PHOTOSYNTHETICA
International Journal for Photosynthesis Research

DOI 10.32615/ps.2026.030 PHOTOSYNTHETICA 64 (3): 186-199, 2026

Environmental conditions dictate the long-term photosynthetic and developmental response of lettuce to cold plasma priming

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Abstract

Cold plasma (CP) seed treatment is a promising non-chemical priming technique, yet its long-term physiological effects remain poorly understood and highly context-dependent. This study evaluated CP treatment (50–150 W) on lettuce seeds grown under controlled phytotron and semi-controlled greenhouse conditions. Germination kinetics, flowering dynamics, shoot biomass, chlorophyll fluorescence (OJIP), and leaf reflectance indices were assessed. CP consistently accelerated germination compared with the untreated control, with only minor differences among plasma power levels. Under stable phytotron conditions, higher plasma power (100–150 W) delayed flowering, increased biomass, and maintained PSII performance, consistent with prolonged vegetative growth. In contrast, greenhouse-grown plants exhibited a distinct physiological response characterised by transient alterations in chlorophyll fluorescence, earlier flowering at the highest plasma power, and no significant biomass increase. Overall, the findings demonstrate that the long-term effects of cold plasma priming depend strongly on post-treatment environmental conditions, highlighting environmental stability as a key determinant of its agronomic potential.

Keywords: biomass; chlorophyll fluorescence; cold plasma; environmental context; photosynthesis efficiency; seed germination.

Introduction

Plants are continuously exposed to a wide array of abiotic and biotic stressors that challenge their survival, development, and productivity (Regassa 2025). In recent decades, the severity and frequency of abiotic and biotic stressors have intensified due to accelerating climate change and increasing environmental pressures, posing major challenges to plant growth and crop productivity (Zayan 2019, Janni *et al.* 2024). Rising global temperatures, altered precipitation patterns, increasing atmospheric CO_2 concentrations (DaMatta *et al.* 2010, Atkin *et al.* 2015), and

Highlights

- Cold plasma accelerated germination regardless of discharge power levels
- Plasma delayed flowering and increased biomass under controlled conditions
- Growth environment determined long-term physiological outcomes after plasma priming

Received 7 April 2026
Accepted 14 September 2026
Published online 2 October 2026

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Abbreviations: CAP – cold atmospheric plasma; ChlF – chlorophyll fluorescence; CP – cold plasma; CRI1 – carotenoid index 1; CRI2 – carotenoid index 2; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; MCARI – modified chlorophyll absorption in reflectance index; NDVI – the normalized difference vegetation index; RNS – reactive nitrogen species; ROS – reactive oxygen species; Φ_{Eo} – the quantum yield of electron transport; Ψ_0 – the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-.

Acknowledgement: This work was supported by grant UJEP-SGS-2024-44-004-3 within the Student Grant Competition at UJEP. The authors thank Ing. Diana Polanská Nebeská, Ph.D., for her valuable comments on the manuscript, including language revision and critical review of the results, and Jarmila Šlechtová for her assistance with plant cultivation and care.
Conflict of interest: The authors declare that they have no conflict of interest.

the prevalence of environmental pollutants significantly affect key plant physiological processes, including photosynthesis, respiration (Centritto *et al.* 2011), water relations, and hormonal signalling. These changes not only influence individual plant performance but also have important consequences for agricultural productivity and ecosystem stability (Tilman *et al.* 2002, Pal *et al.* 2025).

To mitigate the challenges associated with peak pesticide exposure and environmental accumulation (Larsen *et al*. 2019), researchers are actively exploring non-chemical alternatives. One particularly promising approach is the application of cold atmospheric plasma (CAP) – an eco-friendly technology shown to enhance plant stress tolerance (Motyka-Pomagruk *et al*. 2021, Bussmann *et al*. 2023, Gilbert-Eckman *et al*. 2025).

Cold plasma, also called non-thermal or low-temperature plasma, is a partially ionised gas of ions, electrons, neutral particles, and reactive species at near-room temperature (Kanazawa *et al*. 1988, Boulos *et al*. 1994, Eliezer and Eliezer 2001, Burm 2012, López *et al*. 2019). Plasmas are commonly divided into two basic types: "thermal plasma" and "cold (non-thermal) plasma" depending on the comparison of gas (heavy-particle) temperature and electron temperature. In thermal plasmas, electrons, ions, and gas are in thermal equilibrium; hence, all species have similar, very high temperatures. In cold plasma (*e.g*., CAP), electrons remain highly energetic, while ions and the neutral gas stay near ambient temperature, preventing thermal damage to biological material (Lieberman and Lichtenberg 2005). Unlike thermal plasma, only the electrons are highly energetic, allowing CAP to interact with seeds and plant tissues without causing heat damage (Tendero *et al*. 2006, Adhikari *et al*. 2020, Mildaziene *et al*. 2022, Pańka *et al*. 2022). Its non-thermal nature prevents thermal injury and avoids harmful chemical residues, making it an attractive alternative to chemical seed treatments (Cui *et al*. 2019, Starič *et al*. 2020, Šerá *et al*. 2021, Pańka *et al*. 2022). However, excessive exposure can generate high concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS), potentially causing oxidative stress and tissue damage if not carefully controlled (Seol *et al*. 2017, Sivachandiran *et al*. 2017, Gavahian and Khaneghah 2020, Gilbert-Eckman *et al*. 2025).

CAP treatment improves seed germination and seedling growth by modifying the seed coat surface (Paneru *et al*. 2019), increasing wettability (Guragain *et al*. 2022) and permeability, which facilitates faster and more efficient water uptake (Sarinont *et al*. 2016). Plasma-generated reactive species can also break seed dormancy and accelerate germination by altering biochemical pathways (Randeniya and de Groot 2015, Koga *et al*. 2016, Ji *et al*. 2018, August *et al*. 2023). For example, soybean seeds treated with CAP showed germination rate increasing from 1.18 to 66.97%, along with significant root and shoot growth (Volin *et al*. 2000); similar enhancements have been reported in soybean (Li *et al*. 2014), wheat (Starič *et al*. 2020), and millet (Perner *et al*. 2024).

Beyond germination, CAP stimulates seedling vigour (Benabderrahim *et al*. 2024), promoting root and shoot elongation, increasing biomass, and improving long-term development, including flowering and fresh flower mass (Abedi *et al*. 2020). In hydroponic bok choy, DBD cold plasma enhanced salinity tolerance and was linked to elevated H_2O_2 and NO in the solution (Veerana *et al*. 2024). CAP also upregulates antioxidant enzymes such as catalase and superoxide dismutase, mitigating oxidative stress from reactive oxygen species and supporting seedling establishment (Li *et al*. 2015, Cui *et al*. 2019, Sayahi *et al*. 2024).

CAP further reduces microbial contamination on seed surfaces, lowering the risk of seed-borne diseases and improving resistance to insect pests (Afsheen *et al*. 2019, Lee *et al*. 2021, Mravlje *et al*. 2021). It has also been applied directly to fruits, such as cherries, increasing resistance to fungal infections and extending shelf life, likely by inactivating spores and activating plant defence responses (Ambrico *et al*. 2020). In addition, CAP can degrade pesticide residues on seeds, such as chlorpyrifos on soybean, while maintaining seed quality and water uptake (Anbarasan *et al*. 2022). Combining CAP with biorefinery-derived products, such as pyroligneous acid and biochar, improves traits in winter wheat, increasing yield, starch, and protein content (Saberi *et al*. 2022). Recent studies suggest that CAP may also generate RNS from air, offering an eco-friendly nitrogen source (Lamichhane *et al*. 2021, Aceto *et al*. 2024). Beyond plant-focused effects, CAP is a promising tool for water and soil remediation, efficiently degrading organic pollutants and inactivating pathogens through ROS and RNS (Aggelopoulos 2022), and it has also been shown to degrade pesticide residues on food and crop surfaces (Sojithamporn *et al*. 2023).

The effect of CAP has been tested on various plants, but lettuce, which is a common model plant, has not been studied much yet. Song *et al.* (2020) looked at cold plasma mainly as a decontamination tool applied post-harvest to fresh-cut leaves to control microbial spoilage – the focus there was food safety, not plant physiology. More recently, Jankaitytė *et al.* (2025) treated lettuce seeds with cold plasma before growing plants aeroponically and found measurable shifts in physiological and biochemical parameters. Mehrabifard *et al.* (2025) compared several types of plasma-activated water and their effects on germination and early seedling growth. Taken together, these studies point to real effects of CAP (or plasma-activated water) on lettuce, but all three stopped at germination or early biochemical markers. None of them followed the plants further – photosynthetic performance, pigment content, biomass accumulation, or flowering timing remain essentially unexplored in this species. Just as importantly, nobody tested whether the stability of growing conditions changes how plants respond to CAP.

Therefore, in this study, we aimed to move beyond early germination assays to characterise the long-term physiological consequences of CAP seed priming in lettuce, encompassing photosynthetic performance *via* chlorophyll fluorescence and leaf reflectance (Campbell *et al.* 2007, Delalieux *et al.* 2009), biomass accumulation, and flowering dynamics. Critically, we tested these responses under two contrasting cultivation

environments – a stable, fully controlled phytotron and a semi-controlled greenhouse with naturally fluctuating temperature, humidity, and irradiance – to determine whether the stability of growing conditions modulates the physiological and agronomic outcomes of CP treatment.

Materials and methods

Plant material and cold plasma treatment: Seeds of lettuce (*Lactuca sativa* L., purchased from *semena.cz*) were selected for uniformity and the absence of visible defects before experiments. Cold plasma (CP) treatment was performed in a downer-type reactor (Fig. 1) using ambient air at a working pressure of 70–75 Pa. The discharge was generated using an RF generator (*Dressler Cesar 126*) and a matching unit (*MFJ Versa Tuner III*) at a frequency of 13.56 MHz. The quartz tube (inner diameter 20 mm) was surrounded by copper electrodes, and the gas feed (air) was maintained at a flow rate of 40 sccm *via* a mass flow controller (Perner *et al.* 2024).

Seeds were exposed for less than one second during free fall through the plasma zone at three levels of discharge power of 50, 100, and 150 W. The plasma zone was 35–45 cm long (longer for higher power). The time spent in the plasma zone due to free fall is therefore between 0.27 and 0.30 s. The tubing between the end of the plasma zone and the container (cyclone separator) was 50 cm long. Two types of control were included: an untreated control (N) and a vacuum-exposed control (VAC). The VAC treatment involved exposure to reduced pressure (~70 Pa) without plasma discharge to isolate the physical effects of vacuum from the chemical effects of reactive species. All seeds were stored under identical laboratory conditions (22 ± 2°C, 45 ± 5% relative humidity) until further analysis.

Germination: Seeds were placed on five layers of moist filter paper in Petri dishes (Ø 90 mm), with ten seeds per dish and five replicate dishes per treatment (*i.e.*, 50 seeds per treatment per repetition). The dishes were incubated at 24°C in darkness. Germination was defined as radicle protrusion of at least 2 mm. Radicle counts were recorded at 48, 72, and 96 h to monitor germination kinetics. The entire germination experiment was conducted three times independently on different occasions, resulting in a total of 150 seeds per treatment and 750 seeds overall.

Plant growth and cultivation: Following the germination assays, 220 seedlings (22 per treatment) with radicles longer than 2 mm were transplanted individually into 300-mL plastic pots (7 × 7 × 8 cm) filled with a commercial universal horticultural substrate (*KERA GREEN s.r.o.*, Czech Republic). Plants were cultivated for 55 d under either phytotron or greenhouse conditions. Irrigation was performed using distilled water. In the phytotron, plants received 20 mL of water three times per week, whereas greenhouse plants were watered as needed according to prevailing weather conditions.

In the phytotron, environmental conditions were maintained at 24°C with a 16/8-h (light/dark) photoperiod.

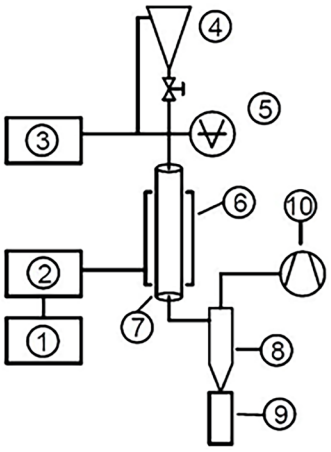


Fig. 1. Schematic diagram of the downer-type RF plasma reactor. Reproduced from Perner *et al.* (2024): 1 – generator; 2 – matching unit; 3 – mass flow controller; 4 – dispenser; 5 – vacuum gauge; 6 – electrodes; 7 – glass tube; 8 – cyclone separator; 9 – collector; 10 – vacuum pump.

Illumination was provided by LED lighting with a PPFD of approximately 231 μmol m^{-2} s^{-1} measured at canopy level. Relative humidity was maintained at approximately 65%.

In the greenhouse, plants were cultivated under natural daylight conditions at the Department of Biology, UJEP (Ústí nad Labem, Czech Republic) during April and May. Air temperature fluctuated between approximately 13 and 39°C during the cultivation period, while relative humidity ranged between 20 and 30%. Light intensity varied substantially depending on weather conditions and time of day, with PPFD values ranging from approximately 12 to 2,231 μmol m^{-2} s^{-1}. PPFD and irradiance values in the greenhouse were estimated from representative measurements performed during the cultivation period and reflect natural variability of environmental conditions.

Photosynthetic efficiency and reflectance measurements: Measurements were performed twice during cultivation, first 20 d after transplanting and again 14 d later.

Chlorophyll fluorescence induction (OJIP): Measurements were recorded using a portable fluorometer *FluorPen FP 110* (*PSI*, Drásov, Czech Republic), which was equipped with a blue LED emitter (455 nm), emitting a maximum light intensity of 3,000 μmol(photon) m^{-2} s^{-1}. The detector is a PIN photodiode with 667–750 nm bandpass filters. Before measurement, leaves were dark-adapted for 20 min using specialised leaf clips. The fluorescence transients (OJIP curves) were plotted as the mean values for individual treatments. Each curve represents the mean of 22 biological replicates per treatment and environment. Various fluorescence indices (F_0, F_m, F_v/F_m, Ψ_0, Φ_{Eo}, Φ_{Pav}, ET_0/RC, PI_{Abs}) were calculated according to Stirbet *et al.* 2018.

Reflectance measurements were performed on the same days as chlorophyll fluorescence measurements. *PolyPen RP 410* (*Photon Systems Instruments*, Brno, Czech Republic) is a handheld passive spectroradiometer

recording reflectance (wavelengths between 380 and 790 nm). In addition, various vegetation indices were calculated to assess plant health and relative pigment content (NDVI, MCARI, CRI1, CRI2) according to the equations listed by El-Hendawy *et al.* (2022).

Measurements were performed from a fixed distance, with a connector applied to a single leaf of each plant. All measurements were conducted inside the growth facilities, ensuring the sample spot was entirely occupied by the lettuce leaves, measuring only actual leaf, thereby preventing any background interference from the soil, container edges, or surrounding facility structures.

Biomass measurement: At 55 d post-germination, the shoot fresh mass (FM) of each plant was measured to determine the aboveground biomass.

Flowering: The relationship between the percentage of flowering individuals and the day of counting was modelled using logistic functions for each treatment and environment separately (greenhouse and phytotron). For each plant, "day zero" (Day 0) was defined as the onset of inflorescence emergence (bolting stage), visually identified by a visible elongation of the main shoot and the differentiation of the apical meristem into flower buds (corresponding to stage 51 of the BBCH scale). Pairwise comparisons of the parameters of the fitted logistic curves were then performed between all treatments within each environment using the Wald test (Wald 1943). To account for multiple comparisons, *p*-values were adjusted using the false discovery rate (FDR) correction.

Statistical data analysis: All statistical analyses were performed in *R 4.6.0* (R Core Team 2026). Generalised linear mixed-effects models (GLMM) were fitted using *glmmTMB* (Brooks *et al.* 2017, McGillycuddy *et al.* 2025), estimated marginal means and contrasts were obtained using *emmeans* (Lenth *et al.* 2026), and post-hoc tests were performed using functions from *stats* and *rstatix* (R Core Team 2026, Kassambara 2023). Statistical significance was assessed at $\alpha = 0.05$.

Germination data were analysed as the number of germinated seeds out of ten seeds per Petri dish. Treatment, time, and their interaction were fitted as fixed effects, with experimental repeat and Petri dish identity included as random effects. A binomial GLMM with a logit link function was used. Fixed effects were tested using type-III *Wald* χ^2 tests. Pairwise treatment comparisons within each time point were performed using *Tukey*-adjusted contrasts. Final germination at 96 h was additionally expressed as % increase relative to the untreated control, and treatment-control comparisons were evaluated using *Dunnett*-adjusted contrasts.

Fast chlorophyll *a* fluorescence induction curves were summarised descriptively as mean OJIP transients for each treatment, environment, and measurement date. Derived chlorophyll fluorescence parameters and leaf reflectance indices were analysed separately for each environment and measurement date. Statistical comparisons were performed on the original unscaled values using *Wilcoxon* rank-sum tests comparing each treatment with the untreated control. *P*-values were adjusted within each panel using the *Benjamini–Hochberg* false-discovery-rate procedure (Benjamini and Hochberg 1995).

Shoot fresh mass was analysed separately for greenhouse and phytotron plants. Normality was assessed using *Shapiro–Wilk* tests and homogeneity of variances using *Bartlett* tests. Because equal variances were not assumed, treatment effects were tested using *Welch*'s one-way *ANOVA*, followed by *Games–Howell* post-hoc comparisons.

Flowering dynamics were analysed separately for each cultivation environment by fitting logistic curves to the percentage of flowering individuals over time. Pairwise comparisons among fitted treatment curves were performed using *Wald* tests (Wald 1943), with *P*-values adjusted by the *Benjamini–Hochberg* false-discovery-rate procedure. Compact-letter displays were used to summarise significant pairwise differences where applicable.

Results

Germination: Germination dynamics differed significantly among treatments and time points (Fig. 2). A generalised linear mixed-effects model (GLMM) confirmed significant main effects of Time (χ^2 = 320.6, df = 2, $P<0.001$) and Treatment ($\chi^2 = 46.7$, df = 4, $P<0.001$), as well as a significant Treatment × Time interaction (χ^2 = 21.1, df = 8, $P=0.007$). At 48 h, control seeds (N) exhibited the lowest germination (mean ~1 seed/dish). In contrast, plasma-treated seeds (50 W, 100 W, 150 W)

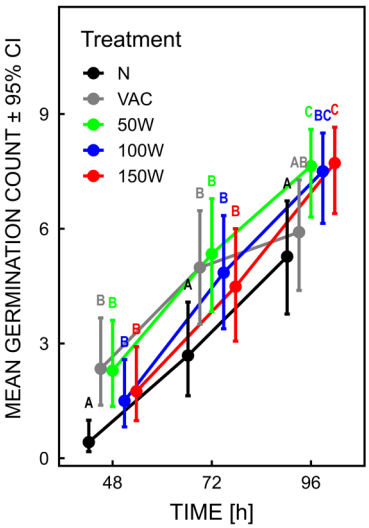


Fig. 2. Germination dynamics of lettuce seeds after vacuum and cold plasma treatment. Points show model-estimated mean germination counts per Petri dish at 48, 72, and 96 h. Error bars represent 95% confidence intervals derived from the fitted binomial GLMM. *Different letters* indicate significant differences among treatments within each time point based on *Tukey*-adjusted pairwise comparisons at $\alpha = 0.05$. N – untreated control; VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively.

showed accelerated germination, reaching mean counts of 2–3 seeds per dish, representing a relative increase of over 40% compared to the control (Fig. 3). By 96 h, the control reached a mean of 5 seeds per dish, while plasma-treated variants achieved significantly higher levels, with means of 7–8 seeds. Across all time points, plasma-treated seeds consistently outperformed the untreated control (N), while differences between plasma power levels (50–150 W) remained minimal, with overlapping standard errors. Overall, the 150 W treatment showed the highest cumulative increase in germination (44.3%), followed by 50 W (43.0%) and 100 W (40.5%), while the vacuum effect alone (VAC) accounted for an 11.4% increase (not statistically significant relative to the untreated control; Fig. 3).

Physiology experiments on mature plants: To further characterise the effects of cold plasma on photosynthetic performance, selected parameters derived from OJIP transients (Fig. 4) were analysed (Fig. 5*A–D*; Fig. 1S, *Supplement*). Treatment-related differences were strongly dependent on both the growth environment and the timing of measurement. Pairwise comparisons revealed more significant deviations in greenhouse-grown plants during the first measurement, while phytotron-grown plants showed only limited differences at the same stage. Conversely, treatment effects became more pronounced in the phytotron during the second measurement, suggesting a delayed physiological response under controlled conditions. The maximum quantum yield of PSII (F_v/F_m) was primarily affected in the greenhouse, where plasma-treated plants exhibited reduced values compared to the control, with the absolute lowest at 100-W treatment. Although partial recovery was observed over time, these values did not return to control levels. In the phytotron, F_v/F_m remained within the optimal range (~0.80) with only minor and insignificant transient fluctuations. Taken together, these data indicate that cold plasma induces a stronger and more immediate alteration in photosynthetic performance under greenhouse conditions, whereas the phytotron promotes a weaker, delayed response characterised by physiological acclimation over time.

F_0 differed significantly between treatments in both environments. While VAC values were comparable to the control, plasma-treated variants, especially 100 W and 150 W, exhibited greater variability. These differences intensified over time in the greenhouse, suggesting a progressive divergence in excitation-related processes. F_m showed contrasting environmental responses. In the greenhouse, plasma treatment (particularly at higher power) reduced F_m during the first measurement, indicating a temporary limitation in PSII performance. Although partial recovery occurred, F_m remained lower than the control. In contrast, phytotron-grown plants showed no initial differences in F_m, but by the second measurement, the 100-W and 150-W treatments showed increased F_m values relative to the control, suggesting enhanced excitation energy utilisation.

In greenhouse-grown plants, the first measurement revealed pronounced differences in the J-step (1–2 ms, reflecting the maximum reduction state of Q_A) among treatments. The N control exhibited a sharply defined and

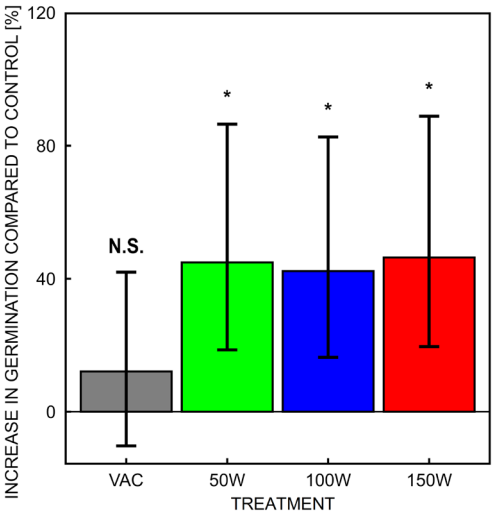


Fig. 3. Model-estimated percentage increase in germination at 96 h relative to the untreated control. Error bars represent 95% confidence intervals for the percentage increase. *Asterisks* indicate *Dunnett*-adjusted treatment-*vs*-control comparisons at 96 h; * $p<0.05$, n.s. – not significant. VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively.

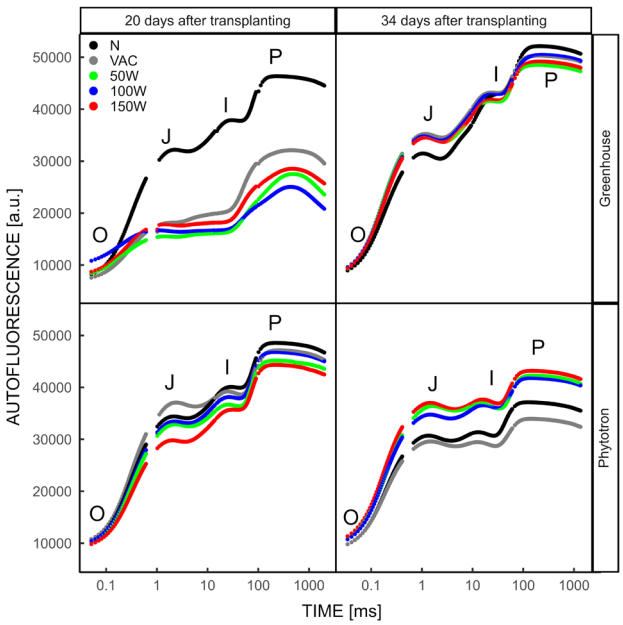


Fig. 4. Fast chlorophyll *a* fluorescence induction curves (OJIP transients) of lettuce plants grown under greenhouse and phytotron conditions. Curves show mean fluorescence values for each treatment. Measurements were performed 20 and 34 d after transplanting. The *x*-axis is shown on a logarithmic scale, and the O, J, I, and P phases of the fluorescence transient are indicated. N – untreated control; VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively.

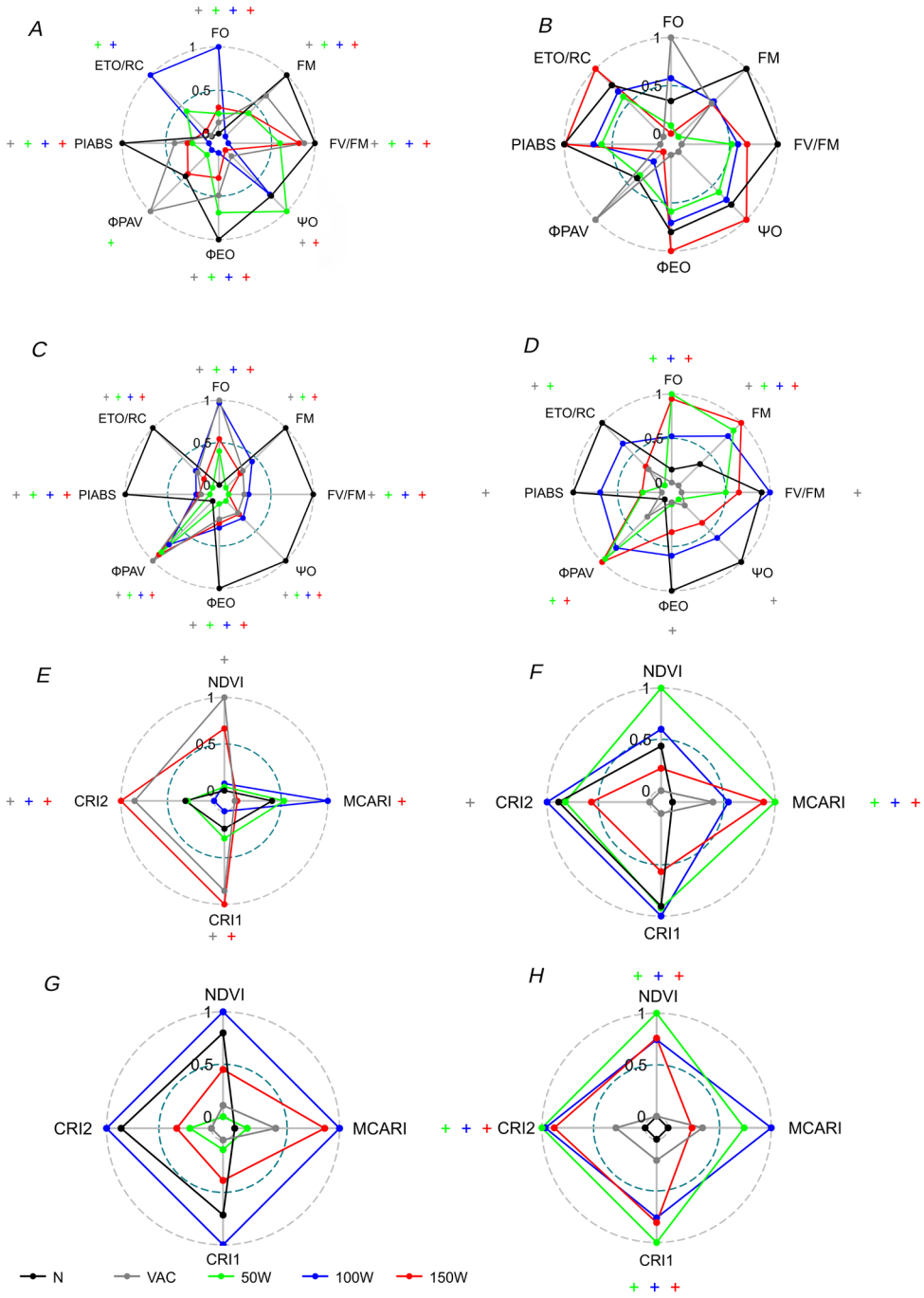


Fig. 5. Spider plots of chlorophyll fluorescence parameters and leaf reflectance indices in lettuce plants grown under greenhouse and phytotron conditions. Each polygon represents treatment medians. For each panel, values of each parameter were independently scaled from 0 to 1 using the minimum and maximum treatment median for that parameter. *Plus signs* indicate treatments significantly different from the untreated control for the given parameter, based on *Wilcoxon* rank-sum tests with *Benjamini–Hochberg* FDR correction within each panel (FDR-adjusted $p<0.05$). Panels *A–D* show chlorophyll fluorescence parameters; panels *E–H* show leaf reflectance indices. Measurements were performed 20 and 34 d after transplanting. (*A*) 1st measurement – greenhouse; (*B*) 1st measurement – phytotron; (*C*) 2nd measurement – greenhouse; (*D*) 2nd measurement – phytotron; (*E*) 1st measurement – greenhouse; (*F*) 1st measurement – phytotron; (*G*) 2nd measurement – greenhouse; (*H*) 2nd measurement – phytotron. N – untreated control; VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively. F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; F_v/F_m – maximal quantum yield of PSII photochemistry; Ψ_0 – probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; Φ_{Eo} – quantum yield of electron transport; Φ_{Pav} – average quantum yield of primary photochemistry; ET_0/RC – electron transport flux per active PSII reaction centre; PI_{Abs} – performance index on an absorption basis; NDVI – normalized difference vegetation index; MCARI – modified chlorophyll absorption in reflectance index; CRI1 – carotenoid reflectance index 1; CRI2 – carotenoid reflectance index 2.

prominent J-step at approximately 30,000 a.u., clearly separated from all other variants. The VAC treatment showed an intermediate J-step of approximately 18,000 a.u., while plasma-treated plants (50 W, 100 W, 150 W) clustered together at the lowest J-step values (~15,000–17,000 a.u.), with the inflexion point less pronounced but still discernible. This ordering – N > VAC > plasma treatments – indicates that the N control experienced the highest degree of Q_A reduction at the J-step timepoint, reflecting greater excitation pressure on PSII and more restricted downstream electron flow – an interpretation supported by the concurrently reduced F_v/F_m and elevated F_0 in the plasma-treated variants at this same timepoint (*see* below).

Plasma-treated plants, by contrast, showed markedly lower J-step and F_m values, reflecting a globally reduced fluorescence yield and diminished light-harvesting capacity rather than a more efficient electron transport state. This pattern, together with the concurrently reduced F_v/F_m in plasma-treated plants at this time point (*see* below), indicates reduced overall photosynthetic activity under the combined influence of CP treatment and greenhouse stress.

Considering the change over time within the greenhouse environment, the J-step architecture changed markedly across all variants. The inflexion point characteristic of the J-step became clearly defined in all treatments. In this context, plasma-treated plants and VAC reached slightly higher values than N in the J-step region (~30,000–34,000 a.u. *vs*. ~30,000 a.u. for N), reversing the hierarchy observed at the first measurement. This convergence and partial reversal suggests that under greenhouse conditions, plasma-treated plants maintained or modestly improved their PSII electron transport characteristics over the 14-d interval relative to the untreated control.

Comparing the two environments at the same (first) time point, J-step values during the first measurement were generally higher and more uniformly expressed across all treatments compared to the greenhouse, reflecting more stable growth conditions. N and the 50-W treatment showed the highest J-step values (~30,000–32,000 a.u.), with 100-W and 150-W slightly lower (~27,000–29,000 a.u.), and VAC the lowest (~25,000 a.u.). Importantly, the J-step was well-defined and clearly shaped in all variants, indicating active PSII photochemistry across treatments. The relatively similar J-step values between N and plasma treatments at this time point, in contrast to the large separation seen in the greenhouse, suggest that phytotron conditions promoted more uniform PSII photochemistry regardless of treatment.

The second phytotron measurement revealed a clear reversal in the J-step hierarchy. Plasma-treated plants maintained the highest J-step values (~32,000–34,000 a.u. for 50 W, 100 W, and 150 W), while N declined to approximately 28,000 a.u. and VAC to ~25,000–26,000 a.u. The J-step remained well-defined in all variants. Considered together with the F_m data, plasma-treated plants at the second phytotron measurement show both a higher J-step and a higher P-step than the declining controls (N, VAC). Rather than being read as straightforward evidence of enhanced photochemical capacity, this pattern is interpreted here as evidence that plasma-treated plants avoided the physiological decline seen in N and VAC. The progressive decline observed in both N and VAC, together with the maintained or increased J-step and F_m values in plasma-treated plants, is consistent with improved maintenance of PSII function under controlled conditions following cold plasma treatment. The OJIP-derived parameters V_j and V_i (Fig. 3S, *Supplement*), together with F_v/F_m, F_0, and F_m (Fig. 4, Fig. 1S), revealed a clear environment and time-dependent pattern of the photosynthetic response to cold plasma treatment. In the greenhouse, the first measurement showed a dose-dependent decline in F_v/F_m, most pronounced at 100 W, accompanied by an increase in F_0, consistent with acute photoinhibition of PSII. V_i in CP-treated variants was also significantly lower than in the N and VAC controls at this time point; however, given the concurrent decline in F_v/F_m and increase in F_0, this lower V_i is interpreted here not as evidence of facilitated electron transport towards PSI, but as part of the same globally reduced fluorescence yield that characterises acute PSII photoinhibition under greenhouse conditions – consistent with the interpretation applied to the J-step data above. A partially similar pattern in V_i was observed during the first measurement in the phytotron, although, in contrast to the greenhouse, plants grown in the phytotron showed no significant change in F_v/F_m at this time point, indicating that this shift in V_i occurred without any concurrent sign of PSII photoinhibition. By the second measurement, F_v/F_m had largely recovered in the greenhouse (all treatments ≥ 0.78), while V_j and V_i were now moderately but significantly elevated relative to N in nearly all treated variants in both environments, indicating a residual, low-magnitude shift in electron transport downstream of Q_A that persisted after the acute effect had resolved.

The effective quantum yield of electron transport (Φ_{Eo}) showed no significant treatment effects in the phytotron at the first measurement; by the second measurement, a significant difference emerged only for the VAC treatment, with no plasma-treated variant (50–150 W) differing significantly from the control. In the greenhouse, by contrast, Φ_{Eo} was significantly reduced relative to the control in all treatments – VAC and all three plasma doses – at both measurement time points. A broadly similar trend was observed for Ψ_0, although treatment responses differed between plasma variants, particularly during the first measurement, suggesting that electron transport beyond Q_A^- was affected under greenhouse conditions, although the response varied among plasma treatments.

Leaf reflectance and vegetation indices: Vegetation indices derived from leaf reflectance revealed treatment-dependent differences in physiological status, which varied significantly by growth environment and measurement time. Consistent with the fluorescence data, a higher number of significant deviations from the control was observed in the greenhouse during the first measurement, while phytotron effects became more pronounced during the second sampling, indicating a delayed physiological

response under fully controlled conditions (Fig. 5*E–H*; Fig. 2S, *Supplement*). However, the MCARI index exhibited the opposite trend in the phytotron, showing a higher response in the first measurement in both environments. NDVI remained relatively stable with fewer significant differences. In the greenhouse, VAC-treated plants often showed the highest values, while plasma variants remained intermediate. In the phytotron, differences appeared only during the second measurement, with the 50-W treatment reaching the highest NDVI values. In the phytotron, plasma-treated variants initially exhibited higher MCARI values than the control. In the greenhouse, differences were significant at both sampling dates, though the magnitude decreased over time. Notably, the 150-W treatment generally showed lower MCARI values, suggesting a potential reduction in chlorophyll-related reflectance at high plasma power. Carotenoid-related indices (CRI1 and CRI2) showed distinct responses among plasma treatments, with the highest values generally observed at 150 W during the first greenhouse measurement. During the first greenhouse measurement, only the highest plasma dose (150 W) was associated with increased CRI values, whereas lower plasma powers produced weaker or inconsistent responses. In the phytotron, these effects emerged during the second measurement; plants exposed to higher plasma power generally exhibited higher CRI values.

Overall, leaf reflectance measurements revealed that cold plasma influenced pigment-related traits, although the magnitude and timing of the responses differed between cultivation environments and among plasma treatments. Greenhouse-grown plants responded more rapidly, whereas changes in the phytotron became more apparent during the second measurement.

Biomass: *Welch*'s *ANOVA* revealed significant differences in plant biomass between treatments in both the phytotron ($F = 7.28$, $p<0.001$) and the greenhouse ($F = 19.43$, $p<0.001$; Fig. 6). In the phytotron, plasma treatment significantly increased biomass at 100 W and 150 W compared with both the untreated control and the VAC treatment ($p<0.05$), whereas the 50-W treatment did not differ significantly from the other groups. In the greenhouse, biomass also increased with plasma power, reaching the highest mean value at 150 W; however, this increase was not significantly different from the untreated control. Overall, the positive effect of cold plasma on biomass accumulation was more pronounced under the controlled conditions of the phytotron than under the more variable greenhouse environment.

Flowering dynamics: Flowering onset showed a striking, environment-dependent reversal in response to plasma treatment (Fig. 7). In the greenhouse, the highest plasma dose (150 W) significantly accelerated bolting relative to the control, consistent with a stress-escape response following the acute PSII photoinhibition observed at this dose. In the phytotron, by contrast, the higher doses (100–150 W) significantly delayed bolting relative to the control, coinciding with the significantly higher final biomass recorded for these treatments. In the phytotron,

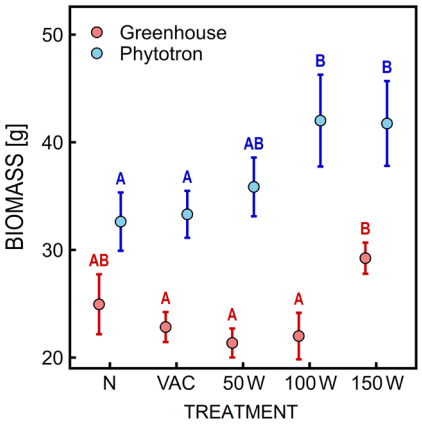


Fig. 6. Effect of cold plasma treatment on shoot fresh mass in lettuce plants grown under greenhouse and phytotron conditions. Points show treatment means, and error bars represent 95% confidence intervals of the means. *Different letters* indicate significant differences between treatments within each cultivation environment based on *Games–Howell* post-hoc tests following *Welch*'s *ANOVA* at $\alpha = 0.05$. N – untreated control; VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively.

the differences between treatments were more pronounced. The control treatments (N and VAC) achieved a higher proportion of flowering individuals earlier than the plasma-treated seeds, while the treatments with higher plasma power (100 W and 150 W) significantly delayed flowering compared to the controls.

Discussion

Enhancement of germination and early development: In this study, germination kinetics was significantly accelerated by cold plasma (CP) treatment, with plasma-treated seeds always outperforming the untreated control at all time points. The 40–45% increase in germination observed at 96 h (Fig. 3) confirms the potential of CP as a potent non-chemical priming tool. Interestingly, differences between plasma power levels (50–150 W) were relatively small and characterised by overlapping standard errors suggesting that the stimulatory effect may reach a plateau within this power range, or that the initiation of germination is triggered by a general threshold of plasma exposure rather than a linear response to discharge power.

The numerically higher germination observed in the vacuum-treated (VAC) group (11.4% increase) did not reach statistical significance relative to the untreated control (Fig. 3), so these germination data alone do not provide clear evidence that physical factors (*e.g.*, pressure changes during the procedure) contribute to the response at this stage. The consistently significant increase in CP-treated seeds, by contrast, indicates that plasma-specific factors – probably the interaction of reactive species with the seed coat – are the primary driver of the accelerated germination observed here.

The present observation of accelerated germination is in accordance with previous reports concerning plasma-

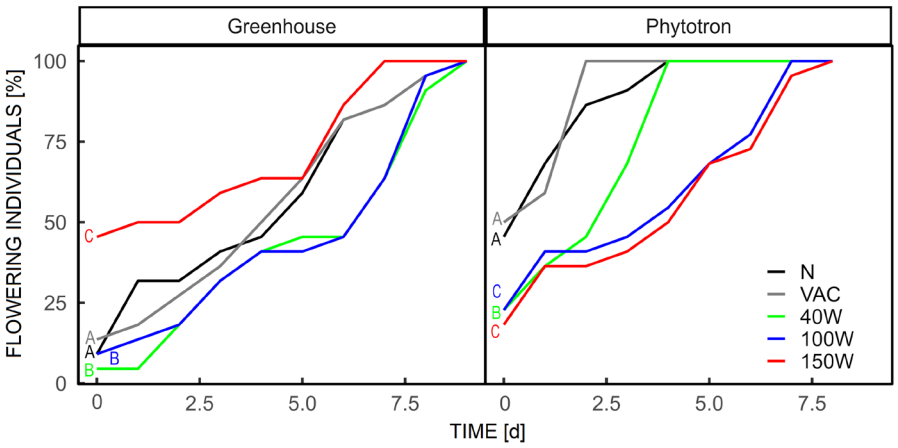


Fig. 7. Flowering dynamics of lettuce plants grown under greenhouse and phytotron conditions. Points show the observed percentage of flowering individuals, and curves show fitted logistic models for each treatment. *Different letters* indicate significant differences between fitted flowering curves within each cultivation environment based on pairwise *Wald* tests with FDR correction at α = 0.05. N – untreated control; VAC – vacuum-treated control; 50 W, 100 W, and 150 W – cold plasma treatments at discharge powers of 50, 100, and 150 W, respectively.

induced changes of the seed surface, including increased hydrophilicity and modified biochemical signalling (Li *et al.* 2015). Reactive oxygen and nitrogen species generated during plasma discharge have been proposed to act as signalling molecules. Mainly involved in dormancy release, modulation of the abscisic acid/gibberellin balance and other physiological processes (Ji *et al*. 2018, Bailly 2019, August *et al.* 2023). Though our study did not directly measure these hormonal changes, the significant increase in germination counts is strong evidence for the occurrence of such physiological priming.

CP has been found to promote biomass and flowering under stable, controlled conditions (Abedi *et al.* 2020, Veerana *et al.* 2024), however, these effects may be dampened in more variable settings. For example, Ahn *et al.* (2019) did not observe significant improvements in the germination or yield of CP-treated maize under field conditions, possibly due to high baseline seed quality or environmental buffering. This shows that the agronomic efficiency of CP treatment is strongly dependent on the type of seeds, initial vigour, and the environmental context of the post-germination growth. Translating these benefits from the laboratory into complex growth environments is still a challenge.

Physiological response to cold plasma treatment depends on the environment: CP treatment increased biomass and prolonged the apparent vegetative phase under stable phytotron conditions (24°C constant, 65% relative humidity, PPFD ~231 µmol m^{-2} s^{-1}), whereas under the greenhouse's markedly fluctuating temperature (13–39°C) and irradiance [12–2,231 µmol(photon) m^{-2} s^{-1}] regime, the same treatments produced no significant biomass gain and, at the highest power, triggered significantly earlier flowering instead. This indicates that CP shifts resource allocation toward reproduction specifically when applied under fluctuating rather than stable abiotic conditions, rather than simply producing a generically "different" outcome depending on environment.

To our knowledge, no previously published study on CP-treated lettuce has reported long-term chlorophyll fluorescence (OJIP) kinetics, biomass accumulation, or flowering dynamics in mature plants; prior work in this species has been limited to microbial decontamination of harvested leaves (Song *et al.* 2020) or germination and early biochemical markers (Jankaitytė *et al.* 2025, Mehrabifard *et al.* 2025). The present findings therefore cannot be directly benchmarked against lettuce-specific data at these later developmental stages, and the comparisons developed below instead draw on CP studies in other species that examined comparable physiological or growth endpoints, following the general framework that plasma–plant interactions must be interpreted in the context of the recipient plant's prevailing physiological state (Ranieri *et al.* 2021).

More broadly, treatment-related divergence in chlorophyll fluorescence and reflectance parameters was significantly more pronounced under greenhouse conditions than in the phytotron. This is consistent with reports that CP and plasma-activated water treatments produce stronger or qualitatively different physiological responses when plants are concurrently exposed to abiotic stress, as shown for osmotic- and saline-stressed *Arabidopsis* (Bafoil *et al.* 2019) and stress-tolerance responses in plasma-activated-water-treated barley (Bradu *et al.* 2020, Gierczik *et al.* 2020) – in each case, the background stress level of the plant, rather than plasma treatment alone, determined the direction and magnitude of the physiological outcome. This environment-dependent outcome in our data likely reflects differences in background stress load: greenhouse plants were simultaneously exposed to fluctuating temperature, variable natural irradiance, and humidity gradients, all of which independently affect PSII redox state and plastoquinone pool turnover. Under such conditions, the additional oxidative load imposed by CP-derived reactive species may interact with existing abiotic stressors, shifting the balance of the response toward a more costly physiological adjustment at higher plasma powers. In the phytotron, where temperature, light intensity, and photoperiod were tightly controlled, plants maintained a lower baseline stress level, potentially widening the window within which CP reactive species act as mild signalling cues rather than as an additional stress load.

Cold plasma as a mild eustressor in phytotron conditions: Considering the change over time within the phytotron environment, CP-treated plants maintained

photosynthetic performance comparable to or exceeding the controls, with the effect becoming more pronounced by the second measurement. Uniform F_0 values across all treatments at both time points confirm that plasma exposure did not cause permanent damage to PSII reaction centres. During the first measurement, N and the 50-W treatment showed the highest J-step and F_m values, with plasma-treated plants at slightly lower but comparable levels and VAC the lowest, suggesting broadly similar PSII photochemistry across treatments under stable conditions. By the second measurement, a clear reversal emerged: plasma-treated plants (50, 100, 150 W) reached the highest J-step and F_m values, while both controls declined markedly – N to approximately 28,000 a.u. and VAC to ~25,000–26,000 a.u. Unlike the greenhouse pattern, where an elevated J-step and F_m co-occurred with reduced F_v/F_m and increased F_0 – a combined signature of acute photoinhibition – the elevated J-step and F_m observed here in CP-treated plants at the second phytotron measurement were accompanied by stable F_v/F_m (~0.80) and uniform F_0 across all treatments, with no indication of photoinhibition. The diagnostic meaning of the J-step/F_m signature therefore depends on its co-occurrence with F_v/F_m and F_0 trends rather than on its direction alone: only when paired with declining F_v/F_m and rising F_0 does an elevated J-step/F_m indicate excitation pressure and restricted downstream electron flow, as observed in the greenhouse. In the present case, the absence of such a stress signature, together with the concurrent decline of J-step and F_m in the untreated and vacuum-treated controls, is more consistent with CP-treated plants avoiding the physiological decline seen in the controls than with impaired electron transport. Notably, Φ_{Eo} showed no significant difference between the plasma-treated variants and the control at this second time point (the only significant deviation being in VAC), so we cannot confirm improved downstream electron transport efficiency in the plasma-treated plants from the fluorescence data alone. This reinforces our interpretation of the maintained or rising J-step and F_m in plasma-treated plants relative to the declining controls as evidence that these plants avoided the physiological decline seen in the untreated and vacuum-treated groups, rather than as direct proof of enhanced photochemical capacity; distinguishing between these two possibilities will require corroborating measurements (*e.g.*, gas exchange) in future work.

Stable F_v/F_m ratios (~0.80) in plasma-treated plants further support the absence of photoinhibition, and increased carotenoid indices (CRI1, CRI2) at higher plasma power additionally point to enhanced photoprotective capacity. Taken together, these responses are consistent with CP acting as a mild eustressor that helps plants maintain functional stability over time under stable growth conditions (Mildažienė *et al.* 2019), though we note this term describes the direction and pattern of the response rather than a demonstrated dose-response relationship.

This pattern of maintained or improved physiological performance under controlled, low-background-stress conditions parallels findings in wheat, where non-thermal plasma treatment altered growth and physiology *via* generated signalling molecules under experimentally controlled conditions without concurrent abiotic stress (Iranbakhsh *et al.* 2017); our phytotron results are broadly consistent with that direction of effect, though the specific fluorescence parameters and species differ.

Acclimation costs under greenhouse conditions: In contrast to the phytotron, greenhouse-grown plants exhibited stronger and more pronounced physiological divergence, particularly at the first measurement. The N control showed by far the highest J-step (~30,000 a.u.) and F_m (~45,000 a.u.), clearly separated from all other variants, while plasma-treated plants clustered at substantially lower values (~15,000–17,000 a.u. at the J-step) and VAC occupied an intermediate position. The high J-step combined with high F_m in the N control reflects strong light-harvesting capacity coupled with restricted downstream electron transport – consistent with the elevated excitation pressure characteristic of variable greenhouse light conditions.

Plasma-treated plants showed markedly lower J-step and F_m values overall at this time point, indicating a globally reduced fluorescence yield rather than a specifically more efficient electron transport state – consistent with reduced light-harvesting capacity under the combined influence of CP treatment and greenhouse stress, as also reflected in their lower F_v/F_m at this time point. The fact that this reduction in F_m was proportional to plasma power – with 100 and 150 W showing the greatest suppression – points to a dose-dependent interaction between CP intensity and the background environmental stress experienced by greenhouse plants.

By the second measurement, the pronounced separation between treatments had largely disappeared, with all variants converging toward comparable F_m values and the distinct J-step inflexion becoming poorly defined across all treatments, indicating partial recovery of PSII function. This pattern of initial suppression followed by convergence over 14 d is consistent with a transient physiological cost: the initial oxidative challenge imposed by CP triggers photoprotective responses – reflected in increased carotenoid indices alongside reduced NDVI and MCARI values – that prioritise stress mitigation over other physiological processes, but which are gradually resolved as plants recover. This is consistent with the general principle that resources allocated to reactive-oxygen-species detoxification and photoprotective pigment synthesis under combined abiotic stress are drawn from the same limited pool that would otherwise support growth (Miller *et al.* 2010).

Notably, this transient suppression and subsequent recovery of PSII function did not translate into a measurable biomass penalty relative to the untreated control, nor did it yield a significant biomass gain (Fig. 6) – in contrast to the sustained biomass advantage observed under phytotron conditions. This suggests that under greenhouse conditions, the resources mobilised for photoprotection, and recovery may have come at the expense of resources that would otherwise have supported the accelerated vegetative growth seen in

the phytotron, although this trade-off was not directly quantified here. Under these variable conditions, higher plasma power levels appear to impose a greater initial physiological cost, potentially explaining the accelerated flowering observed as a stress-avoidance strategy (*see* below).

The role of vacuum treatment and plasma power: Contrary to a purely inert control, the vacuum treatment (VAC) produced measurable physiological effects distinct from both the untreated control (N) and plasma-treated seeds – although the strength of evidence differed between developmental stages. At germination, VAC seeds showed a numerically higher germination rate (11.4%) than untreated seeds. However, this difference was not statistically significant (Fig. 3), so this dataset alone cannot confirm a physical (vacuum-related) contribution to germination. In mature plants, however, VAC produced statistically significant effects distinct from both N and the plasma treatments: an intermediate fluorescence response in the greenhouse, and the most pronounced decline of all treatments in the phytotron by the second measurement.

This indicates that reduced-pressure exposure itself – independent of plasma-generated reactive species – has a measurable physiological effect, most plausibly through mechanical alteration of the seed coat and evacuation of intercellular air spaces, which is known to enhance water and gas exchange during imbibition and to modify seed-surface wettability (Bormashenko *et al*. 2012, Los *et al.* 2019). This distinction between physical (wettability, surface micro-structure) and chemical (reactive-species-driven) mechanisms of plasma seed treatment is well established in the mechanistic literature on plasma–seed interactions (Waskow *et al.* 2021), and is consistent with earlier characterisation of wettability changes in downer-type plasma reactors of the kind used here (Arpagaus *et al.* 2005). The plasma treatments in this study should therefore be understood as combining this physical vacuum effect with an additional, chemically-driven reactive-species effect, rather than the vacuum component being negligible – a distinction supported by the fact that VAC alone diverged significantly from N in mature-plant fluorescence responses, even though the corresponding difference at the germination stage did not reach statistical significance.

Plasma power emerged as a decisive factor for the chemically-driven component specifically: while 50 W generally elicited mild or neutral effects, the 100-W and 150-W treatments induced the strongest physiological responses, the direction and magnitude of which were strictly environment-dependent, as described above. This power-dependent and environment-gated outcome underscores the importance of optimising both treatment parameters and growth conditions when considering CP as an agronomic tool.

Accelerated phenology and biomass response: Cold plasma seed treatment significantly altered flowering dynamics and biomass accumulation, revealing a complex interaction between early-stage priming and post-germination environmental conditions. The effect of CP on flowering timing was neither uniform nor simply "accelerating" – its direction depended on both growth environment and plasma power.

Under phytotron conditions, higher CP power (100 and 150 W) significantly delayed the onset and progression of flowering relative to both controls, reflected in a rightward shift of the fitted logistic curves (later inflexion point, slower initial rise in percentage of flowering individuals). Under greenhouse conditions, only the highest power (150 W) produced the opposite effect – a significantly earlier onset of flowering, reflected in an earlier inflexion point and a steeper initial rise in the fitted curve – while 50 W showed the slowest progression and 100 W was intermediate, overlapping with the controls.

This dose- and environment-dependent divergence indicates that CP does not act as a generic accelerant or generic delayer of phenology. Under stable phytotron conditions, high-power CP appears to prolong the vegetative phase, consistent with the concurrent, significant biomass gain at 100 and 150 W (Fig. 6) and with previous reports that CP-induced priming can extend vegetative growth under optimal conditions, allowing more extensive resource acquisition before the transition to reproduction (Li *et al*. 2015, Mildaziene *et al.* 2021). This biomass-promoting effect under stable, low-stress conditions is consistent with reports of CP-stimulated root and lateral organ growth in sunflower (Tamošiūnė *et al.* 2020) and of enhanced growth and altered chemical composition in basil grown under controlled hydroponic or greenhouse conditions (Auguste *et al.* 2023, Karaayak *et al.* 2023). Notably, in those studies, growth benefits were most clearly expressed where background environmental stress was minimised, supporting the broader pattern that CP-induced biomass gains depend on the stability of the surrounding growth environment rather than being a fixed property of the treatment itself. Since flowering time is under direct hormonal control, this shift may reflect underlying changes in phytohormone balance induced by plasma treatment, as reported for other species (Stolárik *et al.* 2015, Mildažienė *et al.* 2019, Žūkienė *et al.* 2019). We did not measure phytohormone contents directly in this study; however, this remains an inference rather than a demonstrated mechanism.

Under the more variable greenhouse conditions, by contrast, the same high plasma dose appears to trigger an earlier transition to reproduction with no corresponding biomass benefit – a pattern more consistent with a stress-avoidance strategy than with prolonged vegetative growth (Adhikari *et al.* 2020). The lack of significant biomass differences in the greenhouse, despite the accelerated flowering at 150 W, further suggests that CP-induced priming does not necessarily translate into sustained growth advantages under variable environmental stressors (Šerá *et al.* 2021). These findings underscore that the balance between vegetative growth and reproductive timing following CP priming is highly context-dependent, with the same treatment producing opposite phenological

outcomes depending on the stability of the post-germination environment.

Implications: Our study highlights the importance of environmental conditions in determining the long-term response of plants to cold plasma priming. The consistent, environment-independent acceleration of germination contrasts with the strongly environment-dependent and, in some cases, opposite-direction effects on mature-plant physiology, biomass, and flowering timing observed here. This distinction is agronomically important: a treatment effect confirmed only at the germination stage cannot be assumed to translate predictably into field-relevant outcomes.

Future studies should evaluate CP under field conditions to determine its practical applicability, and should use controlled experiments that manipulate individual environmental factors – for example, a phytotron design comparing constant *vs*. fluctuating temperature regimes while holding other factors constant – to isolate which specific components of "environmental context" (temperature stability, light variability, humidity, or their combination) drive the divergent responses reported here. Direct measurement of reactive oxygen/nitrogen species contents, antioxidant enzyme activity, and phytohormone balance would also help distinguish between the physical (vacuum-related) and chemical (reactive-species-related) contributions to the plasma effect identified in this study.

Conclusion: This study demonstrated that cold plasma (CP) seed priming produces effects which are specific to developmental stage and growth environment, rather than a single, generalizable outcome. At germination, CP accelerated radicle emergence consistently across all power levels (40–45% increase at 96 h relative to control), independent of environmental context, since this stage precedes environmental divergence. In mature plants, however, effects were environment- and dose-specific: under stable phytotron conditions, 100-W and 150-W CP significantly increased biomass (*Games–Howell*, $p<0.05$) and delayed flowering onset relative to controls; under fluctuating greenhouse conditions, the same treatments produced no significant biomass gain, and only the highest dose (150 W) significantly accelerated flowering onset. Chlorophyll fluorescence and reflectance responses likewise diverged in direction and timing between environments rather than in magnitude alone. These specific, opposite-direction outcomes – rather than a general claim that CP is "environmentally modulated" – indicate that the agronomic value of CP priming for lettuce depends on matching treatment dose to the stability of the intended cultivation environment, and that recommendations derived from controlled-environment studies alone cannot be assumed to transfer to variable field or greenhouse conditions.

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