Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 170 (2017) 234–241

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Detection of herbicide effects on pigment composition and PSII photochemistry in *Helianthus annuus* by Raman spectroscopy and chlorophyll *a* fluorescence

Petr Vítek, Kateřina Novotná, Petra Hodaňová, Barbora Rapantová, Karel Klem *

Global Change Research Institute CAS, v.v.i., Bělidla 4a, 603 00 Brno, Czech Republic

ARTICLE INFO

Article history:
Received 5 May 2016
Received in revised form 28 June 2016
Accepted 13 July 2016
Available online 15 July 2016

Keywords:
Raman mapping
Chlorophyll fluorescence
Carotenoids
Flavonoids
Photosynthesis
Secondary metabolites

ABSTRACT

The effects of herbicides from three mode-of-action groups – inhibitors of protoporphyrinogen oxidase (carfentrazone-ethyl), inhibitors of carotenoid biosynthesis (mesotrione, clomazone, and diflufenican), and inhibitors of acetolactate synthase (amidosulfuron) – were studied in sunflower plants (*Helianthus annuus*). Raman spectroscopy, chlorophyll fluorescence (ChlF) imaging, and UV screening of ChlF were combined to evaluate changes in pigment composition, photosystem II (PSII) photochemistry, and non-photochemical quenching in plant leaves 6 d after herbicide application. The Raman signals of phenolic compounds, carotenoids, and chlorophyll were evaluated and differences in their intensity ratios were observed. Strongly augmented relative content of phenolic compounds was observed in the case of amidosulfuron-treated plants, with a simultaneous decrease in the chlorophyll/carotenoid intensity ratio. The results were confirmed by in vivo measurement of flavonols using UV screening of ChlF. Herbicides from the group of carotenoid biosynthesis inhibitors significantly decreased both the maximum quantum efficiency of PSII and non-photochemical quenching as determined by ChlF. Resonance Raman imaging (mapping) data with high resolution (150,000–200,000 spectra) are presented, showing the distribution of carotenoids in *H. annuus* leaves treated by two of the herbicides acting as inhibitors of carotenoid biosynthesis (clomazone or diflufenican). Clear signs were observed that the treatment induced carotenoid depletion within sunflower leaves. The depletion spatial pattern registered differed depending on the type of herbicide applied.

1. Introduction

With their proportion in global pesticide consumption of 50–60%, herbicides constitute the most frequently used group of pesticides [1]. Moreover, herbicide use is increasing in global crop production. The herbicide market grew by 39% between 2002 and 2011, and it expanded particularly strongly in developing countries [2]. The high-frequency use of active ingredients with the same mode of action, and sometimes at relatively high doses, leads to numerous problems, including in particular (among others) the selection of resistant weed populations [3]; contamination of soil, surface, and ground water [4]; damage to crops [5]; effects on non-target organisms [6]; and impacts on human health [7]. According to Pimentel and Levitan [8], <0.1% of pesticides reach the target pests, and so the potential for adverse effects on ecosystems and the environment is considerable. Toward addressing this problem, reliable, fast, and low-cost diagnostic methods are needed to assess herbicide uptake, translocation, and biological efficacy in both target and non-target organisms. Methods based on chlorophyll *a* fluorescence (ChlF) were developed to detect photosystem II (PSII) inhibitors in the environment [9]. More recently, ChlF was employed in detecting activity of acetolactate synthase (ALS) inhibitors [10], carotenoid biosynthesis [11], and protoporphyrinogen oxidase (PPO) inhibitors [12].

Raman spectroscopy was established as a nondestructive alternative to conventional chromatographic methods used for qualitative and quantitative analysis of biomolecules in biological samples, including plant tissues [13–17]. Chromatographic methods, in contrast to Raman spectroscopy, required demanding sample preparation and solvent extraction prior to analysis [18,19]. It has been shown that Raman spectroscopy enables detection of the pigments necessary for photosynthetic, photoprotective, and antioxidant activities, in particular chlorophylls, carotenoids, and phenolic compounds [16]. The biosynthetic pathways of these three groups of pigments examined in this study constitute an important target of numerous herbicides.

The occurrences of chlorophyll, a key light-harvesting pigment, and carotenoids are closely linked in the photosynthetic apparatus. The

* Corresponding author.
E-mail addresses: vitek.p@czechglobe.cz (P. Vítek), novotna.k@czechglobe.cz (K. Novotná), rajsnerova.p@czechglobe.cz (P. Hodaňová), rapantova.b@czechglobe.cz (B. Rapantová), klem.k@czechglobe.cz (K. Klem).

http://dx.doi.org/10.1016/j.saa.2016.07.025
1386-1425/

content of chlorophylls can be affected either directly or indirectly by the action of several herbicides, including PSI and PSII inhibitors, PPO inhibitors, and carotenoid biosynthesis inhibitors [20]. Raman spectroscopy of chlorophylls is described in such studies as those of Lutz and Breton [21] and Koyama et al. [22].

Carotenoids are pigments involved in photosynthesis, photoprotection, and membrane stabilization, and they are characterized by a long conjugated double-bond system composed of isoprenoid units. One important role of xanthophyll carotenoids is dissipation of excess excitation energy in the xanthophyll cycle, which is considered a key photoprotective mechanism in higher plants and algae [23,24]. Carotenoid biosynthesis in plants can be inhibited by herbicides at different steps, such as in phytoene desaturase (PDS) [25], hydroxyphenylpyruvate dioxygenase (HPPD) [26], or the chloroplastic isoprenoid pathway [27]. Carotenoids have typical Raman features related especially to the polyene chain [13,28]. The stretching vibrational mode of ν_1(C═C) is located in the region 1490–1540 cm^{-1} and was examined for the purpose of this study. Other corroborative bands of strong and medium intensity, occur in the region 1150–1160 cm^{-1} corresponding to ν_2(C—C) vibrations in the polyene chain and in 1000–1010 cm^{-1}, respectively, where rocking vibrations of methyl groups occur (δ(C-CH_3)) [13].

Phenolic compounds are secondary metabolites that include flavonoids, tannins, hydroxycinnamate esters, as well as structural biopolymer lignin. These are the most abundant secondary metabolites with common origin in the phenylpropanoid biosynthetic pathway [29]. One important subgroup consists of the flavonoid pigments. The subgroup is made up of more than 6000 flavonoids discovered to date, and these are divided into several classes [30–33]. Some molecules are responsible for strong coloration, such as red to blue anthocyanins, while others are colorless (see Gamsjaeger et al. [33], who studied flavonoids and carotenoids using Raman spectroscopy in petals of pansy cultivars *Viola* × *wittrockiana*). The biosynthesis of secondary metabolites can be strongly influenced by herbicides in both directions: biosynthesis suppression and stimulation. For example, the application of ALS inhibitors has been shown to increase the content of cinnamate-derived phenolic compounds [34].

Raman imaging has become an important tool in diverse analytic fields and has been used for spatial mapping of analytes within various biological matrices. These include lignin-cellulose tissues [35–37] and carotenoids within plant leaves [38,39], the later using especially the NIR-FT Raman technique. This study presents data that complement results from point measurements, showing the application of high resolution Raman imaging using visible excitation (514.5 nm) for determining the carotenoid distribution in sunflower plants (*Helianthus annuus*) treated by two different herbicides acting as carotenoid biosynthesis inhibitors. The strength of the green excitation for analysis of carotenoids lies in resonance Raman enhancement [13,15,28,40]. Due to this effect, the excitation wavelength around 500 nm is extremely favorable for detecting carotenoids even at low concentrations down to 0.1 mg kg^{-1} [41,42], and it enables effective detection of carotenoids within a Raman mapping procedure. The potential for evaluating the biological effect of herbicides and their translocation within leaves using Raman spectroscopy is quite high with respect to the major group of compounds detectable by this technique. According to available information however, no reports on such use have yet been published.

The main objective of this study was therefore to compare techniques based on ChlF and Raman spectroscopy in evaluating the effects of herbicides from three groups with different modes of action: PPO inhibitors (carfentrazone-ethyl), ALS inhibitors (amidosulfuron), and carotenoid biosynthesis inhibitors affected at three steps (PDS inhibition by diflufenican, HPPD inhibition by mesotrione, and isoprenoid biosynthesis inhibition by clomazone). The basic hypothesis of the study was that Raman spectroscopy would be complementary to ChlF in detecting biological effects, primarily through detecting changes in the content of carotenoids and phenolic compounds.

2. Materials and methods

2.1. Plant material and herbicide treatments

Sunflower (Alexandria variety) plants were grown in FS-4600 growth chambers (Photon Systems Instruments spol. s r.o., Brno, CZ) under a 15 h day/9 h night regime. During the night, a temperature of 15 °C and relative humidity of 80% were maintained. During the day, temperature, relative humidity, and photosynthetically active radiation intensity were progressively brought up to 25 °C, 60%, and 1000 μmol m^{-2} s^{-1}, respectively, over the first 6 h; maintained at constant levels for 3 h; then progressively changed to their night levels over the next 6 h. Light was provided by super bright white LEDs. Plants were grown for 3 weeks until the second pair of true leaves growth stage in round pots ca 10 cm in diameter and ca 0.5 L in volume. Growth medium was horticultural substrate with basic nutrients (Agro CS, Česká Skalice, CZ).

Herbicides were applied in the second pair of true leaves growth stage. A Solo 402 handheld sprayer (SOLO Kleinmotoren GmbH, Sindelfingen, Germany) was used at a spray volume of 30 mL m^{-2}. The following doses of herbicide active ingredients corresponding to the recommended doses were applied: 2 μg m^{-2} carfentrazone-ethyl (Aurora 50 WG, FMC Corporation, Philadelphia, PA, USA), 19.2 μg m^{-2} mesotrione (Callisto 480 SC, Syngenta Limited, Guildford, UK), 9.6 μg m^{-2} clomazone (Command 48 EC, FMC Corporation), 12.5 μg m^{-2} diflufenican (Sempra, AgriChem B.*V*., Oosterhout, NL), and 3 μg m^{-2} amidosulfuron (Grodyl 75 WG, Bayer CropScience AG, Monheim, DE). These active ingredients represent the following chemical groups and modes of action: Carfentrazone-ethyl, a triazolone herbicide, inhibits PPO, an enzyme that acts during synthesis of protoporphyrin IX, which is a precursor for chlorophyll. Mesotrione, a triketone herbicide, inhibits p-hydroxyphenylpyruvate dioxygenase, which acts during metabolism of carotenoid biosynthesis. Clomazone, an isoxazolidinone herbicide, inhibits isoprenoid (carotenoid) biosynthesis. Diflufenican, a pyridine herbicide, inhibits carotenoid biosynthesis by influencing PDS. Amidosulfuron, a pyrimidinylsulfonylurea herbicide, inhibits ALS, a key enzyme in the pathway for biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine.

After thorough drying of spray liquids on leaf surfaces (ca 4 h), the plants were transferred back into growth chambers for 6 d until Raman spectroscopy, ChlF imaging, and UV screening of ChlF measurements.

2.2. Raman spectroscopy and imaging

Point Raman analysis was undertaken on an *InVia* spectrometer (Renishaw, Wotton-under-Edge, UK) equipped with a Leica confocal microscope. The 785 nm (diode laser) excitation line was used and a 50× magnification Leica objective (NA = 0.75) was employed for point analysis. Spectra were recorded in static mode in the spectral range 650–1750 cm^{-1} as 3 s scans accumulated 5× times using 30 mW laser power at source. The spectra were measured at the adaxial surface of circular pieces of leaves (~6 mm in diameter) taken from the second pair of leaves.

For Raman imaging, the same *InVia* spectrometer was used in streamline mode (linefocus). Prior to mapping acquisition, it was necessary to determine the balance between sufficient Raman signal and heat-induced destruction of the sample. Both are controlled especially by exposure time, laser power, as well as shape and/or size of the laser spot. According to Nasdala et al. [43], the line-by-line mapping used here is advantageous in this regard compared to point-by-point mapping due to its distribution of beam energy over a larger area. The cut leaf was attached to a glass slide using double-sided glue tape to obtain a consistent focal plane and then the leaf's adaxial surface was immediately subjected to mapping. For carotenoid imaging, an Ar laser