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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*

Cristina Alcalde-Eon [a, *], Ignacio García-Estévez [a], Julián C. Rivas-Gonzalo [a], David Rodríguez de la Cruz [b], María Teresa Escribano-Bailón [a]

[a] Grupo de Investigación en Polifenoles, Unidad de Nutrición y Bromatología, Facultad de Farmacia, University of Salamanca, Campus Miguel de Unamuno, E 37007 Salamanca, Spain
[b] Departamento de Botánica, Facultad de Biología, University of Salamanca, Campus Miguel de Unamuno, E 37007 Salamanca, Spain

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ABSTRACT

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

* Corresponding author.
E-mail addresses: crisalcaldeon@usal.es (C. Alcalde-Eon), igarest@usal.es (I. García-Estévez), jcrivas@usal.es (J.C. Rivas-Gonzalo), droc@usal.es (D. Rodríguez de la Cruz), escriban@usal.es (M.T. Escribano-Bailón).

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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

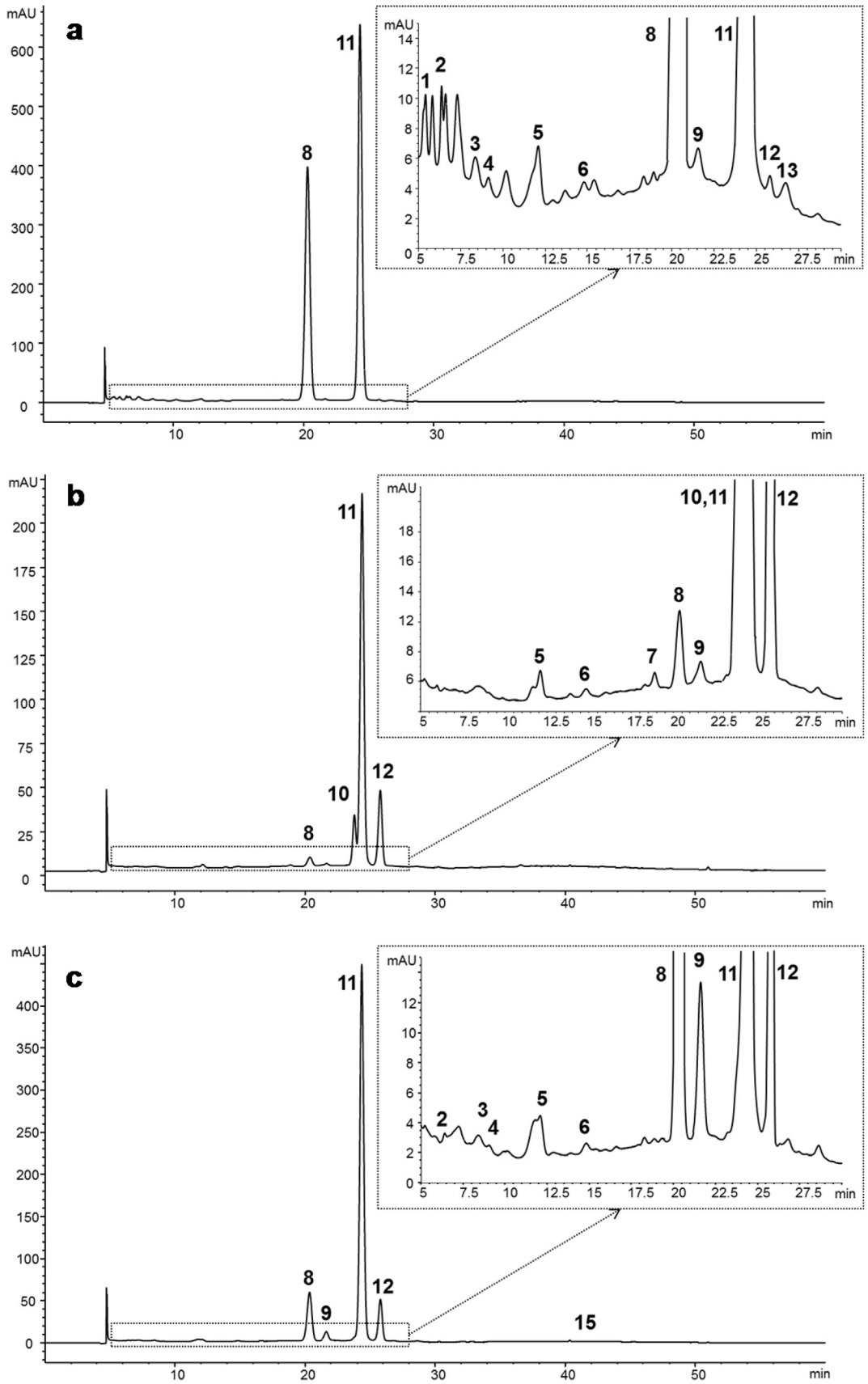

Fig. 1. Chromatograms recorded at 520 nm for *P. nigra* (POP3, a), *P. tremula* (POP11, b) and *P. alba* (POP10B, c) samples. See Table 3 for the features of the samples.

Table 1
Chromatographic, spectral features and fragmentation patterns of the anthocyanins and anthocyanin-derived pigments detected in the *Populus* samples.

	Identity	RT (min)	λ max (nm)	M^+ (*m/z*)	MS^2 fragment ions (*m/z*)	MS^3 fragment ions (*m/z*)
1	F-A^+ GC-Dp-3-glc	6.1	–	769	–	–
2	F-A^+ GC-Cy-3-glc	6.4	288, 440(sh), 526	753	591 $[M-162]^+$(100), 329 $[M-424]^+$(10), 573 $[M-180]^+$(6), 423 $[M-330]^+$(4)	423 $[(M-162)-168]^+$(100), 573 $[(M-162)-18]^+$(70), 329 $[(M-162)-262]^+$(50), 435 $[(M-162)-156]^+$(25), 287 $[(M-162)-304]^+$ (15)
3	F-A^+ C-Dp-3-glc	8.4	440(sh), 532	753	–	–
4	F-A^+ EGC-Cy-3-glc	9.1	527	753	–	–
5	F-A^+ C-Cy-3-glc	12.0	280, 440(sh), 526	737	575 $[M-162]^+$(100), 557 $[M-180]^+$(17), 329 $[M-408]^+$ (15), 423 $[M-304]^+$(10)	423 $[(M-162)-152]^+$(100), 557 $[(M-162)-18]^+$(75), 329 $[(M-162)-246]^+$(65), 435 $[(M-162)-140]^+$(35), 287 $[(M-162)-288]^+$(10)
6	F-A^+ EC-Cy-3-glc	14.8	–	737	–	–
7	Dp-3-samb	18.3	–	597	–	–
8	Dp-3-glc	20.4	276, 348, 440(sh), 524	465	303 $[M-162]^+$(100)	257 $[(M-162)-46]^+$(100), 303 $[M-162]^+$(40), 229 $[(M-162)-74]^+$(40)
9	Dp-3-rut	21.6	278, 348, 440(sh), 524	611	303 $[M-308]^+$(100), 465 $[M-146]^+$(8)	303 $[M-308]^+$(100)
10	Cy-3-samb	23.8	518	581	287 $[M-294]^+$(100)	287 $[M-294]^+$(100)
11a	Cy-3-glc	24.4	280, 328, 440(sh), 516	449	287 $[M-162]^+$(100)	287 $[M-162]^+$(100)
11b	Cy-3-xylrut	24.4	–	727	287 $[M^+-440]$(100), 581 $[M^+-146]$(15)	287 $[M-440]^+$(100)
12	Cy-3-rut	25.8	280, 330, 440(sh), 518	595	287 $[M-308]^+$(100), 449 $[M-146]^+$(9)	287 $[M-308]^+$(100)
13a	Cy-3-hex	26.8	–	449	287 $[M-162]^+$(100)	287 $[M-162]^+$(100)
13b	Pt-3-glc	26.8	–	479	317 $[M-162]^+$(100)	302 $[(M-162)-15]^+$(100)
14	Pg-3-glc	28.6	502	433	271 $[M-162]^+$(100)	271 $[M-162]^+$(100)
15	Unknown-vitisin type	40.4	484(sh), 506	–	–	–

RT: Retention time. Dp: delphinidin. Cy: cyanidin. Pt: petunidin. Pg: pelargonidin. Glc: glucoside. Rut: rutinoside. Samb: sambubioside. Xylrut: xylosylrutinoside. Hex: hexoside. (E)GC: (epi)gallocatechin. (E)C: (epi)catechin. F-A^+: Flavanol-Anthocyanin direct condensation dimer. (sh): shoulder.

single ion at *m/z* 287 by loss of 162 amu (Table 1). The *m/z* of this aglycone and its fragmentation confirmed that compound **11a** was a derivative of cyanidin containing one hexose. Similarly, compound **8**, whose UV–vis spectrum was similar to those of the B-ring tri-substituted monoglucosides and whose molecular ion showed a signal at *m/z* 465 and was fragmented as compound **11a** (loss of 162 amu, originating a single ion at *m/z* 303) (Table 1), was identified as an hexoside of delphinidin. The retention times of these two compounds (compounds **8** and **11a**) were similar to those of the standards of delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside when analysed in the same conditions, which suggested that these could be their identities. Nevertheless, a co-injection of a *P. nigra* sample (POP9, in which compounds **8** and **11a** represented more than 95% of the total area, see Table S1 for further information of the sample) with both standards was carried out in order to confirm their identities. The presence of only two major peaks in the chromatogram of the co-injection together with the spectral data confirmed that compound **8** was delphinidin 3-*O*-glucoside and compound **11a**, cyanidin 3-*O*-glucoside.

Compounds **9** and **12** eluted just after compounds **8** and **11a**, respectively. Their UV–vis spectra were similar to those of the preceding ones, i.e., compound **9**, similar to B-ring tri-substituted anthocyanins and compound **12** to B-ring di-substituted anthocyanins. In both cases, a shoulder was observed at 440 nm, which was indicative of the absence of substitution of the hydroxyl group in position 5 of the anthocyanidin. The fragmentation pattern was the same for compounds **9** and **12**. The molecular ion of compound **9** showed a signal at *m/z* 611 and that of compound **12** at *m/z* 595. They were fragmented in the MS^2 analysis producing, in both cases, two fragment ions. The major one was originated by the loss of 308 amu, whereas the minor one (relative abundance ca. 8) was formed after the loss of 146 amu from the molecular ion. The major fragment ions in the MS^2 spectra of compounds **9** and **12** corresponded to delphinidin (*m/z* 303) and cyanidin (*m/z* 287) respectively and the minor ones to their monohexosides (*m/z* 465 and *m/z* 449, respectively). The residue of 146 amu might correspond either to a *p*-coumaroyl residue or to a rhamnosyl residue. However, the possibility of being a *p*-coumaroyl residue was ruled out on the basis of information supplied by their UV–vis spectra (the absence of an additional shoulder around 309–330 nm in the UV–vis spectrum indicated that there was no acylation of the sugars with hydroxycinnamic acids) and fragmentation pattern. In fact, the fragment ion corresponding to the monohexoside would not be produced if the residue were a *p*-coumaroyl residue since, in the conditions usually employed for mass fragmentation, acyl residues cannot be separated from the sugar moiety (Giusti et al., 1999). Glycosidic bonds among sugar units cannot be cleaved by mass fragmentation either. However, when the disaccharide is a rutinose (6-rhamnosylglucose), a fragment ion corresponding to the loss of the rhamnose moiety can be observed, although with lower relative abundance than the ion corresponding to the aglycone (Giusti et al., 1999; Alcalde-Eon et al., 2013). Taking into account all these data, compounds **9** and **12** were proposed to be delphinidin and cyanidin 3-*O*-rutinosides, respectively. Cyanidin 3-*O*-rutinose has been previously identified in raspberry (*Rubus idaeus* L.) (Mullen et al., 2002a and 2002b, Määtä-Riihinen et al., 2004) and in order to confirm the identity of compound **12**, a raspberry extract was analysed in our laboratory in the same conditions as those employed in this study for *Populus* samples. The chromatographic and spectral data of the compound identified as cyanidin 3-*O*-rutinoside in the raspberry extract were identical to those of compound **12** (see Fig. S1a in supplementary files), thus confirming the identity proposed for it and, indirectly, the identity proposed for compound **9**.

Compound **10** was only detected in *P. tremula* samples. In these samples it accounted for almost 7% of the total anthocyanin content. This compound eluted just before cyanidin 3-*O*-glucoside (compound **11a**) and showed a similar UV–vis spectrum to its. A signal at *m/z* 581 was observed for the molecular ion in the mass spectra, which yielded a single fragment ion in the MS^2 analysis corresponding to cyanidin (*m/z* 287) by the loss of 294 amu. This loss could correspond to the simultaneous loss of one hexose and one pentose (162 + 132 amu). The most usual combination of a hexose and a pentose reported for anthocyanins is sambubioside or 2-xylosylglucoside (Andersen and Jordheim, 2006). Thus, compound **10** was proposed to be cyanidin 3-*O*-sambubioside. A

cyanidin 3-*O*-xylosylglucoside had been previously identified in the female catkins and in the spring and autumn leaves of *P. tremula* (Bendz and Haglund, 1968) but it was not detected in the male catkins. In order to verify if the compound identified in leaves by these authors by means of PC and TCL was the same as compound **10**, autumn leaves from the same *P. tremula* specimens were collected and analysed following the same methodology as that previously employed in the analysis of the anthers of the male catkins. Surprisingly, compound **10** was not present in the autumn leaves of none of the specimens. Cyanidin 3-*O*-glucoside (compound **11a**) was the major compound (more than 90%, see Fig. S2 in supplementary files) detected in autumn leaves and delphinidin 3-*O*-glucoside (compound **8**) was also detected but accounted for low percentages of the total area (see section 2.3 for more details of the anthocyanin composition of the autumn leaves). Cyanidin 3-*O*-sambubioside is also one of the major anthocyanins identified by HPLC-DAD-MS in ripe fruits of *Sambucus nigra* L. (eldeberry) (Hong and Wroldstat, 1990; Nakajima et al., 2004; Lee and Finn, 2007) whose structure was fully elucidated by Andersen et al. (1991) by means of NMR techniques. For this reason, an extract made from ripe elderberry fruits was injected in our laboratory in the same conditions as the *Populus* samples (see Fig. S1b in supplementary files). The chromatographic and spectral features of compound **10** were identical to those of the major compound detected in the *Sambucus* sample, which corresponded to cyanidin 3-*O*-sambubioside. Furthermore, the features of compound **10** were also identical to those of a compound present in the raspberry sample also injected in our laboratory (see Fig. S1a in supplementary files), which, according to previous reports (Mullen et al., 2010; Lee et al., 2012) also corresponds to cyanidin 3-*O*-sambubioside. The presence of the sambubioside derivative of delphinidin (*m/z* 597) might be expected in *P. tremula* samples since the same substitution pattern had been observed for cyanidin and delphinidin-containing anthocyanins in *Populus* samples (compounds **8** and **11a** were glucosides and compounds **9** and **12**, rutinosides). The extracted ion chromatogram (XIC) at *m/z* 597 showed a peak (compound **7**) around 18 min, just before the peak corresponding to delphinidin 3-*O*-glucoside. This chromatographic behaviour is in accordance with what it can be expected for the sambubioside derivative of delphinidin if we take into account what happened in the case of cyanidin 3-*O*-sambubioside: the introduction of an additional xylosyl residue in the anthocyanin structure reduced the retention time in relation to that of cyanidin 3-*O*-glucoside. Thus, from the chromatographic behaviour of compound **7** and from the *m/z* ratio of its molecular ion compound **7** was proposed to be delphinidin 3-*O*-sambubioside.

As previously indicated, two compounds co-eluted in peak 11 in *P. tremula* samples: compound **11a** (cyanidin 3-*O*-glucoside), which was the major one and compound **11b**. The molecular ion of compound **11b** showed a signal in the mass spectrum at *m/z* 727 and was fragmented in the MS^2 analysis yielding two fragment ions: the major one at *m/z* 287, corresponding to cyanidin and the minor one (relative abundance, 15) at *m/z* 581. The latter was originated from the loss of 146 amu and its *m/z* ratio was the same as that of compound **10**. Compound **11b**, therefore, was a cyanidin derivative containing a rhamnose residue (146 amu) and a pentose-hexose disaccharide (294 amu), which was likely to be a sambubioside taking into account the identity of compound **10**. The absence of a fragment ion at *m/z* 433 (rhamnoside of cyanidin) indicated that all the sugars had to be in the same position and the relative abundances of the fragment ions, which were similar to those observed in the fragmentation of the rutinosides of delphinidin and cyanidin, indicated that the rhamnose had to be linked to the glucose and through the same positions as it is linked in rutinosides (6-rhamnosylglucosides). From these chromatographic and spectral data, compound **11b** was proposed to be cyanidin 3-*O*-(2″-*O*-xyloxyl)rutinoside. This anthocyanin is the major pigment in the berries of some black raspberry (*Rubus occidentalis* L.) populations (Dossett et al., 2008, 2010 and 2011), where it has been identified on the basis of its chromatographic and spectral data (Hong and Wrolstad, 1990; Tian et al., 2005; Dossett et al., 2008, 2010 and 2011) and whose structure was verified by NMR (Tulio et al., 2008). The chromatographic and spectral properties of compound **11b** were the same as those previously reported for this compound. Furthermore, in addition to black raspberry, this pigment has also been detected and identified in red raspberry (*Rubus idaeus* L.) by HPLC-DAD-MS/MS (Mullen et al., 2002a, 2010) and HPLC-DAD coupled to a high-resolution Exactive Orbitrap mass spectrometer (HR-MR) (Mullen et al., 2010), although unlike in black raspberry, in red raspberry it is a minor compound (Mullen et al., 2002a). Taking into account this information, we searched for this compound in the raspberry sample that had been injected in our working conditions. The XIC at *m/z* 727 showed a peak at the same retention time of the major compound of this sample, which was cyanidin 3-*O*-glucoside (*m/z* 449). This behaviour was identical to what it was observed in *P. tremula* samples, thus supporting the identity proposed for compound **11b**.

In addition to these delphinidin and cyanidin derivatives, anthocyanins containing other anthocyanidins were also detected. This was the case of compound **14**, which was identified as pelargonidin 3-*O*-glucoside on the basis of the mass analysis results and chromatographic behaviour (Table 1). Furthermore, the retention time, *m/z* of the molecular ion and fragmentation pattern was identical to those of a minor compound detected in the red raspberry sample analysed in our laboratory, which, according to previous studies, corresponded to pelargonidin 3-*O*-glucoside (Mullen et al., 2002a, 2002b and 2010). This compound was present in *P. alba* and *P. tremula* samples, but it was only detected in one of the *P. nigra* samples.

Peak 13 was only detected in two of the *P. nigra* samples. However, the compound eluting in that peak was different in each case. In one of the samples the compound eluting in this peak showed a molecular ion at *m/z* 449 (compound **13a**) whereas in the other, the signal of the molecular ion was observed at *m/z* 479 (compound **13b**). The fragmentation pattern was the same in both cases: 162 amu (one hexose) were lost from the molecular ion, giving rise to the corresponding aglycones (m/z 287, cyanidin and m/z 317, petunidin, respectively). From the fragmentation pattern and chromatographic behaviour, compound **13a** was proposed to be a cyanidin derivative containing a hexose that confers less polarity to the molecule than glucose. Compound **13b** was identified as petunidin 3-*O*-glucoside since its retention time, *m/z* of the molecular ion and fragmentation pattern was the same as those of that pigment in wine samples analysed in the same conditions (Alcalde-Eon et al., 2004).

The identification of compounds **1** to **6,** due to their low concentration, was done on the basis of the mass analyses (*m/z* of the molecular and fragment ions and fragmentation patterns when available) and on the basis of their retention times and elution orders. Only the fragmentation of compounds **2** and **5** could be obtained. Both compounds showed similar fragmentation patterns, which were typical of flavanol-anthocyanin ($F\text{-}A^+$) direct condensation products (Alcalde-Eon et al., 2004; González-Paramás et al., 2006). Compound **2** showed a molecular ion at *m/z* 753, which was fragmented in the MS^2 analysis into a major fragment ion at *m/z* 591 by loss of 162 amu (Table 1). In addition, in the MS^2 spectrum, minor fragment ions appeared at *m/z* 329, *m/z* 573 and *m/z* 423. They were originated by loss of 424, 180 and 330 amu, respectively. These same fragment ions also appeared as major ions in the MS^3 spectrum, thus indicating that they were a part of the aglycon (*m/z*

591). The major one was that of *m/z* 423, originated by loss of 168 amu. This loss and that of 304 amu also observed in the MS^3 spectrum were indicative of the presence of one (epi)gallocatechin in the compound, linked to the rest of the molecule by its C_4 position: the first one corresponded to the fragment released in the retro Diels-Alder cleavage of an (epi)gallocatechin located in the upper position of a flavanol-flavanol or flavanol-anthocyanin dimer and the second one to the loss of the entire upper unit of the dimer after cleavage of the interflavanic linkage (Alcalde-Eon et al., 2006a). The fragment ion at *m/z* 287 was indicative of the presence of cyanidin in the molecule as well as that at *m/z* 329, 42 amu bigger than the anthocyanidin, which is always present in the fragmentation of this kind of compounds (Alcalde-Eon et al., 2004) and which is originated in the cleavage of bonds 2 and 4 of the C-ring of the flavanol. Thus, compound **2** was identified as a direct condensation product of (epi)gallocatechin and a cyanidin hexoside. Furthermore, the chromatographic features of compound **2** were the same as those of the direct condensation product between gallocatechin and cyanidin 3-*O*-glucoside previously identified in wines (Alcalde-Eon et al., 2006b). Consequently, this was the identity proposed for compound **2**.

Compound **5** showed a molecular ion at *m/z* 737, which was fragmented in the MS^2 analysis into one major fragment (*m/z* 575) by loss of 162 amu. This ion was in turn fragmented in the MS^3 analysis giving rise to three major ions at *m/z* 423, *m/z* 329 and *m/z* 557, by loss of 152, 246 and 18 amu, respectively, and to other minor ions among which, that corresponding to cyanidin was also observed (originated by loss of 288 amu). The losses of 152 and 288 amu indicated that a (epi)catechin moiety was present in the compound and that it was located in the upper position of the dimer: the first one corresponded to the fragment released in the retro Diels-Alder cleavage of these flavanols and the second, to the loss of the entire flavanol after cleavage of the interflavanic bond (Alcalde-Eon et al., 2004). From all these data, it can be deduced that compound **5** was a direct condensation product between (epi) catechin and cyanidin 3-*O*-glucoside. According to the results previously obtained in our laboratory for wine samples (Alcalde-Eon et al., 2006b), it seemed that catechin was the flavanol involved in that direct condensation product.

In the case of compounds **1**, **3**, **4** and **6**, it was only possible to know the *m/z* ratio of their molecular ions. Thus, their retention times and elution orders in relation to compounds **2** and **5** were necessary for their identification as well as the data available for this type of compounds obtained from the analysis of wine samples (Alcalde-Eon et al., 2006b). The molecular ions of compounds **3** and **4** showed identical *m/z* ratios (*m/z* 753), which were, in turn, the same as that observed for compound **2** (direct condensation product between gallocatechin and cyanidin 3-*O*-glucoside). This ratio might either correspond to the direct condensation product of epigallocatechin and cyanidin 3-*O*-glucoside or to the direct condensation product of catechin and delphinidin 3-*O*-glucoside. The assignment of the identity of compounds **3** and **4** was first made on the basis of the retention times and the chromatographic behaviour of this type of compounds in wine when analysed in the same chromatographic conditions (Alcalde-Eon et al., 2006b). Thus, the first identity (direct condensation product of epigallocatechin and cyanidin 3-*O*-glucoside) was assigned to compound **4** whereas compound **3** was identified as the direct condensation between catechin and delphinidin 3-*O*-glucoside. In addition, UV–vis spectra of these compounds supplied valuable information for the assignment of the identity. Compound **3** showed a visible absorption maximum at a higher wavelength than compound **4**, whereas this latter showed it at a very close value to that of compound **2**, which was a cyanidin derivative. These differences can be indicative of the anthocyanidin involved in the condensation product. Delphinidin-based anthocyanins and anthocyanin-derived pigments show their absorption maxima at higher wavelengths than cyanidin derivatives (Alcalde-Eon et al., 2006b), as a consequence of the additional substitution in the B-ring of the anthocyanidin. Thus, from the data supplied by their visible spectra it can be concluded that compound **4** contained cyanidin and compound **3,** delphinidin, which is in accordance with the identities proposed for them. Differentiation between gallocatechin or epigallocatechin-containing direct condensation products derived from cyanidin (compounds **2** and **4**) was made from their chromatographic behaviour. In wine samples analysed in these same chromatographic conditions, it has been observed that the F-A^+ dimers that contain epigallocatechin usually elute later than those containing the same anthocyanidin but condensed with gallocatechin (Alcalde-Eon et al., 2006b). Similarly, compound **6** showed the same *m/z* ratio as compound **5** and it was identified as its *epi*-isomer (direct condensation product between epicatechin and cyanidin 3-*O*-glucoside) from its chromatographic behaviour. Compound **1** showed a signal in the mass spectrum at *m/z* 769 which, along with the retention time, was in accordance with those of the direct condensation product between gallocatechin and delphinidin 3-*O*-glucoside previously identified in wines (Alcalde-Eon et al., 2006b).

The detection of this type of anthocyanin-derived pigments (F-A^+) in the anthers of the male catkins of poplars is interesting, since they are usually associated with processed or stored plant-derived foods and beverages. However, their presences in non-processed plant extracts have already been reported (González-Paramás et al., 2006).

In the *P. alba* samples an additional peak (corresponding to compound **15**) was observed at the end of the chromatogram (40.4 min). In some *P. alba* samples, this peak accounted for higher percentages of the total area than some of the F-A^+ dimers. Although this compound co-eluted with some flavonols, which were present at much higher amounts than it, the visible part of the UV–vis spectrum could be obtained, showing a shape and a visible maximum typical of pyranoanthocyanins, a type of anthocyanin derivatives that are formed through cycloaddition of different compounds to the native anthocyanins that gives rise to an additional ring in the anthocyanin structure. However, the co-elution with flavonols made impossible the obtaining of neither the *m/z* ratio of its molecular ion nor the fragmentation pattern even with a deeper evaluation of the mass spectrometry results. In fact, none of the pyranoanthocyanins that could be expected from the main anthocyanins present in the sample were localised in their extracted ion chromatograms (XIC) at the retention time of compound **15**. Although its identity has not been established, compound **15** was considered in this study since it was only present in *P. alba* samples and might be a species-specific compound, useful as chemotaxonomic marker.

In relation to quantitative differences, Table 2 shows for each species the total and individual pigment mean concentration (expressed in mg of cyanidin 3-*O*-glucoside per 100 g of anthers-dry weight) as well as the individual mean percentage over the total content. *P. nigra* samples showed the highest total mean concentration (554.79 mg/100 g) followed by *P. alba* samples (407.96 mg/100 g), although the differences were not significant. On the contrary, significant differences were found between these two species and *P. tremula* samples, which showed a much lower mean content (24.96 mg/100 g) than the former ones. This was in accordance with the colour observed in the catkins: whereas those coming from *P. nigra* and *P. alba* samples showed purple-red anthers, those coming from *P. tremula* samples were much less coloured, with a light red hue in some of the anthers and a pale brown hue in others. From these results, a relationship between the total content of the anthers of the different species and the degree of

Table 2
Individual contents and percentages of the different compounds in the *Populus* samples. Results are the mean values of the contents and percentages determined for all the samples ascribed to a given *Populus* species in accordance with the morphological characters.

Peak	Identity	Content (mg Cy-3-glc/100 g of anthers)			%		
		P. nigra	*P. tremula*	*P. alba*	*P. nigra*	*P. tremula*	*P. alba*
1	F-A$^+$ GC-Dp-3-glc	nq	nq	nq			
2	F-A$^+$ GC-Cy-3-glc	1.30^c	0.00^a	0.25^b	0.23^c	0.00^a	0.07^b
3	F-A$^+$ C-Dp-3-glc	0.72^b	0.00^a	0.92^b	0.15^b	0.00^a	0.23^c
4	F-A$^+$ EGC-Cy-3-glc	0.48^c	0.00^a	0.25^b	0.09^c	0.00^a	0.07^b
5	F-A$^+$ C-Cy-3-glc	2.34^a	0.18^a	4.95^b	0.45^a	0.40^a	1.27^b
6	F-A$^+$ EC-Cy-3-glc	0.62^b	0.05^a	0.63^b	0.11^a	0.07^a	0.14^a
7	Dp-3-samb	0.00^a	0.02^b	0.00^a	0.00^a	0.03^b	0.00^a
8	Dp-3-glc	194.49^b	0.73^a	30.32^a	34.77^b	5.85^a	10.03^a
9	Dp-3-rut	1.51^b	0.28^a	5.72^c	0.27^a	1.89^b	1.77^b
10	Cy-3-samb	0.00^a	2.03^b	0.00^a	0.00^a	6.96^b	0.00^a
11*	Cy-3-glc (+Cy-3-xylrut*)	352.01^b	18.16^a	323.95^b	63.70^a	71.07^{a,b}	77.85^b
12	Cy-3-rut	0.89^a	3.46^a	39.10^b	0.16^a	13.67^c	8.27^b
13a	Cy-3-hex	0.20^a	0.00^a	0.00^a	0.03^a	0.00^a	0.00^a
13b	Pt-3-glc	0.09^a	0.00^a	0.00^a	0.01^a	0.00^a	0.00^a
14	Pg-3-glc	0.12^a	0.03^a	0.47^b	0.02^a	0.04^a	0.11^b
15	Unknown-vitisin type	0.00^a	0.00^a	1.41^a	0.00^a	0.00^a	0.19^b
	Total content	554.79^b	24.96^a	407.96^b			

Dp: delphinidin. Cy: cyanidin. Pt: petunidin. Pg: pelargonidin. Glc: glucoside. Rut: rutinoside. Samb: sambubioside. Xylrut: xylosylrutinoside. Hex: hexoside. (E)GC: (epi) gallocatechin. (E)C: (epi)catechin. F-A$^+$: Flavanol-Anthocyanin direct condensation dimer. nq: not quantified. *: Cy-3-xylrut was only detected as minor compound in *P. tremula* samples. Different lower case letter within each row indicate significant differences ($p < 0.05$; $n = 18$ for *P. nigra* and *P. alba*, $n = 9$ for *P. tremula*).

pilosity of the bracts of the catkins can be established. As previously indicated, the bracts of the catkins from *P. nigra* were glabrous, whereas those of *P. alba* and *P. tremula* were hirsute. However, there were also differences between these two latter ones in relation to the degree of pilosity, with *P. tremula* showing the highest hair density (Fig. 2). Thus, the degree of physical shading of the anthers is much higher for that species than for *P. alba* and *P. nigra* and for this reason, less amounts of anthocyanins are required and, consequently, synthesised for a supposed photoprotective role.

Differences in the anthocyanin profile among the different species were assessed through the mean percentages of the individual compounds (Table 2). *P. nigra* samples were characterised by containing two main compounds that accounted for 98.5% of the total content and some minor compounds with percentages lower than 1% (Table 2 and Fig. 1). To be precise, cyanidin 3-*O*-glucoside (compound **11a**) was the major one (63.7%) and delphinidin 3-*O*-glucoside (compound **8**), the second most abundant compound (34.8%). The rutinosides of cyanidin and delphinidin (compounds **12** and **9**) represented the lowest percentages over the total mean content among the three species. Respecting flavanol-anthocyanin direct condensation products (F-A$^+$, compounds **1**–**6**), some statically significant differences were observed that allow differentiating *P. nigra* from the other species. Whereas the highest percentages of the F-A$^+$ products containing (epi)gallocatechin (compounds **2** and **4**) were observed for *P. nigra* samples, *P. alba* showed the highest percentages of the F-A$^+$ products containing (epi)catechin (compounds **3** and **5**, significant; compound **6**, not significant). For this reason, the mean ratio F-A$^+$ (epi)gallocatechin derivatives/F-A$^+$ (epi)catechin derivatives was higher in the case of *P. nigra* samples (0.67) than in the case of *P. alba* samples (0.09). Conversely, no F-A$^+$ products containing (epi)gallocatechin were detected in *P. tremula* samples.

Respecting *P. tremula* samples, there were five compounds that accounted for 99.5% of the total content. Cyanidin 3-*O*-glucoside (compound **11a**) was also the most abundant compound accounting for 71.1% of the total content. However, in this case, the second and third most abundant compounds were, respectively, the rutinoside (compound **12**) and the sambubioside (compound **10**) derivatives of cyanidin. The glucoside and the rutinoside of delphinidin (compounds **8** and **9**) were the fourth and the fifth most abundant compounds, respectively. Whereas the percentage of delphinidin 3-*O*-glucoside was much lower in *P. tremula* in relation to *P. nigra*, that of delphinidin 3-*O*-rutinoside was higher in *P. tremula*. Among the minor compounds of *P. tremula*, it is worth

Fig. 2. Detail of the male catkins (lyophilised) from *P. nigra* (left), *P. alba* (middle) and *P. tremula* (right) specimens showing the different degree of pilosity of the bracts.

pointing out that only (epi)catechin-containing F-A$^+$ products were detected representing percentages similar to those found in *P. nigra* samples, but lower than those detected in *P. alba* samples. Furthermore, as occurred in the case of compound **10** (cyanidin 3-*O*-sambubioside), compound **7** (delphinidin 3-*O*-sambubioside) and compound **11b** (cyanidin 3-*O*-(2″-*O*-xyloxyl)rutinoside) were only detected in *P. tremula* samples. This means that these three compounds can be considered as chemotaxonomic markers and, consequently, their presence in an unknown poplar sample along with some typical morphological traits will point to the identity of *P. tremula*.

Five compounds also accounted for 99% of the total content in *P. alba*. Cyanidin 3-*O*-glucoside (compound **11a**) was again the major one in this species, showing the highest mean percentage among all the species (77.8%). Similarly to *P. nigra* samples the second most abundant compound was delphinidin 3-*O*-glucoside (compound **8**) but showed lower mean percentage (10%). However, the rutinosides of cyanidin (compound **12**) and delphinidin (compound **9**), that were the third and fourth compound in abundance order, represented much higher percentages (8.3% and 1.8%, respectively) than in the case of *P. nigra*, although lower than in *P. tremula*. Compound **5** (catechin-cyanidin 3-*O*-glucoside direct condensation product) accounted for 1.3% of the total content, which was the highest percentage observed among all the F-A$^+$ compounds and in all the types of samples. *P. alba* samples were also characterised by possessing the highest percentages of pelargonidin 3-*O*-glucoside and by the presence of compound **15**.

2.2. Species differentiation by means of statistical analysis

From the results of the present study it can be seen that there are qualitative and quantitative differences in the anthocyanin composition that might be used to discriminate among the three species. For this reason, a deeper statistical analysis was carried out in the results of the study. Hierarchical cluster analysis (HCA) was performed on the quantitative results as an unsupervised classification technique in order to verify if these differences allow the separation of the different types of samples in different groups or clusters. Furthermore, since the initial classification of the samples was done from morphological characters, the HCA was performed in order to see if there is an agreement between the classification made from the botanical characters and that made from the anthocyanin composition. This statistical technique was selected on the basis of the satisfactory results obtained with anthocyanin composition in other plant genera not only in our laboratory (Alcalde-Eon et al., 2014) but also in other research groups (Wang et al., 2001, 2004; Figueiredo-González et al., 2012; Li et al., 2013). Since cyanidin 3-*O*-glucoside was the major compound and showed much higher percentages than the rest of pigments in all the samples it was not considered for the HCA. Thus, data matrix was constituted by the mean ($n = 3$) percentage of each individual compound (excepting cyanidin 3-*O*-glucoside) in each sample (6 *P. nigra*, 3 *P. tremula* and 6 *P. alba*-like) along with the mean total pigment content in each one. Fig. 3 shows the dendrogram obtained. Two clusters can be observed at the rescaled distance of 25, one containing all the samples that were initially characterised as *P. nigra* and the other containing all the samples characterised as *P. tremula* and *P. alba*. This second cluster was formed, in turn, by two clusters that corresponded each to only one species, *P. alba* and *P. tremula*. These results are very interesting since they reveal that the anthocyanin profile of the anthers of *Populus* male catkins can be used for chemotaxonomic purposes: the hierarchical clustering made from the percentages of the different pigments allowed a separation of the samples in three clusters (safe cutting value at the dissimilarity distance of 20) that were in accordance with the identification of the samples carried out from their morphological features and from comparison to literature (Soriano, 1993). Furthermore, the first initial separation between *P. nigra* samples and *P. tremula* and *P. alba* samples is also in accordance with the most commonly used classification of the different species of the genus *Populus* into different sections carried out by Eckenwalder (1996), that places *P. nigra* in the *Aigeiros* Duby section and *P. tremula* and *P. alba* in the *Populus* (Leuce) Duby section.

Within each of the three clusters corresponding to the different species, separation of samples in sub-clusters also occurred. In the cluster corresponding to the samples initially classified as *P. alba*, two main sub-clusters could be observed. One contained a single sample (POP8) and the other contained the rest of the samples. POP8 was a cultivated specimen whereas the rest of the samples were collected from non-cultivated trees. All the trees were initially classified from their morphologic features and according to literature (Soriano, 1993; Streeter et al., 2011) they were consistent with *P. alba*. However, although it was not an initial aim of the study, the analysis of the anthocyanin composition of the anthers has allowed a further separation of the samples. In order to understand the cause of this separation, the specimens were carefully examined again, not only from the material collected and deposited at the SALA herbarium but also directly at the study site. Small morphologic differences were observed between the cultivated and non-cultivated trees that might be due to differences in the varieties. In fact, POP8 might be an ornamental variety since it was located in a public park. Furthermore, recent studies on *P. alba* based on the use of genetic markers (Amplified fragment length polymorphism-AFPL or microsatellite polymorphism-SSR, among others), have revealed the presence of specimens of *P.* × *canescens* (Ait.) Sm, an hybrid between *P. alba* and *P. tremula*, intermingled with *P. alba* trees in natural *P. alba* populations in several European river valleys (Fossati et al., 2004; Lexer et al., 2005; Van Loo et al., 2008; Santos-del-Blanco et al., 2013), including the river Douro basin, which is not far from the collection sites of the present study. Distinction between *P.* × *canescens* and *P. alba* exclusively on the basis of their morphological features is difficult. Furthermore, the presence of backcrosses of *P.* × *canescens* towards *P. alba*, have also been observed in those sympatric zones (Fossati et al., 2004; Lexer et al., 2005; Santos-del-Blanco et al., 2013), making even more difficult to differentiate between purebreds and hybrids and backcrosses due to the large variance in the phenotypic characters introduced by all this crosses. In our study some of the features of the *P. alba* samples (except POP8) were coincident with those described in literature for *P.* × *canescens* (Soriano, 1993) and similar to those observed in voucher specimens collected not far from our collection sites and also deposited in SALA herbarium. Nevertheless, from these morphologic data it was not possible to indicate if the samples are hybrids or purebreds. In fact, in the study by Santos-del-Blanco et al. (2013) a sample that was initially classified as *P.* × *canescens* on the basis of its morphologic traits was finally identified as purebred *P. alba* on the basis of the molecular markers. In the present study, the hierarchical cluster analysis performed from the anthocyanin composition is pointing out to differences among the *P. alba*-like samples, that might be related to the possible existence of *P.* × *canescens* or even backcrosses towards *P. alba* among them. This aspect will be discussed later. In addition, a further separation was observed in this second sub-cluster into two groups. Interestingly, each group corresponded to a different collection site. POP10 and POB10B were neighbour riparian trees located ca. 30 Km far from the collection site of POP4, POP5 and POP7. This differentiation according to the location can be explained either by the environmental conditions that can influence the anthocyanin synthesis or by the presence of different clones at the different collection sites. However, taking into account the high rates of

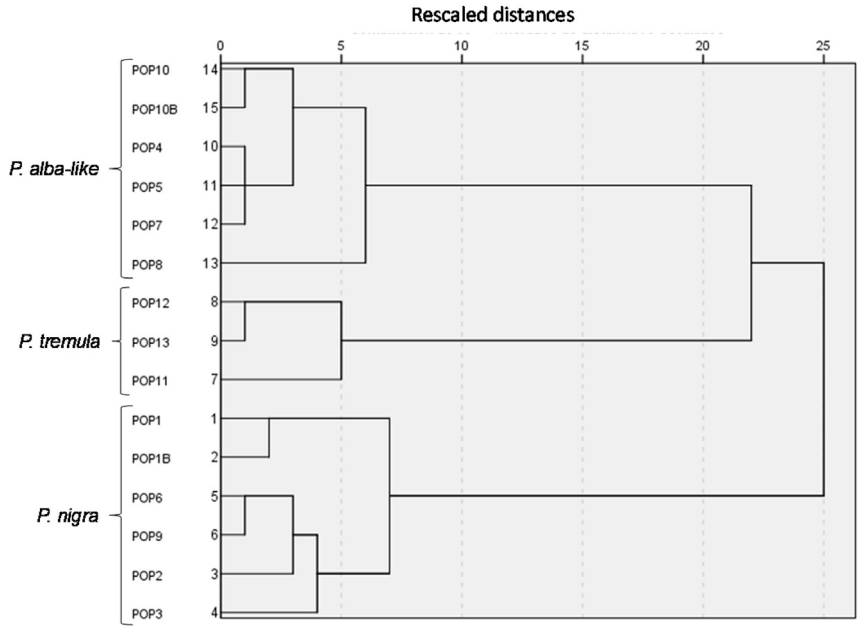

Fig. 3. Dendrogram from Hierarchical Clustering Analysis obtained from the mean individual percentages (except that of the major pigment, cyanidin 3-*O*-glucoside) and from the mean total content in each specimen (n = 3, three samples per specimen). Codification and detail of the samples are reported in Table 3. X-axis represents the rescaled distances between clusters that were calculated by Ward's algorithm. The position of the vertical line on the x-axis scale indicates the distance at which clusters are joined. The classification of the samples initially done on the basis of the morphological characters of the samples has also been included on the left side of the dendrogram in order to compare both classifications.

asexual reproduction in the genus *Populus* and particularly in *P. alba* and *P.* × *canescens* (Santos-del-Blanco et al., 2013), the second possibility seems to be a more important factor. In the case of POP10 and POP10B, it is reasonable to think that they might correspond to a same clone and that they result from vegetative propagation. In the case of POP4, POP5 and POP7, where the distances between individuals were higher than in the other case, the possibility of corresponding to a same clone seems *a priori* more unlikely. However, in the case of *P. alba* and *P.* × *canescens* the maximum distance that has been observed between individuals of the same genet was 150 Km (Santos-del-Blanco et al., 2013). Thus, in our case, POP4, POP5 and POP7 might also be clones.

In the cluster corresponding to *P. nigra*, two clusters could also be observed. The first one contained POP1 and POP1B, which were two samples collected from the same tree but at different elongation phase of the catkin. Thus, their classification within the same sub-cluster seems reasonable. The other sub-cluster contained the rest of the samples classified as *P. nigra*, but unlike in the case of the samples classified as *P. alba*, no further separation related to their collection site could be observed, since POP2 and POP3 were located at the same collection as POP1 and POP1B. No separation between the fastigiated specimens (POP3 and POP6), corresponding to naturalised 'Italica' cultivar specimens, and the two others were observed. It seems that *P. nigra* 'Italica' is a single gene mutant of non-fastigiated *P. nigra* (*P. nigra* var. *typica*) (Adams, 2010) that was probably originated from a spontaneous mutation that occurred in central Asia. It seems that it was probably introduced in Italy during the 18th century and from there it was spread all over the world (Cagelli and Lefèvre, 1995). Since it is otherwise a typical *P. nigra* it has all the characteristics of *P. nigra*, including insect parasites and diseases (Adams, 2010). This is probably why no differences were observed in our study between fastigiated and non-fastigiated trees. On the contrary, on the basis of the hierarchical clustering, the specimen from which samples POP1 and POP1B were collected should be a cultivar or a variety of *P. nigra* different from *typica* or 'Italica'. Although the differentiation among varieties or cultivars was not an objective of the study, individuals of the *P. nigra* cluster were further inspected to detect morphologic features that may be useful for explaining the cause of the separation between POP1 and POP1B from the rest of the samples, as was done for the *P. alba* cluster. The only morphologic difference that could be observed among these samples was the existence of pubescence in the petioles of the specimen from which POP1 and POP1B were collected in contrast to the petioles of the rest of the *P. nigra* samples that were completely glabrous. The hairiness of the leaves, petioles and first year twigs is a feature associated to the varieties *betulifolia* (Pursh) Torr. and *caudina* Ten. (= *P. nigra* var. *pubescens* Parl.) described respectively in Great Britain and France (*betulifolia*) and in Spain, North Africa, central and southern Italy, the Balkans and Iran (*caudina*) (Cagelli and Lefèvre, 1995; Adams, 2010). In our case, POP1 could be a specimen of the *caudina* variety thus explaining the differentiation between POP1 and the rest of the *P. nigra* samples.

These results show the usefulness of carrying out hierarchical clustering with anthocyanin composition data for chemotaxonomic purposes. Previous studies performed in other plant genera that also applied HCA to anthocyanin composition also obtained satisfactory results. In these studies it was possible to classify the samples according to their botanical sections, varieties and even clones, detect possible hybrids and/or determine phylogenetic positions (Wang et al., 2001, 2004; Figueiredo-González et al., 2012; Li et al., 2013; Alcalde-Eon et al., 2014).

In order to know which anthocyanin pigments were responsible for the interspecific and intraspecific discriminations, a Principal Component Analysis (PCA) was carried out from the values corresponding to the mean percentage of each compound and to the mean total content in each sample (Table S1 in supplementary files). Fig. 4 shows the PCA scores plot (left) and the loadings (right) for each of the variables. Factors 1 and 2 (PC1 and PC2, respectively) explained 60.5% of the variance (PC1 35.7% and PC2 24.8%). See

Tables S2 and S3 in supplementary files for further details on the rest of the factors. Samples were first separated along PC1 according to the species. Positive values of PC1 are associated with compounds **2** and **4** (direct condensation products between gallocatechin and cyanidin 3-*O*-glucoside) and compound **8** (delphinidin 3-*O*-glucoside). *P. nigra* samples were those who showed the highest percentages for these three compounds (Table 2 and Table S1) and, for this reason, they showed positive values of PC1. On the contrary, F-A$^+$ dimers containing (epi)gallocatechin were not detected in *P. tremula* and compound **8** showed much lower percentages in *P. tremula* samples (5.9%) than in *P. nigra* samples (34.8%) which has contributed to the separation of *P. tremula* samples towards negative values of PC1. Furthermore, negative values of PC1 are related to high mean percentages of compound **12** (cyanidin 3-*O*-rutinoside) and compound **10** (cyanidin 3-*O*-sambubioside). Compound **10** was only present in *P. tremula* samples and compound **12** showed the highest mean percentage (13.7%) in that species whereas it accounted only for 0.2% of the total area in *P. nigra* samples. These important differences in the composition were, therefore, responsible for the separation of *P. tremula* and *P. nigra* along PC1. *P. alba*-like samples showed intermediate values of PC1 due to the fact that these compounds were present in percentages in-between those found in *P. nigra* and *P. tremula*. However, *P. alba*-like samples were clearly separated from *P. nigra* and *P. tremula* samples along PC2. Positive values in this PC are related to higher percentages of F-A$^+$ dimers containing (epi)catechin (compounds **3** and **5**) and with higher percentages of pelargonidin 3-*O*-glucoside (compound **14**). *P. alba*-like samples showed the highest values of these compounds and although they are minor compounds, they seem to be relevant for the discrimination among species. Compound **15**, which was exclusively found in *P. alba*-like samples, also seemed to be important for the discrimination. In the cases of *P. tremula* and *P. nigra* samples the negative values observed for PC2 are mostly due to the lower percentages of the variables with high positive loadings in PC2. Only in the case of *P. tremula* samples the presence of cyanidin 3-*O*-sambubioside (compound **10**) might have influenced the negative value of PC2. The contribution of the total content (T) to the separation of the samples was quite low for both factors. This means that the percentages of some compounds are more relevant for the discrimination among species than the total anthocyanin content. As can be seen in Fig. 4 and in Table S1, the differences in the anthocyanin composition allowed a clear separation between the *P. alba*-like samples and the *P. tremula* ones. If, as earlier discussed in this work, the *P. alba*-like samples (excepted POP8, which was a cultivated specimen) were F1 hybrids between *P. tremula* and purebred *P. alba* (*P.* × *canescens*) more similarities in the composition between them and *P. tremula* samples would be expected. In fact, Jones and Seigler (1975) studied the flavonoid composition of the leaves of *Populus acuminata* Rydberg, which was supposed to be a hybrid between *Populus angustifolia* James and *Populus sargentii* Dode and they observed that not only the morphologic characters were intermediate of those of the putative parent species but also the flavonoid composition, which was additive of the compositions of both parents. In our study, compounds **7** and **10** (delphinidin and cyanidin 3-*O*-sambubiosides) were exclusively detected in *P. tremula* samples and not in the *P. alba*-like ones. If these latter ones were F1 hybrids, the presence of those compounds should be expected. However, they were not detected in any of the samples not even after performing a XIC at their *m/z* ratios. On the contrary, some morphological characters of the *P. alba*-like samples were similar to those described for *P.* × *canescens* in the literature (Soriano, 1993; Streeter et al., 2011) whereas others fitted well with those described for *P. alba*. Backcrosses between *P.* × *canescens* and their parents have been reported to occur in hybrid zones (Fossati et al., 2004; Lexer et al., 2005; Van Loo et al., 2008) including the Douro Basin (Santos-del-Blanco et al., 2013), which is next to the collection sites of the present study. However, it has been reported that in those zones, the backcrosses with *P. alba* are more abundant than those with *P. tremula* (Fossati et al., 2004; Lexer et al., 2005; Van Loo et al., 2008; Santos-del-Blanco et al., 2013). Successive backcrosses with one of the parents would progressively reduce the contribution of the other parent, which might finally lead to purebreds. Thus, in our case, *P. alba*-like specimens (except POP8) are more

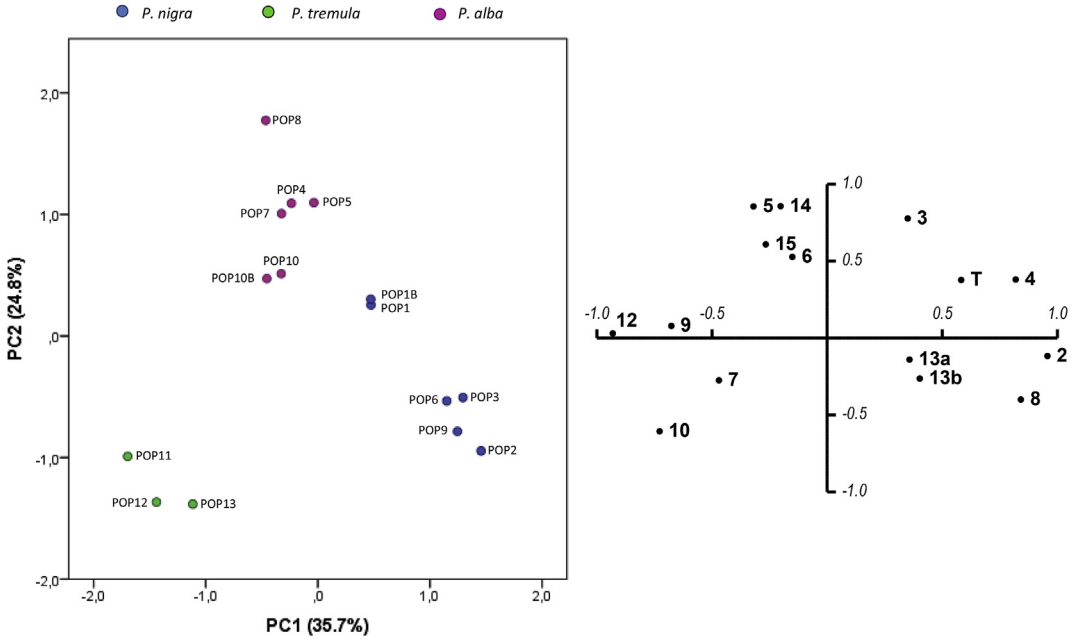

Fig. 4. Plot of the Principal Component Analysis (left) and of the loadings of each of the original variables (right) denoted by the compound number (see Tables 1 and 3 for compound and sample identities). The attribution of the species to each specimen (colour of the circle) was done on the basis of the morphological characters.

likely to be backcrosses between *P.* × *canescens* and *P. alba* than F1 hybrids, which might explain the absence of the specific anthocyanins of *P. tremula*. Independently of the fact of being purebreds or backcrosses, the use of anthocyanin composition combined with chemometrics (HCA and PCA) has allowed the classification of all the *P. alba*-like samples in the same group, completely separated from the samples belonging to the other two species. Indeed, the differentiation between backcrosses and purebreds still remains difficult even with specific genetic techniques (Fossati et al., 2004).

In relation to the intraspecific differentiation of samples that was observed in the HCA (separation of the samples in subclusters), the PCA analysis has revealed that this differentiation is mostly influenced by PC2. *P. alba*-like samples showed high positive PC2 values, and POP8 was the sample with the highest value in this PC. This was mainly due to the higher percentage of compound **15** in relation to the other samples of the cluster and to the lower value of compound **8** (delphinidin 3-*O*-glucoside) (Table S1) which was negatively correlated with PC2. In the case of the *P. nigra* samples, POP1 and POP1B were also separated from the rest of the samples not only along PC2 but also along PC1. The lower percentage of delphinidin 3-*O*-glucoside (compound **8**) observed in these two samples in relation to the rest of them (Table S1) is the main cause of this separation, since it correlates positively with PC1 and negatively with PC2. In addition, the higher percentages observed for compound **5** in POP1 and POP1B (Table S1) may have contributed to the separation along PC2. Regarding the influence of maturity of the catkin in the anthocyanin composition (differences between POP1 and POP1B), slightly higher percentages of compounds **3** and **5** (F-A$^+$ direct condensation products) were detected in POP1B, thus indicating that maturity can increase the content of this type of compounds in the male *Populus* catkins. Furthermore, compound **14** (pelargonidin 3-*O*-glucoside) was detected in the sample collected at lower degree of maturity, but it was absent in POP1B and in the rest of the *P. nigra* samples. However, as it might be expected, these slight differences did not allow the separation of these two samples in the PCA.

2.3. Anthocyanin composition of the autumn leaves from P. tremula

As previously indicated, autumn leaves collected from *P. tremula* trees were also analysed. There was a single compound that accounted for more than 92% of the total area and corresponded to compound **11a**, cyanidin 3-*O*-glucoside. Compound **8** (delphinidin 3-*O*-glucoside) was also detected accounting for less than 2%. Other minor peaks were observed in the chromatogram. However, from the results of the XIC at the *m/z* ratios of the other anthocyanins detected in the anthers, it could be seen that they did not correspond to any of these compounds. Fig. S2 (see supplementary files) shows the comparison between the chromatogram recorded at 520 nm for the male catkins of POP11 and that of the autumn leaves of POP13. Unlike in the study by Bendz and Haglund (1968), which reported the presence of a cyanidin 3-*O*-xylosylglucoside in autumn leaves by means of paper chromatography and TLC, compound **10** (cyanidin 3-*O*-sambubioside) was not detected in the present study. On the contrary, they did not detect the cyanidin 3-*O*-xylosylglucoside in the male catkins, whereas in the present study compound **10** was a major compound in the anthers of the *P. tremula* samples. Although the study of the autumn leaves has revealed differences in the anthocyanin composition between autumn leaves and anthers in *P. tremula* samples, the anthocyanin content calculated for leaf samples, (0.27 mg/g of fresh leaves, expressed in cyanidin 3-*O*-glucoside) was very similar to that observed in the anthers of *P. tremula* samples.

2.4. Possible role of anthocyanins in the anthers of Populus.

In the present study it has been observed that cyanidin 3-*O*-glucoside is the major pigment in both autumn leaves and anthers. Anthocyanin synthesis can be induced by several stimuli and, among them, visible and UVB radiation (Chalker-Scott, 1999). Recent studies have reported that light can induce the expression of genes encoding several enzymes of the anthocyanin biosynthetic pathway (Davies and Schwinn, 2006). Cyanidin is one of the primary anthocyanidins synthesised in the anthocyanin biosynthetic pathway (Strack and Wray, 1994) and for this reason, cyanidin derivatives are commonly the first anthocyanins synthesised in response to light. Anthocyanin synthesis is metabolically expensive (Chalker-Scott, 1999) and this fact might explain why the structures of the anthocyanins synthesised in vegetative tissues are usually simpler that those found in reproductive organs which, in turn, might be related to the different functions associated to anthocyanins in the different tissues (Steyn et al., 2002). Whereas the presence of anthocyanins in vegetative tissues has been related to a photoprotective function (Hoch et al., 2003; Steyn et al., 2002), the presence of anthocyanins in reproductive organs of animal-pollinated plants aims to selectively attract one or another type of pollinators (Harborne and Grayer, 1994). Since poplars are wind-pollinated, there is no need to synthesise complex pigments to selectively attract pollinators. Thus, the presence of cyanidin 3-*O*-glucoside in leaves and anthers might be related to the photoprotective role, necessary in both parts of the plant. In the case of anthers, photoprotection is needed since in these three species flowering takes place before the foliage emerges and thus, aments and consequently anthers and pollen grains are exposed to sunlight. In the case of autumn leaves, anthocyanins shield photosynthetic tissues from excess light (Steyn et al., 2002) thus protecting foliar nutrient resorption during senescence (Hoch et al., 2003). The anthocyanin-coloured autumn leaves from *P. tremula* studied in the present study showed a simple composition with primary anthocyanins containing simple substitution patterns (92% of cyanidin 3-*O*-glucoside and less than 2% of delphinidin 3-*O*-glucoside), which fits well with the supposed photoprotective role of anthocyanins in leaves. Thus, a similar function can be attributed to the anthocyanins of the anthers of *P. nigra* samples, which were mostly composed of these two pigments (63.7% of cyanidin 3-*O*-glucoside and 34.8% of delphinidin 3-*O*-glucoside). This photoprotective role of the anthocyanin pigmentation of the anthers of that species is consistent with the fact that the bracts of the male catkins are glabrous and anthers are more exposed to sunlight than in the other species. *P. tremula* male catkins, on the contrary, possess dense hairs in the bracts that shade anthers almost completely during the elongation of the catkins, allowing a partial exposure to sunlight at the moment of maximum elongation. For this reason, the presence of anthocyanins acting as sunscreens seems not to be as necessary as in the case of *P. nigra* which could explain the much lower contents in *P. tremula* in relation to those detected in *P. nigra* specimens. Apart from cyanidin 3-*O*-glucoside, which was also the major anthocyanin, *P. tremula* anthers contained other anthocyanins with substitution patterns more complex than it could be expected for a photoprotective role and that were not present in the autumn leaves from the same species. It is worth pointing out the presence of sambubiosides and rutinosides of cyanidin and delphinidin and xyloxylrutinoside of cyanidin, which contain xylose, rhamnose or both of them in addition to glucose. Furthermore, *P. tremula* anthers contained pelargonidin 3-O-glucoside. It is interesting to remark that the anthocyanin profile that has been obtained for *P. tremula* anthers (with the exception of the delphinidin derivatives) is quite similar to the anthocyanin profile reported for *Rubus occidentalis* L. berries (black raspberries) (Tulio

et al., 2008; Dossett et al., 2011) despite the fact of belonging to different families (Salicaceae and Rosaceae, respectively) and corresponding to different localisations in the plant. In the studies carried out in black raspberries (Tulio et al., 2008) it has been demonstrated that the antioxidant capacity of cyanidin 3-*O*-rutinoside was higher than that of cyanidin 3-*O*-xyloxylrutinoside and considerable higher than those of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-sambubioside. For this reason, an additional antioxidant function might be attributed to the anthocyanins present in the anthers of *P. tremula*. Taking into account the metabolic cost of the synthesis of anthocyanins (Chalker-Scott, 1999), anthers of this species should be subjected to other stresses different than light that make necessary the additional synthesis of that kind of antioxidants. It would also be possible that this species lacked other compounds that usually play that antioxidant role in the other species and the synthesis of anthocyanins would be shifted towards compounds with higher antioxidant capacities. As the anthers of *P. nigra* samples, those of *P. alba*-like samples lacked xylose derivatives (sambubiosides and xyloxylrutinosides) but unlike them, they contained cyanidin and delphinidin 3-*O*-rutinosides in higher percentages, although they were lower than those determined in *P. tremula*. Interestingly, in this study a direct relationship has been observed between the rutinosides/glucosides ratio (mean ratios: *P. nigra*, 0.003; *P. alba*-like, 0.115; *P. tremula*, 0.202) and the degree of pilosity and physical shading of the anthers (*P. nigra*, no shading; *P. alba*-like, medium shading; *P. tremula*, almost complete shading).

3. Conclusions

The detailed anthocyanin compositions of the anthers of the three main species of the genus *Populus* present in the Iberian Peninsula (*P. nigra*, *P. alba* and *P. tremula*) have been reported here for the first time. Seventeen different compounds have been identified although differences in the anthocyanin profiles among species have been observed. It is worth pointing out that not only have been identified anthocyanin monoglycosides but also di- and triglycosides, with sugars other than glucose, such as rhamnose and xylose. Furthermore, Flavanol-Anthocyanin direct condensation products (F-A^+ dimers) have been detected in all the samples, but with different proportions between those derived from (epi)gallocatechin and those derived from (epi)catechin in the different species. Some of these anthocyanins and anthocyanin-derived pigments might be considered as valuable chemotaxonomic markers, such as cyanidin and delphinidin 3-*O*-sambubiosides and cyanidin 3-*O*-(2″-*O*-xyloxyl)rutinoside that were only detected in *P. tremula* samples or compound **15** (vitisin-type anthocyanin-derived pigment) which was only present in *P. alba*-like samples. This means that the presence of any of these compounds in an unknown poplar sample will be pointing to a certain *Populus* species. Furthermore, the quantitative differences observed in the pigment composition along with the use of HCA or PCA, have allowed a differentiation of the samples that is in accordance with the initial classification of the samples carried out from the morphological traits of the specimens. Moreover, these statistical techniques have also been useful to detect intraspecific differences that might be due to the facts of being different clones or varieties of a same species. From the results of the present study it can be concluded that the anthocyanin composition of the anthers of these three *Populus* species can be used for chemotaxonomic purposes.

The fact that the major anthocyanin in all the samples is cyaninidin 3-*O*-glucoside (>60%) is compatible with the possible photoprotective role of the anthocyanins of the anthers. Furthermore, a reverse relationship between the total anthocyanin content and the degree of physical shading of the anthers supplied by the pilosity of the bracts has been observed, which also supports the proposed photoprotective role. In *P. tremula* samples an additional antioxidant function has been proposed taking into account the complete anthocyanin profile which is similar to those observed in some fruits with high antioxidant capacity.

4. Experimental

4.1. General procedures

HPLC–DAD analyses were performed in a Hewlett-Packard 1200 series liquid chromatograph. An AQUA C18 reverse phase, 5 μm, 150 mm × 4.6 mm column (Phenomenex®,Torrance, CA, USA) thermostatted at 35 °C, was used.

The HPLC–DAD conditions have been previously employed with satisfactory results in our laboratory in the analysis of other plant materials (Alcalde-Eon et al., 2013, 2014). The solvents used were: (A) an aq. soln. (0.1%) of trifluoroacetic acid (TFA) and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 5 min, from 10 to 15% B for 15 min, isocratic 15% B for 5 min, from 15 to 18% B for 5 min and from 18 to 35% B for 20 min, at a flow rate of 0.5 mL/min. Detection was carried out at 520 nm as the preferred wavelength. Spectra were recorded from 220 to 600 nm.

Mass spectrometric analyses were performed in a API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by Analyst 5.1 software. The HPLC system was connected to the mass spectrometer via the UV cell outlet. MS analysis was carried out in positive mode (ESI^+). Zero grade air served as nebulizer (GS1) and turbo gas (GS2) for solvent drying. Nitrogen served as curtain (CUR) and collision gas (CAD). Settings used were optimized by direct infusion of a malvidin 3-*O*-glucoside solution: declustering potential (DP) 41 V, entrance potential (EP) 7.5V, ion spray voltage (IS) 5000V, GS1 40 psi, GS2 50 psi (600 °C), CUR 20 psi, and CAD was set as “High”. Both quadrupoles were set at unit resolution. Mass method consisted of three mass experiments: full mass analysis (EMS mode, collision energy (CE) 10V), MS^2 analysis (EPI mode, CE 25V) and MS^3 analysis (CE 30V, excitation energy (AF2) 50V). Spectra were recorded between *m/z* 150 and 1300.

Quantification of the individual compounds was done from the peak area values obtained in the chromatograms recorded at 520 nm, using a calibration curve of cyanidin 3-*O*-glucoside. Delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside were purchased from Polyphenols Labs (Sandnes, Norway).

4.2. Samples

Male catkins of different *Populus* species (*P. nigra* L., *P. alba* L. and *P. tremula* L.) were collected during flowering season at different locations (Table 3) of the south-western part of the Douro basin (Tormes sub-basin) that were selected in accordance to the available information on the presence of the different species in the Iberian Peninsula (Anthos, 2015). Each species was collected in more than one location. The identification of the *Populus* species was initially done on the basis of the degree of hairiness of the catkins. Other characters like the aspect of the bark, the presence of a fastigiated habit or not, the aspect of the buds, etc., were also used to identify the specimens. Pollen grains were also analysed in order to verify that the selected trees corresponded to *Populus* specimens. For this purpose, the catkins were softly shaken over a microscope slide mounted in fuchsine-stained glycerin gelatin (Galán et al., 2007). The samples were then read on a Nikon Optiphot II microscope, showing a morphology of the pollen grains that was typical of *Populus* genus (Díez, 1987). The identities of the specimens were further confirmed through the study of the morphology of the

Table 3
Code of the samples, species of *Populus* assigned to them on the basis of the morphological characters, UTM coordinates of the collection sites, reference number of the voucher specimens deposited in the SALA herbarium (University of Salamanca) and comments for some of the specimens.

Sample code	Species of *Populus*	Collection sites[a]	Ref. number SALA herbarium	Observations
POP1	*P. nigra*	30TUL0433 Ventosa del Río Almar (SA)	SALA156318	
POP1B	*P. nigra*	30TUL0433 Ventosa del Río Almar (SA)	SALA156318	Same specimen as POP1 at different elongation phase
POP2	*P. nigra*	30TUL0433 Ventosa del Río Almar (SA)	SALA156319	
POP3	*P. nigra*	30TUL0433 Ventosa del Río Almar (SA)	SALA156320	Fastigiated habit (var. *italica*)
POP4	*P. alba-like*	30TTL9441 Babilafuente (SA)	SALA156321	
POP5	*P. alba-like*	30TTL9441 Babilafuente (SA)	SALA156322	Neighbour of POP4
POP6	*P. nigra*	30TTL9441 Babilafuente (SA)	SALA156323	Fastigiated habit (var. *italica*)
POP7	*P. alba-like*	30TTL9441 Babilafuente (SA)	SALA156324	
POP8	*P. alba-like*	30TTL9238 Huerta (SA)	SALA156325	Specimen located in a public garden. Probably ornamental
POP9	*P. nigra*	30TUL1324 Bóveda del Río Almar (SA)	SALA156326	
POP10	*P. alba-like*	30TUL1818 Mancera de Arriba (AV)	SALA156327	
POP10B	*P. alba-like*	30TUL1818 Mancera de Arriba (AV)	SALA156328	Neighbour of POP10
POP11	*P. tremula*	30TUL2603 San Juan del Olmo (AV)	SALA156329	
POP12	*P. tremula*	30TUK2895 Muñana (AV)	SALA156330	
POP13	*P. tremula*	30TUK1684 Navacepedilla de Corneja (AV)	SALA156331	Autumn leaves from this specimen and from a neighbour were analysed in addition to the anthers

[a] (SA): province of Salamanca (Spain). (AV): province of Ávila (Spain).

leaves and comparison to literature (Soriano, 1993; Streeter et al., 2011) and to authenticated herbarium specimens at the University of Salamanca (SALA herbarium). Table 3 shows the codes of the samples, their collection sites, the *Populus* species assigned to them on the basis of the morphological characters and the reference number of the specimens deposited in the SALA herbarium (see Fig. S3 for the location of the samples in a map). POP1 and POP1B samples were collected from the same tree but at different maturity degree: catkins of POP1B were totally elongated (more mature) whereas those of POP1 were still in elongation phase. These two samples were collected at the same collection site as POP2 and POP3. In another location POP4, POP5, POP6 and POP7 were collected. POP4 and POP5 were neighbour trees (less than 20 m) and were 0.5 Km far from POP6 and POP7, which, in turn, were neighbour trees (less than 20 m). POP8 was a *P. alba*-like specimen, probably corresponding to an ornamental variety, that was collected in a public park not far from the collection site of the latter ones (ca. 3 Km). POP9 was a non-fastigiated *P. nigra*-like tree and it was the only specimen collected at its location. POP10 and POP10B were *P. alba*-like neighbour specimens (less than 10 m). POP11, POP12 and POP13 correspond to *P. tremula*-like specimens, but collected at different locations.

Autumn leaves from *P. tremula*-like specimens were collected only from POP13 and from a neighbour specimen of POP13.

Sambucus nigra L. (elderberry) and *Rubus idaeus* L. (raspberry) ripe fruits were collected in the province of Salamanca in specimens previously identified through their morphologic characters (Monasterio-Huelin, 1999; Ruiz-Téllez and Devesa, 2007).

4.3. Sample preparation

Male catkins were first lyophilised making possible an easier separation of the anthers from the rest of the elements of the catkin (axis and bracts) and allowing the quantitative comparison between samples. Anthocyanin extraction was carried out, in triplicated, from ca. 50 mg of anthers from each *Populus* specimen. For each subsample, three macerations in methanol containing 5% of 0.5 N HCl were carried out. The first maceration lasted 24 h and was performed at −30 °C in order to produce cell rupture and to favour anthocyanin extraction to the solvent. The soln. was filtered on a Büchnel funnel and the anthers were extracted again twice, but with shorter maceration times (12 h). Before each maceration, samples were sonicated to increase the extraction. After these three extractions, the anthers were almost colourless. The filtered methanol extracts were gathered and evaporated in a rotary evaporator at 35 °C in order to remove methanol, avoiding the complete dryness by adding ultrapure water. The resulting conc. aq. soln. was diluted with acidified water (pH 1.4, HCl) up to a final volume of 20 mL. Five mL of this soln. (10 mL in *P. tremula* samples) was purified by solid phase extraction in a Waters C-18 Sep-Pak® (500 mg) cartridge (Waters Corp., Milford, MA, USA). The solution was deposited onto the cartridge previously activated with MeOH:HCl 0.1 N (95:5) and equilibrated with ultra-pure water. Sugars and polar substances were removed by passing ultra-pure water and anthocyanin pigments were eluted with MeOH:HCl 0.1 N (95:5). The methanolic extract was evaporated under vacuum and the aq. soln. containing the pigments was taken to a known volume (2 mL) with acidified water (pH 1.4, HCl). HCl was purchased from Panreac® (Castellar del Vallès, Spain) and methanol from Lab-scan (Gliwice, Poland). The ultrapure water was obtained from a Direct-Q™ water purification system equipped with a Millipak® 40 (22 μm) filter unit (Millipore, Billerica, MA, USA).

Samples were filtered through a 0.45 μm hydrophilic PVDF Clarinert™ Syring Filters (Agela Technologies, Wilmington, DE,

19808, USA) prior to the HPLC-DAD analyses.

4.4. Statistical analyses

Total and individual anthocyanin contents as well as the individual percentages over the total content were analysed by means of one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test in order to assess the significance of the differences observed among samples ($p < 0.05$).

Hierarchical cluster analysis (HCA) was used as an unsupervised classification technique in order to verify if these differences allow the separation of the different types of samples in different groups or clusters. A first HCA was carried out with the individual percentages and total pigment content of all of the triplicates of all the samples (15 specimens in triplicate = 45 samples). Samples were grouped as in the dendrogram shown in Fig. 3, but an additional grouping that included all the triplicates of a same specimen in a same group (as expected) was obtained. The use of the values of the triplicates only added complexity to the dendrogram and did not supply additional information. For this reason, the dendrogram shown in Fig. 3 was the result of a second HCA that was carried out from the data matrix containing the mean total pigment content as well as the mean percentages of the individual compounds, excepting that of cyanidin 3-*O*-glucoside, of each specimen (15 cases). After data standardisation, the similarity matrix was calculated using squared Euclidean distances and the Ward algorithm was used to generate the dendrogram.

Another unsupervised pattern recognition method, principal components analysis (PCA), was used to investigate the anthocyanin pigments that were responsible for the interspecific and intraspecific discriminations observed in the HCA. PCA was applied from the correlation matrix of the original variables (mean total pigment content and mean individual percentages of all the pigments that were quantified in the different *Populus* samples excepting cyanidin 3-*O*-glucoside: 15 variables).

The SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA) was used for data processing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.04.004.

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