About the epidermic cells in 'Rosa Narcea'

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Abstract

Epidermic cells of 'Rosa Narcea' are studied at three different moments along the blooming. Qualitative and quantitative studies are carried out. Qualitative research was done by histochemical techniques and transmission electron microscopy (TEM). The quantitative one was done by using images obtained by scanning electron microscopy (SEM). Both adaxial and abaxial epidermic cells in the 'Rosa Narcea' present cuticular striation. The volatile essential oils which determine the 'Rosa Narcea' scent have to cross through the cuticle in order to go out. Under transmission electron microscopy (TEM), some microchannels through which the smell molecules are probably released, are observed. These canaliculi are concentrated in the cuticular striation area. The quantitative analysis indicates that there is a higher number of cells and with thicker striation in the epidermis of those roses blooming at the end of the blossom season. This allows the team to hypothesize that the smell emission will be higher at the beginning of July than at the beginning of May, although nothing may be inferred about the aroma quality.

Keywords: 'Rosa Narcea', ancient rose, histological, petals, lipid components, ultrastructure.

INTRODUCTION

Petals, as well as sepals, carpels and stamens are modified leaves (Evert, 2006; Álvarez, 2015). The petals present underdeveloped adaxial and abaxial epidermis, mesophilics and vascular bundles.

Petals of flowers pollinated by insects, present papilliferous adaxial epidermic cells. However, abaxial epidermic cells are nor papilla-shaped (e.g. Baudino et al., 2007; Martínez et al., 2020). Petals of genus *Rosa* share these characteristics. They present a mesophyll constituted by parenchyma in reserve with conspicuous intercellular spaces. These histological characteristics are common to all *Rosa* cultivars releasing smell and for those which are nor especially aromatic (Bergougnoux et al., 2007).

In every plant organ, epidermic cells synthetize the cuticle (Bargel et al., 2006; Yeasts et al., 2010). It is an acellular layer in the external part of the epidermic cells, in contact with the outer environment. It is mainly formed by lipophilic elements (Leide et al., 2007; Dominguez et al., 2009) isolating and protecting plants from the exterior (Shepherd and Griffiths, 2006; Reina-Pinto and Yephremov, 2009).

Despite the fact that there probably are specific characteristics for every single *Rosa* cultivar (Semyonovna et al., 2016), the main role of the synthesis and, of course the aromas spread specific to these flowers, is usually attributed to the epidermic cells. As to whether rose epidermic cells synthetize and release the odoriferous molecules or if they only work as vehicles transferring the aromas formed in the mesophyll to the exterior, there is no agreement. In this last case, the parenchyma intercellular spaces would take part in it as a storage place for the aromatizing product (Semyonovna et al., 2016).

Given that the aroma is marked by lipidic nature molecules (Caissard et al., 2004), the fact that the transport cell to cell of the mentioned molecules is eased via symplast, interceding lipid transfer proteins, like in the cuticle biosynthesis (Schulz and Frommer, 2004; Kunst et al., 2006; Sheperd and Griffiths, 2006; Panikashvili and Aharoni, 2008, Tafolla

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et al., 2013). But there is also the possibility that the mentioned transportation is carried out via apoplast, from primary wall to primary wall.

One way or another, the molecules responsible for smell, either coming from the cytoplasm or the wall, reach the cuticle: waxen nature layer and permeable to both polar and non-polar compound (Tafolla et al., 2013). Once there, the molecules will spread to the exterior acting as a lure for pollinator insects.

The interest for the study of epidermis has with relation to the odoriferous substances release in the particular case of roses, especially in the cultivar 'Rosa Narcea', seems to be clear. During the present research, petals of 'Rosa Narcea' have been analysed. This is a variety of cultivated ancient rose – identified in Spain – with very few specimens left in the world. Its intense scent - mainly spread by its petals - is characteristic to the point that it is thought to be used in the perfume industry in the future. The mentioned cultivar has been recently described after a multidisciplinary research in which its histologic characterization has been included (Martínez et al., 2020).

From the histological point of view, 'Rosa Narcea' petals present an epidermis with a cuticle with striations. The adaxial epidermis presents papilliferous cells with the mentioned striations converging towards the papillar apex. In the abaxial epidermis – with cubic cells - the striations run somehow parallel to each other (Martínez et al., 2020).

The objective of this work is to deepen in the morphological study of the epidermic cells of 'Rosa Narcea'. On the one hand, bright-field microscope was used for the conventional histochemical studies and others by lecitines. The usage of lecitines may allow the investigators to identify glycoproteins and/or glycolipidsin the epidermic cell walls as well as their cuticles. Besides, ultrastructural details which might make clear the possible morphological mechanism by which the aromas would be transferred to the outside are studied by transmission electron microscope (TEM). On the other hand, epidermic cell attributes (number and thickness of striations and number of papilliferous cells) are studied by scanning electron microscope (SEM). In every case, 'Rosa Narcea' petals harvested in three different moments of their blossom were studied.

MATERIALS and METHODS

'Rosa Narcea' plants bloomed in 2019 along 3-4 weeks.

Petals from 'Rosa Narcea' were taken in three different moments along the blooming from the same setting: initial petals at the beginning of May (05-12-2019), intermediate petals at the end of May (05-25-2019) and final petals at the beginning of June (06-16-2019). Petals from wild rose (*Rosa canina*) were taken too, gathered at mid June (06-16-2019).

Light microscopy

All the petals were taken and later frozen. In order to carry out the microscopic study they were defrosted at room temperature, were fixed in FAA for 48 h, transferred to 70º alcohol, and embedded in paraffin wax following standard protocols. Twelve microns thick sections were cut using a rotatory microtome.

Sections were stained with Safranin and Fast Green to study the general morphology. Conventional histochemical method AB/PAS was performed to distinguish between acidic (AB positive, stained blue) or neutral (PAS positive, stained red) glicoconjugates. Mixed cells would contain both neutral and acid monosaccharide residues (AB/PAS positive, stained purple) (Kiernan, 2008). Moreover, several lectins were used to study the carbohydrate composition of the glicoconjugates. There are lectins that recognize a single monosaccharide residue and others that recognize several with different affinities (Esteban et al., 2009). Consecutive sections from every sample were processed for GSL I-B4 (Griffoniasimplicifoliaisolectin B4) specific for α-galactose, UEA-I (Ulexeuropaeusagglutinin) specific for L-fucose, WGA (*Triticum vulgare* agglutinin) specific for N-acetylglucosamine, SBA (Glycine max agglutinin) specific for N-acetyl-D-galactosamine terminal, PNA (Arachishypogaeaagglutinin) to recognize the sequence galactose (β1-3) N-acetylgalactosamine, Con A (Canavalia ensiformis agglutinin) specific for α-mannose, and SNA

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(*Sambucus nigra* agglutinin) specific for sialic acid, respectively. The protocol followed was the standard protocol for the detection of glycoconjugates through the use of biotinylated lectins. The histochemistry of lectins was performed according to the technique previously described by Molist et al. (2011).

Transmission electron microscopy (TEM)

Petal samples were fixed in 2.5% paraformaldehyde/2% glutaraldehyde in cacodylate buffer for 3 h. They were postfixed in osmium tetroxide, dehydrated in acetone and embedded in Spurr resin. Semithin (1 µm) sections stained with toluidine blue were studied under a bright-field light microscope to locate the area for ultrastructural study. Ultrathin (70 nm) sections were contrasted with uranyl acetate and lead citrate and examined with a Jeol 1010 TEM.

Scanning electron microscope (SEM)

Fragments of the fixed petals were also passed through a dehydrating series of ethanol solutions and after critical point drying were gold-covered and examined using a FEI Quanta 600 environmental scanning electron microscope (ESEM).
From each fragment of petal (initial, intermediate, final and wild) five photographs were taken. The images obtained were used to study:

- number of papillas (cells) in an area 50 x 50µm. Counting about 15 different areas per fragment (Figure 1a);
- in the underside cells, the count of striations present in 50µm was performed due to difficulty of delimitation of the cells. The counting was carried out on about 13 different areas per fragment (Figure 1b);
- striations thickness in the upperside and the underside epidermic cells. The measurement was carried out on some 13 different areas per fragment (Figs. 1c-d).

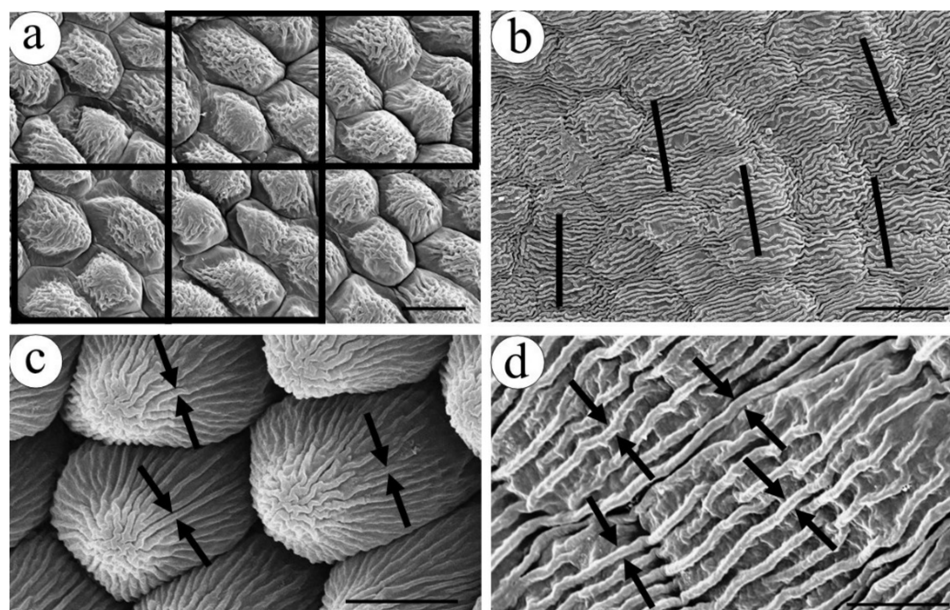

Figure 1. Petals quantitative study. **a** Four areas 50 x 50µmwhere the buds (cells) count was carried out. Adaxial surface of an intermediate 'Rosa Narcea' petal. **b** Five lines 50µm where the striations count was carried out. Abaxial surface of a final petal of 'Rosa Narcea'. **c** Thickness of three striations (arrows). Adaxial side of a wild petal. **d** Four striations thickness (arrows). Abaxial side of a young 'Rosa Narcea' petal. **a-d** SEM. Bars: **a**= 20µm, **b**= 50µm, **c, d**= 10µm.

Statistical analysis

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For each variable and each sample the count and measurement was performed – repeatedly – over an only fragment per petal. That is why all the data are pseudo-replicas. This means that the data variability is lower than what would be found in the nature, as what is registered is the individual variation and not the population. Therefore, the conclusions acquired from this research should be taken cautiously.

In order to compare every type of petals with each other, a Kruskal-Wallis test was performed followed by a post-hoc Wilcox test, with a correction of the *p* values of Benjamini-Hochberg (Benjamini and Hochberg, 1995). All the statistic analysis were performed with R version 3.6.3 (R Core Team, 2020).

RESULTS

Qualitative research

The studied petals morphology is similar in every case: initial petal, intermediate petal, final petal and wild rose petal. They consist of adaxial uniserial epidermis of papilliferous cells: wide at the base and narrow at the apex. Striated cuticle. Parenchyma with big intercellular spaces. Neither crystals nor amyloplasts are observed. Abaxial uniserial epidermis of cubic cells non-papilliferous. Striated cuticle. Small closed collateral vascular bundles (Figs. 2 a-c).

The epidermic cells present a scalloped edge due to the presence of the mentioned striations (Figure 2d). The interior of the cell is observed as non-uniform, presenting a big vacuole displacing the organelles to the periphery.

The wall of the epidermic cells presents a major width than the cuticle being their thickenings considerably bigger in the tangential than in the radial walls. The wall presents a diffuse net of electrodense fibril material (Figure 2e). The filaments are slim and are set in every direction. In the transition area with the cuticle, a major concentration in quantity of that fibril electrodense material is found. The cuticle is slightly more electrodense than the wall. Structures reminding those of narrow branched canals are observed (Figure 2f). These expand from the most external layer of the cell wall towards the exterior. In the cuticular striation areas, a higher concentration of the mentioned canaliculi is observed.

Histochemical differences between the 'Rosa Narcea' along their flowering period are not found either.

The histochemical techniques for glucid detection (AB/PAS) show that the cuticular striations appear in purple colour (Figs. 2b-c), indicating a mixed content both in neutral glycoconjugates (positive PAS) and acids (positive AB). The likely detection of the monosaccharides present in the glycoconjugates, using 7 lecitines which determine every possible monosaccharide group, resulted negative in every case.

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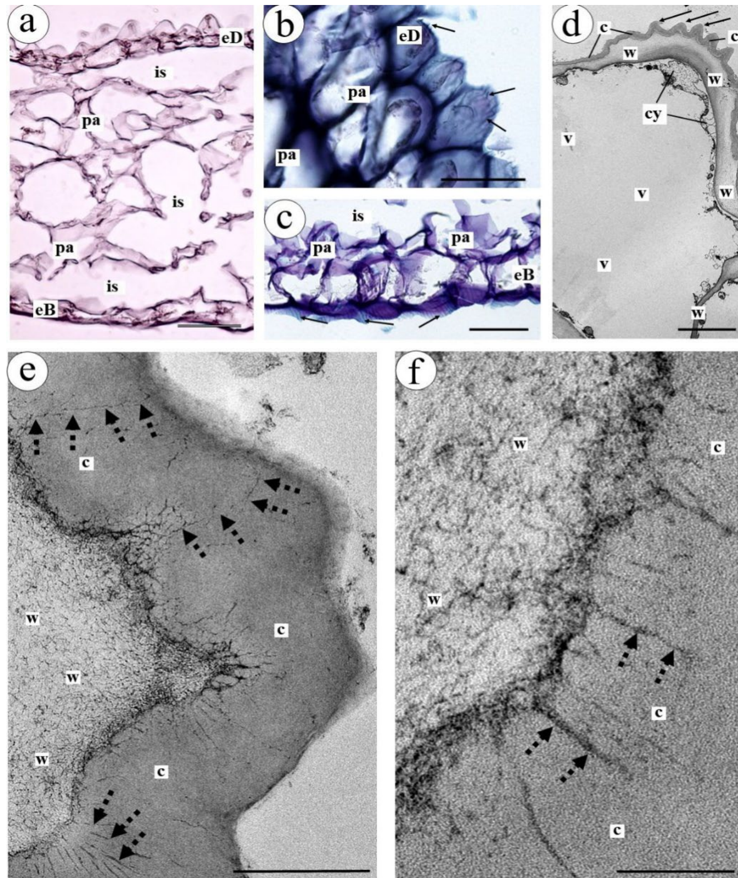

Figure 2. Qualitative research on 'Rosa Narcea'petals. **a** Transverse cut of a petal. Adaxial epidermis cells (aD) are papilliferous whilst abaxial epidermis cells (eB) are not. Observe the presence of big intercellular spaces (is). **b-c** Conventional histochemical reactions. **b** Adaxial epidermis cells where cuticular striations are observed (arrows). **c** Abaxial epidermis cells where parallel cuticular striations (between each others) are observed (arrows). **d** Adaxial epidermic cell. Noticeable thicken cuticle (c) and the presence of striations (arrows). Observe that the tangential cellular wall (w) is thicker than the radial one (inferior right part on the image). **e** Cuticle (c) over the cellular wall(w). A group of canals (arrows) is observed, sometimes more or less three-shaped. Occasionally, the cut allows to observe how the canals cross the whole cuticle, reaching the exterior. **f** Detail of caniculi (arrows) in the area between the cellular wall (w) and the beginning of the cuticle. **a** Safranin fast-green, **b, c** PAS-Alcian blue, **a-c** Optical Microscope light field, **d-f** TEM. Abbreviations: *c* cuticle, *cy* cytoplasm, *eB* abaxial epidermis, *eD* adaxial epidermis, *is* intercellular space, *pa* parenchyma, *v* vacuole, *w* cellular wall. Bars: **a, b**= 50µm, **c**= 100µm, **d**= 5µm, **e**= 0.5µm, **f**= 100nm.

Quantitative research

The petals of initial blooming present significantly less buds (that is less cells) (5.5) than the final blooming lowers petals (8.2) and the wild rose (9.5) (Figure 3).

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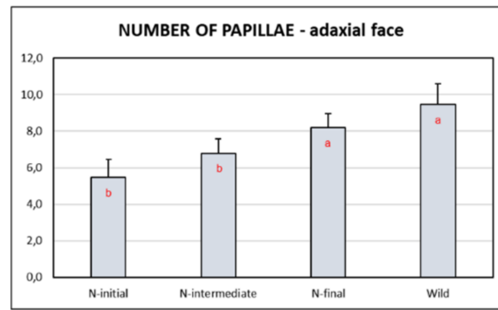

Figure 3. Number of buds (cells) in the adaxial surface of petals: initial (N-initial), intermediate (N-intermediate) and final (N-final) of 'Rosa Narcea' and wild rose (Wild). Different letters indicate significant differences (p= 1,10e – 09).

Significant differences were observed between 'Rosa Narcea' initial and intermediate petals in the number of striations (19 striations average), being much significantly higher (25) in the wild rose (Figure 4).

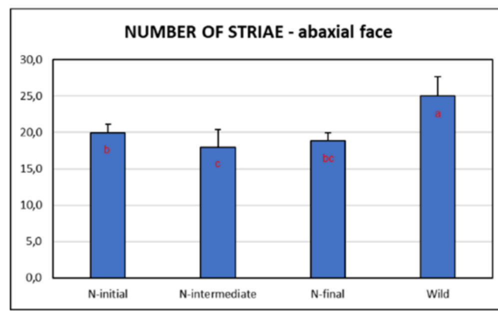

Figure 4. Number of striations in the abaial surface of petals: initial (N-initial), intermediate (N-intermediate) and final (N-final) of 'Rosa Narcea' and wild rose (Wild). Different letters indicate significant differences (p = 1,03e – 06).

With regards to the striations thickness both in adaxial as abaxial epidermic cells, a tendency to thickening when going from initial to final petals is observed (Figure 5). The mentioned thickness is always significantly bigger in 'Rosa Narcea' (average value 1,3µm) than in wild rose (0,8µm).

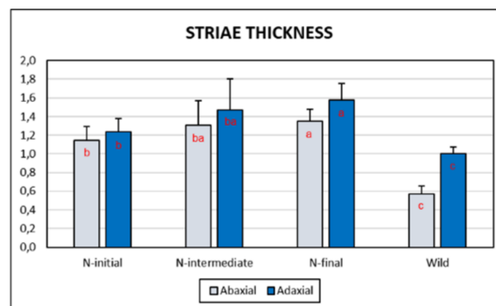

Figure 5. Adaxial and abaxial striation epidermis thickness in petals: initial (N-initial), intermediate (N-intermediate) and final (N-final) of 'Rosa Narcea' and wild rose (Wild). Different letters indicate significant differences (p = 1,73e – 07).

DISCUSSION

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The general morphological structure in the 'Rosa Narcea' petals observed in the present work, is similar to that studied in other genus *Rosa* species (Stubbs and Francis, 1971; Bergougnoux et al., 2007; Sulborska and Weryszko-Chmielewska, 2014; Semyonovna et al., 2016). Adaxial and abaxial epidermis is formed by papilliferous and cubic cells respectively. In both, a parenchyma with big intercellular spaces appears. Both epidermises are coated by a thick cuticle which present striations. These striations converge in the adaxial epidermic cells and are parallel in the abaxial ones (Martínez et al., 2020).

This general structure in the 'Rosa Narcea' endures along the blooming season studied in the present research. The histochemical location with Sudan black (Martínez et al., 2020) and oil red (personal observations) indicates that the cuticular striations are the places where neutral lipids accumulate. The molecules spreading smell are released to the outside via cuticle (Baudino et al., 2007; Bergougnoux et al., 2007). Hence, it is possible to assume that the striations in the cuticle are specifically the primal places where the aroma release is produced.

In the TEM study of Moraes et al. (2009) on six different species of genus *Simira* (Rubiaceae), the existence of three zones in the wall in the external tangential wall of the epidermic cells: 1/ the most internal one, rich in polysaccharides and mainly composed by cellulose, 2/ the intermediate one, with a tree-shaped aspect, presenting cutin rich in polysaccharides, and 3/ the cuticle itself. On another Rubiacea (*Plectroniella armata*), Tilney et al. (2012) observed in a domatium´s epidermis a cuticle similar to the middle layer described by Morais et al. (2009), assuming also that it is rich in polysaccharides. The observations (by TEM) - in the present research - of a branched fibril net in the 'Rosa Narcea' petals cuticle, reminds of the described by these authors. Besides, the conventional histochemical techniques for carbohydrates used in this work show that the cuticular striations area contains acid and neuter glycoconjugates. The negative results observed with the histochemical techniques with lecitines, did not allow the research team to be more accurate in these glycoconjugates composition.

Bearing in mind that the cuticular fibril net is mainly concentrated in the cuticular striations, we could assume, as Morais et al. (2009) describe, that the fibrils net observed is rich in polysaccharides. In the case of nectar, this fibril net in the cuticle was interpreted microcanals through which nectar would be secreted (Radice and Galati, 2003; Nepi, 2007). Our observations seem to agree with these last authors. Despite this, in the present work, the canaliculi which seem to cross the cuticle may be the way by which the odoriferous substances are released to the outside.

Whilst the general structure of the petals studied keeps up along the flowering period, this does not happen with regards to the quantitative aspects analysed: quantitative differences as for the number of epidermic cells and the striation thickness between the 'Rosa Narcea' petals and with regards to the wild rose. If we assume – as formerly pointed – that the striations are the areas which petals release the smell through, we understand that the specialization in smell production of 'Rosa Narcea' is provided with thicker striations than those in wild roses. The fact that there are significantly more cells in the final roses epidermis, and their striations are thicker, allows us to hypothesize that the smell emission will be higher at the beginning of July than at the beginning of May, although nothing can be inferred about the mentioned smell quality.

CONCLUSIONS

The present work shows that either adaxial and abaxial 'Rosa Narcea' petals epidermis are involved in the secretion of volatile oils responsible for the scent spread by these flowers. In addition, it is very possible that these substances are released to the outside through the microcanals that cross the cuticle and concentrate in the cuticular striations. The wider thickness of these striations as well as the number of cells, makes us think that the smell emission varies along the blooming season.

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