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Detecting Phytochemicals on Plant Surfaces: Non-Destructive Fluorescence Methods

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Phytochemicals compounds such as polyphenols are of large interest because of the multiple roles they can play in plants and for their antioxidant properties beneficial for the human beings. For this, the possibility to control in situ their concentrations rapidly and non-destructively can be a useful tool for plant physiology studies as well as to assess vegetable and fruit quality.

Within the last 20 years, a new fluorescence spectroscopic method, aimed to estimate phenolic compounds on plant superficial tissues, has been developed. It is based on the filtering effect of phenolic compounds present in leaf epidermises and fruit skins that reduce the incoming light impinging on the under-laying chlorophyll. The more the phenolic concentration, the less the light, at specific wavelengths, seen by Chl and the less the Chl fluorescence (ChlF) detected. Because of its straightforwardness, the method was lately called the ABC (Agati-Bilger-Cerovic) fluorescence method, referring to the authors who proposed and validated it by laboratory studies [1-3]. The derived index of a particular class of compounds is related to the comparison between the ChlF signals excited at two excitation wavelengths: one absorbed by the compound to be detect (for example in the UV-A for flavonols and in the green for anthocyanins) and one not absorbed (in the red), as reference.

It is worth noting that the ABC fluorescence (ABC-fluo) method represents a different use of Chl fluorescence with respect to the PAM technique, largely employed to assess the photosynthetic activity of leaves by measuring the fluorescence induction kinetics occurring during the transition from dark to light [4].

The great advantage of the ABC-fluo technique, as any rapid non-invasive optical technique, is that it allows for the monitoring of phenolic compounds accumulation and changes in the same sample over time. It is, therefore, more powerful than destructive methods, where data from different samples are compared, and more representative of the process under study, being the fluorescence data collected much higher in number than those usually acquired from the chemical analysis.

A limit of the method is that it can detect only surface-located compounds and not the internal ones. Consequently, it gives significant information only when superficial concentrations are correlated to the total ones, as proved for leaf flavonoids in some species [5, 6].

The ABC-fluo method works properly when the compound to be detected is spatially well separated by the first Chl layer. In leaves, the sub-epidermal localization of Chl

allows for detection of transmittance (absorbance) of compounds in the epidermis. Therefore, only phenols present in the epidermis can be assessed. Localization of phenolic compounds by multispectral fluorescence microscopy in cross sections of grapevine leaves proved that flavonols but not hydroxycinnamic acids can be detected non-destructively by the fluorimetric method [6].

In fruits, it may occur that flavonoids are mostly localized above Chl [7, 8] or they are partially co-localized with Chl, as is the case of anthocyanins in olives [9] or grapevine berries [1]. In this evenience, only a fraction of the flavonoid layer can be quantified by the ABC-fluo technique.

The ABC-fluo approach was first applied by recording the whole ChlF excitation spectra from leaves of Arabidopsis thaliana wild type and mutants deficient in flavonoids or/and sinapate esters [10]. The comparison of the ChlF excitation spectra of an intact leaf and the same leaf devoid of the epidermis clearly showed the UV absorption properties of the superficial leaf layers [2, 3]. Spectral application of the method to adaxial and abaxial sides of bifacial leaves, or to older and younger segments of monocotyledonous leaves allowed for the assignment of the type of UV-screening compounds present in the leaf epidermis [3].

The increase in leaf epidermal absorbance with increasing solar irradiance was found in three woody species belonging to different chemotaxons, (Morus nigra L., Prunus mahaleb L. and Lagerstroemia indica L.) acclimated to different light microclimates and related to flavonoids and hydroxycinnamic acids [11]. The in vivo absorption spectra of Phyllirea latifolia L. and Myrtus communis L. were characterized by a band centered at 360–380 nm, which was greater in sun than in shade leaves and in the adaxial than in the abaxial surfaces. By HPLC analysis of leaf extracts, it was concluded that quercetin, luteolin and myricetin derivatives were responsible for the observed light-induced changes in the spectral features of examined tissues [12].

During the last decades several instruments applying the ABC-fluo method to detect UV transmittance of leaf epidermis associated with its phenolic content have been developed. Self-assembled apparatus were used to detect non-destructively flavonoids on marketable broccoli heads [13]. The Xe-PAM fluorometer (Xe discharge lamp and Pulsed Amplitude Modulation, Walz, Effeltrich, Germany) provides three bands of excitation, in the UV-B (λmax 314 nm), UV-A (λmax 366 nm) and blue-green (400–550 nm) spectral ranges, and detects ChlF above 700 nm. It has been used by several authors [5, 14-17] to investigate the flavonoids accumulation due to UV-B radiation. Applications of the ABC-fluo technique by the Xe-PAM device concerned: i) the effects of enhanced and reduced solar UV-B on leaf epidermis UV transmittance [14]; ii) the decrease of epidermal UV-transmittance induced specifically by low temperature in the absence of UV-B radiation in different species [17]; iii) the estimation of UV photo protection efficiency in natural phytoplankton, ascribed to UV absorption by mycosporine-like amino acids [18]; iv) the influence of environmental factors, such as UV-B radiation and temperature, on epidermal UV-B screening of several arctic plants [19] and alpine plants along a latitudinal gradient in Europe [20]; v) longitudinal distribution of UV-absorbing screening pigments in barley leaves [21].

Since the Xe-PAM fluorometer operates on detached leaves, it may not be considered non-invasive. To overcome to this limit, the UV-A PAM fluorometer (Gademann Instruments, Würzburg, Germany) for assessing epidermal compounds on attached leaves in the field was developed [22]. It provides two bands of excitation in the UV-A (peak 375 nm) and blue (peak 400 nm) spectral regions and detects ChlF above 650 nm. The UV-A-PAM sensor was used to study UV-A protection in mosses during their acclimation to different light regimes [23]. It was employed by Barnes et al. (2008) to detect the rapid, diurnal changes in epidermal UV-A transmittance for three herbaceous species (Verbascum thapsus, Oenothera stricta, Vicia faba) grown in naturally high UV environments in tropical subalpine habitats. Krause et al. (2003), instead, utilized the UV-A-PAM to compare the UV-A shielding in sun and shade leaves of tropical forest plants. UV-A-PAM measurements on curly kale leaves were found to be well correlated with the quercetin content determined by HPLC [24]. Other recent

applications of the ABC-fluo method by the UV-A-PAM sensor comprised: i) the non-destructive detection of flavonoids response to UV exposure in the Sinapis alba L. and Nasturtium officinale L. Brassicaceae [25]; ii) the measurement on a daily basis of kinetics of epidermal UV shield (%) buildup in soybean leaves receiving full (VIS + UV) or UV-attenuated (VIS) solar radiation [26]; iii) studying of the effects of UV radiation exposure on broccoli plants related to the interactions with herbivorous insects [27]; iv) the evaluation of pre-harvest UV-B treatments at 22 °C and 9 °C and controlled storage conditions on epidermal UV-A absorbance in pak choi (Brassica campestris L. ssp. chinensis var. communis) leaves [28]. Berry skin UV absorbances in the berries of the Bacchus white grape cultivar were investigated by the Xe-PAM and UV-A-PAM fluorimetric methods. Comparison of non-destructive data with those from wet chemistry on skin extracts suggested that UV screening in berries depends almost exclusively on flavonol content [8].

Using the blue-excited ChlF as reference signal limits the application of both the Xe-PAM and UV-A-PAM fluorometers to sample free of blue-absorbing compounds in the epidermis, as evidenced by Barnes et al. (2000) and Pfündel et al. (2007) in anthocyanin-containing leaves. For these, the Dualex Scientific+ (Force-A, Orsay, France) sensor, providing excitation in the UV-A (375 nm), in the green (520 nm) and in the red (657 nm), can be used. Dualex applications included: i) the finding of a longitudinal gradient of flavonoids content in wheat leaves [29]; ii) the detection of epidermal UV transmittance on dicots and monocots species [30-32]; iii) the investigation of the epidermal phenols photo protection against natural UV light in oak leaves during the growth season, proposing an optical signature of tree phenology to probe in vivo the maturity state of leaves [30]. Meyer et al. (2006), combining Dualex epidermal polyphenol measurements with chlorophyll and leaf dry mass per area data, proposed a signature of the switch of plant investment from protein into polyphenol production. The Dualex in vivo estimation of the epidermal flavonoid contents in Betula pendula Roth leaves of different ages under altered UV radiation showed that silver birch leaves modulate their protective pigments in response to changes in the UV environment during their expansion [33]. The Dualex meter was also used to select in the field sun-exposed and shaded grapevine leaves with higher and lower preformed flavonoids, respectively, and study their role in the response of plants to Plasmopara viticola inoculation [6]. The Dualex and the UV-A-PAM fluorometers were compared on leaves of a wide range of different species [34].

Applications of the ABC-fluo technique to relatively large (up to 8-cm in diameter) and thick samples was then possible with the development of the Multiplex sensor (Force-A, Orsay, France) [35]. It provides excitation in the UV-A (375 nm), blue (450 nm), green (520 nm) and red (630 nm) spectral regions and detects over three channels in the blue-green, red and far-red spectral regions, these two last at 680–690nm and 730–780 nm, respectively, corresponding to the two emission peak of chlorophyll.

The Multiplex sensor provided an index of flavonols and flavons in the whole apical part, three leaf-pairs for each shoot, of Ligustrum vulgare potted plants at 30% or 85% full sunlight in the presence or in the absence of UV radiation. This index was greater in leaves growing at 85% than at 30% sunlight, irrespective of UV irradiance. When plants acclimated for 3 weeks to 30% sunlight, in the presence or in the absence of UV irradiance, were transferred to 85% sunlight, the flavonoid index exponentially increased, reaching a maximum within 10 days [12]. The natural temporal increase in epidermal flavonols was detected by the Multiplex sensor in Hybrid poplar (Populus × canadensis) saplings to study the seasonal effects on the plant response to UV-A irradiance concerning photosynthesis and isoprene emission [36].

The Multiplex sensor was employed to assess the flavonol content in apple [37] and kiwifruit [38] exocarps, as well as to detect anthocyanins accumulation in grapevine bunches in the vineyards [39, 40].

The non-destructive detection of epidermal flavonols by the Dualex and Multiplex devices was also used to define an indicator of leaf nitrogen status superior to those based on the detection of chlorophyll alone [29, 41-43].

It is also worthy to mention the application of the ABC-fluo method as imaging technique for: i) detecting the spatial distribution of phenolic compounds over the leaf lamina [44]; deriving the distribution of UV-shielding in broad Fagus sylvatica L. leaves [45]; assessing flavonols in white grape berries [46] and anthocyanins in whole red grape bunches [47].

In conclusion, there is now a huge amount of data that validated the ABC-fluo method for the non-destructive assessment of epidermal compounds absorbing in the UV or in the visible (anthocyanins). The method has been used both in the laboratory by spectrofluorimeters or filter-based bench devices and in the field by using optical portable sensors. Examples of mapping of flavonoids spatial distribution obtained using sensors as manual proximal sensing or mounted on a utility vehicle exist.

The method cannot substitute wet chemistry when the composition of phenolic compounds is wanted or if the total amount of compounds, in the case they are uncorrelated to those localized into the superficial tissues, is searched. However, the possibility to perform a large number of parallel measurements with minimal sample preparation on the same target over time, considering also the large variability of vegetation attributes, makes the ABC-fluo an important tool of investigation in plant science.

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