

# Breeding for Drought Tolerance by Monitoring Chlorophyll Content

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## ABSTRACT

Crop yields have increased substantially during the last 50 years, but the traits that drove these remarkable improvements, such as plant architecture, have a little remaining potential for improvement. New traits such as photosynthesis, as the ultimate determinant of yield, must be explored to support future demands. However, improving photosynthetic efficiency has played only a minor role in improving crop yield to date. Chlorophylls are the pigments allowing light to be transformed into carbohydrates, and therefore help to maintain crop yield under stress. Chlorophyll content correlates with higher yields in diverse conditions. In this review, we discuss using chlorophyll content as the basis of screens for drought tolerance. We review chlorophyll-related responses to drought in different plants and summarize the advantages and disadvantages of current methods to measure chlorophyll content, with the ultimate goal of improving the efficiency of crop breeding for drought tolerance.

**Keywords:** Photosynthetic Pigments, Crop Breeding, Spectrophotometry, Spectral Reflectance, Determination Methods

## INTRODUCTION

The unrelenting growth of the global population leads to an ever-increasing demand for food. The average yield of the main crops (such as wheat, maize, rice, and soybean) has increased substantially in the last 50 years [1], supporting The Green Revolution. However, the traits that drove the remarkable yield increases, such as optimization of shoot architecture and biomass partition to harvested product, appear to have a little remaining potential for further improvement [2]. Therefore, new and previously unappreciated traits should be (re)evaluated for their potential contribution to crop yield.

Plant biomass is largely derived from photosynthetically captured carbon. Variations in the efficiency of photosynthesis can lead to variations in growth rate and productivity, which are important factors in crop yield [3]. Directly improving photosynthetic efficiency has played a minor role in increasing yield to date, most likely due to the observation that yield was more limited by the strength of the sinks than by photosynthetic capacity itself [4-6]. Then the yield is limited by the ability to transport the carbon products to the grain more than the light interception capacity and carbon fixation rate. However, more recently, researchers have demonstrated that increased rates of photosynthesis can lead to higher yields in soybean and rice, grown under high CO<sub>2</sub> concentrations [6-8], supporting

the possibility to increase photosynthesis efficiency even more in those crops. Additionally, it is worth mentioning that other crops still have a large potential to improve yields by the optimization of light interception capacity, the conversion efficiency to biomass, and carbon partition to harvested products [2,6].

Plant stress, such as drought, negatively impacts plant growth and yield. For instance, between 1983 and 2009, global harvested areas for rice, maize, rice, and soybean were affected by drought in a 75, 82, 62, and 91% (respectively) [9], and researches estimate further reductions of crop yield in the next century (around 10-20% in case of moderate to severe droughts for the same crops) [10]. In the antenna complex of the chloroplasts, chlorophylls are the critical pigments that capture the light to be transformed into carbohydrates during photosynthesis. The use of chlorophyll content as a trait may contribute to increase light interception and efficiency of conversion, and therefore to maintain/increase crop yield under stress [11,12]. Photosynthesis-related traits, and particularly chlorophyll content, have been studied for decades, but they have been under-utilized as traits to screen genotypes for drought tolerance. However, a myriad of studies has found that chlorophyll content is reduced in response to drought and that chlorophyll maintenance correlates with drought tolerance; however, only a few of them explicitly calculate the correlation between the chlorophyll content with yield under drought [13-38]

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(Table 1). Here we propose that screening for chlorophyll content is a simple and effective strategy to identify drought-tolerant crops, to complement other traits.

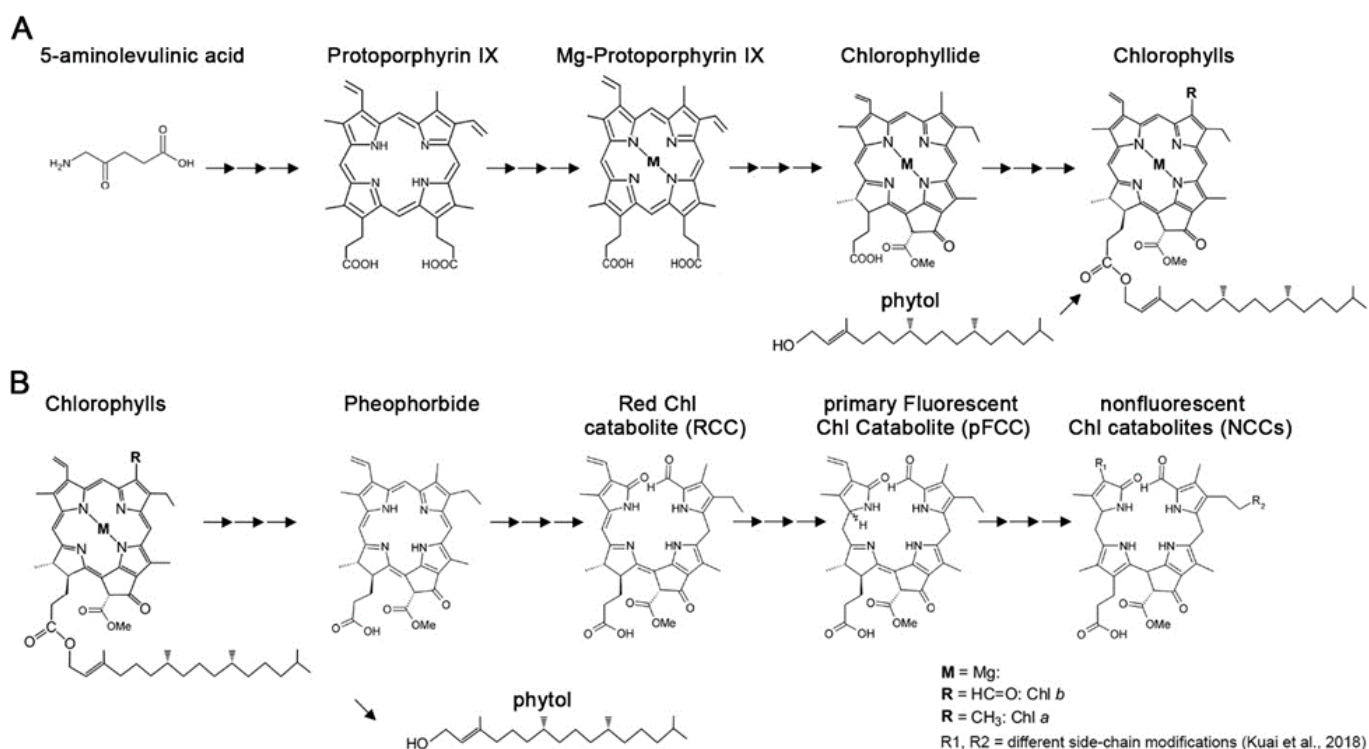
## CHEMICAL AND PHYSICAL PROPERTIES OF THE CHLOROPHYLLS

The predominant chlorophylls found in photosynthetic tissues of higher plants are reduced porphyrins (dihydro porphyrins) that contain a centrally bound  $Mg^{2+}$  ion and are linked to a long hydrophobic phytol tail through the esterification of the acid group at C-17 [39,40] (Figure 1). During photosynthesis, antenna pigments in chloroplasts absorb solar radiation, and the resulting excitation is directed to the reaction center pigments, which release electrons and initiate the photochemical process [41,42]. Chlorophyll absorbs strongly in the red (650-700 nm) and blue (400-500 nm) regions, although absorption in the blue by carotenoids often prevents this region from being useful in chlorophyll estimation. The most abundant chlorophylls are types a and b, which absorb most of the light on the two bands of the visible spectrum in a complementary way: chlorophyll a absorbs more in the red-orange band (640-670 nm), whereas chlorophyll b absorbs more in the blue-purple band (430-460 nm). Besides, the presence of a methyl group in chlorophyll a instead of an aldehyde group in chlorophyll b shifts the absorption peaks in the red (669 nm vs. 644 nm) and blue (432 nm vs. 455 nm) [39], which may allow for discrimination between these two types of chlorophylls. Both chlorophyll types have a minimum of around 550 nm, which makes plants look green.

Leaf total-chlorophyll content (chlorophyll a+b) positively correlates with the amount of solar radiation absorbed by the leaves [43] and with the photosynthetic rate [44,45]. Additionally, chlorophyll content also correlates well with nitrogen content in a wide range of plant species, including crops [46-50]. Indeed, about 75% of the total nitrogen is found in chloroplasts [51]. These correlations reinforce the idea that greener plants will have higher photosynthesis rates [52], and in turn, yield.

The steady-state level of chlorophylls is determined by the relative rates of anabolism and catabolism in chloroplasts (reviewed in [53] Figure 1). Chlorophylls are synthesized via the magnesium (Mg) branch of tetrapyrrole biosynthesis [40,54,55]. The first step is the formation of 5-aminolevulinic acid. Six enzymes subsequently convert eight molecules of 5-aminolevulinic acid into the precursor for chlorophylls, protoporphyrin IX. An Mg-chelatase catalyzes the insertion of  $Mg^{2+}$  into protoporphyrin IX, followed by a series of reactions that generate chlorophyllide. Chlorophyll synthase esterifies chlorophyllide to form the hydrophobic chlorophyll a, which can be further converted into chlorophyll b by chlorophyllide a oxygenase. Finally, newly synthesized chlorophylls a and b are rapidly integrated into the chlorophyll-binding proteins of light-harvesting chlorophyll a/b-protein complexes [56].

On the other hand, chlorophyll breakdown is initiated by conversion of chlorophyll b into chlorophyll a by reductases and oxygenases in the so-called "chlorophyll cycle" (reviewed by [57,58]).  $Mg^{2+}$  is removed from chlorophyll a by a dechelatease to form pheophytin a. The phytol in pheophytin a is hydrolyzed to generate pheophorbide a, and the porphyrin ring is then cleaved to generate a series of fluorescent compounds (such as the red chlorophyll catabolite, RCC, and the



**Figure 1:** Main compounds in the synthesis (A) and degradation (B) pathways of chlorophylls.

Based on [55,57,61-64]. Chl, chlorophylls; M, Magnesium; Me, methyl; R, side-chain modifications

primary fluorescent chlorophyll catabolite, pFCC). These compounds are finally isomerized in the vacuole into non-fluorescent products (nonfluorescent chlorophyll catabolites, or NCCs) [59-61].

Chlorophyll degradation predominates during leaf senescence and fruit ripening and is induced by drought and other stress conditions. The dynamics of chlorophyll turnover influence plant growth and productivity reducing the light interception capacity, but also enabling nutrient remobilization during leaf senescence. In addition, free chlorophylls and their metabolic intermediates generate singlet oxygen and toxic radicals upon illumination, which compromise tissue integrity [65]. Therefore, chlorophyll degradation is important to detoxify free chlorophylls [53,66]. However, protecting chlorophyll from degradation induced by stress (such as oxidative stress) might support drought tolerance [67].

## MAINTAINING CHLOROPHYLL CONTENT PREDICTS DROUGHT TOLERANCE

In most plant species, water deficit due to drought induces the degradation of the thylakoid membrane within chloroplasts, negatively affecting chlorophyll and other photosynthetic pigments (reviewed by [68]). As a consequence of the stress, the photosynthetic rate, leaf area, and ultimately yield, are reduced [69,70]. Drought generally leads to lower total chlorophyll content and an altered proportion of chlorophyll a/b. Increased degradation of chlorophyll a under drought led to a reduction of the chlorophyll a/b ratio in barley [28,71] and in *Vigna radiata* [72].

However, not all plants under drought reduce their chlorophyll content (Table 1). Some drought-tolerant grasses maintain their chlorophyll content under drought [35,73], and this response has also been reported in soybean (ADM 5009 RG, [24]) and potato (UNICA, [74]). The ability to maintain chlorophyll content may vary with the genotype, and stress duration and intensity. Overall, plants that maintain a relatively higher chlorophyll content under drought use light energy more efficiently [75], which indicates higher drought tolerance.

Interestingly, the chlorophyll a/b ratio decreases during leaf senescence [67]. Senescence is induced in response to drought and might in turn trigger chlorophyll degradation. Delayed-senescence crops, also called “stay-green” crops [76], have been screened in an effort to increase yield by delaying the normal senescence process and prolonging photosynthesis [77,78]. The development of stay-green genotypes has contributed to increased yield under stressful conditions in grasses, such as wheat, maize, rice, sorghum, and barley [71,79-84] and in legumes, such as soybean [85], among other crops. However, senescence can be delayed by diverse mechanisms, such as high cytokinin levels, low ethylene perception, and better maintenance of water intake. Thus, stay-green crops may have a “functional” phenotype with a high photosynthesis rate or a “cosmetic” phenotype with reduced photosynthesis but high levels of non-functional pigmented catabolites [80,81]. Therefore, monitoring chlorophyll content alone may not ensure drought tolerance, but this limitation could be overcome by examining the photosynthesis rate of drought-tolerant candidates.

In some cases, delayed senescence and chlorophyll maintenance might not improve the yield under stress [86]. For example, the drought tolerance of some wheat cultivars depends on the remobilization of carbon from stems to fill the grain, prioritizing grain fill over plant cycle length. In these cases, the stay-green phenotype should be avoided because it delays or inhibits the ability to use the vegetative biomass to

fill the grains [86], and does not result in higher yield under drought. We infer that the delay of senescence and chlorophyll degradation can be a tolerance response, depending on the genotype. These observations reinforce the idea that screening for chlorophyll content under specific conditions (such as under drought) could significantly contribute to breeding programs.

Overall, diverse mechanisms could protect the chlorophylls from degradation under drought. A priori, a relatively higher chlorophyll content under stress may directly cause higher photosynthesis rate and yield, or it could be the result of another mechanism (such as the activation of the antioxidant system). Then the higher (or stress-insensitive) chlorophyll content would be correlated with, but not causing, the tolerance. However, a high correlation exists for some species and we support it can be used to easily identify drought tolerance.

## METHODS TO QUANTIFY CHLOROPHYLLS

Most of the quantification methods are based on chlorophylls' ability to reflect or absorb light. A decrease in chlorophyll content reduces the absorbance and, concomitantly, enhances the reflectance in the visible and infrared bands. Four of the most frequently used methods are described in Table 2.

Both spectrophotometry and High-Performance Liquid Chromatography (HPLC) destroy the sample, impeding monitoring responses in the same leaf over time. Also, removing or injuring a leaf may substantially affect plant performance under certain conditions, such as during seedling establishment or under severe stress. Both methods involve chlorophyll extraction by a solvent, the determination of absorbance by the chlorophyll solution, and conversion from absorbance to concentration using standard formulas [12,74]. These methods can discriminate between a and b chlorophyll, and therefore can be used to calculate the a/b ratio. Different solvents (such as acetone, methanol, or ethanol) vary in their ability to extract pigments and alter the absorption peaks of both a and b chlorophylls. Increasing polarity and/or water content will shift the absorption peaks to longer wavelengths [51]. Spectrophotometry is the simpler and more accessible method, whereas HPLC is more accurate but expensive. Chlorophylls can also be measured by other analytical methods, such as Nuclear Magnetic Resonance, with even higher precision and cost. Overall, these destructive methods are not amenable to large-scale experiments and natural community studies [28,41,87].

Handheld meters, such as SPAD-501/2 (Minolta), CL-01 (Hansatech), CCM-200 (Optiscience), and Dualex (Force-A) [41], measure the absorbance of the chlorophylls in situ, without destroying the sample. Actually, they require intact tissue, which limits the possibility to pool samples. They measure the chlorophyll absorbance in the red peak (~620-650 nm, with a higher contribution from chlorophyll a). Also, a reference wavelength in the near-infrared (~760 nm or ~940-950 nm, according to the instrument) is used to determine the contribution of leaf structures such as cell walls [41,67,88]. However, some of these leaf structures, such as vein distribution, leaf anatomy, or water content, do not scale linearly and therefore chlorophyll quantification must be validated with an extractive method [21,85,87-106]. Table 3 shows correlations between different handheld meters and extraction methods in the most important crops. Several of those correlations, such as for Poaceae and Solanaceae families, needs power or exponential functions due to the non-linear influence of leaf structures. Comparing chlorophyll content among species may be inaccurate due to heterogeneity, although high correlations were found for some grasses, legumes, and green leafy vegetables [95,98,99,105].

**Table 1:** Chlorophyll content in crops under drought stress. Handheld and liquid extracts measurements correspond to SPAD and spectrophotometer unless specified.

Family	Species	Common name	Water deficit treatment	Chlorophyll determination method	Chlorophyll content response to drought	Chlorophylls vs. yield correlation	Reference
Amaranthaceae	<i>Beta vulgaris</i> subsp. <i>vulgaris</i> var. <i>altissima</i>	Sugar beet	Field, supplemental irrigation for controls	Handheld meter	Reduction, less reduction in tolerant genotypes	n.d.	13
Apiaceae	<i>Daucus carota</i> L.	Carrot	Greenhouse, controlled irrigation reduction	Liquid extract	Reduction of total content and ratio a/b, less reduction in tolerant genotypes	n.d.	14
Arecaceae	<i>Elaeis guineensis</i>	Oil palm	Greenhouse, water withhold	Liquid extract	Reduction of total content and ratio a/b	n.d.	15
Asteraceae	<i>Helianthus tuberosus</i> L.	Artichoke	Greenhouse, water withhold	Handheld meter	Increase	n.d.	16
Brassicaceae	<i>Brassica napus</i>	Rapeseed	Greenhouse, water withhold	Handheld meter	Reduction	yes, n.d.	17
	<i>Brassica campestris</i> , <i>B. carinata</i> , <i>B. juncea</i> , and <i>B. napus</i>	Sarson, Ethiopian mustard, Brown mustard, and Oilseed rape, respectively	Greenhouse, water withhold	Liquid extract	Reduction, less reduction in tolerant genotypes	n.d.	18
Cucurbitaceae	<i>Cucumis sativus</i> L. cv. <i>Jinyou No.1</i>	Cucumber	Greenhouse, PEG treatment, hydroponics	Handheld meter	Reduction	n.d.	19
	<i>C. lanatus</i> var. <i>lanatus</i>	Watermelon, drought-sensitive genotype Y34	Greenhouse, controlled irrigation reduction	Liquid extract	Reduction	n.d.	20
Fabaceae	<i>Arachis hypogaea</i>	Peanut	Field, controlled irrigation reduction	Liquid extract and Handheld meter	Reduction of total content and increase of chlorophyll density	n.d.	21
			Field, controlled irrigation reduction	Handheld meter	n.d.	r <sup>2</sup> =0.43, for seed size	22
	<i>Phaseolus vulgaris</i>	Common bean accessions	Growth chamber, water withhold	Handheld meter	No reduction in the tolerant and reduction in the sensitive genotype	n.d.	23
	<i>Glycine max</i> Merr.	Soybean accessions and cultivars	Greenhouse, controlled irrigation reduction	Liquid extract	No reduction in the tolerant and reduction in the sensitive genotypes	n.d.	24
	<i>Glycine max</i> Merr. cv. <i>Gongxuan</i>	Soybean	Greenhouse, water withhold	Liquid extract	Reduction of a and a/b ratio	n.d.	25
<i>Cicer arietinum</i>	Chickpea	Field, supplemental irrigation for controls	Handheld meter	n.d.	r <sup>2</sup> =0.34/0.32 (2005/2006)	26	
<i>Lens culinaris</i> ssp. <i>culinaris</i> Medikus	Lentil	Field,	Handheld meter	n.d.	r <sup>2</sup> =0.30 for seed weight and number per plant	27	

Poaceae	<i>Hordeum vulgare</i> <i>L. cv.</i>	Barley	Greenhouse, controlled irrigation reduction	Handheld meter	No or less reduction in the tolerant and higher reduction in the sensitive genotypes	$r^2=0.67$	28
			Greenhouse	Handheld meter	Increase in most of the genotypes	n.d.	29
	<i>Triticum aestivum</i> <i>L.</i>	Bread wheat	Field, supplemental irrigation for controls	Handheld meter	No reduction to reduction, according to the genotype	$r^2=0.54$	30
			Field, supplemental irrigation for controls	Liquid extract	Reduction of total content and a/b ratio	$r^2=0.843$	31
	<i>Triticum durum</i> <i>Desf.</i>	Durum wheat	Field, supplemental irrigation for controls	Handheld meter	No reduction to reduction, according to the genotype	n.d.	32
			Field, supplemental irrigation for controls	Handheld meter	n.d.	$r^2=0.39$	33
	<i>Zea mays</i>	Corn	Field, supplemental irrigation for controls	Handheld meter	No reduction in the tolerant and higher reduction in the sensitive genotypes	n.d.	34
			Growth chamber, controlled irrigation reduction	Liquid extract	Increase	n.d.	35
	<i>Sorghum bicolor</i>	Sorghum	Field, supplemental irrigation for controls	Handheld meter	Reduction	$r^2=0.64$	36
	<i>Saccharum spp.</i>	Sugarcane	Field, controlled irrigation reduction	Handheld meter	No reduction in the tolerant and higher reduction in the sensitive genotypes	$r^2=0.36-0.33$	37
Solanaceae	<i>Solanum tuberosum</i> <i>var. Désirée, Unica, and Samav</i>	Potato	Greenhouse, controlled irrigation reduction	Handheld meter	Increase	n.d.	38

\*n.d., not determined or informed by the original report

**Table 2:** Common methods to determine chlorophyll content in crops.

Method	Spectrophotometric	HPLC	Handheld meters	Remote sensors
Principle	Absorbance	Separation based on the interaction with the fixed and mobile phases, plus absorbance	Absorbance	Spectral reflectance
Sampling method	Destructive	Destructive	Non-destructive (require intact tissue)	Non-destructive
Accuracy	High	High	Intermediate	Low
Scale	Sub-leaf to the individual or pooled leaf level	Sub-leaf to the individual or pooled leaf level	Sub-leaf to the individual leaf level	Fraction of individual leaf to canopy
Time and cost per sample	High	High	Intermediate	Low to very low
Training investment	Small	Intermediate/Large	Small	Large
Other characteristics			Possible non-linear correlation with Chl content	Several algorithms available
Disadvantages			Affected by leaf structure	Affected by leaf structure, environment, and surroundings



**Table 3:** Correlations between chlorophyll content measured by handheld meters and liquid extract methods among crops under different experimental conditions. Handheld and liquid extracts measurements correspond to SPAD and spectrophotometer unless specified.

Family	Species	Common name	Experimental condition	Determination method	Function type	Reference
Asteraceae	<i>Lactuca sativa</i> cv. <i>Lores</i>	Butterhead lettuce	Greenhouse, no treatment	Handheld meter/liquid extract	Linear, $r^2=0.92$	91
	<i>Lactuca sativa</i> L. var. <i>longifolia</i>	Romaine lettuce 'Green Star'	Field, in tunnels, no treatment	Handheld meter/liquid extract	Linear, $r^2=0.97$	92
Brassicaceae	<i>Raphanus sativus</i>	Radish	Greenhouse, no treatment	Handheld meter (CL-1)/liquid extract	Linear function, $r^2=0.65$	93
Cucurbitaceae	<i>Cucumis sativus</i>	Cucumber	Greenhouse, no treatment	Handheld meter (CL-1)/liquid extract	Linear function, $r^2=0.64$	
Fabaceae	<i>Arachis hypogaea</i>	Peanut	Field, limited irrigation	Handheld meter/liquid extract (Chl density)	Linear, $r^2=0.76/0.94/0.96$ (at 20/40/60 days after emergence, respectively)	21
		Soybean accessions	Field, no treatment	Handheld meter/liquid extract	Linear/power, $r^2=0.36/0.93$	94
		<i>Glycine max</i> Merr.	Soybean RILs	Field, no treatment (different developmental stages and parts of the canopy)	Handheld meter/liquid extract	Linear, $r^2=0.57-0.87$
Poaceae	<i>Oryza sativa</i> L. <i>japonica</i> var. <i>Koshihikari</i>	Rice	Field, N fertilization	Spectral reflectance/liquid extract	Linear function, $r^2=0.947$	95
	<i>Oryza sativa</i> / <i>Triticum aestivum</i>	Rice/wheat	n.d.	Handheld meter/liquid extract	Power/linear, $r^2=0.97/0.93$	96
	<i>Sorghum bicolor</i>	Sorghum	Field, limited irrigation	Handheld meter/liquid extract	Linear, $r^2=0.91$	97
	<i>Triticum aestivum</i> L. cv. <i>Isengrain</i>	Winter wheat	Field, N fertilization	Handheld meter/liquid extract	Power, $r^2=0.91$	98
	<i>Triticum aestivum</i> / <i>Zea mays</i>	Wheat/Maize	Greenhouse/field and greenhouse	Handheld meter (SPAD-CCR-Dualex)/liquid extract	Homographic / exponential/linear, $r^2=0.94/0.91/0.96$ respectively	69
	<i>Zea mays</i>	Corn	Greenhouse, no treatment	Handheld meter (CL-1)/liquid extract	Linear function, $r^2=0.74$	99
Poaceae /Fabaceae	<i>Zea mays</i> / <i>Glycine max</i> Merr.	Maize / Soybean	Field, no treatment	Handheld meter/liquid extract	Exponential, $r^2=0.94$	100
Solanaceae	<i>Lycopersicon esculentum</i>	Tomato	Greenhouse, no treatment	Handheld meter/liquid extract	Exponential/linear, $r^2=0.75/0.74$	101
	<i>Nicotiana tabacum</i> L. cv. <i>Samsun</i>	Tobacco	Growth chamber, blue light (chloroplast movement induction)	Handheld meter/liquid extract	Cubic, $r^2=0.86$	102
	<i>Solanum tuberosum</i> cv. <i>Bintje</i>	Potato	Field, open-top-chamber, high CO <sub>2</sub>	Handheld meter/liquid extract	Quadratic, $r^2=0.95$	103
			Field, no treatment	Handheld meter/liquid extract	Exponential, $r^2=0.58$	66
Theaceae	<i>Camellia sinensis</i> L. cv. <i>Jiukeng</i>	Tea	Field, no treatment	Handheld meter/liquid extract	Exponential, $r^2=0.84-0.88$	104
Rosaceae	<i>Fragaria</i> sp.	Strawberry	n.d.	Handheld meter/liquid extract	Linear, $r^2=0.92$	105

Green leaf species	<i>Brassica oleracea</i> var. <i>alboglabra</i> Bailey, <i>Brassica rapa</i> L. var. <i>parachinensis</i> , <i>Amaranthus spinosus</i> L., <i>Ipomoea aquatica</i> Forsk., <i>Manihot utilissima</i> Pohl, <i>Lactuca sativa</i> L., <i>Brassica rapa</i> L. subsp. <i>chinensis</i> , <i>Ocimum citriodorum</i> Vis., <i>Lactuca sativa</i> L. var. <i>augustana</i> , <i>Brassica oleracea</i> L. var. <i>capitata</i>	Chinese kale, Chinese flowering cabbage, spinach, water spinach, cassava, green leaf lettuce, Chinese cabbage pak-choi, basil, sword-leaf lettuce, head cabbage, respectively	n.d.	Handheld meter/ liquid extract (HPLC)	Linear, $r^2=0.74$ (all species together)	106
Various crop species	<i>O. sativa</i> L. <i>japonica</i> , <i>T. aestivum</i> L., <i>Z. mays</i> L., <i>G. max</i> (L.) Merr., <i>B. vulgaris</i> L.	Rice, wheat, corn, soybean, sugarbeet	Field, no treatment	Spectral reflectance (Ratio Spectral Index)/liquid extract	Linear function, $r^2=0.89$	95

\*n.d. not determined or reported in the text or supplemental material

In general, a handheld meter can be used in controlled environments as well as in field conditions, they measure a small area per sample, but the measurements are quick, do not require high training to process the data, and can be repeated in several leaves of the same plant (such as different parts of the canopy), in different plants, or even on the same leaf area to assess temporal dynamics. Overall, this method is very useful to compare cultivars of the same species, in similar conditions, in analogous leaves, and the same part of the leaf (excluding veins). This approach has been used to examine different developmental stages and different portions of the canopy for rice and soybean [87,89,90]. Besides, handheld measurements might be used as a proxy for photosynthesis rate, which is effective for primary gross productivity in forest communities [107]. In the latter case, the same precautions, such as sampling the same leaf and excluding veins, are required. While these considerations may imply a higher initial time investment, handheld meters require an intermediate amount of time per sample.

Finally, remote sensors quantify chlorophylls based on the changes in spectral reflectance. Overall, these methods detect the wavelength range between  $\sim 400$  to  $2500$  nm, varying in spectra resolution (the highest current resolution is  $\sim 0.5$  nm). Chlorophyll peaks can be analyzed in combination with other regions to decipher reflectance affected by other factors mentioned above, such as leaf structures. There are four main remote sensing techniques, classified according to the operating spectral regions: optical, thermal, radar, and Light Detection and Ranging (LIDAR) (reviewed by [77,108]). Optical remote sensing is the most well-established approach for vegetation and chlorophyll mapping [94]. In general, spectral reflectance measurements can be made in controlled environments as well as in field conditions, across a broad range of spatial scales from sub-leaf to the whole canopy in the field. These kinds of measurements are quick, can be repeated on the same sampling area to assess temporal dynamics, and substantially reduce the time per sample, but require more training to process the data. The sensors can be carried on different instruments, from airborne (e.g., satellites, planes, drones) to portable spectroradiometers, which makes them very versatile and allows for high-scale data acquisition, but also can add errors that need to be considered and corrected. The reflectance spectrum

is influenced not only by the leaf but also by variations in soil background reflectance, light scattering by surrounding objects, 3-D canopy and plant architecture, atmospheric conditions (such as clouds and dust suspension), as well as by the angles of the sensor and the incident sunlight, which varies with the hour and the season of the measurement [109,110].

Spectral data analysis is usually simplified by the calculation of indices that use only a discrete band combination (often 2 to 4 bands) and aim to cancel the internal reflection effects. Several spectral indices and models have been developed to assess the chlorophyll content. Most of the currently available models were reviewed by [25] and the indices are listed in several reviews such as [111,112]. Those indices consider one or two bands that vary under the studied condition (e.g. 690-700 nm) and an extra strong quantitation band that is invariable (e.g. near-infrared, NIR) [39,113,114], in a similar way that the handheld meters do. One of the most frequently used indices to characterize chlorophyll content is the Normalized Difference Vegetation Index (NDVI). NDVI is based on the reflectance contrast between the red and the NIR bands [115]. Although broadband NDVIs can discern broad differences in vegetation conditions [116,117], they are not effective in assessing detailed chlorophyll content of the canopy due to their saturation at a high leaf area index [111]. Most indices vary with the specific environmental and developmental conditions for each species and therefore need to be calibrated and validated with previously established methods for that condition [108,118].

Overall, the use of indices has a cost in accuracy [118]. Some researchers have proposed that using the whole spectrum could increase the validity of the correlations under a wider range of conditions, but this approach would require much more time, training, and informatic resources for the data analysis (which could be worth it for high throughput projects). Nevertheless, remote monitoring of chlorophylls under drought stress by spectral reflectance is achievable and will likely be more accessible in the near future. The potential of remote sensing to capture simultaneous and massive data on the field may be a turning point for the conventional techniques and thus contribute to increasing the genetic gain efficiency [77].

## BREEDING FOR DROUGHT TOLERANCE BY MONITORING CHLOROPHYLL CONTENT

After considering the chlorophyll responses to drought, they indicate that plants which maintain relatively higher chlorophyll content than plants under optimal water availability will be more tolerant. We expect that those plants with higher chlorophyll contents will be more likely to have a higher photosynthesis rate and yield. However, that correlation needs to be demonstrated for most of the crops. Additionally, some experimental aspects may affect the final relevance of the screening to monitor chlorophyll content.

First, if the goal is to map QTLs for drought tolerance, it was proposed that the screenings and heritability calculation need to be done under the specific conditions (drought, in this case). For instance, chlorophyll content in barley exhibited high heritability when mapped independently for well-water and water-restricted conditions, but the heritability was reduced when calculated for both conditions altogether [71]. These differences in heritability suggest that different biochemical mechanisms contribute to chlorophyll content under the specific environmental conditions.

Second, most of the secondary characteristics (characteristics correlated with yield, such as chlorophyll content) used for phenotyping in breeding programs may vary with the duration and intensity of water shortage, the phenological stage when the crop is exposed to the stress, among other factors [119], as mentioned before. Therefore, it is critical to define and plan the experimental conditions and the phenological stage of the crop, to carry out an effective screening.

The experimental conditions will limit the kind of drought that the genotype will experience in the screening. The screening can be carried out in the field (in a dry region or under a rain-exclusion shelter) or some analogous treatment in greenhouse or growth chambers. Different options have been carefully analyzed for rice by [120], which could be easily extrapolated to other crops. There are some mimicking strategies of water restriction, like treatments with osmotic agents such as sorbitol or polyethylene glycol (PEG) [121,122], in vitro or hydroponic systems. PEG treatment has been proposed to be useful to study the physiological effects of some components of drought stress [121]. However, drought is more complex stress, e. g. the water in the soil has a gradient of availability, and the plant responds accordingly to it. Then, the use of mimicking systems will require validation with a soil-based water deficit treatment. Therefore, each scale has their own advantages. Field experiments are highly variable and require the repetition in several years, but it could be done in a similar or the same environment where the crop will be cultivated. In vitro and hydroponic systems are the least variable and allow very fast screenings, however, they are the least comparable to fields. In between, greenhouses and chambers, allow a fast selection with a controlled drought, excluding most of the environmental variation, and ensure to study the tolerance responses to the specific stress. Depending on the crop cycle, those semi-controlled conditions allow to carry out the screening process in several cycles per year which reduce the time to identify tolerant genotypes and reduce the time required for breeding.

Then, to choose the phenological stage when the plant will be exposed to the stress, we can try to mimic the moment when the stress more often happened on the fields for that crop. The

screening design needs to be different for one crop exposed to drought during the seedling establishment than other crops that are exposed at the flowering stage. In particular, chlorophylls may be more useful for leafy crops or for grain crops suffering the stress at vegetative or flowering stages, in contrast with crops exposed to post-flowering stress when most of the chlorophylls were already degraded.

Lastly, it is essential to consider the chlorophylls are not homogeneously distributed across the leaf blade or along with the plant shoot [39,87,123]. This variability needs to be monitored, and equivalent samples (same leaf or in a similar developmental stage) can be collected to compare genotypes. In all cases, it is recommended to sample fully expanded (mature) leaves excluding veins, grown under the stress. These considerations are valid for measuring chlorophyll content by using extractive methods or handheld meters, as well as when sampling to validate spectral reflectance approaches.

## CONCLUSION

Monitoring chlorophyll content, together with other traits, is a promising but underexplored strategy to breed for drought tolerance, and adaptable to economic availability depending on the approach. High-throughput technologies to monitor crop traits (such as chlorophyll content) are key to increase productivity and meet the increasing worldwide demand for plant products. However, for low-income countries, which tend to be the most affected by food scarcity and in need of accessible solutions, affordable technologies are critical and some of the methods presented here may be useful to increase yield at a low cost. Lastly, studies that validate different instruments and methods are needed to obtain comparable and robust results for most of the crops.

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