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Note

Precision phenotyping of imidazolinone-induced chlorosis in sunflower

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Chlorosis level is a useful parameter to assess imidazolinone resistance in sunflower (*Helianthus annuus* L.). The aim of this study was to quantify chlorosis through two different methods in sunflower plantlets treated with imazapyr. The genotypes used in this study were two inbred lines reported to be different in their resistance to imidazolinones. Chlorosis was evaluated by spectrophotometrical quantification of photosynthetic leaf pigments and by a bioinformatics-based color analysis. A protocol for pigment extraction was presented which improved pigment stability. Chlorophyll amount decreased significantly when both genotypes were treated with 10 μM of imazapyr. Leaf color was characterized using Tomato Analyzer® color test software. A significant positive correlation between color reduction and chlorophyll concentration was found. It suggests that leaf color measurement could be an accurate method to estimate chlorosis and infer chlorophyll levels in sunflower plants. These results highlight a strong relationship between imidazolinone-induced chlorosis and variations in leaf color and in chlorophyll concentration. Both methods are quantitative, rapid, simple, and reproducible. Thus, they could be useful tools for phenotyping and screening large number of plants when breeding for imidazolinone resistance in this species.

Key Words: carotenoids, chlorophylls, chlorosis, color analysis software, *Helianthus annuus* L., sunflower.

Introduction

Chlorosis is a plant condition in which pigments levels are reduced. Chlorophylls and carotenoids are the principal pigments and the major constituents of the photosynthetic apparatus in land plants and green algae. Chlorophyll a (Chl_a) is in nearly all oxygenic photosynthetic organisms (Nakamura *et al.* 2003) and is essential in photochemistry. Chlorophyll b (Chl_b) is necessary for stabilizing the major light-harvesting complexes within the pigment antenna system (Lichtenthaler *et al.* 1981). Carotenoids have many physiological functions in plants and one of the most important is to allow plants to overcome the negative effects of stress on their growth and development (Kopsell and Kopsell 2008).

Chlorophyll content in plants is affected by developmental stage and by numerous environmental factors. Chlorophyll content of barley leaves was reported to change throughout the growing season and to start decreasing at the beginning of leaf senescence (Matile *et al.* 1988). Decrease in chlorophyll levels due to adverse conditions is used as a marker for external damage in plants (Kara and Mujdeci 2010). Also, the Chl_a/Chl_b ratio is known as a quantitative indicator of the degree of adaptation of the photosynthetic apparatus to the illumination conditions (Kitajima and Hogan 2003). One of the most common methods for the quantification of chlorophylls and carotenoids is by measuring their absorption of light. Destructive quantification methods require pigment purification by organic solvents like acetone or DMSO (MacKinney 1941). There are also some non-destructive techniques to estimate leaf chlorophyll content, such as chlorophyll meters, which have been extensively used in agriculture because they provide instantaneous readings although, the accuracy of the measurement can be affected by leaf veins and variations in leaf thickness (Brito *et al.* 2011).

Sunflower (*H. annuus*) is one of the most important oil crops worldwide. Weeds compete with sunflower for moisture, nutrients, and, depending on the species, for light and space. Broadleaf weeds are known to cause considerable yield losses in sunflower production (Blamey *et al.* 1997). The most common method for weed control is herbicide treatment. It is known that herbicide application induces stress signals in non-target species. For this reason, trait

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development for herbicide resistance in sunflower, particularly imidazolinones (IMI) and sulfonylureas (SU), has been an active area of research during the past decade (Sala *et al.* 2012). IMI and SU are among the five chemical families of herbicides that inhibit the enzyme acetohydroxiacid synthase (AHAS; EC 4.1.5.18). AHAS is a critical enzyme for the biosynthesis of branched-chain amino acids in plants (Tan *et al.* 2006). AHAS inhibitors have been revolutionary to the herbicide market because they are potent, effective, and environmentally safe. IMI and SU herbicides control weeds by blocking the biosynthesis of valine, leucine and isoleucine (Duggleby *et al.* 2008). Crop injury after herbicide treatment includes symptoms such as chlorosis, stunting, yellowing, reduction of biomass production, and yield loss. The effect of these herbicides on chlorophyll levels has been evaluated in several species such as corn, chickpea, soybean, green bean, and green algae (Aamil *et al.* 2004, Alonge 2000, Cayon *et al.* 1990, Couderchet and Vernet 2003, Hoseiny-Rad and Jagannath 2011, Matocha and Hopper 2001, Saghir Khan *et al.* 2006, Shim *et al.* 2003, Wilson and Wilson 2010).

Imidazolinone herbicide application has been reported to cause initial chlorosis and necrosis of meristematic tissues, followed by a slow necrosis of mature tissues (Shaner *et al.* 1984). In previous studies, different levels of chlorosis have been observed in IMI-resistant and susceptible sunflower inbred lines when they were treated with imidazolinones (Breccia *et al.* 2011). However, chlorosis was only described but was not quantified. Chlorosis assessments were carried out using visual qualitative scales, which are highly dependent on the operator. Tomato analyzer® color Test program (TACT) is a recently developed bioinformatics tool that allows an objective quantification of color and color uniformity (Rodríguez *et al.* 2010). This software application was designed for measuring internal fruit color and shape of tomatoes with a colorimeter and/or from scanned digital images. This tool allows association of phenotypic color variation with genotypic variances by comparing colorimetric data (Darrigues *et al.* 2008). Tomato analyzer® has also been used for the colorimetric and volumetric characterization of other fruits and vegetables (Darrigues *et al.* 2008), and in our group it has been applied for leaf and roots analyses (Breccia *et al.* 2012). To the best our knowledge, there are no publications about its implementation for color characterization. The objectives of this study were to (i) evaluate imidazolinone-induced chlorosis using both image analysis and the quantification of photosynthetic pigments, and (ii) determine the correlation between these methods.

Materials and Methods

Plant material and growth condition

The two sunflower (*H. annuus*) inbred lines used for the study were HA425 and 1058-1, developed by USDA-ARS (United States Department of Agriculture, Agricultural Research Service) in cooperation with the North Dakota Experiment Station, United States. According to the digenic model proposed by Bruniard and Miller (2001) in which a major semidominant gene (I_{mr1}) interacting with a second modifier gene (I_{mr2}) confers resistance to imidazolinones in sunflower, the inbred lines HA425 ($I_{mr1}I_{mr1}I_{mr2}I_{mr2}$) (Miller and Al-Khatib 2002) and 1058-1 ($I_{mr1}I_{mr1}i_{mr2}i_{mr2}$) have been classified by these authors as resistant and intermediate, respectively.

Seeds were sown in plastic pots (4 cm wide, 5.5 cm high) filled with commercial perlite as described by Vega *et al.* (2009). The pots were placed in plastic trays and watered by capillarity with nutritive solution consisting in Murashige Skoog salts (1.1 gl^{-1}) plus herbicide imazapyr (Clearsol® BASF). Two treatment concentrations were evaluated (2.5 uM and 10 uM). The control treatment consisted in watering the plantlets with nutritive solution free of herbicide. All trays were watered by capillarity from sowing to collection dates. Pots were incubated in a growth chamber under controlled temperature (25 ± 2°C), photoperiod (16 h light and 8 h dark), and light intensity (100 µmol m^{-2} s^{-1}) for 15 days. After the incubation period all plantlets presented two expanded leaves (V2 stage) (Schneiter and Miller 1981) which were excised from seedlings and immediately used for pigment and color test determinations.

Measurement of chlorophylls and carotenoids concentration

The pigment extraction protocol was based on Porra (2002) and Meléndez-Martínez *et al.* (2007), with modifications. We significantly reduced the working volume. One distal 1 cm^2 section was cut from one expanded leaf of fifteen-days-old sunflower seedlings. Each section was weighed and then pulverized in a microtube with micropestle and 1 ml of acetone 80% v/v. Leaf extract was quickly vortexed and spun for 5 min at 4000 rpm, and the supernatant transferred to a glass tube. The pellet was re-extracted with 1 ml of acetone 80% v/v, vortexed and spun again, and the supernatant added to the first extract. Final volume of extract solution was approximately 6 ml. Absorbance was measured at 750, 664, 647 and 470 nm in a Perking Elmer Lambda Bio+ spectrophotometer. Background effects were corrected for by subtracting the 750 nm absorbance determinations. A_{664} and A_{647} were used in the equations described by Porra *et al.* (2002) to estimate concentrations of Chl_a, Chl_b, and total Chl in treated sunflower leaves. A_{470} values were used in Meléndez-Martínez *et al.* (2007) carotenoids equations.

Bioinformatic color tests determinations

Leaves of each genotype and herbicide treatment were removed from seedlings and immediately imaged with a HP Scanjet G3010 flatbed scanner. TIFF images (300 dpi) were analyzed using Tomato Analyzer (http://www.oardc.ohiostate.edu/vanderknaap/tomato_analyzer.htm). Leaf color was estimated with the *hue* angle parameter.

Experimental design and data analysis

The experimental design was a randomized block design with six replications and each replication consisted in four plantlets. One leaf was sampled from each replication and a 1-cm sample from distal region of these leaves was used for the pigment extraction determinations. For colorimetrical determinations, three leaves from each replication were imaged.

Chlorophylls, carotenoid concentrations, and *hue* angle values were analyzed by two-way analysis of variance. Normality of the empirical distribution of each variable was assessed by the Shapiro and Wilk's *W* statistic test. Homogeneity of variance was evaluated using the Levene's test. Treatment means were compared by Tukey's test. Correlation values among all pigment concentrations and *hue* angles were also estimated. Statistical analyses were performed using *agricolae* and *car* packages of R software (R Development Core Team 2010).

Results

A slight yellowing uniform chlorosis was observed in imidazolinone-treated leaves of both sunflower genotypes. Leaf area also decreased, even in the imidazolinone-resistant genotype (Fig. 1). Imidazolinone-induced chlorosis was evaluated by chlorophyll and carotenoid quantifications obtained by spectrophotometric methods and by color variations obtained by bioinformatic color analysis. Pigment concentrations and colorimetric values were analyzed by two-way analysis of variance (ANOVA) and correlation values were also estimated.

Pigments concentration analysis

Amounts of Chl_a, Chl_b, and total Chl in leaf samples were determined for each genotype. The ANOVA showed significant herbicide effect on the concentration of Chl_a, Chl_b, and total Chl ($p < 0.0001$; $p < 0.0029$; and $p < 0.001$ respectively). Chl_a and total Chl were significantly lower than those of the controls in imidazolinone treated plants of both genotypes (Table 1). Chl_a content decreased by 44.9% in HA425, and 46.3% in 1058-1 compared to control leaves. Also, total Chl decreased by 42.5% in HA425, and 43.5% in 1058-1.

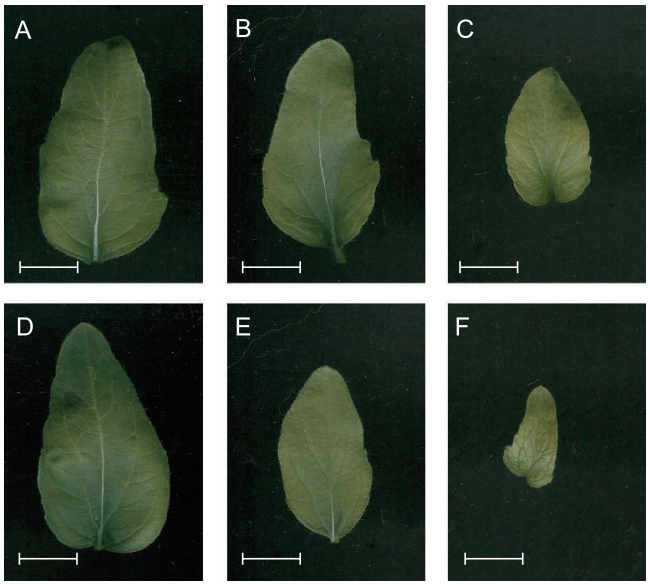

Fig. 1. Scanned sunflower leaves of resistant (HA425) and intermediate (1058-1) genotypes exposed to imazapyr: Panels A, B and C show genotype HA425 exposed to 0 μM (control), 2.5 μM and 10 μM of imazapyr, respectively. Panels D, E and F show genotype 1058-1 treated with 0 μM (control), 2.5 μM and 10 μM of imazapyr. Bars: 1 cm.

No significant decrease was found in Chl_b, Chl_a/Chl_b ratio, or carotenoids contents (Table 1).

An interesting result of the present study was that no block effects were detected for all studied variables by ANOVA. This result points out the robustness of the modified pigments extraction protocol.

Bioinformatics color analysis

Changes in leaf coloration were determined with the *hue* angle parameter, using TACT. The *hue* angle is associated with the green color component of tissues, with a 90° *hue* value representing yellow, and 180° representing a bluish green color. *Hue* angle of treated leaves was significantly lower than that of untreated leaves for both genotypes (Table 1). ANOVA showed a significant effect of herbicide, genotype, and the interaction between them ($p < 0.0001$, p

Table 1. Pigment content (μg g^{-1} of tissue) and *hue* angle values (grades) of resistant (HA425) and intermediate (1058-1) genotypes treated with imazapyr

Genotype	Imazapyr (μM)	Chl_a	Chl_b	Chl_a/Chl_b ratio	Total Chl	Carotenoids	*hue* angle
HA425	0	752.80 (±31.72)^{a}	198.15 (±18.31)^{ab}	3.92 (±0.28)^{a}	950.95 (±46.50)^{a}	140.91 (±5.3)^{a}	139.07 (±0.32)^{a}
	2.5	665.4 (±46.23)^{ab}	205.49 (±28.02)^{a}	3.78 (±0.94)^{a}	870.89 (±58.49)^{a}	145.66 (±14.34)^{a}	134.73 (±0.26)^{c}
	10	414.64 (±24.73)^{c}	131.10 (±3.25)^{ab}	3.17 (±0.22)^{a}	545.75 (24.19)^{b}	119.99 (±2.69)^{a}	129.16 (±0.25)^{d}
1058-1	0	744.5 (±20.17)^{a}	218.84 (±26.06)^{ab}	3.59 (±0.31)^{a}	967.35 (±45.27)^{a}	136.67 (±1.28)^{a}	136.55 (±0.13)^{b}
	2.5	603.93 (±28.73)^{b}	240.38 (±35.89)^{ab}	2.73 (±0.31)^{a}	844.51 (±36.34)^{a}	119.53 (±15.19)^{a}	133.92 (±0.51)^{c}
	10	399.92 (±16.72)^{c}	145.63 (±6.00)^{a}	2.75 (±0.09)^{a}	545.55 (±21.28)^{b}	118.23 (±5.96)^{a}	128.87 (±0.23)^{d}

Each value is the mean ± standard error. Mean values with the same letter are not significantly different at the 0.05 probability level (Tukey's multiple comparison test). Chl_a/Chl_b ratio data was square root-transformed: mean values are based on untransformed data. Letters refer to transformed mean values.

< 0.0001 and $p < 0.0056$, respectively). Seedlings exposed to the higher herbicide concentration showed the greatest decrease in *hue* angle. This decrease is directly related to an increase in the yellowish colorimetric component and thus with a chlorosis increase. Consistent with the other variables tested, there were no significant effects of repetition.

Correlation studies

Correlation analyses showed a strong relationship between different concentrations of pigments and bioinformatic color determinations with a positive correlation between Chl_a concentration and *hue* angle values ($r = 0.81$; $R^2 = 0.64$; $p < 0.0001$). These results indicate that there is a strong association between colorimetric determinations and pigment concentration for the imidazolinone-induced chlorosis.

Discussion

Herbicide application aims at eliminating crop competitive weeds. However, herbicides can cause abiotic stresses in non-target species, increasing the levels of oxidative metabolites which hinder normal plant development. The effects of these metabolites may cause symptoms such as chlorosis, necrosis, and decreased growth, among others (Kopsell *et al.* 2007). Previous studies have evaluated the influence of herbicides affecting photosystem II and of chlorosis-inducing herbicides on the synthesis and accumulation of chlorophylls and carotenoids in cyanobacteria (González-Barreiro *et al.* 2004) and algae (Prado *et al.* 2011). Atrazine was reported to cause a significant decrease in the Chl_a content in *Synechococcus elongatus* (González-Barreiro *et al.* 2004). Also, paraquat treatment induced chlorosis in *Chlamydomonas moewusii* (Prado *et al.* 2011).

Chlorophyll content is commonly used as a parameter to evaluate the physiological condition of plants (Kara and Mujdeci 2010). Measurement of these pigments is most frequently done by spectrophotometric methods. In intact plant organs, however, this measurement is greatly hindered by scattering and non-specific absorption (Brito *et al.* 2011). It is therefore more accurate to analyze chlorophyll extracts, but the stability of the chlorophylls *in vitro* has to be considered. Most long-standing extraction methods use solvents that are mutually miscible with water and non-polar liquids, e.g. acetone (Bruisna 1961, MacKinney 1941). During extraction and subsequent absorbance measurement, chlorophyll can be degraded by long exposure to oxygen, by chlorophyllase activity, and by the action of cell organic acids, among others factors (Bruisna 1961). Thus, chlorophyll stability must be ensured before doing spectrophotometric measurements. In our work we proposed some interesting modifications to a commonly used protocol. Extract exposure to air and the time required to complete the extraction procedure were markedly reduced by using micropestles to disrupt a small sample of fresh leaf tissue in a microtube. Also, the extraction time was considerably shortened by replacing the commonly used vacuum purification step with centrifugation in microtubes. No repetition effects between determinations for each treatment and genotype were detected by statistical analysis; therefore, the protocol modifications did not affect the repeatability of this test.

The effects of imidazolinones and sulfonylureas on chlorophyll levels have been evaluated in several species. Treatments with sulfonylureas have been reported to reduce chlorophyll content in corn, green pea, and chickpea leaves (Aamil *et al.* 2004, Matocha and Hopper 2001, Shim *et al.* 2003). Treatments with imidazolinone caused internerval chlorosis and reduced plant height in green pea and soybean (Alonge 2000, Cayon *et al.* 1990, Wilson and Wilson 2010). Chlorophyll concentration decreased significantly in soybean leaves treated with high doses of imazaquin, mainly after post-emergence applications (Alonge 2000, Cayon *et al.* 1990). Imazethapyr-treated chikpea leaves showed a significant decrease in total chlorophyll content when treated with high herbicide concentration (e.g. 10 ppm) (Hoseiny-Rad and Jagannath 2011). All these studies are consistent with our results. Imidazolinone reduced Chl_a, Chl_b, and total Chl concentrations in sunflower. The higher dose of imazapyr induced a significant decrease in Chl_a, Chl_b, and total Chl from 33% to 46% for both genotypes. The sunflower genotypes used in this work differ in their resistance to imidazolinones; however, slightly differences were detected between them under this experimental condition. Further analysis including higher herbicide concentrations or longer incubation periods will be of interest to establish conditions for screening imidazolinone-resistance in this species. Reductions in chlorophyll content could be due to a decrease in chlorophyll synthesis or to an increase in pigment degradation. AHAS-inhibiting herbicides are neither direct suppressors of pigment synthesis nor principal inductors of oxidative stress, but these symptoms are associated with herbicide treatment. Some authors propose that the decrease in chlorophyll concentration in soybeans treated with imazaquin may be related to an increase in the photo-oxidation of chlorophylls due to lack of carotenoids (Alonge 2000, Cayon *et al.* 1990, Duke 1985). In our work, by contrast, carotenoid concentration remained unchanged in both control and treated leaves. This result suggests that the decrease in chlorophyll content could be brought about by mechanisms which are independent from the photo-protection mediated by carotenoids.

Identification of herbicide-resistant sunflower phenotypes involves spraying the herbicide onto plants grown in the field or greenhouse at early stages of development, usually V2–V4, for subsequent selection of resistant genotypes (Sala *et al.* 2012). For this reason, early phenotypic selection for herbicide resistance is usually tested with soil-less bioassays, which allow fast and inexpensive screening of large numbers of individuals (Beckie *et al.* 2000). Previous studies in this species showed that root growth parameters in seed germination bioassays were useful for early imidazolinone resistance screening (Vega *et al.* 2009). The present study broadens the spectrum of analysis for imidazolinone

resistance in sunflower by considering chlorosis characterization. Due to the low imazapyr concentrations evaluated, chlorosis increase in treated leaves was barely visible (Fig. 1). However a significant *hue* angle reduction was detected by TACT analysis. The significant positive correlation between *hue* angle and Chl_a concentration agrees with Majer *et al.* (2010). These authors found a significant linear correlation between *hue* angle and chlorophyll content in tobacco and grapevine leaves having different colours at different stages of senescence. In our study, colorimetric analysis with TACT allowed quantifying the increase in leaf yellowing as seedlings were treated with increasing imazapyr concentrations. This work is the first report on the implementation of this software in leaf color characterization. Leaf color measurement by TACT could be an accurate, simple, fast and cost-effective method to measure chlorosis in leaves of sunflower plants. It also allows inferring Chl_a without using toxic chemicals so that makes analyses more environmentally friendly. The new application of this bioinformatic tool could be useful in sunflower-breeding programs.

In conclusion, our results show that both chlorophyll content determinations and TACT image analysis are valuable tools for the quantitative assessment of imidazolinone-induced chlorosis in sunflower leaves. TACT image analysis has potential for high-throughput screenings, thus reducing the time and labor required for the characterization of large numbers of plants. The protocols described could be useful for the phenotypic characterization of the imidazolinone resistance trait. Moreover, they could also be applied to other biotic or abiotic stresses involving this symptom.

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Literature Cited

Aamil, M., A. Zaidi and M.S. Khan (2004) Effect of herbicides on growth, nodulation and yield of chickpea (*Cicer arietinum* L.). Ann. Plant Protect. Sci. 12: 186–191.

Alonge, S.O. (2000) Effect of imazaquin applications on the growth, leaf chlorophyll and yield of soybean in the Guinea Savanna of Nigeria. J. Environ. Sci. Health B. 35: 321–336.

Beckie, H.J., I.M. Heap, R.J. Smeda and L.M. Hall (2000) Screening for herbicide resistance in weeds. Weed Technol. 14: 428–445.

Blamey, F.P.C., R.K. Zollinger and A.A. Schneiter (1997) Sunflower production and culture. *In*: Schneiter, A.A. (ed.) Sunflower Technology and Production, ASA, CSSA and SSSA, Madison, Wisconsin, pp. 595–670.

Breccia, G., T. Vega, G. Nestares, M.L. Mayor, R. Zorzoli and L. Picardi (2011) Rapid test for detection of imidazolinone resistance in sunflower (*Helianthus annuus* L.). Plant Breeding 130: 109–113.

Breccia, G., M. Gil, T. Vega, R. Zorzoli, L. Picardi and G. Nestares (2012) Effect of cytochrome P450s inhibitors on imidazolinone resistance in sunflower. *In*: 18th International Sunflower Conference, Mar del Plata and Balcarce, Argentina.

Brito, G.G., V. Sofiatti, Z.N. Brandão, V.B. Silva, F.M. Silva and D.A. Silva (2011) Non-destructive analysis of photosynthetic pigments in cotton plants. Acta Sci. Agron. 33: 671–678.

Bruisna, J. (1961) A comment on the spectrophotometric determination of chlorophyll. Biochim. Biophys. Acta 52: 576–578.

Bruniard, J.M. and J.F. Miller (2001) Inheritance of imidazolinone-herbicide resistance in sunflower. Helia 24: 11–16.

Cayon, D.G., N.F. Lopes, M.A. Oliva and J.F. Silva (1990) Teores de clorofilas e de proteína bruta em soja (*Glycine max* (L.) merril) tratada com imazaquin. Rev. Bras. Fisiol. Veget. 2: 33–40.

Couderchet, M. and G. Vernet (2003) Pigments as biomarkers of exposure to the vineyard herbicide flazasulfuron in freshwater algae. Ecotoxicol. Environ. Saf. 55: 271–277.

Darrigues, A., J. Hall, E. van der Knaap and D.M. Francis (2008) Tomato Analyzer-color Test: A new tool for efficient digital phenotyping. J. Amer. Soc. Hort. Sci. 133: 579–586.

Duggleby, R.G., J.A. McCourt and L.W. Guddat (2008) Structure and mechanism of inhibition of plant acetohydroxyacid synthase. Plant Physiol. Biochem. 46: 309–324.

Duke, S.O. (1985) Effects of herbicide on nonphotosynthetic biosynthetic process. *In*: Duke, S.O. (ed.) Weed Physiology. Herbicide Physiol. 2, CRC Press, Boca Raton, pp. 91–112.

González-Barreiro, O., C. Rioboo, A. Cid and C. Herrero (2004) Atrazine-induced chlorosis in *Synechococcus elongatus* cells. Arch. Environ. Contam. Toxicol. 46: 301–307.

Hoseiny-Rad, M. and S. Jagannath (2011) Effect of herbicide Imazethapyr (pursuit™) on chickpea seed germination. Arch. Phytopathology Plant Protect. 44: 224–230.

Kara, B. and M. Mujdeci (2010) Influence of late-season nitrogen application on chlorophyll content and leaf area index in wheat. Sci. Res. Essays 5: 2299–2303.

Kitajima, K. and K.P. Hogan (2003) Increases of chlorophyll *a/b* ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. Plant Cell Environ. 26: 857–865.

Kopsell, D.A., T.C. Barickman, C.E. Sams and J.S. McElroy (2007) Influence of nitrogen and sulfur on biomass production and carotenoid and glucosinolate concentrations in watercress (*Nasturtium officinale* R. Br.). J. Agric. Food Chem. 55: 10628–10634.

Kopsell, D.A. and D.E. Kopsell (2008) Genetic and environmental factors affecting plant lutein/zeaxanthin. Agro Food Ind. Hi-Tech. 19: 44–46.

Lichtenthaler, H.K., U. Prenzel, R. Douce and J. Joyard (1981) Localization of prenylquinones in the envelope of spinach chloroplasts. Biochim. Biophys. Acta 641: 99–105.

MacKinney, G. (1941) Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315–322.

Majer, P., L. Sass, G.V. Horváth and É. Hideg (2010) Leaf hue mesurements offer a fast, high-throughput initial screening of photosynthesis in leaves. J. Plant Physiol. 167: 74–76.

Matile, P., S. Ginsburg, M. Schellenberg and H. Thomas (1988) Catabolites of chlorophyll in senescing barley leaves are localized in the vacuoles of mesophyll cells. Proc. Natl. Acad. Sci. USA 85: 9529–9532.

Matocha, J.E. and F.L. Hopper (2001) Influence of Terfubos and Nicosulfuron on iron chlorosis and growth of corn. J. Plant Nutr.

24: 1445–1455.

Meléndez-Martínez, A.J., I.M. Vicario and F.J. Heredia (2007) Carotenoid pigments: structural and physicochemical considerations. Arch. Latinoam. Nutr. 57: 109–117.

Miller, J.F. and K. Al-Khatib (2002) Registration of imidazolinone herbicide-resistant sunflower manteiner (HA 425) and fertility restorer (RHA 426 and RHA 427) germplasms. Crop Sci. 42: 988–989.

Nakamura, A., A. Akai, E. Yoshida, T. Taki and T. Watanabe (2003) Reversed-phase HPLC determination of chlorophyll *a′* and phylloquinone in Photosystem I of oxygenic photosynthetic organisms. Universal existence of one chlorophyll *a′* molecule in Photosystem I. Eur. J. Biochem. 270: 2446–2458.

Porra, R.J. (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. Photosynth. Res. 73: 149–156.

Prado, R., C. Rioboo, C. Herrero and Á. Cid (2011) Characterization of cell response in *Chlamydomonas moewusii* cultures exposed to the herbicide paraquat: Induction of chlorosis. Aquat. Toxicol. 102: 10–17.

R Development Core Team (2010) R Foundation for Statistical Computing, Austria.

Rodríguez, G.R., J.B. Moyseenko, M.D. Robbins, N.H. Morejón, D.M. Francis and E. van der Knaap (2010) Tomato Analyzer: A useful software application to collect accurate and detailed morphological and colorimetric data from two-dimensional objects. J. Vis. Exp. 37: 1856.

Saghir Khan, M., A. Zaidi and P. Qamar Rizvi (2006) Biotoxic effects of herbicides on growth, nodulation, nitrogenase activity, and seed production in chickpeas. Comm. Soil Sci. Plant Anal. 37: 1783–1793.

Sala, C.A., M. Bulos, E. Altieri and M.L. Ramos (2012) Genetics and breeding of herbicide tolerance in sunflower. Helia 35: 57–70.

Schneiter, A.A. and J.F. Miller (1981) Description of sunflower growth stages. Crop Sci. 21: 901–903.

Shaner, D.L., P.C. Anderson and M.A. Stidham (1984) Imidazolinones, potent inhibitors of acetohydroxyacid synthase. Plant Physiol. 76: 545–546.

Shim, S.I., B.M. Lee, E.I. Ryu and B.H. Kang (2003) Response of leaf acetolactate synthase from different leaf positions and seedling ages to sulfonylurea herbicide. Pestic. Biochem. Physiol. 75: 39–46.

Tan, S., R. Evans and B. Singh (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino Acids 30: 195–204.

Vega, T., G. Breccia, G. Nestares, M.L. Mayor, R. Zorzoli and L. Picardi (2009) Soil-less bioassays for early screening for resistance to imazapyr in sunflower (*Helianthus annuus* L.). Pest Manag. Sci. 65: 991–995.

Wilson, P.C. and S.B. Wilson (2010) Toxicity of the herbicides bromacil and simazine to the aquatic macrophyte, *Vallisneria americana* Michx. Environ. Toxicol. Chem. 29: 201–211.