A new method for determination of the photosynthetic pathway in grasses

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Received: 25 February 2019 / Accepted: 8 May 2019 / Published online: 15 May 2019
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Abstract
An easy and inexpensive method of determining the photosynthetic pathway in grasses using a dye widely used in microscopy. To evaluate the efficiency of a new histochemical test for determination of the photosynthetic pathway in grasses (Poacea). Leaves of 58 grass species were sectioned transversally, and the sections treated with a 2% sodium hypochlorite solution to clarify the tissue. After discoloration, sections were washed with distilled water and double-stained with astra blue and safranin (1% each in 50% ethanol) for 1 min. Sections were then mounted between microscopy glass slides and coverslips using water. Grass species showing red staining of the bundle sheath cells were considered C4, and species with translucent bundle sheath were considered C3. The results of the histochemical test were then compared with results from carbon isotope composition analysis and the relevant scientific literature. Observations from the histochemical test were congruent with results from δ13C isotope composition analysis, and with data previously presented in the scientific literature. The proposed histochemical test proved efficient for characterization of the photosynthetic pathway in the tested grasses; however, the method should be further tested in a greater number of grass species, encompassing, preferably, all Poacea subfamilies. Future studies may elucidate if the proposed method can effectively be used in other botanical families. Furthermore, additional investigations may determine whether the phenolic compounds indicated by the histochemical test are exclusive to the bundle sheath of C4 grasses and if possible relations exist between these phenolic compounds and the C4 photosynthetic pathway in grasses.

Keywords Poaceae · Histochemistry · Safranin · C4 · Phenolic · Grasses

Introduction
The C4 photosynthetic route evolved in plants tens of millions of years ago in response to environmental changes, characterizing an advantage under high light, high temperature, or draught conditions (Ehleringer et al. 1991; Bowes 1993; Sage 2004).

There are 19 botanical families with C4 species (Sage et al. 2012). Among them, Poaceae is the most diverse with 4500 C4 species (Christin et al. 2008; Vicentini et al. 2008; Bouchenak-Khelladi et al. 2009; Christin and Bernard 2009), which comprises ca. half of all C4 species (Sage et al. 1999). Within grasses, all C4 lineages occur in branches denominated PACMAD, including the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Dantoideae (Kellogg 1999, 2001; Sage et al. 2011).

The distinction between C3 and C4 grasses is not often apparent, although some characteristics can aid in the process, with emphasis on: anatomic characters; carbon isotope composition and CO2 compensation point; and the activity of specific enzymes. Many authors have attempted to use anatomical characteristics (Hattersley and Watson 1975, 1976; Hattersley and Browning 1981; Hattersley 1984; Prendergast and Hattersley 1987; Prendergast et al. 1987; Dengler et al. 1994), however, evaluation by anatomical traits alone led to much confusion. Carbon isotope composition (δ13C) is also used as a distinction criterion between C3 and C4 plants. Values between –10 and –14‰ indicate a
C4 plant, while values between −20 and −35‰ indicate a C3 plant (Cerling 1999); however, this method requires expensive, high-maintenance equipment. Other methods were employed for the determination of photosynthetic pathways in plants (Edwards and Walker 1983; Hatch 1987; Sage et al. 1987), but always with caveats related to time and cost issues.

Neto (2017) proposed a histochemical method for determination of the photosynthetic pathway in grasses using Safranin O. The test is positive for C4 grasses when cells of the bundle sheath stain strongly red. In C3 grasses, cells of the bundle sheath do not stain red. Confirmation of the efficiency of the histochemical test would provide an additional—as well as convenient and cheap method for determination of the photosynthetic pathway in grasses. However, validation of the test requires evaluation of its efficiency in a larger number of grass species, as well as confirmation of whether similar results can be obtained among the three types of C4 grasses [NAD-dependent malic enzyme (NAD-ME), PEP-Carboxikinase (PCK), and NADP-dependent malic enzyme (NADP-ME)], comparing the results from the histochemical test with carbon isotope composition analysis and information within the relevant literature.

Materials and methods

Plant material collection and preparation

Control leaf samples were collected from grasses with known photosynthetic pathways (rice—C3; maize and sugarcane—C4 NADP-ME). Leaves of 15 identified forage species and 14 identified bamboo species, of several genera, were collected from plants cultivated at Universidade Federal de Santa Catarina’s Fazenda Experimental da Ressacada, Florianópolis, Santa Catarina, Brazil. Additional leaf samples were obtained from 12 grass species cultivated at Embrapa’s Amazonia Oriental experimental area in Belém, Pará, Brazil, and 13 wild grass species in Santa Catarina state. Dried specimens from grasses collected in Belém were deposited at the Museu Paraense Emílio Goeldi’s herbarium (Herbarium MG), where specimens were identified by means of botanical comparison, and dried specimens of grasses collected in Santa Catarina were deposited at Universidade Federal de Santa Catarina’s herbarium (Herbário Flor), where specimens were also identified by means of comparison.

Leaves collected for determination of carbon isotope composition were placed in paper bags for subsequent dehydration, and leaves used in the histochemical test were fixated in 70% ethanol.

Determination of stable carbon isotope composition (δ¹³C) in grasses

Leaf samples were dehydrated in a drying stove (50 °C, until constant mass). Afterwards, leaves were ground in liquid nitrogen with a mortar and pestle until a fine powder was obtained. Ground leaf samples were analyzed at the Laboratório de Ecologia Isotópica—CENA/USP (Piracicaba, SP). About 2.5 mg of leaf material were conditioned inside aluminium capsules and inserted in an element analyser (EA CHN Carlo Erba—modelo E-1110, Milan, Italy), for determination of the total carbon concentration. The gases generated by the samples’ combustion were purified and injected directly into a mass spectrometer (IRMS Delta Plus, Thermo Scientific, Bremen, Germany), for determination of isotope ratios. The natural abundance of ¹³C is expressed as the notation delta (δ) and as a value given in parts per thousand (‰), being determined by means of the equation:

\[ δ^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \]

where \( R_{sample} \) and \( R_{standard} \) are the molar ratios \( ^{13}C/^{12}C \) of the sample and the standard, respectively. The employed standard was the fossil limestone PeeDeeBelamnitte (PDB). At each analysis round reference leaf material of known carbon concentration and \( δ^{13}C \) was used (sugarcane leaves, \( δ^{13}C = -12.7 \; \text{‰}; \%C = 43.82\% \)). The acceptable analytical error for carbon concentration determination is 0.3% and for \( δ^{13}C \) is 0.3‰.

Histochemical test for determination of the photosynthetic pathway in grasses

Leaves fixed in 70% ethanol were sectioned transversally at their middle portion by hand with stainless steel blades. Leaf sections were treated with 2% sodium hypochlorite solution for tissue clarification. After discoloration, sections were washed with distilled water and double-stained with astra blue and safranin (1% each in 50% ethanol) for 1 min. Sections were then mounted between microscopy glass slides and coverslips, with a drop of water between them. Photomicrographs were obtained using an Olympus BX40 optical microscope attached to an Olympus DP71 camera.

Results

Results from the histochemical test differed between C3 and C4 grasses. C4 grasses presented bundle-sheath cells stained red, while C3 did not. Results for Arundo donax and
Uruchloa brizantha clearly illustrate the distinction (Fig. 1). These results are compatible with those provided in Neto (2017).

Table 1 shows the results of the carbon isotope composition analysis and of the histochemical test of 58 grass species, representing 40 genera, 12 tribes and 6 subfamilies. The results from isotope composition ($\delta^{13}\text{C}$) analysis of all evaluated grasses correlated with the histochemical test. All grasses with isotope composition values between $-26.09$ and $-29.26$ indicated a C3 plant, and presented a negative result in the histochemical test. In contrast, all plants with isotope composition values between $-9.88$ and $-14.21$ indicated a C4 plant, and presented a positive result in the histochemical test.

The histochemical test showed a negative result for all evaluated bamboos, and confirmed the photosynthetic pathway of maize and sugarcane (C4 type NADP-ME, with positive results) and rice (C3, with a negative result), and positive to the three photosynthetic pathway grasses (PDK, NAD-ME and NADP-ME) (Table 1).

Among the forage grasses, only Festuca aurora, Festuca arundinacea, and Holcus lanatus showed negative results. Among the tested wild grasses, Homolepsis aturensis, H. isocylicia, Steinchis malaxum, and A. donax showed negative results, indicating a C3 photosynthetic pathway. All other tested species of forage and wild grasses presented a positive result, indicating they have C4 photosynthetic systems.

Leaf transversal sections that underwent dehydration during performance of the histochemical test presented a slight detachment of the cell wall membrane, and this allowed inferring that the safranin-reactive phenolic substance is located in C4 grasses’ bundle sheath cells’ protoplasts, and not in the cell wall (data not shown).

**Discussion**

Safranin O is the least specific histochemical indicator of lignin because it not only stains lignin, but all related phenolic substances (Lewis and Yamamoto 1990). The red staining exclusively observed in cells from the bundle sheath of C4 grasses indicates the existence of a phenolic substance only in these cells.

Many plant species present hydroxycinnamic acids (p-coumaric, sinapic and ferulic acids) attached to cell walls (Chen et al. 1998; Brett et al. 1999; Bunzel et al. 2003). Derived from the phenilpropanoid metabolism (Ou and Kwok 2004), ferulic acid (4-hydroxy-3-methoxycinnamic) is abundant in plant epidermis, xylem, sheath bundle, and sclerenchyma cells (Faulds and Williamson 1999; Lambert
Table 1 Results of the histochemical test (H.T.) and isotopic composition (δ^{13}C, average of three replicates and SD) of 58 grass species

| Categories/species | Subfamily     | Tribe             | P.P.                  | H.T.                  | δ^{13}C‰                  |
|--------------------|---------------|-------------------|-----------------------|-----------------------|--------------------------|
| **Forage**         |               |                   |                       |                       |                          |
| Axonopus cothinasis| Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -12.39 ± 0.29            |
| Brachiaria brizantha| Panicoideae   | Paniceae          | PCK                   | +                     | -11.77 ± 0.17            |
| Brachiaria decumbens| Panicoideae   | Paniceae          | PCK                   | +                     | -12.27 ± 0.27            |
| Cynodon nlemfensis | Chloridoideae | Cynodonteae       | NAD-ME (G)            | +                     | -13.46 ± 0.16            |
| Festuca arundinacea| Pooidae       | Poeae             | C3                    | -                     | -26.09 ± 0.30            |
| Festuca aurora     | Pooidae       | Poeae             | C3                    | -                     | -28.14 ± 0.12            |
| Hemitrichia altissima| Panicoideae  | Andropogoneae     | NADP-ME (G)           | +                     | -11.68 ± 0.24            |
| Holcus lanatus     | Pooidae       | Poeae             | C3                    | -                     | -29.26 ± 0.06            |
| Panicum maximum    | Panicoideae   | Paniceae          | PCK (E)               | +                     | -12.45 ± 0.15            |
| Paspalum atratum   | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -12.06 ± 0.12            |
| Paspalum notatum   | Panicoideae   | Paniceae          | NADP-ME (S)           | +                     | -11.99 ± 0.13            |
| Pennisetum americanum| Panicoideae  | Paniceae          | NADP-ME (G)           | +                     | -12.54 ± 0.12            |
| Pennisetum purpureum| Panicoideae  | Paniceae          | NADP-ME (S)           | +                     | -9.88 ± 0.14             |
| Pennisetum setaceum| Panicoideae  | Paniceae          | NADP-ME (G)           | +                     | n                        |
| Setaria anceps     | Panicoideae   | Paniceae          | NADP-ME (S)           | +                     | -11.04 ± 0.12            |
| Sorghum sudanense  | Panicoideae   | Andropogoneae     | NADP-ME (G)           | +                     | -12.80 ± 0.09            |
| **Bamboo**         |               |                   |                       |                       |                          |
| Bambusa oldhamii   | Bambusoideae  | Bambuseae         | C3                    | -                     | -28.66 ± 0.23            |
| Chimonobambusa quadrangulares| Bambusoideae | Bambuseae         | C3                    | -                     | -31.01 ± 0.10            |
| Chusquea leptophyla| Bambusoideae  | Bambuseae         | C3                    | -                     | -27.92 ± 0.18            |
| Dendrocalamus asper| Bambusoideae  | Arundinariae       | C3                    | -                     | -26.42 ± 0.12            |
| Drepanostachyum falcatum| Bambusoideae| Arundinariae       | C3                    | -                     | -29.12 ± 0.12            |
| Fargesia gaolinensis| Bambusoideae | Arundinariae       | C3                    | -                     | -27.71 ± 0.07            |
| Guadua chacoensis  | Bambusoideae  | Bambuseae         | C3                    | -                     | -30.83 ± 0.19            |
| Parodiolyra micrantha| Bambusoideae| Olyraceae         | C3                    | -                     | -28.70 ± 0.20            |
| Phyllostachys nigra nigra| Bambusoideae| Bambuseae         | C3                    | -                     | -29.36 ± 0.16            |
| Pleioblastus simonii| Bambusoideae | Bambuseae         | C3                    | -                     | -28.05 ± 0.15            |
| Pseudosasa japônica| Bambusoideae  | Bambuseae         | C3                    | -                     | -29.15 ± 0.15            |
| Raddia soderstromii| Bambusoideae  | Olyraceae         | C3                    | -                     | -29.16 ± 0.08            |
| Sasa palmate       | Bambusoideae  | Bambuseae         | C3                    | -                     | -29.91 ± 0.18            |
| Thysostachys siamensis| Bambusoideae| Bambuseae         | C3                    | -                     | -28.99 ± 0.09            |
| **Collected**      |               |                   |                       |                       |                          |
| Arundo donax       | Arundinoideae | Arundineae        | C3                    | -                     | -30.30 ± 0.09            |
| Cenchrus echinatus  | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -12.12 ± 0.14            |
| Cymbopogon citratus| Panicoideae   | Andropogoneae     | NADP-ME (G)           | +                     | -12.4 ± 0.20             |
| Digitaria ciliates  | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -11.99 ± 0.18            |
| Digitaria horizontalis| Panicoideae| Paniceae          | NADP-ME (G)           | +                     | -12.0 ± 0.20             |
| Echinochloa colona  | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -13.02 ± 0.14            |
| Echinochloa crus-pavonis| Panicoideae| Paniceae          | NADP-ME (G)           | +                     | -12.64 ± 0.16            |
| Echinochloa polystachya| Panicoideae| Paniceae          | NADP-ME (G)           | +                     | -11.88 ± 0.26            |
| Eleusine indica    | Chloridoideae | Cynodonteae       | NAD-ME (G)            | +                     | -13.3 ± 0.09             |
| Ergrogris tenfolia | Chloridoideae | Ergrostideae      | NAD-ME (G)            | +                     | -14.21 ± 0.07            |
| Homolepis atrensis | Panicoideae   | Paniceae          | C3                    | -                     | -28.8 ± 0.12             |
| Homolepis isocaylica| Panicoideae  | Paniceae          | C3                    | -                     | -26.3 ± 0.13             |
| Hymenachne amplexicaulis| Panicoideae| Paniceae                  | C4                    | +                     | -12 ± 0.14               |
| Megathyrsus maximus | Panicoideae   | Paniceae          | C4 (G)                | +                     | -13.47 ± 0.24            |
| Paspalum conjugatum | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -12.3 ± 0.13             |
| Paspalum maritimum  | Panicoideae   | Paniceae          | NADP-ME (G)           | +                     | -11.6 ± 0.18             |
| Paspalum melanospermum | Panicoideae| Paniceae          | NADP-ME (G)           | +                     | -11.8 ± 0.09             |
et al. 1999), while also being present on primary and secondary cell walls in grasses (Harris and Hartley 1976).

The red stain observed in bundle sheath cells may indicate the presence of hydroxicinamic acid. However, the observation that the phenolic substances revealed by the histochemical test are located in the protoplasts of bundle sheath cells, and not in the cell wall, suggests such substances are likely to perform another important function inside the cells. The presence of these substances exclusively in cells of C4 grasses' bundle sheaths may indicate an antioxidant function in view of the capacity antioxidant of these substances. According to Faulds and Williamson (1999) hydroxycinamic acids, such as ferulic, sinapic, caffeic and p-coumaric acid, are found both covalently attached to the plant cell wall and as soluble forms in the cytoplasm.

The results of the histochemical test of bamboos were expected, given that bamboos are included in the Bambusoideae subfamily, which is composed exclusively of C3 species, distributed between the Bambuseae, Olyreae and Arundinarieae tribes.

The genera Festuca and Holcus are included in the Pooidae subfamily, which is composed exclusively by C3 species (Sage and Monson 1999). Homolepis aturensis, Homolepis isocalycia e Steinchis malaxum are part of the Panicoidae subfamily, Panicoea tribe. The Panicoea tribe presents C3, C4 (of all biochemical subtypes) and intermediary C3–C4 plants. Arundo donax is included in the Arundinarieae subfamily (composed of 60 genera, of which five are C4) and within the Arundinarieae tribe, composed exclusively of C3 species (Sage et al. 1999).

The genera Axonopus, Cenchrus, Cymbopogon, Digitaria, Hemarthria, Paspalum, Pennisetum, Setaria and Sorghum are C4 of the NADP-ME type. The photosynthetic pathway NADP-ME occurs in the forage species Setaria anceps, Pennisetum purpureum, and Paspalum notatum. The genera Brachiaria and Urochloa are C4 of type PCK, and genera Cynodon, Eragrostis and Sporobolus are C4 of type NAD-ME. The genus Panicum is the only genus to present C3, C4 of all types, and intermediary C3-C4 species, with Panicum maximum being PCK (Sage et al. 1999).

The results from the histochemical test and the carbon isotope composition analysis are in accordance with previous reports concerning the plant genera represented in this study, indicating that the proposed histochemical test can be employed as an additional criterion for the determination of the photosynthetic pathway in grasses. The convenience and low cost of the proposed method allows its utilization as a first indicator of the photosynthetic pathway in grasses. However, its use is recommended only with fresh samples, given that its performance with dry samples is not feasible.

Future studies may confirm the applicability of this method in other botanical families with C4 member species. In Cyperaceae, preliminary studies did not validate use of the test (data not shown). Additionally, the isolation and identification of the phenolic compounds detected by the histochemical test in bundle sheath cells of C4 grasses may elucidate a possible relation between the presence of these substances and the C4 photosynthetic pathway.

Acknowledgements Our most sincere thanks to the Laboratório de Ecologia Isotópica do Centro de Energia Nuclear na Agricultura—CENA/USP, represented by Prof. Dr. Luiz Antônio Martinelli, for the isotope analyses. We also thank the herbariums Herbários Flor, of Universidade Federal de Santa Catarina and Herbário MG, of Museu paraense Emílio Goeldi, for the plant species identification.
Author contributions MAMN and MPG performed the experiments. MAMN analyzed and interpreted the results. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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