

## Portable spectrometer-based cold stress detection in C3 and C4 plants

### Detekcija stresa na niske temperature kod C3 i C4 biljaka pomoću prijenosnog spektrometra

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#### ABSTRACT

Plant stress detection is crucial for enhancing crop yield, particularly in the face of climate change. Cold stress significantly affects photosynthetic activity and pigment composition in soybean (a C3 plant) and maize (a C4 plant), underscoring the need to identify stress signals before irreversible damage occurs. This study used portable spectrometers to non-destructively detect the early signs of cold stress-related processes in these crops. The spectral reflectance indices were measured to identify crop-specific stress responses. According to the results, pigment-based indices have better potential for crop-specific stress detection than general stress response indices. The normalized phaeophytinization index (NPQI) and normalized pigment chlorophyll index (NPCI) exhibited diverse responses in C4 maize compared to C3 soybean, indicating the potential for discerning stress-induced changes in chlorophyll and other pigment contents. The photochemical reflectance index (PRI), modified to specific wavelengths, emerged as a crucial tool for stress detection in both crops, showing strong correlations with photosynthetic parameters and biomass traits. Specifically, the strongest correlation was found between  $PRI_{(525, 570)}$  and fresh mass in maize and  $PRI_{(555, 570)}$  and fresh mass in soybean. This approach underscores the importance of advanced spectral techniques in understanding and improving crop responses to cold stress, highlighting their potential for precision agriculture.

**Keywords:** spectral reflectance indices, photochemical reflectance index, maize, soybean

#### SAŽETAK

Detekcija stresa kod biljaka ključna je za povećanje prinosa usjeva, posebno u kontekstu klimatskih promjena. Stres na niske temperature značajno utječe na fotosintetsku aktivnost i sastav pigmenta kod soje (C3 biljka) i kukuruza (C4 biljka), što naglašava potrebu za otkrivanjem znakova stresa prije nego što dođe do nepovratne štete. U ovom istraživanju korišteni su prijenosni spektrometri za otkrivanje ranih znakova stresa izazvanog niskim temperaturama bez uništavanja biljnog materijala kod navedenih kultura. Mjerenjem spektralnih indeksa identificirani su specifični odgovori na stres ovisni o kulturi. Prema rezultatima, indeksi za procjenu sadržaja pigmenta pokazali su veći potencijal u otkrivanju stresa specifičnog za biljnu vrstu u odnosu na opće indekse odgovora na stres. Normalizirani indeks feofitinizacije (NPQI) i normalizirani indeks omjera ukupnih pigmenta i klorofila (NPCI) pokazali su različite odgovore kod C4 kukuruza u usporedbi s C3 sojom, što ukazuje na potencijal za prepoznavanje stresom izazvanih promjena u sadržaju klorofila i drugih pigmenta. Fotokemijski indeks reflektivnosti (PRI), modificiran na specifične valne duljine, pokazao se kao vrijedan alat ključan za detekciju stresa kod obje kulture, pokazujući jake korelacije s fotosintetskim parametrima i svojstvima biomase. Specifično, najjača korelacija pronađena je između  $PRI_{(525, 570)}$  i svježe mase kod kukuruza, te  $PRI_{(555, 570)}$  i svježe mase kod soje. Ovaj pristup naglašava važnost naprednih spektralnih tehnika za razumijevanje i poboljšanje odgovora usjeva na stres izazvan niskim temperaturama, ističući njihov potencijal za primjenu u preciznoj poljoprivredi.

**Ključne riječi:** spektralni indeksi, fotokemijski indeks reflektivnosti, kukuruz, soja

## INTRODUCTION

Plant stress detection is considered one of the most critical areas for improving crop yield under compelling global climate change. Climate change brings various challenges to agricultural production, affecting both producers and plant breeders who face demanding goals. More frequent drought years with high temperatures necessitate the shifting of sowing dates to earlier spring when there is a risk of low temperatures that are unfavorable to growth and development. Additionally, due to climate change, unpredictable occurrences of low temperatures even within optimal sowing dates are becoming more common (Shivanna, 2022).

Cold stress can significantly affect plant growth, particularly during the early growth stages (Hussain et al., 2018). This occurs when plants are exposed to temperatures below the optimal range for their growth and physiological functions. This environmental condition can trigger a range of negative effects, including slowed growth, reduced photosynthesis, cell membrane damage, and, in extreme scenarios, tissue freezing and death (Hu et al., 2017; Li et al., 2019; Zhou et al., 2022). Cold stress poses a significant threat to photosynthetic activity, adversely affecting photosynthetic pigments (Freitas et al., 2019). Leaf chlorosis, a common chilling stress symptom, results from inhibited chlorophyll synthesis and the destruction of existing chlorophyll (Soufi et al., 2015). In the context of C3 and C4 plants, understanding their unique responses to cold stress is crucial for devising targeted strategies. C3 and C4 plants are two different forms of photosynthesis that have evolved to adapt to different environments. C3 plants perform photosynthesis by using the Calvin cycle to convert carbon dioxide into sugar (Wang et al., 2019). C4 plants have an additional mechanism to concentrate CO<sub>2</sub> in specialized cells, which allows them to perform photosynthesis more efficiently under hot and dry conditions (Cui, 2021). This key difference in photosynthetic pathways means that novel diagnostic methods must consider the inherent distinctions between C3 and C4 responses to cold stress.

Therefore, understanding the impact of cold stress on soybean (*Glycine max* L. Merr.; a C3 plant) and maize (*Zea mays* L.; a C4 plant) requires a comprehensive exploration of their distinct responses.

Although quantitative methods offer highly sensitive indicators of most stressors, their disruptive approach hinders continuous monitoring and dynamic studies. Qualitative methods, including fluorescence, thermography, and VIS/NIR reflectance, offer a non-destructive perspective on the impact of stressors, even across extensive fields (Galieni et al., 2021). The exploration of these responses using advanced non-destructive techniques is instrumental in identifying early indicators of stress in soybean and maize under cold stress conditions.

Chlorophyll *a* fluorescence (ChlF) has been proven useful for assessing plant conditions under stress in numerous studies (Franić et al., 2018, 2020a, 2020b; Galić et al., 2019, 2020). However, this approach requires a period of dark adaptation for samples, which is a limiting factor for the throughput of measurements (Mazur et al., 2023). Using portable spectral measurement instruments for reflectance measurements could offer an advantage over this approach because of its potential for faster and simpler data acquisition (Arias et al., 2021). Extensive research has been conducted on leaf reflectance under various biotic and abiotic stress conditions. Environmental stress induces changes in the spectral line shape (Carter, 1993). Various spectral reflectance indices (SRIs) can effectively describe these alterations. Numerous SRIs are associated with stress responses, among which the phytochemical reflectance index (PRI) and Carter indices (Ctr1 and Ctr2) are recognized as general stress response indicators (Carter, 1994; Zhang et al., 2016; Sukhova and Sukhov, 2019).

The aim of this study was to use portable spectral measurement instruments for reflectance measurements as an advanced non-destructive technique to identify

early indicators of cold stress in C3 and C4 plants. By exploring the unique responses of soybean and maize to cold stress through these methods, this research sets the stage for targeted agricultural approaches and provides crucial insights for breeding programs and resource-efficient stress management strategies in the face of climate change.

## MATERIALS AND METHODS

The plant trial was conducted in an Aralab Bio single-tier plant growth room (Fitoclima 10.000 HP, Aralab, Rio de Mouro, Portugal) under fully controlled conditions. The plant material included 12 soybean cultivars from the 0–I maturity group and 14 maize parental lines previously evaluated for cold stress tolerance (Mazur et al., 2024). These materials were developed and owned by the Agricultural Institute Osijek (AIO, Osijek, Croatia).

The experiment comprised two treatments: control (T1) and cold stress treatment (T2). Five seeds of each genotype were sown in four biological replicates per treatment. Each treatment was grown in separate time series to achieve the required conditions. Under T1 conditions, the day/night parameters were set to a 16/8 h photoperiod, 25/18 °C temperature, 70/90% relative humidity (RH), and 300  $\mu\text{mol}(\text{photon})/\text{m}^2\text{s}$  light intensity. T2 conditions mirrored those of the control until 25 days after sowing (DAS) in soybean and 17 DAS in maize. At these points, cold stress was induced and maintained for three days under the following day/night conditions: 16/8 h photoperiod, 10/5 °C temperature, 70/90% RH, and 300  $\mu\text{mol}(\text{photon})/\text{m}^2\text{s}$  light intensity.

The plants were grown in trays measuring 510 x 350 x 200 mm. Each tray was loaded with 5.5 kg of soil characterized by pH ( $\text{CaCl}_2$ ) = 5.7, N ( $\text{NH}_4^+ + \text{NO}_3^-$ ) = 70 mg/L, P ( $\text{P}_2\text{O}_5$ ) = 50 mg/L, K ( $\text{K}_2\text{O}$ ) = 90 mg/L, and EC = 40 mS/m. These trays were sectioned into 12 rows for soybean with 3 and 2 planting spaces alternating, and 7 rows with 5 planting spaces for maize. In each soybean tray, six genotypes were accommodated with two trays per replicate and eight trays per treatment. For maize, each tray featured seven genotypes with two trays per

replicate and eight trays per treatment. The genotype sequence within each replicate was randomized, and the trays were shuffled randomly in the growth room daily. All plants were watered with tap water every other day. The growth conditions were monitored daily and the temperature and humidity conditions of the growth room were recorded using a FitoLog9000 data logger (Aralab, Rio de Mouro, Portugal).

The spectral reflectance of leaves was measured using a handheld instrument PolyPen RP 410 (Photon Systems Instruments, Drásov, Czech Republic) in the UVIS and NIR response ranges (380–790 nm, 640–1050 nm respectively). The devices are equipped with an internal light source (Xenon incandescent lamp) and measure reflectance with a spectral resolution of 1.9 nm. ChlF was determined using a FluorPen FP 110 (Photon Systems Instruments, Drásov, Czech Republic) by the saturation pulse method (Kalaji et al., 2016). Both type of measurements were performed in the middle of the last fully developed leaf for maize and on the middle leaflet of the last fully developed trifoliolate for soybean. Two measurements per genotype were made for each replicate and treatment, totaling 8 measurements per genotype per treatment. Measurements were taken for three consecutive days in both treatments, one hour after lights were turned on. For soybean, this began on the 26<sup>th</sup> DAS and for maize, on the 18<sup>th</sup> DAS. This schedule represented 24, 48, and 72 hours of cold stress in the T2 treatment. In total, 576 measurements were obtained for soybean and 672 for maize for both types of measurements.

Data recorded by measuring the spectral reflectance of the leaves were processed with SpectraPen software (Drásov, Czech Republic) provided with the measuring devices. The spectral reflectance indices used in this study are listed in Table 1. The data recorded by measuring ChlF were used to calculate the maximum quantum yield of photosystem II (PSII) photochemistry ( $F_v/F_m$ ) and performance index ( $PI_{\text{abs}}$ ) according to Strasser et al. (2004).

**Table 1.** The spectral reflectance indices used for the cold stress detection

Abbreviation	Index name	Equation	Reference
Vegetation indices			
NDVI	Normalized difference vegetation index	$(R_{NIR} - R_{RED}) / (R_{NIR} + R_{RED})$	Rouse et al. (1974)
RDVI	Renormalized difference vegetation index	$(R_{780} - R_{670}) / [(R_{780} + R_{670})^{0.5}]$	Roujean and Breon (1995)
SR	Simple ratio	$R_{NIR} / R_{RED}$	Rouse et al. (1974)
G	Greenness index	$R_{554} / R_{677}$	Smith et al. (1995)
TVI	Triangular vegetation index	$0.5[120(R_{750} - R_{550}) - 200(R_{670} - R_{550})]$	Broge and Leblanc (2001)
Stress response reflectance indices			
Ctr1	Carter index	$R_{695} / R_{420}$	Carter (1994)
Ctr2		$R_{695} / R_{760}$	
Lic1	Lichtenthaler index	$(R_{790} - R_{680}) / (R_{790} + R_{680})$	Lichtenthaler et al. (1996)
Lic2		$R_{440} / R_{690}$	
GM1	Gitelson and Merzlyak index	$R_{750} / R_{550}$	Gitelson and Merzlyak (1996)
GM2		$R_{750} / R_{700}$	
Pigment related reflectance indices			
ZMI	Zarco-Tejada and Miller index	$R_{750} / R_{710}$	Zarco-Tejada et al. (2001)
SIPI	Structure insensitive pigment index	$(R_{790} - R_{450}) / (R_{790} + R_{650})$	Peñuelas et al. (1995)
MCARI	Modified chlorophyll absorption in reflectance index	$[(R_{700} - R_{670}) - 0.2(R_{700} - R_{550})] (R_{700} / R_{670})$	Daughtry et al. (2000)
MCARI1		$1.2[2.5(R_{790} - R_{670}) - 1.3(R_{790} - R_{550})]$	Haboudane et al. (2004)
TCARI	Transformed chlorophyll absorption in reflectance index	$3[(R_{700} - R_{670}) - 0.2(R_{700} - R_{550})] (R_{700} / R_{670})$	Haboudane et al. (2002)
NPCI	Normalized pigment chlorophyll index	$(R_{680} - R_{430}) / (R_{680} + R_{430})$	Peñuelas et al. (1994)
SPRI	Simple ratio pigment index	$R_{430} / R_{680}$	Peñuelas et al. (1995)
NPQI	Normalized phaeophytinization index	$(R_{415} - R_{435}) / (R_{415} + R_{435})$	Barnes et al. (1992)
CRI1	Carotenoid reflectance index	$(1/R_{510}) - (1/R_{550})$	Gitelson et al. (2002)
CRI2		$(1/R_{510}) - (1/R_{700})$	
ARI1	Anthocyanin reflectance index	$(1/R_{550}) - (1/R_{700})$	Gitelson et al. (2001)
ARI2		$R_{800} [(1/R_{550}) - (1/R_{700})]$	
PRI <sub>(531, 570)</sub>	Photochemical reflectance index	$(R_{531} - R_{570}) / (R_{531} + R_{570})$	Gamon et al. (1992)
PRI <sub>(λ, 570)</sub>	Modified photochemical reflectance index	$(R_{λ} - R_{570}) / (R_{λ} + R_{570})$	Sukhova and Sukhov (2020)

λ=510, 515, 520, 525, 535, 540, 545, 550, 555

Aboveground biomass of each soybean cultivar and maize inbred line in four replicates was weighed on a four-decimal laboratory scale and designated as fresh mass (FM). Plants were dried for 24 hours at 80 °C before weighting for dry mass (DM), which was expressed as percentage of FM.

Data were analyzed using the statistical software package JASP (JASP Team, 2023). The normality of the data was checked using the Shapiro-Wilk test ( $P < 0.05$ ) before conducting the statistical tests. The effects of treatments and genotypes for each crop were examined using analysis of variance (ANOVA). Tukey's HSD post-hoc test ( $P < 0.05$ ) was used to test the differences between the control and three durations of cold stress for the chosen parameters. All replicates considered in this study were independent biological replicates originating from different plants. Because there were no statistically significant differences among the measured values under control conditions, measurements from three consecutive days were aggregated before ANOVA to simplify data representation. The relationships between spectral reflectance indices and photosynthetic parameters, as well as biomass traits, were evaluated using Pearson's correlation coefficients. The strength of the correlation was assessed using the scale established by Evans (1996).

## RESULTS

Analysis of variance revealed significant differences among treatments and genotypes for most examined SRIs in both maize and soybean (Tables 2 and 3). Among all SRIs, apart from PRI and its modifications, ARI1 and ARI2 in maize exhibited the greatest changes under cold stress compared to the control (Table 4). The impact of stress duration on maize was not pronounced for most indices, but was evident in ARI1 and ARI2. ARI values increased with the duration of stress (ARI1 increased by 24.4%, 34.0%, and 34.1%; ARI2 increased by 22.8%, 28.8%, and 34.7% after 24, 48 and 72 h of low temperatures, respectively). NPQI values sharply increased by 23.0% after 24 h of stress, followed by a decline to control levels after 72 h of cold (Table 4). In maize, most indices responded to low temperatures within 24 h, whereas

soybean exhibited a different response. Many indices in soybean remained at control levels after 24 h of low temperatures, with changes occurring only after 48 or 72 h (Table 5). On the other hand, some indices in soybean showed an opposite reaction after 24 h compared with 48 and 72 h of low temperatures. This trend was most pronounced for the NPCI, which decreased by 41.0% compared to the control after 24 h at low temperatures, and then began to approach the control values. The greatest change under cold stress in soybean was observed in NPQI, which increased by an average of 317.4% in T2 compared to control, followed by ARI1 and ARI2. In soybean, ARI1 increased by 46.0%, 121.9%, and 130.9% after 24, 48, and 72 h of low temperatures, respectively. ARI2 showed a similar trend (Table 5).

Changes in PRI and its modifications at the measuring wavelengths of 510, 515, 520, 525, 535, 540, 545, 550, and 555 nm induced by cold stress were analyzed (Figure 1). In maize, all investigated PRIs, except  $PRI_{(510, 570)}$ , significantly decreased ( $P < 0.01$ ) after only 24 h of cold stress (Figure 1A). Similarly, significant differences ( $P < 0.01$ ) between the control and cold stress conditions were observed for all PRI modifications in soybean (Figure 1B). The greatest decline under low temperature conditions in both crops occurred in modified  $PRI_{(525, 570)}$ , with an average reduction of 89.4% in maize and 66.9% in soybean compared with the control. Typical  $PRI_{(531, 570)}$  decreased under cold stress by 27.2%, 27.5%, and 37.8% in maize and by 34.7%, 43.8%, and 53.1% in soybean after 24, 48 and 72 h of exposure to low temperatures (Figure 1).

Cold stress induced changes in SRIs were hypothesized to be associated with changes in photosynthetic activity and reflected in the accumulation of aboveground biomass. To investigate the relationship between the indices and selected ChlF parameters, a correlation analysis was conducted among all SRIs,  $F_v/F_m$ ,  $PI_{abs}$ , and biomass traits to identify appropriate SRIs for cold stress detection. In maize, moderately high positive correlations were observed between photosynthetic parameters and FM, whereas moderately high negative correlations were found with DM.

**Table 2.** Analysis of variance for spectral reflectance indices of maize

	Source of Variability			
	Treatment (T)	Genotype (G)	T × G	Residuals
NDVI	0.037***	0.010***	0.001	$9.097 \times 10^{-4}$
RDVI	0.014***	0.005***	$6.937 \times 10^{-4}$	$5.920 \times 10^{-4}$
SR	6.549***	1.474***	0.168	0.145
G	0.069**	0.206***	0.014	0.013
OSAVI	0.021***	0.006***	$8.823 \times 10^{-4}$	$6.726 \times 10^{-4}$
TVI	33.283***	10.614***	2.502	2.414
Ctrl1	0.018***	0.014***	0.003	0.002
Ctrl2	0.028***	0.007***	$7.987 \times 10^{-4}$	$6.161 \times 10^{-4}$
Lic1	0.03***	0.007***	0.001	$7.501 \times 10^{-4}$
Lic2	0.01***	0.007***	0.001	0.001
GM1	1.181***	0.615***	0.029	0.029
GM2	2.977***	0.803***	0.057	0.055
ZMI	0.648***	0.386***	0.018	0.018
SIPI	0.031***	0.008***	$9.743 \times 10^{-4}$	$7.877 \times 10^{-4}$
MCARI	0.003***	0.006***	$2.173 \times 10^{-4}$	$2.610 \times 10^{-4}$
MCARI1	0.024***	0.008***	0.002	0.002
TCARI	0.006***	0.002***	$1.384 \times 10^{-4}$	$1.309 \times 10^{-4}$
NPCI	0.002***	0.002***	$2.896 \times 10^{-4}$	$2.492 \times 10^{-4}$
SPRI	0.01***	0.011***	0.002	0.001
NPQI	$4.816 \times 10^{-4}$ *	$7.946 \times 10^{-4}$ ***	$1.322 \times 10^{-4}$	$1.415 \times 10^{-4}$
CRI1	2.649***	1.148***	0.215	0.191
CRI2	0.091	0.863***	0.122	0.105
ARI1	2.006***	0.254***	0.027	0.027
ARI2	0.457***	0.07***	0.007	0.007
PRI <sub>(531, 570)</sub>	0.004***	$6.564 \times 10^{-5}$ ***	$2.604 \times 10^{-5}$	$1.900 \times 10^{-5}$
df	3	13	39	392

Values present mean squares. \*, \*\*, \*\*\* significant at the  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively

**Table 3.** Analysis of variance for spectral reflectance indices of soybean

	Source of Variability			
	Treatment (T)	Genotype (G)	T × G	Residuals
NDVI	0.011***	0.001***	$5.904 \times 10^{-4}$ *	$3.854 \times 10^{-4}$
RDVI	$2.941 \times 10^{-4}$	0.001***	$2.737 \times 10^{-4}$	$2.919 \times 10^{-4}$
SR	5.212***	0.567***	0.272*	0.18
G	0.498***	0.42***	0.053*	0.032
OSAVI	0.002***	$4.526 \times 10^{-4}$ ***	$2.327 \times 10^{-4}$ *	$1.456 \times 10^{-4}$
TVI	16.136***	9.384***	2.893***	1.364
Ctrl	0.344***	0.045***	0.019***	0.009
Ctrl	0.015***	0.001***	$5.642 \times 10^{-4}$ *	$3.499 \times 10^{-4}$
Lic1	0.005***	0.001***	$2.290 \times 10^{-4}$	$1.563 \times 10^{-4}$
Lic2	0.062***	0.007***	0.004***	0.002
GM1	1.489***	0.39***	0.104	0.071
GM2	3.302***	0.46***	0.13*	0.082
ZMI	0.554***	0.114***	0.027*	0.018
SIPI	0.006***	$7.044 \times 10^{-4}$ ***	$1.912 \times 10^{-4}$	$1.573 \times 10^{-4}$
MCARI	0.058***	0.017***	0.004**	0.002
MCARI1	0.014***	0.008***	0.002***	0.001
TCARI	0.022***	0.008***	0.002***	$9.754 \times 10^{-4}$
NPCI	0.006***	0.001***	$6.474 \times 10^{-4}$ ***	$2.930 \times 10^{-4}$
SPRI	0.029***	0.007***	0.003***	0.001
NPQI	0.001***	0.002***	$2.406 \times 10^{-4}$	$2.033 \times 10^{-4}$
CRI1	1.68***	2.375***	0.105	0.156
CRI2	1.814***	2.191***	0.11	0.16
ARI1	2.175***	0.102***	0.015*	0.01
ARI2	0.618***	0.028***	0.005	0.003
PRI <sub>(531, 570)</sub>	0.010***	$2.239 \times 10^{-4}$ ***	$8.567 \times 10^{-5}$	$6.949 \times 10^{-5}$
df	3	11	33	336

Values present mean squares. \*, \*\*, \*\*\* significant at the  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively

**Table 4.** Effects of different cold stress durations on leaf spectral reflectance indices of maize

	T1	T2 24 h	T2 48 h	T2 72 h
NDVI	0.631 ± 0.003 <sup>a</sup>	0.599 ± 0.003 <sup>b</sup>	0.599 ± 0.003 <sup>b</sup>	0.589 ± 0.004 <sup>b</sup>
RDVI	0.526 ± 0.002 <sup>a</sup>	0.511 ± 0.003 <sup>b</sup>	0.518 ± 0.002 <sup>ab</sup>	0.500 ± 0.003 <sup>c</sup>
SR	4.456 ± 0.043 <sup>a</sup>	4.020 ± 0.040 <sup>b</sup>	4.023 ± 0.037 <sup>b</sup>	3.910 ± 0.044 <sup>b</sup>
G	1.869 ± 0.014 <sup>a</sup>	1.829 ± 0.013 <sup>b</sup>	1.820 ± 0.012 <sup>b</sup>	1.814 ± 0.013 <sup>b</sup>
TVI	26.99 ± 0.14 <sup>b</sup>	27.10 ± 0.16 <sup>b</sup>	27.67 ± 0.15 <sup>a</sup>	26.34 ± 0.17 <sup>c</sup>
Ctrl1	1.057 ± 0.004 <sup>a</sup>	1.067 ± 0.005 <sup>ab</sup>	1.079 ± 0.005 <sup>b</sup>	1.085 ± 0.005 <sup>bc</sup>
Ctrl2	0.250 ± 0.002 <sup>a</sup>	0.278 ± 0.003 <sup>b</sup>	0.278 ± 0.003 <sup>bc</sup>	0.287 ± 0.003 <sup>c</sup>
Lic1	0.675 ± 0.003 <sup>a</sup>	0.646 ± 0.003 <sup>b</sup>	0.647 ± 0.003 <sup>b</sup>	0.638 ± 0.003 <sup>b</sup>
Lic2	1.051 ± 0.003 <sup>a</sup>	1.038 ± 0.004 <sup>b</sup>	1.035 ± 0.003 <sup>b</sup>	1.028 ± 0.004 <sup>b</sup>
GM1	2.720 ± 0.022 <sup>a</sup>	2.531 ± 0.019 <sup>b</sup>	2.544 ± 0.019 <sup>b</sup>	2.487 ± 0.021 <sup>b</sup>
GM2	3.260 ± 0.028 <sup>a</sup>	2.974 ± 0.025 <sup>b</sup>	2.963 ± 0.025 <sup>bc</sup>	2.890 ± 0.028 <sup>c</sup>
ZMI	2.237 ± 0.017 <sup>a</sup>	2.103 ± 0.015 <sup>b</sup>	2.105 ± 0.015 <sup>b</sup>	2.062 ± 0.017 <sup>b</sup>
SIPI	0.643 ± 0.003 <sup>a</sup>	0.613 ± 0.003 <sup>b</sup>	0.614 ± 0.003 <sup>b</sup>	0.605 ± 0.003 <sup>b</sup>
MCARI	0.096 ± 0.002 <sup>b</sup>	0.105 ± 0.002 <sup>a</sup>	0.108 ± 0.002 <sup>a</sup>	0.105 ± 0.002 <sup>a</sup>
MCARI1	0.719 ± 0.004 <sup>b</sup>	0.720 ± 0.004 <sup>b</sup>	0.736 ± 0.004 <sup>a</sup>	0.700 ± 0.004 <sup>c</sup>
TCARI	-0.150 ± 0.001 <sup>a</sup>	-0.163 ± 0.001 <sup>b</sup>	-0.166 ± 0.001 <sup>b</sup>	-0.163 ± 0.001 <sup>b</sup>
NPCI	-0.084 ± 0.001 <sup>b</sup>	-0.078 ± 0.002 <sup>ab</sup>	-0.076 ± 0.001 <sup>a</sup>	-0.075 ± 0.002 <sup>a</sup>
SPRI	1.183 ± 0.003 <sup>a</sup>	1.171 ± 0.004 <sup>ab</sup>	1.165 ± 0.003 <sup>b</sup>	1.162 ± 0.005 <sup>b</sup>
NPQI	0.019 ± 0.001 <sup>b</sup>	0.024 ± 0.001 <sup>a</sup>	0.022 ± 0.001 <sup>ab</sup>	0.020 ± 0.001 <sup>ab</sup>
CRI1	2.799 ± 0.050 <sup>a</sup>	2.519 ± 0.044 <sup>b</sup>	2.466 ± 0.041 <sup>b</sup>	2.498 ± 0.043 <sup>b</sup>
CRI2	1.696 ± 0.036	1.635 ± 0.034	1.645 ± 0.033	1.678 ± 0.032
ARI1	-1.103 ± 0.020 <sup>c</sup>	-0.884 ± 0.015 <sup>b</sup>	-0.821 ± 0.017 <sup>a</sup>	-0.820 ± 0.018 <sup>a</sup>
ARI2	-0.555 ± 0.009 <sup>c</sup>	-0.452 ± 0.008 <sup>b</sup>	-0.431 ± 0.009 <sup>ab</sup>	-0.412 ± 0.009 <sup>a</sup>
PRI	0.0311 ± 0.0004 <sup>a</sup>	0.0219 ± 0.0004 <sup>b</sup>	0.0215 ± 0.0004 <sup>b</sup>	0.0180 ± 0.0005 <sup>c</sup>

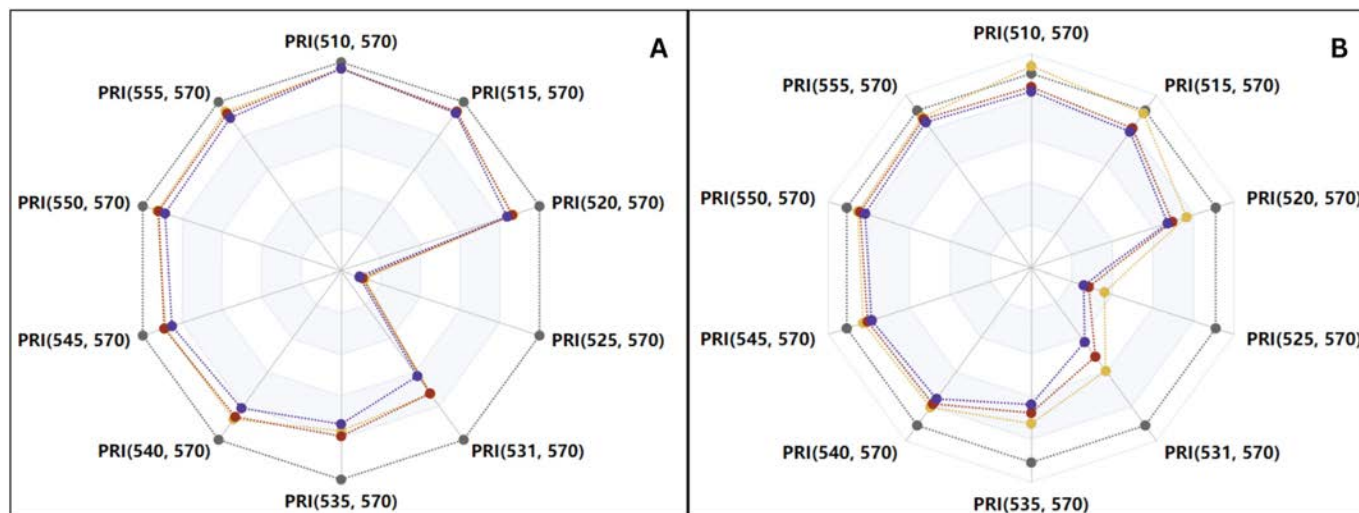
T1 – control; T2 24 h, T2 48 h, T2 72 h – 24, 48, and 72 h of cold stress treatment, respectively; full names of SRIs are given in Table 1; different superscript letters indicate significant differences ( $P < 0.05$ ) between the treatments; values present means ± standard error,  $n = 112$ .



**Table 5.** Effects of different cold stress durations on leaf spectral reflectance indices of soybean

	T1	T2 24 h	T2 48 h	T2 72 h
NDVI	0.708 ± 0.002 <sup>a</sup>	0.706 ± 0.002 <sup>a</sup>	0.692 ± 0.002 <sup>b</sup>	0.686 ± 0.002 <sup>b</sup>
RDVI	0.594 ± 0.001	0.594 ± 0.001	0.594 ± 0.001	0.591 ± 0.003
SR	5.887 ± 0.048 <sup>a</sup>	5.818 ± 0.042 <sup>a</sup>	5.514 ± 0.046 <sup>b</sup>	5.405 ± 0.046 <sup>b</sup>
G	2.254 ± 0.023 <sup>a</sup>	2.097 ± 0.018 <sup>b</sup>	2.212 ± 0.024 <sup>a</sup>	2.244 ± 0.021 <sup>a</sup>
TVI	31.33 ± 0.15 <sup>c</sup>	31.60 ± 0.10 <sup>bc</sup>	32.25 ± 0.15 <sup>a</sup>	31.99 ± 0.13 <sup>ab</sup>
Ctrl1	1.388 ± 0.013 <sup>a</sup>	1.290 ± 0.009 <sup>b</sup>	1.408 ± 0.010 <sup>a</sup>	1.422 ± 0.0110 <sup>a</sup>
Ctrl2	0.214 ± 0.002 <sup>b</sup>	0.215 ± 0.002 <sup>b</sup>	0.233 ± 0.002 <sup>a</sup>	0.238 ± 0.0020 <sup>a</sup>
Lic1	0.746 ± 0.001 <sup>a</sup>	0.737 ± 0.001 <sup>b</sup>	0.731 ± 0.002 <sup>c</sup>	0.729 ± 0.0020 <sup>c</sup>
Lic2	0.914 ± 0.005 <sup>b</sup>	0.959 ± 0.004 <sup>a</sup>	0.911 ± 0.004 <sup>b</sup>	0.902 ± 0.0040 <sup>b</sup>
GM1	3.063 ± 0.031 <sup>b</sup>	3.173 ± 0.029 <sup>a</sup>	2.946 ± 0.030 <sup>c</sup>	2.895 ± 0.0280 <sup>c</sup>
GM2	3.366 ± 0.035 <sup>a</sup>	3.385 ± 0.031 <sup>a</sup>	3.087 ± 0.031 <sup>b</sup>	3.028 ± 0.0300 <sup>b</sup>
ZMI	1.973 ± 0.016 <sup>b</sup>	2.026 ± 0.016 <sup>a</sup>	1.886 ± 0.015 <sup>c</sup>	1.863 ± 0.0140 <sup>c</sup>
SIPI	0.737 ± 0.001 <sup>a</sup>	0.725 ± 0.001 <sup>b</sup>	0.722 ± 0.001 <sup>bc</sup>	0.719 ± 0.0020 <sup>a</sup>
MCARI	0.183 ± 0.005 <sup>b</sup>	0.168 ± 0.004 <sup>b</sup>	0.214 ± 0.006 <sup>a</sup>	0.220 ± 0.0050 <sup>a</sup>
MCARI1	0.823 ± 0.004 <sup>b</sup>	0.824 ± 0.003 <sup>b</sup>	0.848 ± 0.004 <sup>a</sup>	0.840 ± 0.0040 <sup>a</sup>
TCARI	-0.174 ± 0.004 <sup>b</sup>	-0.162 ± 0.003 <sup>a</sup>	-0.191 ± 0.004 <sup>c</sup>	-0.194 ± 0.0040 <sup>c</sup>
NPCI	-0.024 ± 0.002 <sup>a</sup>	-0.041 ± 0.002 <sup>b</sup>	-0.027 ± 0.002 <sup>a</sup>	-0.025 ± 0.0020 <sup>a</sup>
SPRI	1.050 ± 0.004 <sup>b</sup>	1.087 ± 0.004 <sup>a</sup>	1.056 ± 0.004 <sup>b</sup>	1.052 ± 0.0040 <sup>b</sup>
NPQI	0.002 ± 0.001 <sup>b</sup>	0.010 ± 0.002 <sup>a</sup>	0.009 ± 0.002 <sup>a</sup>	0.010 ± 0.0020 <sup>a</sup>
CRI1	5.200 ± 0.041 <sup>a</sup>	4.885 ± 0.044 <sup>c</sup>	4.989 ± 0.045 <sup>bc</sup>	5.060 ± 0.0580 <sup>b</sup>
CRI2	4.632 ± 0.039 <sup>bc</sup>	4.496 ± 0.046 <sup>c</sup>	4.734 ± 0.045 <sup>ab</sup>	4.814 ± 0.0570 <sup>a</sup>
ARI1	-0.568 ± 0.013 <sup>c</sup>	-0.389 ± 0.013 <sup>b</sup>	-0.256 ± 0.011 <sup>a</sup>	-0.246 ± 0.0110 <sup>a</sup>
ARI2	-0.308 ± 0.007 <sup>c</sup>	-0.216 ± 0.007 <sup>b</sup>	-0.143 ± 0.006 <sup>a</sup>	-0.135 ± 0.0060 <sup>a</sup>
PRI	0.0370 ± 0.0005 <sup>a</sup>	0.0220 ± 0.0009 <sup>b</sup>	0.0180 ± 0.0010 <sup>c</sup>	0.0140 ± 0.0009 <sup>d</sup>

T1 – control; T2 24 h, T2 48 h, T2 72 h – 24, 48, and 72 h of cold stress treatment, respectively; full names of SRIs are given in Table 1; different superscript letters indicate significant differences ( $P < 0.05$ ) between the treatments; values present means ± standard error,  $n = 96$ .



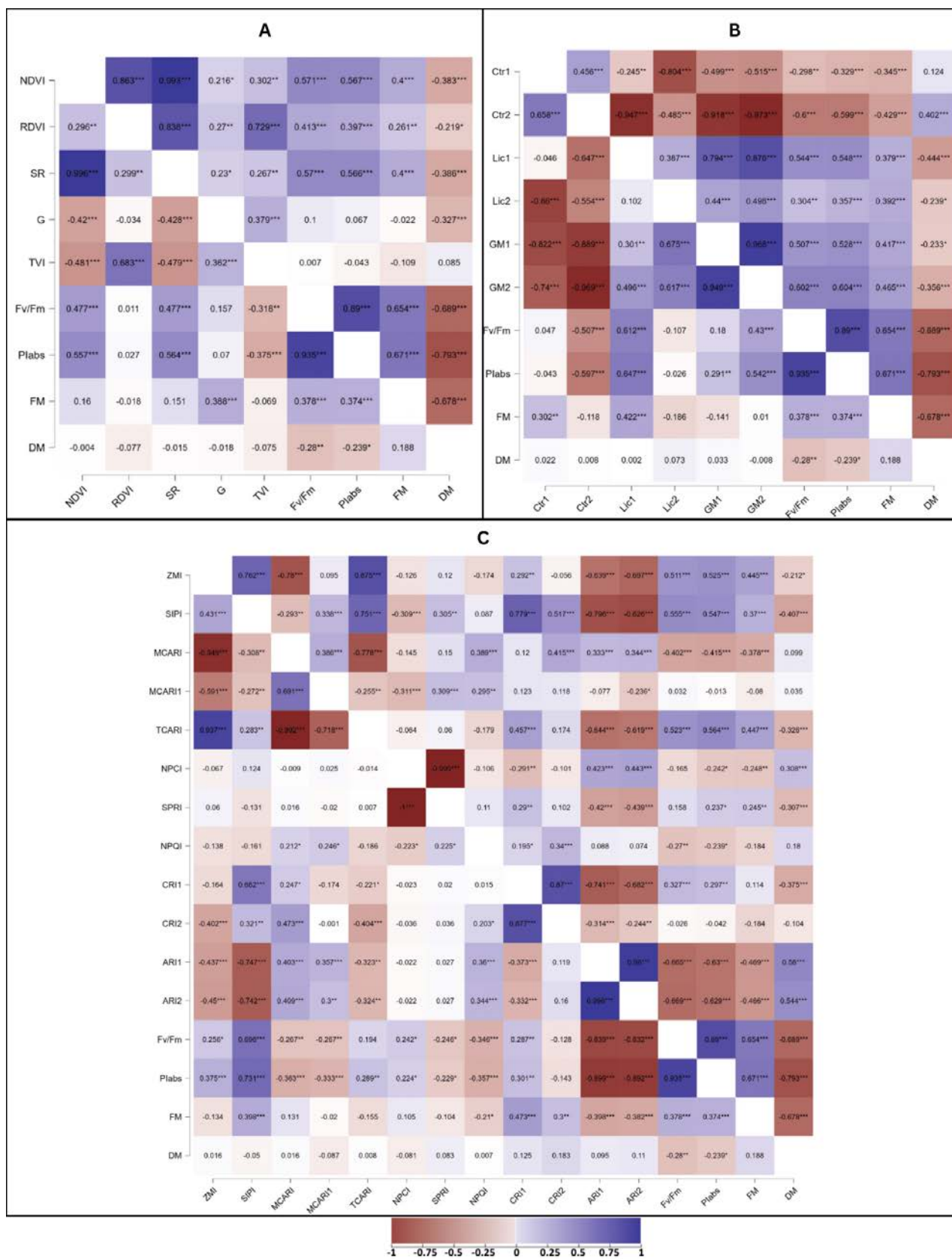
**Figure 1.** Changes in the photochemical reflectance index (PRI) and its modifications (descriptions can be found in Table 1) induced by cold stress in maize (A) and soybean (B). The values represent the means relative to the control, where the control was set to 1 ( $n = 112$  for maize and 96 for soybean). Significant differences among treatments at  $P < 0.01$  level were detected for all PRIs except  $PRI_{(510, 570)}$  in maize. T1 – control; T2 24 h, T2 48 h, T2 72 h – 24, 48, and 72 h of cold stress treatment, respectively.

In soybean, photosynthetic parameters showed weak significant positive correlations with FM and weak significant negative correlations with DM in soybean (Figure 2).

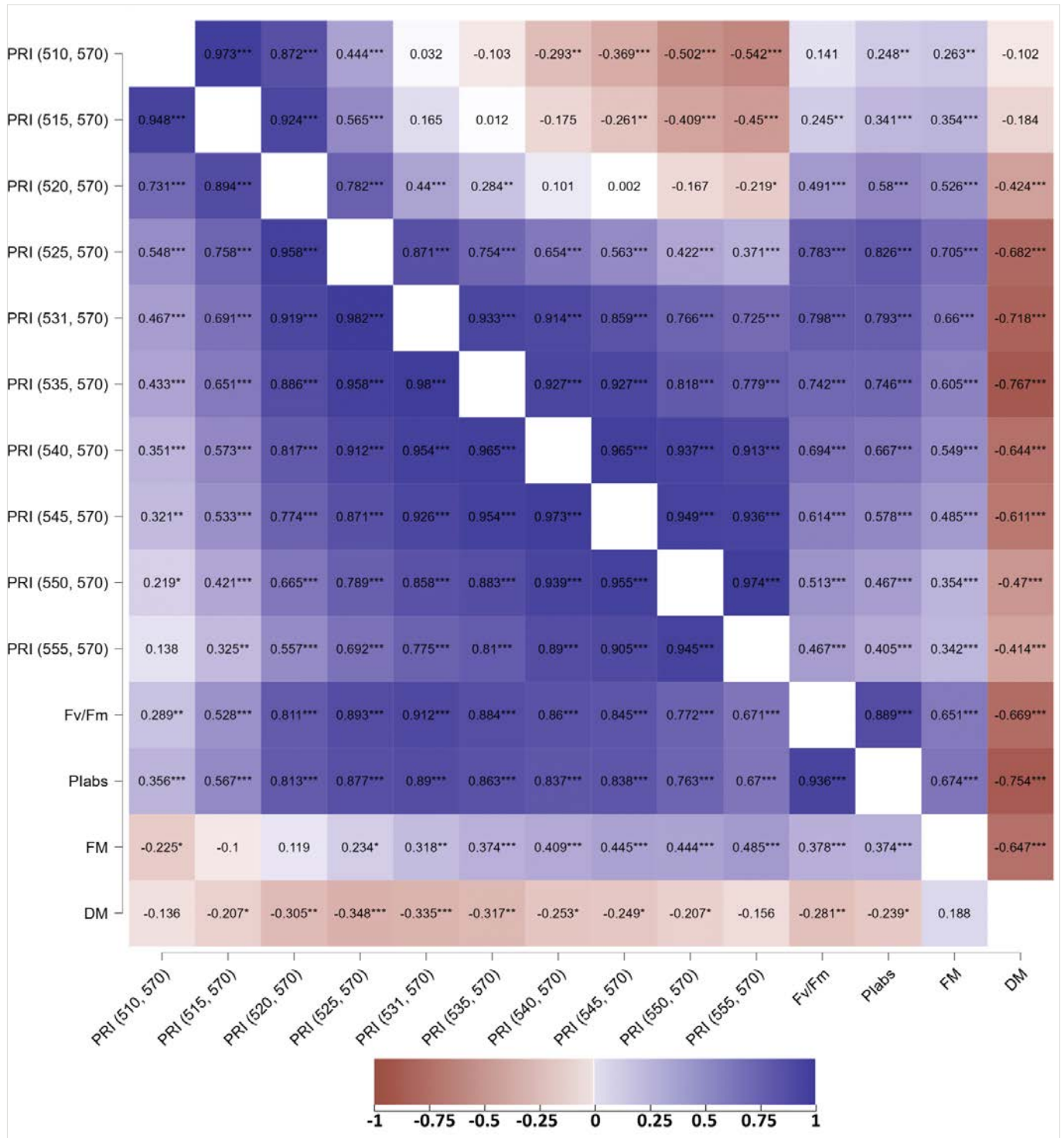
In maize, weak to moderate significant correlations were observed between most SRIs and biomass traits (Figures 2A-C). The strongest correlation between any SRI and FM in soybean was a moderately positive correlation with CRI1 (Figure 2C). A moderately positive correlation was also observed between Lic1 and FM in soybean (Figure 2B). Other statistically significant correlations with FM were weak (Figure 2A-C). Among all investigated SRIs, only PRI showed a weak significant negative correlation with DM in soybean (Figure 3). PRI exhibited weak to moderate correlations with FM in soybean, depending on the measuring wavelength. The strongest significant positive correlation was found between PRI

using reflectance at 555 nm and FM ( $r = 0.485, P < 0.001$ ) (Figure 3). In maize, changes in measuring wavelength significantly affected the relationship between PRI and biomass traits. The strongest significant positive correlation in maize was found between  $PRI_{(525, 570)}$  and FM ( $r = 0.705, P < 0.001$ ), and the strongest significant negative correlation was found between  $PRI_{(535, 570)}$  and DM ( $r = -0.767, P < 0.001$ ) (Figure 3).

Compared with the correlations among photosynthetic parameters and biomass traits, certain SRIs in soybean exhibited stronger correlations with FM. In maize, the correlations among vegetation indices, except for PRI at specific wavelengths, and biomass traits were weaker than the correlations among photosynthetic parameters and biomass traits but were generally higher than those in soybean (Figures 2, 3).



**Figure 2.** Pearson's correlation matrix of spectral reflectance indices (vegetation indices (A); stress-related indices (B); pigment-related indices (C) - descriptions can be found in Table 1), photosynthetic parameters (maximum quantum yield of PSII photochemistry -  $F_v/F_m$ ; performance index -  $PI_{abs}$ ) and biomass traits (fresh mass - FM; dry matter content - DM) for maize (above diagonal) and soybean (below diagonal). Statistical significance is indicated by \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ).



**Figure 3.** Pearson's correlation matrix of modified photochemical reflectance indices (PRIs), photosynthetic parameters (maximum quantum yield of PSII photochemistry -  $F_v / F_m$ ; performance index -  $PI_{abs}$ ), and biomass traits (fresh mass - FM; dry matter content - DM) for maize (above diagonal) and soybean (below diagonal). Statistical significance is indicated by \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ) and \*\*\* ( $P < 0.001$ ).

## DISCUSSION

Maize and soybean are economically important crops that serve as sources of food, feed, and biofuel (Kim et al., 2020; Skoufogianni et al., 2020). Both species are susceptible to low temperatures, particularly during the early growth stages, which significantly affect their growth and development. Increasing cold tolerance has the potential to extend the growing season, reduce environmental impact, and enhance yields. Plants grown under suboptimal and optimal temperatures exhibit different growth responses and biomass accumulation due to variations in leaf chlorophyll and carotenoid content, and other chemical and morphological attributes (Lee et al., 2002; Obeidat et al., 2018).

In recent years, significant attention has been focused on evaluating stress-induced changes in photosynthetic traits, as photosynthesis is highly sensitive to low temperatures (Stewart et al., 2016). Soybean and maize exhibit differences in their photosynthetic pathways. While C3 soybean is well-adapted to temperate environments, C4 maize utilizes an additional mechanism to concentrate CO<sub>2</sub> in specialized cells, allowing for more efficient photosynthesis under hot and dry conditions (Cui, 2021). ChlF serves as a direct tool for detecting PSII efficiency (Adams et al., 2013). It has been used to detect cold stress in various plant species (Heerden and Krüger, 2000; Fracheboud et al., 2002; Mishra et al., 2011; Adams et al., 2013; Soufi et al., 2016). Changes in  $F_v/F_m$  values are often used as reliable indicators of a plant's response to various stress types (Sharma et al., 2012).  $PI_{abs}$  is another frequently used photosynthetic parameter reflecting the efficiency of both PSI and PSII under different conditions (Živčák et al., 2008). Both  $F_v/F_m$  and  $PI_{abs}$  declined in response to cold stress, suggesting that cold stress induces photoinhibition to prevent photodamage.

Measuring ChlF requires prior sample adaptation to darkness, leading to the proposal of spectral reflectance indices as reliable and faster substitutes due to their high correlation with photosynthetic parameters (Székely et al., 2023). However, atmospheric and soil background can strongly influence the sensitivity of various SRIs,

reducing their informativity (Baret et al., 1993; Kior et al., 2021; Tayade et al., 2022). In this study, the experiment was conducted entirely under controlled conditions using direct leaf reflectance measurements to minimize the influence of atmospheric conditions and soil factors on measurement accuracy. These controlled conditions allow more precise assessments of the physiological responses of plants. Leaf reflectance measurements have an advantage over canopy-level measurements because they are faster than fluorescence measurements and reduce the impact of background and soil, especially when plants are young and the canopy does not cover a significant portion of the ground.

Chlorophyll, the main photosynthetic pigment, degrades under oxidative stress (Kuai et al., 2018), prompting the development of numerous indices for studying variations in chlorophyll content. In this study, MCARI increased under cold stress in maize, whereas it initially decreased after 24 h of cold stress in soybean and then increased with longer cold stress durations (Tables 4 and 5). However, MCARI's sensitivity to low chlorophyll content and the influence of non-photosynthetic pigments (Tayade et al., 2022) raise questions about its effectiveness as a cold stress indicator. Therefore, SRIs that study chlorophyll ratios to other pigments are better estimators of stress changes in plants. The degradation of chlorophylls occurs faster under stress than carotenoids (Merzlyak et al., 1999; Sims and Gamon, 2002). The slower degradation of carotenoids in response to cold stress may reflect the role of the xanthophyll cycle in releasing thermal energy and protecting PSII reaction centers (Adams et al., 2006). The ratio of chlorophyll to other pigments can be observed in indices such as SPRI, SIPI, and NPQI (Barnes et al., 1992; Peñuelas et al., 1995; Merzlyak et al., 1999). The NPQI, corresponding to chlorophyll degradation through pheophytin occurrence, sharply increased after 24 hours of cold stress in maize and then declined, reaching control levels after 72 hours (Table 4). In soybean, NPQI increased by an average of 317.4% under cold stress compared with the control, and the increase continued with the duration of cold exposure (Table 5). Pheophytinization disrupts

photosynthetic electron transport, affecting plants' ability to photosynthesize efficiently under cold conditions (Agathokleous et al., 2023). An increase in NPQI indicates greater chlorophyll degradation, whereas a substantial decrease after 48 hours of low temperatures in maize that chlorophyll degradation to pheophytin has been slowed. The underlying reasons remain unclear, but prolonged cold stress may activate protective mechanisms against chlorophyll degradation (Agathokleous, 2021) or other breakdown pathways, such as direct oxidative damage by reactive oxygen species (Agathokleous et al., 2023). Furthermore, NPCI, indicating the ratio between total pigments and chlorophyll, increased in maize under low temperatures, confirming the altered total pigment-to-chlorophyll ratio (Table 4). This indicates a progressive decrease in the chlorophyll proportion of total pigments under prolonged cold stress in maize, highlighting the importance of considering multiple indices to avoid misleading conclusions. Peñuelas et al. (1994) also reported that NPCI was higher in stressed leaves and negatively correlated with chlorophyll content.

Anthocyanins are pigments that accumulate under stress conditions. They are derived from the flavonoids of higher plants and are responsible for the red coloration of plants (Kim et al., 2017). They protect plants from the detrimental effects of excess light by absorbing high-energy quanta (Gould, 2004) and stimulating the plant antioxidant system, neutralizing reactive oxygen species and radicals (Sims and Gamon, 2002). ARI1 and ARI2 were proposed by Gitelson et al. (2001) as non-destructive methods for predicting anthocyanin content. Both ARI1 and ARI2 levels increased under cold stress in both crops (Tables 4 and 5), indicating the accumulation of anthocyanins in leaves. The increase in ARI under stress conditions underscores the protective role of anthocyanins, making these indices useful for monitoring stress responses and developing agricultural management strategies.

Additionally, several SRIs are related to the stress response, including Ctr1, Ctr2, Lic1, Lic2, GM1, and GM2. Although changes in the values of these indices under cold stress were significant in this study (Tables 2 and

3), the response was not as pronounced as that of some pigment-related indices or was unclear (Tables 4 and 5). Thus, SRIs based on the concentration of leaf pigments appear to have better potential for detecting cold stress than general stress response indices like Ctr1 and Ctr2, which are often used as stress response indicators (Carter, 1994; Zhang et al., 2016; Sukhova and Sukhov, 2019).

Among all SRIs, PRI is the most used parameter for stress detection (Gamon et al., 1992). Assesses green vegetation reflectance and is sensitive to variations in carotenoid pigments, particularly xanthophyll, which are closely related to photosynthetic efficiency. Changes in carotenoid pigments indicate the energy absorbed during photosynthesis as light use efficiency or the rate of carbon dioxide absorbed by green vegetation, reflecting leaf fluorescence and photosynthesis (Kim et al., 2017). Consequently, PRI has been utilized to detect various stresses in crops (Li et al., 2018; Cao et al., 2019). PRI serves as a pigment index dependent on the concentrations of photosynthetic pigments and the ratio between the concentrations of carotenoids and chlorophylls (Wong and Gamon, 2015; Zhang et al., 2016).

However, changes in PRI can also result from short-term processes strongly associated with photosynthetic changes (Evain et al., 2004; Filella et al., 2009). Short-term PRI changes relate to the activity of the xanthophyll cycle, which is an essential mechanism for rapid photosynthetic adaptation to stressors (Garbulsky et al., 2011). The typical PRI is based on the reflectance at a measuring wavelength of 531 nm, which is the center of the green shift in reflectance due to the de-epoxidation of violaxanthin to zeaxanthin (Filella et al., 2009). Reflectance at 531 nm decreases rapidly in response to excess energy dissipation by xanthophyll de-epoxidation. This process is driven by increased zeaxanthin concentration and chloroplast shrinkage following an increase in thylakoid  $\Delta$ pH, which is insensitive to short-term changes at 570 nm (Sukhov et al., 2021). Therefore, reflected light at 570 nm is usually used as the reference wavelength for PRI calculations (Gamon et al., 1992, 1997). Given these complex mechanisms of PRI changes, modifying the measuring wavelength (531 nm) can influence the

sensitivity of this index to stress and photosynthetic parameters. Using modified photochemical reflectance indices ( $PRI_{(\lambda,570)}$ , where  $\lambda$  is the modified measuring wavelength, equaling 510, 515, 525, 535, 545, or 555 nm), different relationships between different  $PRI_{(\lambda,570)}$  and photosynthetic parameters under cold stress were observed (Figure 3), which is consistent with earlier findings by Sukhova and Sukhov (2020).

In the present study, reductions in  $F_v/F_m$  and  $PI_{abs}$  in response to cold stress showed strong correlations with Lic1 and SIPI in soybean, and ARI1, ARI2, Ctr2, and GM2 in maize (Figure 2), as well as PRI and its modifications in both crops (Figure 3). Reductions in chlorophyll and carotenoid contents in response to cold stress are closely correlated with  $F_v/F_m$  and  $PI_{abs}$  parameters (Hajihashemi et al., 2018). Several studies have also shown a strong correlation between PRI and the  $F_v/F_m$  ratio (Zhang et al., 2016; Peng et al., 2017). Additionally, other indices, such as SIPI, have also been correlated with photosynthesis parameters (Zhang et al., 2011).

According to Pearson's correlation coefficient, PRI provided the most accurate biomass estimation. Among the spectral reflectance indices correlated with biomass traits, the highest correlation coefficients were observed for  $PRI_{(535, 570)}$  and DM, followed by  $PRI_{(525, 570)}$  and FM in maize (Figure 3). These indices also exhibited strong correlations with photosynthetic parameters, with stronger correlations observed with biomass traits compared to those between photosynthetic parameters and biomass traits. Conversely, in soybean, correlations with biomass traits were generally weaker, both for SRIs and photosynthetic parameters. The strongest correlation was recorded between  $PRI_{(555, 570)}$  and FM ( $r = 0.485$ ,  $P > 0.001$ ), as well as between  $PRI_{(525, 570)}$  and DM ( $r = -0.348$ ,  $P < 0.001$ ) (Figure 3). Although these correlations were weak to moderate, PRI achieved stronger correlations than  $F_v/F_m$  and  $PI_{abs}$  in soybean.  $PRI_{(525, 570)}$  primarily reflects changes in the xanthophyll cycle, whereas  $PRI_{(555, 570)}$  primarily reflects changes in chloroplast shrinkage (Gamon et al., 1997; Sukhova and Sukhov, 2020).

The data collected in this study can be applied to improve crop management practices under stress conditions. By identifying the most sensitive indices for cold stress detection, producers can more effectively monitor crop health, leading to timely interventions and potentially higher yields. Additionally, this research contributes to advances in scientific knowledge by providing insights into the mechanisms of stress response in different crops, facilitating the development of more resilient crop varieties through breeding programs and resource-efficient stress management strategies.

## CONCLUSION

In conclusion, SRIs provide valuable insights into the responses of maize and soybean plants to cold stress, revealing distinct differences in their responses. Pigment-related SRIs, especially NPQI and NPCI, showed great potential for capturing stress-induced variations, emphasizing differences in crop-specific responses to stress. The NPQI increased significantly under cold stress conditions, particularly in soybean, indicating greater chlorophyll breakdown. In maize, NPQI initially increased but later returned to control levels, suggesting the activation of protective mechanisms against chlorophyll degradation. Additionally, the increase in NPCI in maize under cold stress underscores the altered pigment composition, with a progressive decrease in chlorophyll content. Anthocyanin accumulation, as indicated by increases in ARI1 and ARI2, indicates the protective role of these pigments against stress.

Among SRIs, PRI has emerged as a prominent index for stress detection, showing strong correlations with photosynthetic parameters and biomass traits, particularly in maize. Modifications in PRI measuring wavelength offer nuanced insights into the stress responses. Overall, understanding the intricate relationships between spectral reflectance indices and plant physiological processes is crucial for devising effective strategies to mitigate cold stress and enhance crop productivity in maize and soybean cultivation.

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