Carbon isotope composition and leaf anatomy as a tool to characterize the photosynthetic mechanism of *Artemisia annua* L.

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Leaves of *Artemisia annua* L. are a plentiful source of artemisinin, a drug with proven effectiveness against malaria. The aim of this study was to classify the photosynthetic mechanism of *A. annua* through studies of the carbon isotope composition ($\delta\ ^{13}C$) and the leaf anatomy. *A. annua* presented a $\delta\ ^{13}C$ value of - 31.76 ± 0.07, which characterizes the plants as a typical species of the C_3 photosynthethic mechanism, considering that the average $\delta\ ^{13}C$ values for C_3 and C_4 species are -28 and -14, respectively. The leaf anatomy studies were consistent with the $\delta\ ^{13}C$ results, where, in spite of the existence of parenchymatic cells forming a sheath surrounding the vascular tissue, the cells do not contain chloroplasts or starch. This characteristic is clearly different from that of the Kranz anatomy found in C_4 species.
Key words: C_3 and C_4 plants, isotope discrimination, Kranz anatomy, photosynthesis.

Composição dos isótopos do carbono e anatomia foliar como ferramenta para caracterizar o mecanismo fotossintético de *Artemisia annua* L.: As folhas de *Artemisia annua* L. são fonte abundante de artemisinina, uma droga que apresenta ação efetiva contra a malária. O objetivo deste trabalho foi classificar o mecanismo fotossintético de *A. annua* mediante estudos da composição dos isótopos do carbono ($\delta\ ^{13}C$) e da anatomia foliar. *A. annua* apresentou uma $\delta\ ^{13}C$ = - 31.76 ± 0.07, valor tópico de espécies com mecanismo fotossintético C_3, que apresentam, em média, valores de $\delta\ ^{13}C$ = - 28, enquanto espécies C_4 apresentam, em média, valores de $\delta\ ^{13}C$ = - 14. Os estudos da anatomia foliar confirmaram os resultados encontrados para a $\delta\ ^{13}C$, onde, a despeito da existência de células parenquimáticas formando um anel ao redor do feixe vascular, essas não apresentaram cloroplastos e amido. Tal observação descaracteriza a existência de anatomia Kranz, típica de espécies C_4, em *A. annua* .
Palavras-chave: anatomia Kranz, fotossíntese, discriminação isotópica, plantas C_3 e C_4.

A. annua (Asteraceae) is a native Chinese herbaceous plant acclimatized in Brazil. The leaves are a plentiful source of artemisinin, a sesquiterpene lactone, with proven effectiveness against *Plasmodium* resistant strains, the parasitic causal agent of malaria (Klayman, 1985; Geldre et al., 1997). Chemical synthesis of artemisinin is complex, involving many stages and with low yields (Chan et al., 1995; Geldre et al., 1997). In view of the high costs of chemical synthesis, the isolation of artemisin from the *A. annua* plant is the preferred way to obtain the drug (Woerdenbag et al., 1991; Ferreira and Janick, 1996; Geldre et al., 1997).

A. annua is considered a short-day plant (Ferreira et al.,1995) either in a qualitative or absolute sense (Marchese et al., 2002) while some genotypes can present a requirement for low temperatures or vernalization to accelerate flowering (Marchese et al., 2002). Apart from its photoperiodic behaviour and the effect of water supply and different temperatures on the artemisinin content (Marchese, 1999; Marchese and Rehder, 2001), little other information is available in the literature relating to the physiology of *A. annua*. Due to the importance of *A. annua* as a source of artemisinin, in many countries where malaria occurs efforts

are underway to introduce and acclimatize the plant (Mueller et al., 2000).

Information on the photosynthetic mechanism can be useful for the introduction of any species, but especially for an exotic medicinal plant, such as *A. annua*. In general, plants with the C_4 photosynthetic mechanism are better adapted for hot climates, while C_3 plants are more appropriate for temperate regions (Loomis and Connor, 1992; Rudall, 1994; Hall and Rao, 1995; Körner and Bazzaz, 1996; Lambers et al., 1998; Lawlor, 2001).

The photosynthetic mechanism or biochemical pathways of photosynthesis are highly conserved. The majority of plants are C_3 plants, in which the first product of photosynthesis is the three-carbon compound phosphoglyceric acid. A second biochemical pathway, that leads to the concentration of CO_2 in leaves, is found in C_4 plants, which initially fix inorganic carbon in mesophyll cells into the four-carbon compound oxaloacetic acid. In C_4 plants, oxaloacetate is converted into malate or aspartate, which then diffuses into the bundle sheath cells that surround the vascular system where descarboxylation supplies high concentrations of CO_2 for Rubisco. In higher plants, the C_4 pathway involves both biochemical and anatomical modifications, but it is not clear which of these modifications evolved first. Some plants, that possess characteristics of both C_3 and C_4 plants, have been classified as C_3-C_4 intermediates, and these plants may represent transitional stages in the evolution of C_4 photosynthesis from C_3 photosynthesis (von Caemmerer, 1992; Lawlor, 2001; Hibberd and Quick, 2002).

Plants discriminate carbon isotopes during photosynthesis. The carbon dioxide in the earth's atmosphere is composed of different carbon isotopes. The principal carbon isotope is $^{12}CO_2$ (98.9 atom %) while only about 1.1 atom % of the total CO_2 in the atmosphere is $^{13}CO_2$ and an even smaller fraction (10^{-10} atom %) is the radioactive species $^{14}CO_2$. Modern ecophysiological research makes abundant use of the fact that the isotope composition of plant biomass differs from that of the atmosphere. Furthermore, isotope composition differs between plants, according to their photosynthetic pathway (Lambers et al., 1998).

The chemical properties of $^{13}CO_2$ are identical to those of $^{12}CO_2$, but because of the slight difference in mass (2.3%), plants use less $^{13}CO_2$ than $^{12}CO_2$. C_3 plants ($\delta‰^{13}C$ about –28 ‰) discriminate more $^{13}CO_2$ than the C_4 plants ($\delta‰^{13}C$ about –14 ‰). The largest isotope discrimination step is the carboxylation reaction catalyzed by Rubisco, the primary CO_2 fixation enzyme of C_3 plants, which has an intrinsic discrimination value ($\Delta^{13}C$) of –30 ‰. On the other hand, PEP carboxylase, the primary CO_2 fixation enzyme of C_4 plants, has a much smaller isotope discrimination effect ($\Delta^{13}C$ = –2 to 5.7 ‰) (Sternberg et al., 1984; Farquhar et al., 1989; O'Leary et al., 1992; O'Leary, 1993; Lambers et al., 1998; Condon et al., 2002).

There are also differences in leaf anatomy between C_4 and C_3 plants. A cross section, of a typical C_3 leaf reveals one major cell type with chloroplasts, the mesophyll. In contrast, a typical C_4 leaf has two distinct chloroplast-containing cell types: mesophyll and bundle sheath cells (Mauseth, 1988; Rudall, 1994; Lawlor, 2001). The aim of this investigation was to classify the photosynthetic mechanism of *A. annua* through studies of carbon isotope composition ($\delta‰^{13}C$) and leaf anatomy.

Plants of a genotype of *A. annua* originating from Vietnam plant population (CPQBA 2/39x1V) and developed by the Breeding Program of the Centre of Chemical, Biological and Agricultural Research of the State University at Campinas (CPQBA/UNICAMP) were used in this study. For carbon isotope composition ($\delta‰^{13}C$) measurements the leaves were dried at 80^0C overnight and powdered in a cryogenic mill (-196^0C). They were then analysed, in triplicate, in a mass spectrometer coupled to an elemental analyser for the determination of the ratio *R* ($R = {}^{13}CO_2/{}^{12}CO_2$). The standard ratio is that of Pee Dee belemnite (PDB). Carbon isotope composition ($\delta‰^{13}C$) is a measure of the $^{13}C/^{12}C$ ratio in a sample of plant relative to the value of the same ratio in an accepted international standard, the limestone Pee Dee belemnite (PDB). Thus,

$$\delta‰^{13}C = [(R_p/R_s) - 1] \times 1000$$

where $\mathbf{R_p}$ is the $^{13}C/^{12}C$ ratio measured in plant material and $\mathbf{R_s}$ is the ratio of standard (PDB). Carbon isotope composition provides a means of relating samples of diverse origin. Samples of contemporary plant material have negative values of $\delta‰^{13}C$ because the $^{13}C/^{12}C$ ratio in the atmosphere is less than in Pee Dee belemnite and because there is a net discrimination against ^{13}C by plants during uptake and fixation of CO_2 into plant dry matter (O'Leary et al., 1992; O'Leary, 1993; Condon et al., 2002; Dawson et al., 2002).

For leaf anatomical studies, the leaves were fixed in FAA_{50}, and stored in ethanol 70% (Johansen, 1940). The samples were embedded in historesin (Leica®) according to Gerrits (1991) and stained with toluidine blue (O'Brien et al., 1964). Sections for light microscopy were obtained using a rotatory microtome at a thickness of 8 µm, and treated with PAS (Periodic acid/Shiff reagent) (Pearse, 1968). This

general test for carbohydrate gives a rose to purple coloration for starch or structural wall carbohydrates. The sections were covered with synthetic resin (Permount®) and a cover -slip.

A. annua produced values for $\delta‰^{13}C$ of - 31.76 ± 0.07 (table 1), a result that characterizes the plant as a typical species of the C_3 photosynthetic mechanism, considering that the average $\delta‰^{13}C$ values for C_3 and C_4 species are -28 and – 14 respectively (Sternberg et al., 1984; Farquhar et al., 1989; O'Leary et al., 1992; O'Leary, 1993; Lambers et al., 1998; Condon et al., 2002). The ratio of $^{13}C/^{12}C$ in dry matter of C_3 plants is the result of discrimination against ^{13}C during several processes. These include: during diffusion of CO_2 through the stomata; at Rubisco during the process of CO_2 fixation; and at some downstream metabolic steps and (possibly) respiration (Condon et al., 2002).

The leaf anatomy studies (figure 1) were in agreement with the results of $\Delta‰^{13}C$. The parenchyma cells which form a bundle sheath surrounding the vascular tissue contain no chloroplasts or starch. The leaf is amphistomatic and the mesophyll is dorsiventral, with little differentiation into palisade and spongy parenchyma. Starch (figure 1) is present throughout the mesophyll, indicating no specific areas for its production. Such leaf characteristics are not found in the Kranz anatomy of a C_4 species, but are typical of C_3 species (Mauseth, 1988; Rudall, 1994; Lawlor, 2001).

The existence of an evident parenchyma bundle sheath with little differentiation of the mesophyll cells may represent a transitional stage in the evolution of C_4 photosynthesis from C_3 photosynthesis (Lawlor, 2001; Condon et al., 2002), but, in general, the Kranz anatomy bundle sheath consists of a single layer of large cells containing large chloroplasts and starch (Mauseth, 1988; Rudall, 1994). The test with PAS produced a negative reaction for the vascular bundle sheath cells to starch, indicating the absence of chloroplasts in these cells. The results of $\delta‰^{13}C$ together with the absence of chloroplasts and starch in parenchyma vascular bundle sheath cells suggest that leaves of *A. annua* have a C_3 photosynthetic mechanism.

Table 1. Mean average carbon isotope composition $(\delta\ ^{13}C)_{PDB}$, for *A. annua* (hybrid CPQBA 2/39x1V).

Sample	$(\delta‰^{13}C)_{PDB}$
01	-31.69
02	- 31.82
03	-31.78
*mean ± standard deviation	- 31.76 ± 0.07

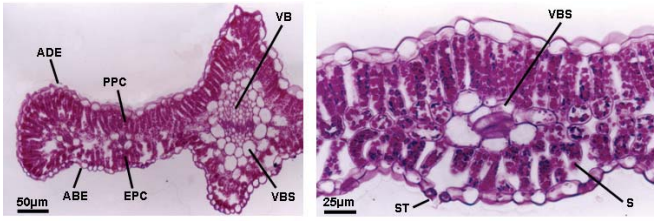

Figure 1. PAS test on cross sections of the *A. annua* leaf with palisade parenchyma cells **(PPC)**; spongy parenchyma cells **(EPC)**; vascular bundle **(VB)**; vascular bundle sheath **(VBS)**; adaxial epidermis **(ADE)**; abaxial epidermis **(ABE)**; stomata **(ST)**; starch **(S)**.

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