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# Anthocyanins of the anthers as chemotaxonomic markers in the genus *Populus* L.. Differentiation between *Populus nigra*, *Populus alba* and *Populus tremula*

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Three main species of *Popululs* L. (Salicaceae) have been reported to occur in the Iberian Peninsula: *Populus nigra* L., *Populus alba* L. and *Populus tremula* L. The degree of pilosity of the bracts of the male catkins is a key character for their differentiation. The anthers of these poplar species possess anthocyanins that provide them a red colouration. Since these poplars are wind-pollinated and, consequently, do not need to attract pollinators, anthocyanins in the anthers might be acting as photoprotectors, shielding pollen grains from excessive sunlight. In order to verify this hypothesis, the first objective of this study was to establish if there is any relationship between the degree of pilosity of the bracts (related to the physical shading of the pollen grains) and the levels and types of anthocyanins in the anthers of these three species. This study also aimed to check the usefulness of the anthocyanins of the anthers as chemotaxonomic markers, through the study of the differences in the anthocyanin composition between these poplar species. Anthocyanins were identified from the data supplied by HPLC-DAD-MS$^n$ analyses. Seventeen different compounds, including mono-, di- and triglycosides and anthocyanin-derived pigments (F-A$^+$ dimers) have been identified. Cyanidin 3-*O*-glucoside was the major compound in all the samples (>60% of the total content), which may be in accordance with the photoprotective role proposed for them. However, qualitative and quantitative differences were detected among samples. Cyanidin and delphinidin 3-*O*-sambubiosides have been detected only in the anthers of *P. tremula* as well as cyanidin 3-*O*-(2″-*O*-xyloxyl)rutinoside, making them valuable chemotaxonomic markers for this species. Hierarchical Cluster and Principal Components Analyses (HCA and PCA) carried out with the anthocyanin percent composition data have allowed a separation of the samples that is in accordance with the initial classification of the samples made from the morphological characters of the specimens. Furthermore, these analyses have revealed intraspecific differences among samples that point out to different clones or varieties of a same species.



## 1. Introduction

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They belong to the flavonoid family, being responsible for most of the red, pink, purple and blue colours of flowers and fruits (Strack and Wray, 1994). In these localisations, their major role is signalisation. In the case of flowers, the colour of the corolla is one of the floral traits that constitute the pollination syndrome of the plant. This means that the colour of the petals along with other floral traits condition the type of pollinator that is attracted to the flower and involved in the sexual reproduction of the plant (Harborne and Grayer, 1994; Miller et al., 2011; Alcalde-Eon et al., 2013). In the case of fruits, the colour provided by anthocyanins is involved in seed dispersal since it is a signal for animals of its maturity (Miller et al., 2011). Apart from flowers and

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fruits, anthocyanins also supply colour to other parts of the plants such as stems or leaves in which their main function has been proposed to be the photoprotection (Steyn et al., 2002). In senescing leaves, a resorption protective role of anthocyanins has been reported (Hoch et al., 2003). In this case, anthocyanins facilitate the recovery of foliar nutrients by protecting senescing leaves from excess light (Hoch et al., 2003). It has also been suggested that the colouration of vegetative tissues might be a defence mechanism against herbivores by undermining their camouflage and making them visible to their predators (Lev-Yadun et al., 2004). Furthermore, anthocyanins are responsible for the colouration of the carpels, styles or anthers of some plants (Nakayama et al., 1999; Strack and Wray, 1994; Wheldale, 1916). In addition, anthocyanins have been reported in pollen grains or in nectars from other plants (Hansen et al., 2007; Miller et al., 2011) being also detectable in hive products such as bee pollen (di-Paola et al., 2004). Although these latter floral traits seem to be related to animal-pollination (Hansen et al., 2007; Nakayama et al., 1999; Miller et al., 2011), some of them have also been observed in wind-pollinated plants. This is the case of the male catkins of some species of the genus *Populus* L. which present purple anthers. This genus belongs to the Salicaceae family and comprises ca. 29 species grouped into six sections based on relative morphological similarity and crossability (Eckenwalder, 1996). In the Iberian Peninsula, Soriano (1993) has reported the presence of three main species of *Populus*: *Populus nigra* L., *Populus alba* L. and *Populus tremula* L. Morphological characters have traditionally been used to differentiate the three species. Among them, the degree of pilosity of the bracts of the male catkins is a key character that allows a first distinction between *P. nigra* (glabrous) and *P. alba* and *P. tremula* (heary) (Soriano, 1993; Aizpuru et al., 2000; Streeter et al., 2011) and a subsequent differentiation between the latter ones, *P. tremula* showing longer and denser hairs in the bract than *P. alba*. In these three species of poplars, flowering takes place before foliar emergence and, consequently, anthers and pollen grains are exposed to sunlight. Bracts of the aments partially shade anthers and the pilosity of the bracts might provide additional sunlight protection. Anthocyanins in the anthers might, therefore, be performing a photoprotective role, contributing to avoid the damage of the male gametes by excessive sunlight. For this reason it would be interesting to establish if there is any relationship between the degree of pilosity and the levels of anthocyanins in the anthers. Furthermore, it would also be interesting to determine the qualitative anthocyanin composition of the anthers in order to check if it is compatible with a photoprotective function, since, for a photoprotective role, plants synthesise anthocyanins simpler in structure than those synthesised for a signalisation role (Steyn et al., 2002). This is the first objective of the present study, i.e. the determination of the anthocyanin composition of the anthers of the three main species of the genus *Populus* present in the Iberian Peninsula and the study of their possible role of sunscreens for the pollen grains. The second objective is to check if there are differences in the anthocyanin profiles of the anthers of these three species that might be used in their classification and differentiation, i.e., to check the usefulness of the anthocyanins of the anthers as chemotaxonomic markers. Classical taxonomic analysis, based on morphological characteristics and crossability encounters sometimes difficulties due to the high intraspecific phenotypic variability observed within broadly distributed *Populus* species, to the high natural crossability among members of the genus leading to hybrids and to the convergent morphology shown by hybrids and their parental species (Eckenwalder, 1996; Cervera et al., 2005; Šiler et al., 2014). Morphometrics characters of the leaves have also been employed to assess the variability within the genus *Populus* (Kovačević, 2014) although molecular markers (microsatellites (SSR) and Amplified Fragment Length Polymorphism (AFLP), above all) have nowadays become a widely used approach for taxonomic and phylogenetic purposes (Fossati et al., 2003, 2004; Cervera et al., 2005; Smulders et al., 2008; Jelić et al., 2014). In addition, different kinds of flavonoids present either in the bud exudates (Wollenweber, 1975; Greenaway et al., 1989a, 1989b; Kurkin et al., 1990; Rivera et al., 1997) or in the leaves (Jones and Seigler, 1975) of the poplars have been employed to differentiate among different species and clones. However, studies on the anthocyanin composition are scarce in the genus *Populus* despite the positive results of the use of anthocyanin profiles for taxonomic purposes that have been reported for some plant families. In fact, during the last three decades several works (Hrazdina, 1982; Harborne and Grayer, 1988; Strack and Wray, 1994; Andersen and Jordheim, 2006) have aimed to summarise all the studies dealing with anthocyanin composition that had been done until that moment revealing relationships and similarities between the anthocyanin profiles of genera belonging to the same families. In addition, the application of statistical methods such as hierarchical clustering or Principal Components Analysis (PCA) to the data matrix constituted by the anthocyanin composition has been proved to be an useful tool for chemotaxonomic purposes allowing the differentiation among different species of the same genus or even among varieties and clones of the same species (Mattivi et al., 1990; Wang et al., 2001, 2004; Figueiredo-González et al., 2012; Li et al., 2013; Alcalde-Eon et al., 2014). Nevertheless, in the case of the genus *Populus* and to our knowledge, only the anthocyanin composition of the leaves and catkins of *P. tremula* and the male catkins of a *Populus* hybrid (*P. alba* × *P. tremula*) (Bendz and Haglund, 1968) and the anthocyanin composition of autumn leaves of *Populus tremuloides* Michx (Chang et al., 1989). have been reported. Although these studies are a valuable starting point for the present study, it has to be noted that the anthocyanin composition was determined by means of TLC and paper chromatography. For this reason, a detailed study of the anthocyanin composition is required in order to establish the usefulness of the pigment profile as chemotaxonomic marker. For this purpose, analyses of the only coloured part of the male catkins, the anthers, have been performed in the present study by means of HPLC-DAD-ESI/$MS^n$.

## 2. Results and discussion

Fig. 1 shows the chromatogram recorded at 520 nm of one sample from each of the *Populus* species studied in the present work (Fig. 1a, *P. nigra*; Fig. 1b, *P. tremula*; Fig. 1c, *P. alba*). 17 different compounds (Table 1) were detected and identified through their chromatographic properties, UV–vis and mass spectra and fragmentation patterns and by comparison to those of standards. In some cases, identities were corroborated through the injection of other plant material whose anthocyanin composition is well established. As can be seen in Fig. 1, peak 11 was the major peak in the anthers from all the types of poplars analysed in the present study, representing more than 50% of the total area in all the samples. However, quantitative and qualitative differences in the anthocyanin composition can be observed among the three types of samples.

### 2.1. Qualitative and quantitative analyses.

A first difference among samples was observed in peak 11: whereas it contained only one compound in *P. nigra* and *P. alba* (compound **11a**), another minor compound (compound **11b**) was co-eluting with compound **11a** in *P tremula*. Compound **11a** showed a UV–vis spectrum similar to those of the B-ring di-substituted monoglycosides (Table 1). Its molecular ion originated a signal at *m/z* 449 and was fragmented in the $MS^2$ analysis giving rise to a

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