mitigate Cd toxicity, suggesting an adverse ammonium–Cd interaction. OJIP analysis revealed severe oxygen-evolving complex damage under combined treatment, marked by prominent K- and L-bands. These findings highlight the localised nature of Cd stress and reveal responses missed by whole-leaf measurements.

Keywords: cadmium ion stress; chlorophyll fluorescence; OJIP; *Solanum lycopersicum*; spatial heterogeneity.

Introduction

Chlorophyll fluorescence (ChlF) is widely used as a tool to investigate the effects of abiotic stress on plants and other photoautotrophs (Krause and Weis 1991, Maxwell and Johnson 2000, Murchie and Lawson 2013, Kalaji *et al.* 2017). Several ChlF techniques, such as the slow Kautsky kinetics and fast OJIP kinetics, provide valuable insights into different components of the primary photosynthetic

machinery (Strasser *et al.* 2004, Baker 2008). These methods enable assessment and evaluation of the functional state of PSII, the photosynthetic electron transport chain, and the activation of stress-responsive mechanisms (Genty *et al.* 1989, Baker and Rosenqvist 2004, Moustakas *et al.* 2022).

However, conventional ChlF measurements are limited by the assumption that photosynthetic metabolism is spatially uniform across the leaf surface. In reality,

Highlights

- Cd stress is spatially heterogeneous, concentrating at leaf veins and tips
- Spatial heterogeneity can be quantified for several ChlF parameters
- Pixel distribution analysis reveals stress that leaf averages miss

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Abbreviations: ABS/RC – absorbed energy per reaction centre; DI₀/RC – dissipated energy flux per reaction centre; ET₀/RC – electron transport flux per reaction centre; F_v/F₀ – water-splitting complex efficiency; F_v/F_M – maximum (potential) quantum yield of photosynthetic processes in PSII; NPQ – nonphotochemical quenching; PI_{ABS} – performance index; Rfd – chlorophyll fluorescence decrease ratio (vitality index); TR₀/RC – the trapped energy flux per reaction centre; Φ_{PSII} – effective quantum yield of photosynthetic processes of PSII.

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photosynthetic activity is rarely uniform and often exhibits spatial heterogeneity (Genty and Meyer 1995), and the conventional measurements of ChlF can sometimes obscure localised stress responses. This limitation is addressed by ChlF image analysis, a technique that can capture the spatiotemporal variability of photosynthetic efficiency and stress-related photosynthetic limitation across the leaf area (Sperdouli and Moustakas 2012, Chang *et al.* 2025).

Recently, ChlF imaging has advanced, with studies that accurately quantify its spatio-temporal heterogeneity (Bresson *et al.* 2015). However, many studies focus exclusively on F_v/F_m analysis, omitting other equally important parameters such as NPQ, effective quantum yield Φ_{PSII} , relative fluorescence decrease ratio Rfd, *etc.* Some studies about the spatial heterogeneity of chlorophyll fluorescence have been conducted on plants exposed to several stressors such as drought, thermal stress, salinity, heavy metals, and pathogens (Martínez-Peñalver *et al.* 2011, Bresson *et al.* 2015, Huang *et al.* 2023, Yang *et al.* 2025). The novelty of the image analysis, such as the one employed in this study, lies in its ability to detect and quantify subtle spatial variations in ChlF parameters across the leaf surface under cadmium stress of different intensities. Therefore, this study aims: (1) to develop and implement a full spatial ChlF imaging pipeline on tomato (*Solanum lycopersicum*) under cadmium (Cd^{2+}) stress, together with the addition of extra ammonium sulfate; (2) evaluate and quantify spatial heterogeneity of different ChlF parameters, other than F_v/F_m , such as Φ_{PSII} , NPQ, and Rfd; (3) demonstrate the added sensitivity of distribution-level analysis over leaf mean-value comparisons; (4) complement the imaging pipeline with OJIP transient analysis (JIP-test) and organ-specific Cd^{2+} quantification *via* square-wave anodic stripping voltammetry (SWASV) polarography to provide a mechanistic context. Cadmium stress was selected as a validation system in this study because: (1) its effects on PSII are well documented, providing a robust case study for the scope of this study; and (2) extra ammonium sulfate nutrition can alter both Cd translocation and photosynthetic performance, providing a more complex context in which to detect treatment-specific spatial shifts.

Heavy metal contamination is a growing concern in agricultural and natural ecosystems, with cadmium (in the form of Cd^{2+}) representing one of the most toxic and persistent elements affecting plant growth and productivity. Cd accumulation in agricultural systems results from anthropogenic activities such as excessive use of phosphate fertilisers, pesticides, sewage sludge and wastewater (Srivastava *et al.* 2017, Khatun *et al.* 2022), as well as mining activities (Shi *et al.* 2022).

Cadmium in soil is absorbed by crops, raising food safety concerns as it enters the food chain and poses a risk to human health (Suhani *et al.* 2021). Negative effects of Cd stress and the corresponding tolerance mechanisms have been widely studied in plants. Cd pollution in soil disrupts the uptake of essential micronutrients such as calcium ions (Ca^{2+}), copper ions (Cu^{2+}), iron ions (Fe^{2+}), zinc ions (Zn^{2+}), and magnesium ions (Mg^{2+}), leading

to competitive inhibition (Khaliq *et al.* 2019, Qin *et al.* 2020). Cd-induced stress affects plants at morphological, physiological, and molecular levels. Common symptoms include limited growth and development, reduced stomatal conductance and gas exchange, overproduction of reactive oxygen species (ROS) with consequent oxidative damage to cell components, lipid peroxidation, protein degradation, and impaired photosynthetic processes (El Rasafi *et al.* 2020, Mushtaq *et al.* 2025, Song *et al.* 2025). Plants typically respond with an upregulation of the ROS-scavenging antioxidant system involving both enzymatic and nonenzymatic antioxidants. Moreover, several osmotically active compounds and Cd-chelating compounds are synthesised and accumulated in high amounts to provide increased tolerance against Cd stress (Majumdar *et al.* 2025).

Agricultural practices, as a crucial part of abiotic stress mitigation (Hamada and Hamada 2020, Hossain *et al.* 2020), fertilisation, and nutrient management in the soil can make a difference in Cd mobility, availability, uptake, and stress in plants (Jalloh *et al.* 2009, Hattab *et al.* 2014, Xu *et al.* 2021). The sulfur (S) addition can mitigate Cd uptake and its negative effects on plants (Gaafar *et al.* 2012, Adhikari *et al.* 2018, Liu *et al.* 2020). An underlying physiological mechanism is the synthesis of sulfur-rich phytochelatins, short Cys-rich peptides with repeating γ -Glu-Cys motifs, which are capable of complexing Cd into their molecules to make them less harmful for plants (Wątył *et al.* 2021). These complexes are transported into the vacuole, where allocated, which helps lower the toxic concentration of Cd in other cellular compartments (Marques *et al.* 2025). Similarly, a pivotal role has been attributed to nitrogen (N) nutrition as well (Khan *et al.* 2016); many studies have shown that different forms of nitrogen can affect Cd translocation and/or speciation (Wang *et al.* 2004, Cheng *et al.* 2016, Tian *et al.* 2022).

To quantify Cd accumulation in different organs of plants reliably, polarography was employed in our study as a highly sensitive electrochemical technique easily applicable to complex biological matrices. The study employed polarography to measure Cd^{2+} ion concentrations in different organs of *Solanum lycopersicum*, *i.e.*, leaves, stems, and roots. Moreover, integrating polarographic techniques with ChlF imaging and JIP-test gave a systemic image of cadmium's physiological impact.

Materials and methods

Plant materials: Tomato (*Solanum lycopersicum*, cherry variety Rapido F1) seeds were surface sterilised using a 30% v/v commercial Clorox solution; they were agitated in the solution for 10 min, then rinsed 5 times with distilled sterilised water. Afterwards, the seeds were placed in a germination unit, *i.e.*, a 40 × 40 cm tray with inorganic and sterile sand. Germination took place in a greenhouse with the following microclimate parameters: average temperature of 22°C, minimum temperature of 17°C, and maximum temperature of 28°C; 30% RH during the day and 65% during the night; light intensity of 200 ± 10 $\mu\text{mol}(\text{photon})\text{m}^{-2}\text{s}^{-1}$; 14/10-h day/night photoperiod.

When the first true leaf appeared, the seedlings were transferred to a hydroponic system consisting of 60×15 cm trays filled with Hoagland nutrient solution [in mg L^{-1} : $\text{Ca}(\text{NO}_3)_2$, 284.9; KNO_3 , 160.5; KH_2PO_4 , 51.85; MgSO_4 , 133.1; Na-EDTA , 29.7; NH_4NO_3 , 400.2; H_3BO_3 , 2.473; MnSO_4 , 0.676; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.121; CuSO_4 , 0.075; ZnSO_4 , 0.489; K_2SO_4 , 139.4; and CaCl_2 , 133.2], and an air pump for oxygenation. Four treatments (16 replicates each) were applied: control, 5 mM ammonium sulfate [$(\text{NH}_4)_2\text{SO}_4$, AS], 100 μM cadmium (CdCl_2 , Cd-only), and a combination of both (AS+Cd). Treatments were applied at the 4th leaf stage, and above- and below-ground biomass was harvested and measured 8 d later. At harvest, plants were first washed with deionized water and the excess water was dried using filter paper; the root and shoot fresh mass (FM) was recorded on an analytical scale, while some plants that were used for the Cd^{2+} ion content analysis were separated into roots, stems, and leaves and put directly in the oven. Eight plants for each treatment group were dried in an oven overnight at 80°C ; afterwards, the root and shoot dry mass (DM) was recorded, and the dry matter content (DMC) was calculated as follows: $\text{DMC} = (\text{DM}/\text{FM}) \times 100$. Cd^{2+} content analysis in roots, stems, and leaves was conducted as described in section “ Cd^{2+} ion content determination” below.

Chlorophyll fluorescence image analysis: Chlorophyll fluorescence (ChlF) image analysis was performed using an *Open FluorCam* imaging system and *FluorCam7* software (type *FC 800-O/1010*, *Photon Systems Instruments*, Drásov, Czech Republic) using the protocol described in [Giordano *et al.* \(2025\)](#). The measurements were conducted for all experimental groups (*i.e.*, control, ammonium sulfate, cadmium, and combined) after 8 d. Eight leaves per treatment were randomly selected among the healthiest 3rd leaves.

Prior to the measurement, leaves were dark-adapted for 15 min, then cut off at the petiole from the plant and placed on a plastic plate under the *Open Fluorcam* chamber; a drop of water was applied at the cutting point to prevent rapid loss of water from the leaf.

For each leaf, the mean value and standard deviation were calculated for F_v/F_m , Φ_{PSII} , nonphotochemical quenching (NPQ), and fluorescence decline ratio (Rfd). Pixel-wise images were exported to evaluate spatial heterogeneity in photosynthetic performance; visual assessment was conducted on images by thresholding F_v/F_m values, masking pixels with values below 0.60 (considering 0.6 as the photoinhibition threshold; [Quiles 2005](#)); the most representative leaf of each treatment is showed in [Fig. 1](#).

From the average leaf values and standard deviations, the coefficient of variation (CV) was calculated according to the formula: $\text{CV} = (\text{std}/\text{mean}) \times 100$.

Histograms representing pixel frequency distributions for each ChlF parameter were exported for further analysis. We evaluated the distribution characteristics, including mean, skewness, and kurtosis; multimodality was assessed using *Hartigan's* dip test; the *REBMIX* algorithm was used to characterise each mode of the multimodal distributions,

as described by [Bresson *et al.* \(2015\)](#). [Fig. 5](#) shows the average distributions (among eight replicates) of each treatment.

Data were processed using the *FluorCam* software (*ver. 1.5.0.46*), and then further analysed using *MS Excel 365* and the *Python* programming language (*see* section “Statistical analysis” for further description).

JIP-test: Fast kinetics OJIP were recorded using a *PAR-FluorPen* portable fluorimeter (*Photon Systems Instruments*, Drásov, Czech Republic) equipped with detachable leaf clips. A total of eight leaves for each group of treatment was dark-adapted for 15 min using the fluorimeter leaf clips. Then the OJIP protocol was used to measure ChlF. It induced a saturating light pulse of $3,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$, and the fast rise of ChlF was recorded for 2,000 ms, as described by [Strasser *et al.* \(2004\)](#). The ChlF parameters related to PSII functioning were derived from the OJIP analysis; their description is available in Table 1S (*Supplement*). Data were processed using the *FluorPen* software (*ver. 1.1.2.6*), and then further analysed using *MS Excel 365* and the *Python* programming language.

Cd^{2+} ion content determination: The pH measurements were performed using a *WTW Inolab* pH meter with a calibrated *WTW* pH electrode. Dry plant material (leaves, stems, and roots) was ground to powder and approximately 1.0 g of each sample was digested in a microwave oven using 5.0 mL of H_2SO_4 (6.0 M) and 2.0 mL of H_2O_2 (30%, added in two steps). The resulting solution was neutralised to pH 4.5 with NaOH (6 M) and diluted to a final volume of 20.0 mL with deionised water.

Cadmium ions (Cd^{2+}) were quantified by square-wave anodic stripping voltammetry (SWASV) using a *Metrohm 663 VA* stand connected to an *Autolab PGSTAT204* potentiostat. A three-electrode cell was employed, consisting of a hanging mercury drop electrode (HMDE) as the working electrode, an Ag/AgCl (3 M KCl) reference electrode, and a platinum auxiliary electrode. Before measurements, dissolved oxygen was removed by N_2 purging. Cd^{2+} was pre-concentrated at -1.1 V for 120 s under stirring, followed by a 5-s equilibration, and re-oxidised by scanning from -1.1 to -0.4 V (frequency:

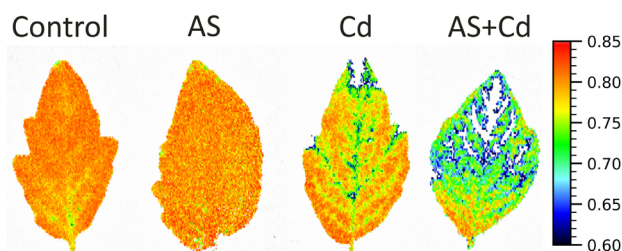


Fig. 1. Representative pixel-wise ChlF images of the leaf areas. Each leaf is a representative among the eight replicates for each treatment. Treatments displayed are Control, ammonium sulfate addition (AS), cadmium addition (Cd), and combined treatment (AS+Cd). Images were created by thresholding the F_v/F_m , masking pixels with values below 0.6.

25 Hz; modulation amplitude: 20 mV). A calibration curve was constructed from triplicate measurements at various Cd^{2+} concentrations, and sample concentrations were additionally verified by the standard addition method (additions of 5, 10, 15, and 20 ppb). The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 3 and 10 s m^{-1} , respectively, where s is the standard deviation at the lowest calibration point, and m is the calibration slope. Accuracy was assessed by recovery analysis of spiked samples. All measurements were performed in triplicate, and results are reported as mean $\pm$ confidence interval at 95% (t -distribution, $n = 3$).

The results were reported with confidence limits calculated as:

$$\bar{X} \pm \frac{t_{n-1} \times s}{\sqrt{n}}$$

Where $\bar{X}$ represents the mean (Miller and Miller 2000), s denotes the standard deviation of the repeated measurement, and n is the number of repetitions (3). The value of t was obtained from the t -distribution table corresponding to 2 degrees of freedom ($n - 1 = 3 - 1$) at a confidence level of 95% ($P=0.05$).

Statistical analysis: Differences between groups in ChlF parameters, CV values, distribution metrics, JIP-test parameters, biometric data, and Cd^{2+} concentrations were assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p \leq 0.05$). Data preprocessing was performed in Excel 365 and Python; dip test and REBMIX analyses were performed in R using “dipTest” and “rebmix” libraries; all statistical analyses and visualisations were produced in Python.

Results

Biometrical analysis: Biometric results are presented in Fig. 2. In shoots, the AS treatment resulted in the lowest DMC values, ranging from 8–9%, compared to the control and other treatments, which showed values between 13–14%.

In roots, the highest DMC values were observed in the Cd treatment, both when applied alone and in combination with AS. In contrast, the control and AS treatments exhibited lower DMC values, indicating higher water uptake in the roots. Moreover, the AS treatment appeared to promote greater water translocation to the aerial parts of the plant, contributing to stronger shoot growth. However, this positive effect was mitigated by the addition of Cd to the nutrient solution.

As further evidenced by the fresh and dry masses of both shoots and roots (Fig. 2), Cd exposure led to a noticeable reduction in overall plant growth.

ChlF image analysis: Fig. 3 presents the parameters obtained from the ChlF image analysis, highlighting the effects of Cd and AS treatments on photosynthetic performance of PSII, i.e., F_v/F_m and Φ_{PSII} , NPQ, and Rfd. Cd exposure impaired photosynthetic metabolism, and

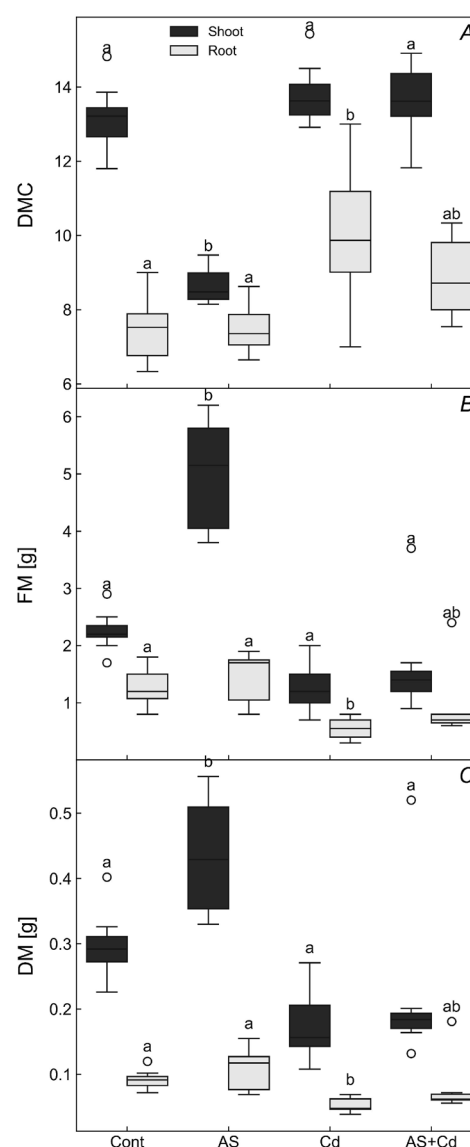


Fig. 2. Biometric analysis derived parameters: dry matter content, DMC (A), fresh mass, FM (B), dry mass DM (C). Treatments displayed are Control (Cont), ammonium sulfate addition (AS), cadmium addition (Cd), and combined treatment (AS+Cd). Letters above the boxes represent the significant difference between treatments for shoot and for roots (differences were considered significant for p -values < 0.05).

this negative effect was not alleviated by the addition of ammonium sulfate; in fact, the combined AS+Cd treatment appeared to further exacerbate photosynthetic decline in PSII and photosynthetic linear electron flow.

F_v/F_m decreased by approximately 0.1 in Cd-only and 0.15 in AS+Cd-treated plants, compared to the control and AS-only treatments, both of which maintained optimal values. Effective quantum yield (Φ_{PSII}) and chlorophyll fluorescence decline ratio (Rfd) followed similar trends; the highest values were found in AS-only and the lowest in AS+Cd plants.

Nonphotochemical quenching (NPQ) under Cd treatments was similar to the control, while the AS-only

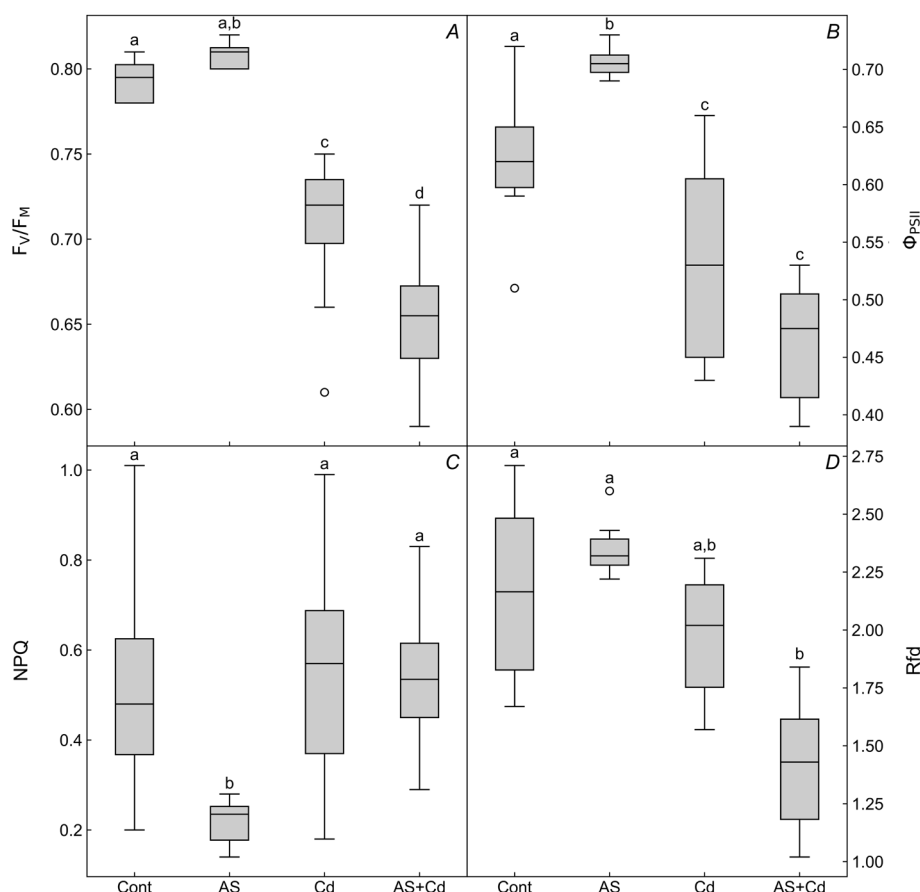


Fig. 3. ChlF derived parameters: maximum (potential) quantum yield of photosynthetic processes in PSII, F_v/F_m (A), effective quantum yield of photosynthetic processes of PSII, Φ_{PSII} (B), nonphotochemical quenching, NPQ (C), chlorophyll fluorescence decrease ratio (vitality index), Rfd (D). Treatments displayed are Control (Cont), ammonium sulfate addition (AS), cadmium addition (Cd), and combined treatment (AS+Cd). Letters above the boxes represent the significant difference between treatments for each leaf area; differences were considered significant for p -values < 0.05.

treatment exhibited lower NPQ values than the control, in line with the other parameters, indicating a healthier status.

Regarding spatial heterogeneity in ChlF, Cd stress induced a heterogeneous impairment of PSII and net photosynthesis, as seen from the increase in CV of these parameters (Fig. 4). On the opposite, NPQ showed a high baseline level of heterogeneity (~65% in control), and Cd treatment decreased this heterogeneity, even if not statistically different from control (Fig. 4), suggesting a more systematic activation of NPQ, in contrast with the more heterogeneous impairment of PSII.

Pixel-wise images (Fig. 1) clearly display this trend, showing a stronger stress-induced inhibition at the vascular level and in close-to-tip regions, showing the increase in heterogeneity of limitation of primary photosynthesis induced by Cd and combined AS+Cd.

Histograms are displayed in Fig. 5, and Table 1 shows the distribution metrics calculated from them. Over a single leaf area, pixels are non-uniformly distributed even under control, consistent with an intrinsic spatial heterogeneity where the distribution of ChlF-related pixels is not perfectly bell-shaped. *Hartigan's* dip test detected deviations from unimodality in all treatments ($p < 0.001$), a result which may partly reflect the large number of pixels contributing to each histogram; nevertheless, visual inspection of histograms revealed a dominant primary peak with only minor secondary physiological “patches”. Distribution metrics complete the framework: all treatments exhibited

negatively skewed F_v/F_m , Φ_{PSII} , and Rfd distributions and were positive for NPQ, indicating the presence of a long tail of pixels with reduced photosynthetic efficiency – or higher NPQ values – while maintaining a main peak.

Cd treatment mainly affected the mean and mode values, but also caused a moderate change in the symmetry as well; kurtosis did not change significantly. The change in mean and skewness is consistent with the occurrence of a higher number of regions of photoinhibition compared to control values. Rfd mean values ranged from 1.41 to 2.34; while most samples were positively skewed, Cd-only displayed a slight negative skew (–0.18 in Cd-only) or lower than control (0.31 in AS+Cd vs. the 0.51 of the control, *see* Table 1), suggesting a shift toward lower values of fluorescence decline ratios compared to higher values and a right-leaning distribution in control.

The detected and characterised modes from the *REBMIX* algorithm (Fig. 6) provide a complementary illustration of what is displayed from the distribution metrics. Healthy leaves from the AS treatment showed the most homogeneous distribution, while control leaves, even if in healthier condition compared to Cd and SC, present a higher heterogeneity than AS heterogeneity. Important in this study are the results from Rfd: Cd treatment did not change the mean Rfd values or mode significantly, but we still observed a significant shift to lower values in the distribution compared to control (negative skewness vs. a positive skewness in control),

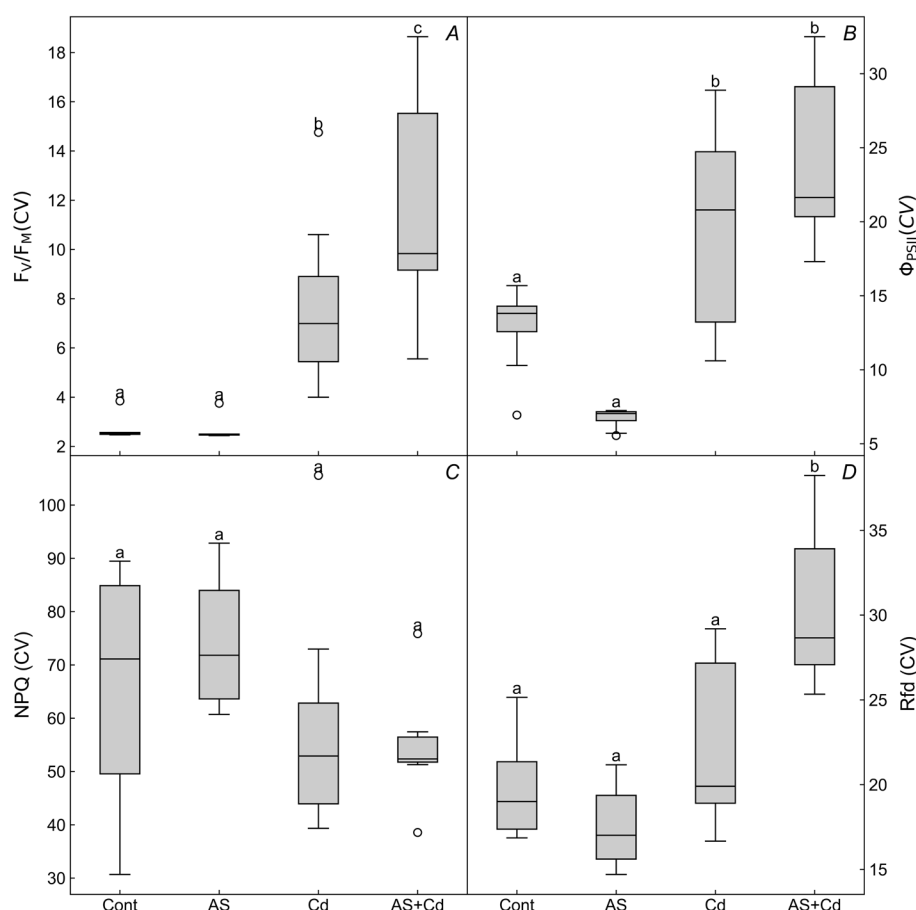


Fig. 4. ChlF derived parameters' coefficient of variation (CV): maximum (potential) quantum yield of photosynthetic processes in PSII, F_v/F_m (A), effective quantum yield of photosynthetic processes of PSII, Φ_{PSII} (B), nonphotochemical quenching, NPQ (C), chlorophyll fluorescence decrease ratio (vitality index), Rfd (D). Treatments displayed are Control (Cont), ammonium sulfate addition (AS), cadmium addition (Cd), and combined treatment (AS+Cd). Letters above the boxes represent the significant difference between treatments for each leaf area; differences were considered significant for p -values < 0.05.

suggesting a shift towards lower Rfd values was not detected from the mean value analysis.

JIP-test: JIP-test provided a complementary, more mechanistic insight into Cd-induced photosynthetic impairment. A distinct K-band (Fig. 3S, *Supplement*) appeared at $\sim 300 \mu s$ in the Cd-only and AS+Cd treatments, while the AS-only treatment resembled the control and showed no K-band. A similar pattern occurred for the L-band: the AS-only treatment did not differ from the control, whereas Cd-only and AS+Cd treatments showed increased amplitudes.

Fig. 4S (*Supplement*) presents a radar plot of JIP-test parameters normalised to the control. Both F_v/F_0 and F_v/F_m decreased under Cd treatment, with a stronger reduction in AS+Cd. Since F_v/F_0 reflects the efficiency of the water-splitting complex on the donor side of PSII, the decrease suggests impairment of the oxygen-evolving complex under Cd stress. The lowest F_v/F_0 values occurred in AS+Cd, which differed significantly from the control and AS-only treatment, while Cd-only did not differ significantly from the other groups.

Cd treatment increased energy dissipation (DI_0/RC and ΦDo) relative to the control and AS-only groups, with the highest values in AS+Cd, indicating greater stress-related energy loss. Cd exposure also increased TR_0/RC and ABS/RC . ET_0/RC increased under Cd treatment but decreased in AS+Cd. These changes

may reflect limitations of the OEC and impairment of the antenna complex (Franić *et al.* 2017).

Finally, the performance index (PI_{ABS}), a comprehensive indicator of photosynthetic vitality, declined significantly in Cd-only and AS+Cd treatments, with the largest decrease in AS+Cd, indicating reduced overall photosynthetic performance under cadmium stress.

Cd²⁺ ion content analysis: SWASVs of Cd²⁺ ions in acetate buffer (pH 4.5) exhibited a well-defined anodic peak at $-0.58 V$. The reoxidation peak of accumulated Cd to Cd²⁺ was used to plot a calibration curve, demonstrating a linear relationship between peak current and Cd²⁺ concentration within the range of 5.0–50.0 ppb. The good linearity of the calibration graph is evident from the correlation coefficient (0.9997) (Fig. 1S, *Supplement*).

The possibility of using SWASV with HMDE for determination of Cd²⁺ ions in the prepared samples was studied by the validation method. The LOD and LOQ of the SWASV method were calculated to be 0.02 ppb and 0.08 ppb, respectively. The low values of repeatability (0.17 %) and reproducibility (0.40 %) indicate good precision, while the value of the recovery percentage close to 100% shows the accuracy and reliability of the method. Analytical parameters of the SWASV are listed in Table 3S (*Supplement*). The optimised SWASV was successfully applied for the determination of Cd²⁺ ions in plant samples. The quantification of Cd²⁺ ions in the prepared sample

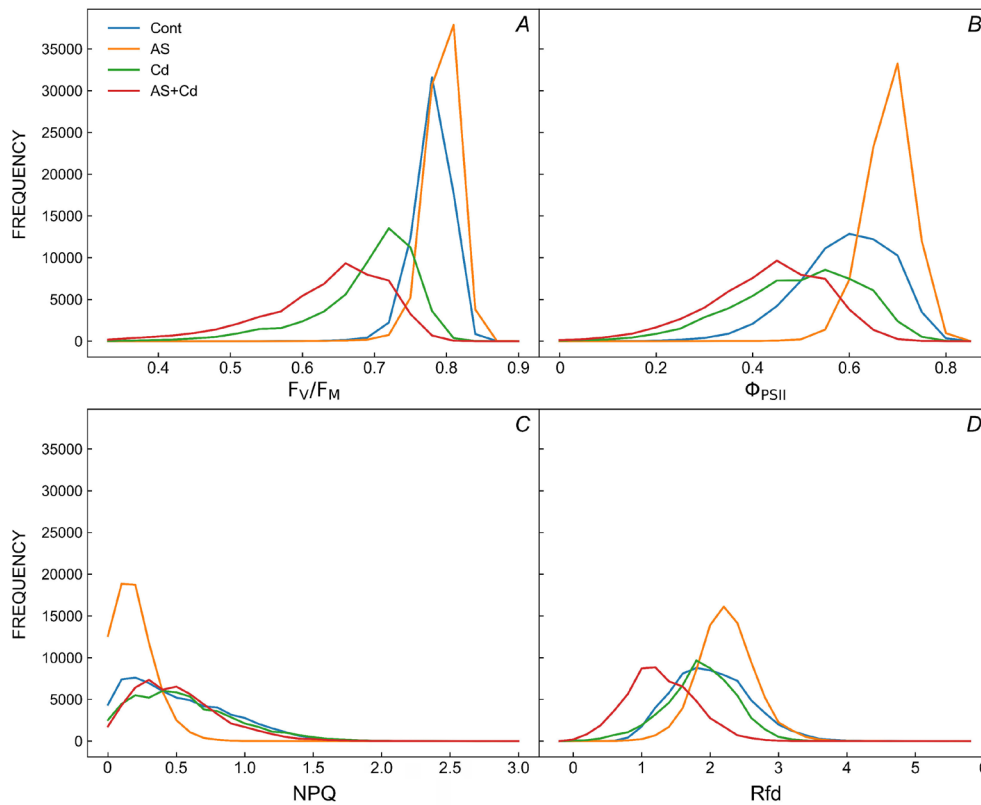


Fig. 5. Histograms showing the pixel distribution across ChlF parameters: maximum (potential) quantum yield of photosynthetic processes in PSII, F_v/F_m (A), effective quantum yield of photosynthetic processes of PSII, Φ_{PSII} (B), nonphotochemical quenching, NPQ (C), chlorophyll fluorescence decrease ratio (vitality index), Rfd (D). For each treatment, the distribution displayed is the calculated average among eight replicates. Treatments displayed are Control (Cont, blue), ammonium sulfate addition (AS, yellow), cadmium addition (Cd, green), and combined treatment (AS+Cd, red).

Table 1. Distribution metrics of ChlF-related histograms; each value represents the average from eight replicates $\pm$ standard deviation. Letters on the right of each value show significant differences between treatments ($p < 0.05$). For the dip test, p was considered significant when < 0.001 . *Proportion of replicates with significant multimodality from the dip test ($n = 8$). F_v/F_m – maximum (potential) quantum yield of photosynthetic processes in PSII; NPQ – nonphotochemical quenching; Rfd – chlorophyll fluorescence decrease ratio (vitality index); Φ_{PSII} – effective quantum yield of photosynthetic processes of PSII.

Parameter	Treatment	Mean	Mode	Variance	Skewness	Kurtosis	Multimodal proportion*
F_v/F_m	Control	0.80 ± 0.01^a	0.80 ± 0.01^a	0.000 ± 0.000^a	-0.81 ± 0.44^a	2.35 ± 1.91^a	1
	AS	0.81 ± 0.01^a	0.81 ± 0.00^a	0.000 ± 0.000^a	-0.82 ± 0.41^a	2.51 ± 1.84^a	1
	Cd	0.71 ± 0.05^b	0.73 ± 0.03^b	0.003 ± 0.002^a	-1.47 ± 1.25^a	6.44 ± 8.79^a	1
	AS+Cd	0.65 ± 0.04^c	0.68 ± 0.04^c	0.006 ± 0.004^b	-0.99 ± 0.43^a	1.48 ± 1.61^a	1
Φ_{PSII}	Control	0.62 ± 0.06^a	0.65 ± 0.07^a	0.006 ± 0.002^a	-0.86 ± 0.29^a	1.74 ± 1.23^a	1
	AS	0.71 ± 0.01^b	0.71 ± 0.02^a	0.002 ± 0.000^b	-0.60 ± 0.18^a	1.60 ± 1.00^a	1
	Cd	0.53 ± 0.09^c	0.56 ± 0.08^b	0.010 ± 0.004^c	-0.64 ± 0.20^a	1.00 ± 0.45^a	1
	AS+Cd	0.46 ± 0.05^c	0.48 ± 0.06^b	0.012 ± 0.003^c	-0.62 ± 0.18^a	0.83 ± 0.62^a	1
NPQ	Control	0.53 ± 0.27^a	0.40 ± 0.28^a	0.097 ± 0.032^a	0.92 ± 0.48^a	1.81 ± 1.78^a	1
	AS	0.22 ± 0.05^b	0.22 ± 0.10^a	0.029 ± 0.005^b	0.46 ± 0.13^b	0.83 ± 0.43^a	1
	Cd	0.55 ± 0.25^a	0.50 ± 0.26^a	0.093 ± 0.054^a	0.52 ± 0.18^b	0.65 ± 0.47^a	1
	AS+Cd	0.54 ± 0.18^a	0.48 ± 0.17^a	0.088 ± 0.035^a	0.70 ± 0.20^{ab}	1.24 ± 0.53^a	1
Rfd	Control	2.17 ± 0.41^a	2.06 ± 0.39^a	0.180 ± 0.036^a	0.51 ± 0.30^a	1.49 ± 1.01^a	1
	AS	2.34 ± 0.12^a	2.26 ± 0.14^a	0.172 ± 0.034^a	0.60 ± 0.77^a	8.14 ± 19.5^a	1
	Cd	1.97 ± 0.29^a	2.01 ± 0.26^a	0.184 ± 0.037^a	-0.18 ± 0.40^b	1.80 ± 1.17^a	1
	AS+Cd	1.41 ± 0.29^b	1.46 ± 0.36^b	0.185 ± 0.062^a	0.31 ± 0.31^a	1.78 ± 1.44^a	1



Fig. 6. Detected and characterized modes from the REBMIX algorithm of ChlF parameter pixel distribution: maximum (potential) quantum yield of photosynthetic processes in PSII, F_v/F_m (A), effective quantum yield of photosynthetic processes of PSII, Φ_{PSII} (B), nonphotochemical quenching, NPQ (C), chlorophyll fluorescence decrease ratio (vitality index), Rfd (D). Modes were characterized for value, standard deviation, and weight; each dot represents a mode, dot size represents the mode's weight.

Table 2. The content of Cd²⁺ ions [$\mu\text{g g}^{-1}$] found in dried plant samples using the SWASV method at HMDE by standard addition and calibration curve methods. 1 – leaf; 2 – stem; 3 – root. The confidence level was calculated by $t \times s/\sqrt{n}$, where t is the critical value of Student's t for a two-tailed test at $\alpha=0.05$, s is the standard deviation of the results of three independent measurements, and n is the degree of freedom.

Treatment	Plant organ	Amount of Cd [$\mu\text{g g}^{-1}$] found using standard addition	Amount of Cd [$\mu\text{g g}^{-1}$] found using calibration curve
Control, AS	1, 2, 3	Below detection limit	Below detection limit
Cd	1	34.18 ± 0.20	34.34 ± 0.46
Cd	2	59.75 ± 0.53	57.07 ± 0.90
Cd	3	$7,553.58 \pm 281.44$	$7,407.11 \pm 233.86$
AS+Cd	1	27.20 ± 0.63	27.72 ± 0.84
AS+Cd	2	132.65 ± 0.73	131.63 ± 0.77
AS+Cd	3	$3,602.73 \pm 68.88$	$4,148.91 \pm 166.03$

was carried out using the standard addition method and the calibration curve method. The results from the Cd content analysis are shown in Table 2; Cd²⁺ contents in control and AS-only treatments were below the detection limit in all plant parts for both quantification methods, indicating either the absence of cadmium content or too low to be reliably detected by SWASV. In contrast, the Cd-only group showed an increasing Cd²⁺ accumulation from leaf to root: $34.18 \pm 0.20 \mu\text{g g}^{-1}$ in leaf, $59.75 \pm 0.53 \mu\text{g g}^{-1}$ in stem, and a very high concentration of $7,553.58 \pm 281.44 \mu\text{g g}^{-1}$ in root based on standard addition. The combined treatment, AS+Cd, revealed slightly altered Cd²⁺ distribution: $27.20 \pm 0.63 \mu\text{g g}^{-1}$ in leaf, $132.65 \pm 0.73 \mu\text{g g}^{-1}$ in stem, and $3,602.73 \pm 68.88 \mu\text{g g}^{-1}$ in roots. These results suggest that cadmium primarily accumulated in roots of experimental plants and that sulfur may mitigate root-level Cd²⁺ accumulation. A comparison between the two quantification methods, which are presented in Table 2, showed satisfactory agreement, confirming the reliability of the measurements and indicating reliable detection and minimal matrix effects. The recorded SWASVs of the samples and corresponding standard addition graph and concentration dependence equation are given in Fig. 2S (Supplement).

Discussion

Our study showed that spatial heterogeneity is intrinsic, not only in the whole plant but also at the leaf level, where a functional base heterogeneity is always present. Under non-stressed conditions, F_v/F_m remained highly homogeneous across the leaf surface ($CV \sim 2\%$), reflecting a uniform structural integrity of PSII reaction centres and antenna complexes. This observation is consistent with the physiological interpretation of F_v/F_m as an indicator of maximal photochemical efficiency associated with the trapping efficiency of all the absorbed light to reduce the primary quinone electron acceptor of PSII (Q_A) (Strasser and Tsimilli-Michael 1998). In contrast, parameters more closely related to effective photosynthetic performance, such as Φ_{PSII} and vitality index (Rfd), exhibited greater spatial variability even in control plants. This intrinsic heterogeneity likely reflects functional differences

between leaf regions, including variations in electron transport activity, carbon assimilation capacity, and anatomical organisation across distinct functional areas of the leaf (*i.e.*, veins or mesophyll) (Walter *et al.* 2004, Xiong and Flexas 2020). Additional variability may also arise from the heterogeneous distribution and organisation of PSII complexes within thylakoid membranes: some are in stacked grana while others are located in more exposed regions of the stroma (Cao and Govindjee 1990, Lavergne and Leci 1993).

The central methodological contribution of this study is the demonstration that distribution metrics derived from pixel-wise ChlF imaging provide a more sensitive and more informative characterisation of photosynthetic stress responses than conventional leaf mean-value comparisons. This was most clearly illustrated by the Rfd distributions under Cd stress: mean Rfd values were statistically indistinguishable between Cd-only and control leaves, yet the distribution skewness shifted from positive to negative: a change that reflects a redistribution of pixels toward lower Rfd values that is invisible to mean-based analysis. This result has direct implications for stress-detection sensitivity: relying solely on mean Rfd values would conclude that net photosynthesis is unaffected by Cd treatment at the tested exposure level, whereas the distributional analysis reveals a spatial shift in the photosynthetic activity over the leaf area. The values, in the case of stronger stress, instead, shift the mean significantly (and the mode), while the skewness is positive again, reflecting a further change in shape compared to Cd-only, in this case more similar to control, but with the main peak shifted to lower values.

This phenomenon can be understood as a form of survivor bias in mean-value analysis: the majority pixel population, corresponding to relatively unaffected leaf tissue, dominates the mean and effectively masks the signal from the minority population of photoinhibited pixels, concentrated at vascular and close-to-tip regions. Our approach overcomes this by treating the entire pixel population as a structured distribution rather than collapsing it into a single summary statistic. The REBMIX-based mixture model extends this further by explicitly characterising co-existing ChlF subpopulations within

a single leaf, providing a more quantitative tracking of the weight and position of modes across treatments. This approach was first proposed by [Bresson *et al.* \(2015\)](#) for drought stress in *Arabidopsis* and is here extended and validated for a different stressor (cadmium), a different crop species (tomato), a more complex multi-treatment experimental design and ChLF parameters different from F_v/F_m . However, the sensitivity of the algorithm should be considered. Secondary peaks presented a weight close to 0, where physiologically they could not be considered as a subpopulation, but a statistical artefact ([Nagode 2015](#)). The threshold images identified vascular tissues and leaf tips as the primary sites of Cd-induced photoinhibition. This spatial organisation is consistent with findings in poplar and willow ([Pietrini *et al.* 2010](#)) as well as white mustard exposed to thallium stress ([Mazur *et al.* 2016](#)) and suggests a connection with Cd transport physiology: cadmium entering the plant *via* roots is transported through the xylem to aerial tissues, resulting in the highest local Cd concentrations at sites of xylem unloading ([Song *et al.* 2017](#)). Even if the SWASV results confirmed an elevated Cd accumulation in stems in AS+Cd plants, suggesting the transportation of Cd^{2+} ions from the shoots could be part of a detoxification process ([van Belleghem *et al.* 2007](#)), and combined with the imaging data, they show a more extensive vascular photoinhibition in this treatment, validating this transport-driven spatial hypothesis, a more specific Cd quantification of vascular vs. mesophyll tissue and correlation with ChLF spatial data would certainly confirm this hypothesis.

Interestingly, NPQ heterogeneity did not significantly change across the leaf surface, while also average NPQ values remained statistically similar to those of control plants. This pattern suggests two main things: (1) NPQ is not necessarily correlated with a heterogeneous decline of F_v/F_m , being more systematically activated across the leaf area; and (2) that Cd exposure did not trigger a strong upregulation of regulated photoprotective thermal dissipation. Similar observations have been reported in plants exposed to high Cd concentrations ([Wang *et al.* 2013](#)). OJIP analysis complemented these observations and helped complete the frame by revealing the appearance of L- and K-bands, mirroring negative effects on the connectivity between PSII and the light-harvesting complex (LHCs), as well as donor-side limitations consistent with impairment of the OEC – as reflected also by reduced F_v/F_0 – and damage to proteins such as the D1 ([Figlioli *et al.* 2019](#), [Dobrikova *et al.* 2021](#), [Giordano *et al.* 2025](#)). Our results are consistent with recent knowledge on Cd effects on OJIP-derived parameters related to the O–J phase of the OJIP curve, since the appearance of K- and L-bands is Cd dose- ([Perreault *et al.* 2011](#)) and stress duration-dependent ([Zhou *et al.* 2024a](#)). Furthermore, the increase in DI_0/RC and ϕDo in Cd-treated plants compared to control suggests that a larger fraction of absorbed light energy is being dissipated rather than utilised in photochemistry, which is likely associated with reaction centre inactivation and restricted electron transport ([Zhou *et al.* 2024b](#)). This confirms the interpretation that energy dissipation

in Cd-stressed leaves is driven primarily by PSII damage rather than by regulated photoprotective thermal dissipation.

The results of this study demonstrate Cd-induced impairment of plant growth and photosynthetic efficiency, while ammonium sulfate supplementation alone appeared to enhance morphological and physiological performance in tomato. However, when ammonium sulfate was combined with Cd, a contrasting response was observed: it mitigated the uptake of Cd from roots, strongly reducing the accumulation of the heavy metal in roots and leaves and, on the other hand, its allocation to the upper vascular system, but at the same time worsening the negative effect on photosynthesis. This outcome contrasts with several studies reporting a mitigating effect of sulfur nutrition on Cd stress ([Adhikari *et al.* 2018](#), [Liu *et al.* 2020](#), [Chen and Lai 2025](#)).

While a full mechanistic investigation of ammonium sulfate influence on Cd stress was beyond the scope of this study, a possible explanation of this could lie in the complexity of the interaction between ammonium sulfate supply, Cd concentration, and plant-specific physiological responses. In this study, ammonium (NH_4^+) had a key role in the reduction in photosynthetic performance under Cd stress; previous studies have shown that NH_4^+ nutrition can enhance Cd translocation from roots to shoots ([Wang *et al.* 2004](#), [Cheng *et al.* 2016, 2017](#); [Nogueirol *et al.* 2018](#)). Our results are consistent with this mechanism, as adding ammonium sulfate promoted Cd allocation to aerial tissues despite reducing its overall uptake.

In contrast, sulfur nutrition is generally associated with increased Cd-detoxification capacity through the synthesis of sulfur-containing compounds such as glutathione and phytochelatins, which play key roles in Cd chelation and compartmentalisation ([Ahmad *et al.* 2019](#), [Li *et al.* 2020](#), [Peng *et al.* 2025](#)). These mechanisms often contribute to reduced Cd toxicity and improved physiological performance. However, mitigating effects of sulfur are typically reported at lower Cd concentrations (approximately 4–50 μM) than the level used in this study (100 μM) ([Afef *et al.* 2012](#), [Li *et al.* 2020](#), [Lyčka *et al.* 2023](#)). At such elevated Cd exposure, detoxification mechanisms may become insufficient to fully counteract toxicity.

Additional factors may also contribute to the observed response: Cd exposure can inhibit enzymes involved in NH_4^+ assimilation, such as glutamine synthetase, leading to elevated intracellular ammonium concentrations under Cd stress ([Chien and Kao 2000](#), [Dguimi and Alzahrani 2023](#)). The accumulation of NH_4^+ may therefore act as an additional physiological stress factor, potentially exacerbating the negative impact on photosynthesis.

Furthermore, nutrient composition may influence Cd speciation within plant tissues. Chelated forms of Cd are generally less harmful to photosynthetic metabolism, whereas higher proportions of free Cd^{2+} ions may lead to more serious physiological damage ([Tian *et al.* 2022](#)). Changes in nutrient availability could therefore alter Cd chemical forms within leaf tissues and influence

the extent of photosynthetic inhibition. Further studies will be necessary to better understand the speciation of Cd inside the leaf and the dynamics behind nutrient composition and Cd stress in tomato.

The decline in photosynthetic performance observed in the combined treatment may also reflect increased metabolic investment in detoxification processes, such as the synthesis of phytochelatins and/or antioxidant compounds, which can divert resources from growth and photosynthetic activity (Waheed *et al.* 2022).

Overall, these results suggest that the combined effects of ammonium-enhanced Cd translocation and high Cd exposure may override the protective role typically associated with sulfur nutrition. In order to further investigate this particular response, a study focusing on the role of ammonium alone in Cd stress will be needed. This highlights the importance of nutrient composition and metal concentration in determining plant responses to heavy metal stress.

Future studies may certainly improve our knowledge; investigating a wider range of Cd concentrations could further clarify how spatial photosynthetic heterogeneity develops under more moderate stress conditions. Furthermore, integrating a detailed mechanistic analysis – such as molecular or biochemical markers – would provide a more comprehensive understanding of the pathways driving the observed physiological responses.

Conclusions: This study presents and validates an integrated pixel-wise chlorophyll fluorescence imaging analysis pipeline for the quantitative characterisation of spatial heterogeneity in photosynthetic stress responses. The pipeline moves beyond the standard mean-value ChlF analysis to extract distribution-shape metrics from full-leaf pixel distributions, providing substantially greater sensitivity for stress detection than standard approaches.

The findings suggest that spatial heterogeneity is intrinsic at the leaf level, and the response to stress is heterogeneous as well; different functional areas of the leaf may respond in a different way. Each ChlF parameter presents a peculiar spatial pattern in response to stress, demonstrating that, compared to leaf average analysis, a deeper spatial evaluation may provide a more extensive view of the stress physiology of photosynthesis, as well as help with earlier stress detection.

The distributional analysis validated here may be fully generalizable to other stressors and crop species. The integration of distribution-shape metrics with mixture model decomposition represents a methodological advance with direct applicability to precision agriculture stress monitoring, where early, sensitive, and spatially resolved detection of photosynthetic impairment may have a key practical importance.

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