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Near-infrared reflectance spectroscopy allows rapid and simultaneous evaluation of chloroplast pigments and antioxidants, carbon isotope discrimination and nitrogen content in *Populus* spp. leaves

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A B S T R A C T

Poplar species are economically important sources of timber and bioenergy and they also have a valuable application in phytoremediation and in forest restoration programmes. The characterization of their responses in the face of environmental constraints is essential to ascertain their adaptation capacity. Quantification of chloroplast pigments and antioxidant compounds, nutrients and carbon isotope discrimination ($\Delta^{13}C$) of a large set of samples in ecophysiological studies is important for determining the plant's physiological responses. $\Delta^{13}C$ and nitrogen are parameters of special interest for providing information on photosynthesis, water relations and nutritional characteristics in forest stands. Photoprotective and antioxidant compounds participate in the physiological defence of plants subjected to stressful environmental conditions. The measurement of these leaf components by traditional techniques is laborious and expensive. To evaluate them, a calibration model was obtained using Near-Infrared Reflectance Spectroscopy (NIRS) and chemical analyses of *Populus* spp. leaves sampled from different clones in different years under different physiological conditions. Predictive calibration equations for the concentration of chloroplast pigments (Chlorophylls *a* and *b*, lutein, neoxanthin, β-carotene (β-Car), VAZ (violaxanthin + antheraxanthin + zeaxanthin), epoxidation index (EI = (0.5·antheraxanthin + violaxanthin)/VAZ)), antioxidants (total phenolics (TPhe), ascorbate and α-tocopherol), $\Delta^{13}C$ and N content were established using a partial least squares regression algorithm. Results showed a remarkable accuracy of the calibration equations for quantifying several antioxidative and photoprotective defence compounds such as neoxanthin ($R^2 = 0.844$), β-Car ($R^2 = 0.827$), VAZ ($R^2 = 0.846$), EI ($R^2 = 0.831$) and excellent accuracy for TPhe ($R^2 = 0.957$), N ($R^2 = 0.963$) and $\Delta^{13}C$ ($R^2 = 0.922$). As shown, results can be obtained on multiple physiological attributes with a single measurement, saving time and money and demonstrating the suitability of NIRS for ecophysiological purposes and bioremediation programs.

1. Introduction

Human activities are altering and degrading biotic systems. Direct human interventions, (timber removal, water extractions, and polluting fertilizer additions, among others) and indirect human interventions (environmental pollution and climate change) are currently recognized as notable threats to human health and ecosystem survival (Mooney et al., 2009). The important ecological role of the forest ecosystem in mitigating the impacts of these environmental menaces on a global and local scale has long been documented (Lindner et al., 2010). In this context, forest plant species have been commonly used for environmental assessment and restoration projects because of their potential for biomonitoring and bioremediation (Schröter et al., 2005).

Among forest plant species, particular efforts have been made to evaluate the suitability of poplar (*Populus* spp.) as a model for researching the biological potential in monitoring and counteracting adverse environmental factors. Poplars are fast-growing trees widely distributed in the northern hemisphere, where they colonize many different ecosystems. Cottonwoods (*Populus* spp.) are

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ecological pioneers that colonize barren riparian sites and in semi-arid environments they are the dominant and often exclusive riparian tree species (Patten, 1998). Riparian cottonwoods provide stream-bank stabilization and wildlife habitat, contribute to aquatic ecosystems and provide the parental genotypes for natural and artificial interspecific hybrids (Stettler et al., 1996). Natural hybrids are important for ecosystem biodiversity (McIntyre and Whitham, 2003). Cottonwood forests are particularly vulnerable to the degradation of riparian zones as a result of livestock production, tree harvesting for fibre and fuel, and clearance for agriculture, human settlement and transport corridors (Muller et al., 2002). Due to their great adaptability to different climate conditions, their capacity for vegetative propagation and their high biomass yield, artificial intersectional poplar hybrids are widely cultivated for different purposes, ranging from the timber products to biomass for energy generation (Scarascia-Mugnozza et al., 1997; Kauter et al., 2003; Al Afas et al., 2008; Werner et al., 2012; Benetka et al., 2014). Furthermore, the complete sequencing of the *P. trichocarpa* genome (Tuskan et al., 2006), together with a highly informative genetic mapping of the *P. nigra* species (Gaudet et al., 2011; Iori et al., 2015), have led to the poplar being considered as a model tree for forest plant genomics (Jansson and Douglas, 2007). In recent years, the value of poplars in terms of ecological services has been highlighted (Tognetti et al., 2013), both in terms of sequestration of atmospheric CO_2 (Calfapietra et al., 2010) and the reduction of inorganic and organic pollution in soils and water (Fernàndez et al., 2012; Fernàndez-Martínez et al., 2013). For these reasons, the involvement of poplar species in bioenergy and phytoremediation applications, and in general in environmental restoration projects, has become notably important. It is therefore of interest to evaluate the poplar physiological responses to biotic and abiotic constraints to provide information on their growth and tolerance capacity under these conditions.

Plants subjected to environmental stress may lead to the formation of reactive oxygen species (ROS) in chloroplasts that can be highly reactive (Asada, 1996) and result in damage to the photosynthesis apparatus and the degradation of cell components. For this reason, information on variations in leaf chloroplast pigments, antioxidants and nitrogen are necessary to establish the photosynthetic and tolerance capacity in front of environmental changes or limitations. In addition, leaf carbon isotope discrimination ($\Delta^{13}C$) is a valuable tool that provides information concerning the physiological state of leaves (e.g. photosynthetic limitants, water use efficiency (WUE)).

Techniques and experimental procedures which allow the evaluation of a large set of parameters in a short time are of special interest to ascertain plant's responses to environmental constraints. Traditional methods of organic compound analysis, through extraction and spectrophotometric or HPLC measurement, limit the measurement to a few leaf components. The techniques are also time-consuming and expensive, making assessment of the overall vegetation health of landscapes and ecosystems impractical. Near-Infrared Reflectance Spectroscopy (NIRS) is a rapid, cheap technique that uses the same leaf sample for analysing multiple components. NIRS analyses the diffuse reflectance of samples (Williams, 1975), and can be used to examine the biochemical composition of foods and plant material. The absorption of light between 780 and 2,500 nm, as a result of molecular vibrations of bonds like O—H, C—N, N—H and C═O characteristic of organic matter, produces a unique spectral signature for each sample analysed (Osborne et al., 1993). The spectrum of light reflected by a sample therefore provides details on the chemical composition (i.e., number and nature of bonds present) of that material (Birth and Hecht, 1987). A calibration model based on the relationship between chemical measurements and spectral features using NIRS is required to determine the concentration of a particular organic constituent. NIRS allows rapid, repeatable, and accurate measurement of hydrocarbonated molecules and nutrient fractions in plant material (McLellan et al., 1991; Joffre et al., 1992; Foley et al., 1998; Gillon et al., 1999; Takahashi et al., 2011; Windley and Foley, 2015). So far, data from NIRS analyses of *Populus* foliage are available from two studies, in which the authors determined nitrogen, sugar, starch, tannin and phenolic glycoside content (Rubert-Nason et al., 2013) and condensed tannins and salicinoids (Couture et al., 2016). NIRS has been applied in other species to analyse $\Delta^{13}C$ (Kleinebecker et al., 2009), chloroplast pigments (Pintó-Marijuan et al., 2013) and antioxidants (Stolter et al., 2006; Gäb et al., 2006; Pintó-Marijuan et al., 2013). To be most useful in ecological studies, NIRS calibrations should determine organic components in leaves of trees stressed by environmental conditions and should be generalizable across species and leaf ontogenetic and phenological development stages. NIRS calibrations of compounds involved in photosynthesis performance in different *Populus* clones and species would ascertain the usefulness of this method for predicting the content of leaf components and their applicability in a wide range of conditions and genotypes.

The aim of this study was to demonstrate the suitability of NIRS (Near-Infrared Reflectance Spectroscopy) for the quantification of chloroplast pigments, photoprotective and antioxidant systems, N content and carbon isotope discrimination ($\Delta^{13}C$) in leaves of the model tree *Populus* spp. in order to be applied in ecological studies. We therefore performed: (1) the laboratory determination of chloroplast pigments (chlorophylls *a* and *b*, lutein, neoxanthin, β-car, total content of VAZ cycle pigments (violaxanthin + zeaxanthin + antheraxanthin)), antioxidants (total phenolics, ascorbate and α-toc), carbon isotope discrimination ($\Delta^{13}C$) and nitrogen, (2) NIRS application to the samples that had been previously analysed by biochemical methods, (3) correlation of biochemical quantification with NIRS records and construction of a reliable and reproducible model with the calibration equations between NIR spectra and the concentrations of the selected compounds.

2. Material and methods

2.1. Plant material, experimental site and sampling

Adult plants of several poplar clones grown since 2001 in the IBAF-Institute experimental field near Rome in the Tevere valley (42°06′20.9″N 12°38′15.8″E) were used for this study. From a 50-clone collection, we selected the poplar clones *Populus deltoides* Batr. (Cottonwood) – Lux clone; *Populus nigra* L. (black poplar) – 58–861 and Jean Pourtet clones; *Populus deltoides* x *maximowiczii* – Eridano clone and *Populus x canadensis* Mönch. (hybrid black poplar) Luisa Avanzo, Adige and I-214 clones. Each clone was represented by 10–30 individuals; all were female except Jean Pourtet and Eridano, which were male.

In order to construct accurate calibration models between chemical constituents and spectral absorbance, the widest range of values should be obtained for the compounds of interest. Thus, to obtain likely differences between groups in leaf chloroplast pigments, antioxidants, $\Delta^{13}C$ and N content, leaf samples were obtained from different poplar species and clones, as explained above, and across the leaf phenological development. Samples were collected throughout the growing season from leaves at their initial growth stage (spring: 18–25 May), at their greatest productivity period (early summer: 6–13 July), and at the senescence period (late summer: 14–21 September). In order to obtain an expansion of the trait and spectral space collected necessary to capture the dynamic range of trait and spectral information, another sampling campaign was carried out in the summer of the following year (12–19 July). Leaf samples (approximately 1 g