Effect of Heat Stress on the Relationship between SPAD and Chlorophyll Content in Indian Mustard Genotypes

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Authors’ contributions

This work was carried out in collaboration between both authors. Author PC helped in conceptualization of research, analysis of data and interpretation, preparation of manuscripts. Author PS designed the experiments, contributed in experimental materials and prepared the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2021/v40i1531412

Editor(s):
(1) Dr. Chen Chin Chang, Hunan Women’s University, China.

Reviewer(s):
(1) Lydia Mugao, University of Embu, Kenya.
(2) Seyede Roghie Ghadimezhad Shiade, Sari Agricultural Science and Natural Resources University, Iran.

Complete Peer review History: http://www.sdiarticle4.com/review-history/69892

Received 24 April 2021
Accepted 30 June 2021
Published 02 July 2021

ABSTRACT

\textbf{Aims: }To find the relationship between the non-destructive and destructive methods for the evaluation of photosynthetic pigments under field condition concerning heat stress conditions.

\textbf{Study Design: }The crop was sown in factorial randomized block design in three replication.

\textbf{Place and Duration of Study: }The experiment was conducted during the (2016-2018) rabi crop season for two years. The crop was sown under timely and late sown condition in the research farm of Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana

\textbf{Methodology: }In the present investigation forty-nine advanced breeding lines of Indian mustard were sown under two planting conditions. The data recorded for photosynthetic pigments by calorimetric method by non-destructive methods at three crop stages i.e vegetative, flowering and siliqueing. The relationship between these parameters was carried out by correlation and regression analysis.

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**1. INTRODUCTION**

The leaf photosynthetic capacity can be determined by chlorophyll, which is the main light-harvesting pigment. Chlorophyll is strongly influenced by nitrogen fertilizer [1]; however, the content can vary with leaf position, species, crop types, crop growth stage and crop management [2]. Chlorophyll measurement can be done by destructive and non-destructive measurement and both these methods rely on light adsorption/transmission [3]. Wet chemical methods were conducted for destructive measurement in a lab [4]. Leaves were harvested from the plant and chlorophyll was extracted using organic solvents (e.g., acetone, methanol, ethanol, dimethyl sulphoxide (DMSO), or N-dimethyl formamide (DMF)) [5]. Other methods, including spectrophotometric, fluorometer, or high-performance liquid chromatography (HPLC) are also used to measure light absorptions at particular wavelength range [1]. The disadvantage in these techniques is that the lab-based approach is costly, labour intensive and time consuming. It was also observed that destructive sampling does not allow for tracking the temporal dynamics of these pigments of the same leaves [6]. The portable chlorophyll meters provide simple quick and non-destructive methods for measuring greenness in plants. The Soil Plant Analysis Development (SPAD-502) (Konica Minolta Co, Tokyo, Japan) meter is widely used to guide N management practices in agriculture system as well as greenness. The mechanism of N allocation is important for consideration as 80% of leaf N invested in chloroplast and 20% in photosynthetic protein, only 1% of leaf N is allocated to chlorophyll depending on the plant’s growth environment and species [7].

SPAD meter worked on two transmission values: Red light transmission at 650 nm, which is absorbed by chlorophyll and infrared light transmission at 940 nm at which no chlorophyll absorption occurs. To assess the leaf chlorophyll concentration SPAD meter is commonly used, but calibrating SPAD reading into the direct unit of chlorophyll concentration is difficult and an understanding of these two parameters is necessary [8]. Many researchers have found variability among SPAD value and chlorophyll estimated value per leaf in interspecific and intraspecific cultivars [9,10]. This is due to variability in measurement conditions [11] and also variation present in different light reflections or scattering effects caused by structural differences among leaves. These outcomes suggest that the link between SPAD reading and chlorophyll content per leaf area remains to be established.

Many studies from our laboratory indicated variation in chlorophyll content with respect to growth stage of *Brassica juncea* [12-16] in response to various abiotic stresses. The decrease in photosynthetic pigment due to heat stress has been studied in *Brassica juncea* [17-20], wheat [21] and in sorghum [22].

Studies elucidating the relationship between chlorophyll content and SPAD-502 value at different growth stages in response to terminal heat stress are still insufficient in literature. The mathematical correlation between SPAD value and chlorophyll content can be important to optimize the advanced interpretation of data from the chlorophyll meter. The present investigation gives an insight to determine the realistic relationship between chlorophyll content and SPAD value in Brassica leaves at different crop growth stages and sowing dates. The mathematical relationship will provide more precise, reliable and easier method for estimation of leaf chlorophyll content.

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**Results:** Regression analysis indicated a non-linear relationship at vegetative stage under both sowing conditions, but at flowering stage, slope of the relationship increased with increasing SPAD and a linear relationship was observed (R²= 0.0362) as compared to vegetative stage. A significant and strong correlation was observed between total chlorophyll (siliquing stage) and SPAD value (flowering stage) in timely sown crop. Positive correlation existed between SPAD value and total chlorophyll at flowering stage and carotenoid content at siliquing stage.

**Conclusion:** The variation in the SPAD value and chlorophyll value is strongly related with the species, distribution of chlorophyll in leaf and hence chloroplast also. The lower value of regression coefficient and insignificant values of correlation might due to non-destructive SPAD values under field condition and sampling for calorimetric estimations.

**Keywords:** SPAD; chlorophyll; high temperature; correlation; regression.
2. MATERIAL AND METHODS

2.1 Plant Material and Experimental Sites

The data set was collected from the research area of oilseed section at Punjab Agricultural University, Ludhiana. The forty-nine advanced breeding lines of *Brassica juncea* were sown under timely (October) and late sown (November) conditions in factorial randomized block design with three replications for two crop seasons (2016-18). The Soil Plant Analysis Development (SPAD) unit of Minolta Camera Co., Japan has developed SPAD 502 chlorophyll meter as a hand held self-convenient and light weight device for non-destructive estimation of chlorophyll present in the leaves. The SPAD reading were taken at three growth stages (vegetative, flowering and siliquing). For each observation from each plot, second and third fully extended top leaf was randomly selected from five plants. The mean value of ten reading represents the SPAD value. Care was taken that midrib of the leaf did not fall under sample area /sensor of the instrument.

2.2 Principle of SPAD Meter

The portable chlorophyll meter exploited the beer’s law which is expressed in terms of intensity of the incident monochromatic light ($I_0$) and the intensity of the transmitted light ($I$).

$$a = \log \frac{I_0}{I}$$

The SPAD meter worked on two-band frequency based on the light transmittance, index band which lies in a chlorophyll absorption region (650nm) while reference band is located in the NIR region (940 and 960 nm). To compensate the mechanical differences caused by leaf structure such as leaf thickness or leaf density, there is no light absorption in the reference band. The efficient light is absorbed in red light region (650nm) by chlorophyll molecules. The transmittance at a reference band or wavelength is insignificant (940 and 960 nm). The SPAD-502 meter calculated an output SPAD value (M) according to following equation.

$$M = k \log \frac{I_{663}^{\text{volume}}}{I_{645}^{\text{volume}}}$$

2.3 Analysis of Photosynthetic Pigments [23]

For chlorophyll estimation by calorimetric method, the leaf sample (0.1g) was stored overnight in dimethyl sulphoxide (5ml) in vial at room temperature where no chlorophyll degradation takes place in the solvent being used and no maceration is required. The vials were kept in the boiling water bath at 65°C for 1hour. Absorbance was recorded at 645 nm, 663 nm and 480 nm by using UV 2600 spectrophotometer (Techcomp). The concentration of chlorophyll a, b and total chlorophyll were calculated by using Arnon's equations, whereas the concentration of carotenoids was computed using Kirk and Allen [24] (1965) formula.

$$\text{Chl}_a (\text{mg/g FW}) = 12.7 \times \frac{A_{663} - 2.69 \times A_{645}}{1000 \times \text{weight (g)}}$$

$$\text{Chl}_b (\text{mg/g FW}) = 22.9 \times \frac{A_{645} - 4.68 \times A_{663}}{1000 \times \text{weight (g)}}$$

$$\text{Total Chl (mg/g FW)} = 20.2 \times \frac{A_{645} + 8.02 \times A_{663}}{1000 \times \text{weight (g)}}$$

$$\text{Carotenoids (mg/g/FW)} = 1000 \times \frac{A_{480} - 1.29 \times \text{Chl}_a - 53.78 \times \text{Chl}_b}{1000 \times \text{weight (g)}}$$

2.4 Statistical Analysis

To test the relative performance of genotypes and the significance of treatments, the data recorded in the field and laboratory at various crop growth stages and sowing dates were statistically analyzed using computer programme CPCSI [25]. Pearson’s correlation coefficient was analyzed by online statistical software (OPSTAT). Regression analysis between positively correlated traits was calculated in excel worksheet (2007) using data analysis tool.

3. RESULTS AND DISCUSSION

3.1 Photosynthetic Pigments

The declining trend was observed for SPAD and chlorophyll content during first and second crop season in the late sown condition. Overall, the chlorophyll content was lower at vegetative stage, increase exponentially at flowering stage
and then declined at siliqueing stage under both sowing dates (Fig. 1). The maximum chlorophyll content was observed at flowering stage both in the normal and late plantings however the photosynthetic pigments including carotenoids, the accessory pigment were lower in the late sown condition. This is attributed to reduced growing period with shortened reproductive phase. Timely sown genotypes of *B. juncea* had higher photosynthetic pigments including carotenoids as compared to late planting. Chlorophyll a and b content reduced by 14.1% and 19.6% at vegetative stage, 12.0% and 15.2% at flowering stage, 24.9% and 23.6% at siliqueing stage respectively. Total chlorophyll content decreased by 14.7%, 13.1 %, 24.0% and the carotenoid content reduced by 15.5%, 14.8%, and 29.2% at vegetative, flowering and siliqueing stage respectively in the late sown crop over the timely sown. All the photosynthetic pigments including accessory pigments were higher at the three studied crop growth stages during the two sowing dates except for chlorophyll b during vegetative and siliqueing stages in the timely sown crops. Similarly, SPAD value also declined at vegetative stage by 6.04%, at flowering by 6.71% and at siliqueing by 16.05% as indicated by pooled mean of both year (Table 1).

The above results are in agreement with Din et al. [26] where 23.8% reduction in chlorophyll a+b content was recorded in canola cultivars. Earlier Kauser et al. [27] noticed similar reduction in chlorophyll pigment in *Brassica napus*. The decrease in chlorophyll content under drought
stress was due to reduced chlorophyll synthesis encoded cab gene family [28] or destruction of light harvesting chlorophyll a or b pigment protein complexes which protect the photosynthetic apparatus or may be due to oxidative damage of chloroplast lipids, pigment and protein [29]. Leaf senescence may speed up the inhibition of chlorophyll biosynthesis under high temperature (≥34°C) in wheat [21]. Another evidence in Brassica juncea also proved that chlorophyll content was higher in tolerant genotypes [19]. Chlorophyll content decreased in plant in second and third date of sowing as reported by Jangid and Srivastava [18] as compared to first date of sowing in wheat cultivar under heat stress conditions.

**Table 1. Variation in mean values of portable SPAD meter and leaf chlorophyll by calorimetric method under timely and late sown condition in different crop growth stages for two-crop season**

<table>
<thead>
<tr>
<th>Leaf chlorophyll</th>
<th>Stages</th>
<th>Timely sown</th>
<th>Late sown</th>
<th>CV (%)</th>
<th>Timely sown</th>
<th>Late sown</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2016-17</td>
<td>2017-18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Portable SPAD meter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPAD 502 (Minolta Camera Co, Japan)</td>
<td>Vegetative</td>
<td>43.3</td>
<td>40.7</td>
<td>4.06</td>
<td>44.3</td>
<td>41.6</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>Flowering</td>
<td>45.7</td>
<td>42.9</td>
<td>3.53</td>
<td>47.8</td>
<td>44.3</td>
<td>3.93</td>
</tr>
<tr>
<td></td>
<td>Siliquing</td>
<td>38.4</td>
<td>31.8</td>
<td>5.35</td>
<td>39.3</td>
<td>33.4</td>
<td>6.16</td>
</tr>
<tr>
<td><strong>Calorimetric estimations method</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)Chlorophyll a (mg/g FW)</td>
<td>Vegetative</td>
<td>1.44</td>
<td>1.26</td>
<td>6.78</td>
<td>1.40</td>
<td>1.18</td>
<td>5.26</td>
</tr>
<tr>
<td></td>
<td>Flowering</td>
<td>1.62</td>
<td>1.43</td>
<td>9.00</td>
<td>1.53</td>
<td>1.34</td>
<td>4.99</td>
</tr>
<tr>
<td></td>
<td>Siliquing</td>
<td>1.31</td>
<td>1.02</td>
<td>8.75</td>
<td>1.22</td>
<td>0.88</td>
<td>7.56</td>
</tr>
<tr>
<td>(b)Chlorophyll b (mg/g FW)</td>
<td>Vegetative</td>
<td>0.28</td>
<td>0.23</td>
<td>10.59</td>
<td>0.28</td>
<td>0.22</td>
<td>10.24</td>
</tr>
<tr>
<td></td>
<td>Flowering</td>
<td>0.34</td>
<td>0.28</td>
<td>11.24</td>
<td>0.31</td>
<td>0.27</td>
<td>9.56</td>
</tr>
<tr>
<td></td>
<td>Siliquing</td>
<td>0.24</td>
<td>0.20</td>
<td>9.52</td>
<td>0.26</td>
<td>0.18</td>
<td>9.86</td>
</tr>
<tr>
<td>(c)Total chlorophyll (mg/g FW)</td>
<td>Vegetative</td>
<td>1.72</td>
<td>1.49</td>
<td>7.19</td>
<td>1.67</td>
<td>1.41</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td>Flowering</td>
<td>1.95</td>
<td>1.71</td>
<td>9.23</td>
<td>1.86</td>
<td>1.61</td>
<td>8.85</td>
</tr>
<tr>
<td></td>
<td>Siliquing</td>
<td>1.56</td>
<td>1.22</td>
<td>7.57</td>
<td>1.45</td>
<td>1.06</td>
<td>12.19</td>
</tr>
<tr>
<td>(d)Carotenoids content (mg/g FW)</td>
<td>Vegetative</td>
<td>0.47</td>
<td>0.40</td>
<td>6.89</td>
<td>0.44</td>
<td>0.36</td>
<td>7.84</td>
</tr>
<tr>
<td></td>
<td>Flowering</td>
<td>0.52</td>
<td>0.50</td>
<td>10.39</td>
<td>0.45</td>
<td>0.42</td>
<td>8.27</td>
</tr>
<tr>
<td></td>
<td>Siliquing</td>
<td>0.42</td>
<td>0.30</td>
<td>7.40</td>
<td>0.40</td>
<td>0.27</td>
<td>10.84</td>
</tr>
</tbody>
</table>

**Fig. 1. Variation in photosynthetic pigments and SPAD values at different stages of crop growth stages.**
Table 2. Correlation analysis of photosynthetic pigment at three crop stages measured by calorimetric estimation and SPAD portable meter

<table>
<thead>
<tr>
<th></th>
<th>Vegetative</th>
<th>Flowering</th>
<th>Siliquing</th>
<th>SPAD value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total chl</td>
<td>Carotenoid</td>
<td>Total chl</td>
<td>Carotenoid</td>
</tr>
<tr>
<td>Total chl</td>
<td>1</td>
<td>0.930</td>
<td>-0.043</td>
<td>-0.040</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>0.909**</td>
<td>1</td>
<td>0.001</td>
<td>-0.040</td>
</tr>
<tr>
<td>Total chl</td>
<td>0.313</td>
<td>0.246</td>
<td>1</td>
<td>0.803</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>0.182</td>
<td>0.136</td>
<td>0.814**</td>
<td>1</td>
</tr>
<tr>
<td>Total chl</td>
<td>0.574</td>
<td>0.454*</td>
<td>0.538</td>
<td>0.453</td>
</tr>
<tr>
<td>Carotenoid</td>
<td>0.427**</td>
<td>0.402**</td>
<td>0.230</td>
<td>0.227</td>
</tr>
<tr>
<td>Vegetative</td>
<td>0.153</td>
<td>0.144</td>
<td>0.173</td>
<td>0.101</td>
</tr>
<tr>
<td>Flowering</td>
<td>0.026</td>
<td>-0.034</td>
<td>0.190</td>
<td>0.277</td>
</tr>
<tr>
<td>Siliquing</td>
<td>0.258</td>
<td>0.180</td>
<td>0.222</td>
<td>0.197</td>
</tr>
</tbody>
</table>
Similar results in oilseed species under water stress have been reported by Faneri et al. [30] leading to decrease in SPAD value from mid flowering stage to end of siliquing indicates of the stage sensitivity to heat stress. The decline in SPAD value at successive developmental stage was due to accelerated degradation of chlorophyll molecules with enhanced effect in late planting. These results are further in confirmation with the findings in *B. juncea* [31], *Triticum aestivum* [32] and *Ocimum basilicum* [33]. The higher reduction percentage was reported in SPAD value at 90 DAS and 120 DAS as compared to 30 DAS and 60 DAS in Indian mustard [34]. Similar finding was reported in wheat that SPAD value increased up to 12 days after anthesis and then decline sharply [35].

3.2 Regression and Correlation Analysis

Despite of plant stage, a linear mathematical relationship fitted best in relationship between chlorophyll content and SPAD value.

Where, \( \text{SPAD} \) (response) = Constant + coefficient 1 (predictor) + coefficient 2 (predictor)

Positive and strong relationship existed between total chlorophyll at vegetative stage and SPAD value at siliquing stage \((r=0.258)\), total chlorophyll at flowering stage and SPAD value at siliquing stage \((r=0.222)\). Significant and strong correlation was also observed between total chlorophyll at siliquing stage with SPAD value at flowering stage \((r=0.315^*)\) and siliquing stage \((r=0.293^*)\) in timely sown conditions (Table 2). In case of late planted conditions positive correlation was observed between SPAD value and total chlorophyll at flowering stage \((r=0.221)\) and carotenoid at siliquing stage \((r=0.267)\) only.

Non-significant regression was observed indicated non-linear relationship at vegetative stage under both planted dates. At flowering stage the slope of the relationship increased with increasing SPAD indicating linear relation \((R^2 =0.0362)\) for total chlorophyll, while carotenoid content showed non-linear relation in timely sown condition (Fig 2). In case of late sown condition, non-linear relation was observed at flowering and siliquing stages. Similar trend was recorded under siliquing stage. This may be due to the more degradation of chloroplast in late sown condition or more mobilization of dry matter to the developing part to sustain grain weight. The variation in irradiation condition would cause changes in chlorophyll component e.g. the ratio of chlorophyll a and chlorophyll b and hence total chlorophyll, which contributes to a relatively stable coefficient to SPAD value.

The correlation and regression analysis showed that coefficient was largely declined between SPAD and chlorophyll value. It may be because leaf condition would affect the accuracy of correlation analysis as supported by Jiang et al [36-38]. Xiong et al. [39] reported that SPAD reading correlate non-linearly with chlorophyll content per leaf area. A photon reaching to leaf is absorbed, reflected or transmitted and its fate is substantially affected by the distribution of grana within chloroplast, chloroplasts within cells, and cells within tissue layers [40]. Non-significant difference exist between SPAD and chlorophyll content per unit leaf area in monocot and dicot species as reported by Xiong et al. [39] and Parry Blonquist [9] in monocot, dicot, deciduous and annual species.
4. CONCLUSION

In the present study, portable chlorophyll meter and calorimetric method of analysis give significant values individually and the prominent values of photosynthetic pigments. The insignificant relationship between these parameters at various growth stages was due to several reasons like time of SPAD measurement, environmental irradiance and growth stages. Evidence also proved that the distribution of chloroplast plays crucial role in determining the relationship between these two parameters. There is need for a reliable method that provides the realistic measurement instead of the dynamics and insignificant values.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.
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