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NEW METHODS IN CELL BIOLOGY

Technique for the identification of osmophores in flowers of herbarium material (TIOFH)

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Abstract

The histochemical studies that search for osmophores differ in the protocol they follow for fixation and discoloration of the samples, and also in the type and number of stains utilized. Despite these differences, all the studies have one point in common: the use of fresh material either collected directly in the field or cultivated in botanical gardens and greenhouses. This is an obvious limitation for the studies of osmophores. Flower parts of herbarium specimens of different dates of collection and different plant families were exposed to variable times and percentages of discoloration agents and under different stains for finding if it is possible to positively test osmophores in this type of material. We obtained positive results discoloring the samples with ethyl alcohol 96° and sodium hypochlorite, and staining with Lugol, Oil Red O, and Neutral Red (TIOFH). A protocol (TIOFH3) for osmophore testing that combines these three stains into a single procedure is proposed.

Keywords Floral osmophores · Protocol · Herbarium material · Histochemical technique

Introduction

Osmophores in flower parts are engaged in scent production and differ from other secretory structures (e.g., hydathodes, nectaries, resin ducts) by their product, site, duration, and anatomical structure (Vogel 1990). The fragrant substances secreted by osmophores are mainly volatile low terpenes, which are lipophilic substances. They occur in the form of minute droplets in the cytoplasm of the epidermal and neighboring parenchyma cells. At an appropriate temperature, the droplets diffuse in gaseous form from the cytoplasm through the cell wall and cuticle to the outside. With the diffusion of droplets, new ones are constantly produced. Below the epidermis the cells are filled with starch grains which, used as a source of energy, reveal the secretory activity of the osmophores (Fahn 1979). Osmophores may be multilayered or the osmophoric function may be fulfilled only by epidermis (Vogel 1990), sometimes papillose or with trichomes, and are commonly found in certain floral and inflorescence parts such as petals and tepals, spatha of Araceae, staminodes, and stamens (Singh et al. 1996; Weryszko-Chmielewska et al. 2007; Weryszko-Chmielewska and Sulborska 2012).

The literature is quite abundant in studies of osmophores of Dicot and Monocot families mainly Orchidaceae (e.g., Stern et al. 1987; Wiemer et al. 2009; Cabral de Melo et al. 2010; Kowalkowska et al. 2015) and Araceae (e.g., Méndez and Obeso 1992; Singh et al. 1996; Hadacek and Weber 2002), but also Aristolochiaceae (Vogel 1990), Asclepiadaceae (Vogel 1990; Aliscioni et al. 2017), and Solanaceae (Sazima et al. 1993; Cocucci 1996), among other families. In general, the histochemical studies that search for osmophores differ in the protocol they follow for fixation and discoloration of the samples, and also in the type and number of stains utilized. The following are some stains and their combinations that we found in papers for testing osmophores: (1) Sudan III, Sudan black B (lipids), and Lugol (starch); (2) Neutral Red (vacuole content and cell walls), Sudan IV (lipids), and Lugol; (3) Sudan Red B (lipids) and Lugol; (4) Neutral Red; (5) Sudan Black B and Lugol; (6) Sudan III and Lugol; (7) Neutral Red and Lugol; (8) Sudan III,

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Auramine O (lipids), and Lugol; (9) Neutral Red, Sudan III, and Lugol. This multiplicity of approaches reflects that a comprehensive analysis of an osmophore-staining technique is still lacking.

Despite these differences, all the studies have one point in common: the use of fresh material either directly collected in the field or cultivated in botanical gardens and greenhouses. This is probably because the experiments with vital staining in the keystone works on osmophores of Vogel (1963, 1990) using mainly Neutral Red (but also other stains) were the basis for further studies. Vogel (1990: 184) mentioned that with the help of elective vital staining, the fragrance fields can be made visible macroscopically. Neutral Red has been extensively used for vital staining of vacuoles (Guilliermond 1930; Drawert 1968). It penetrates cells and reaches vacuoles with lipophilic and other contents easily and its red staining can be clearly seen with the naked eye and with stereoscopic and light microscopes. The sole use of Neutral Red, however, is not sufficient indicative of the presence of osmophores. Stern et al. (1987) concluded that neither staining reaction is considered an absolute indicator of osmophore tissue, but Sudan Black B and in vivo staining with Neutral Red could be useful to establish presumptive evidence for these structures.

The exclusive use of living material is an obvious limitation for the studies of osmophores. For example, Curry et al. (1991) performed a phylogenetic analysis of *Stanhopea* and *Sievekingia* (Orchidaceae) based on characters of the florets and particularly of the osmophores, and mentioned that they could not analyze all species but only those maintained in greenhouses. Therefore, we recognize that a positive testing of osmophores in desiccated, herbarium specimens using histochemical methods will be a very useful tool for researchers. Curatorial treatment of herbarium specimens by freezing, desiccation in stoves, insecticides and antifungal substances, may cause physical, physiological, and chemical changes in the plant tissues. We propose to experiment with flowers of herbarium specimens of different dates of collection and different plant families, exposing them under variable times and percentages of discoloration agents and under different stains for finding if it is possible to test the presence of osmophores. Simultaneously, we will apply the same treatment to fresh and fixed material for comparing the results.

To achieve this objective, we will consecutively follow these steps: (1) to test which is the best treatment for fresh, fixed and herbarium material with hydration and discoloration agents; (2) to test the presence of osmophores by employing some of the most-used stains according to the literature, i.e., Neutral Red, Sudan IV, Sudan Black B, and Lugol, and also Nile Blue A and Oil Red O; (3) to select the most suitable and informative stains for osmophore testing, and to compare the results between fresh, fixed and herbarium material; and (4) to combine the selected stains into a single procedure for generating a simple protocol for osmophore testing.

Materials and methods

As a general approach, we will test the presence of floral osmophores by comparing the results of the techniques in the same species under four different conditions: fresh material, fresh material conserved in a fixation agent (stored ca. 1 month), herbarium material, and herbarium material conserved in a fixation agent (stored ca. 1 month). In addition, we will test the techniques in species without osmophores to discard false positives.

The presence or absence of floral osmophores for selecting the species in this study was corroborated with smell registration in living specimens and with the literature. Accordingly, the following species have floral osmophores and/or evident flower fragrance emission: *Narcissus tazetta* L. (Amaryllidaceae; Arai 1994), *Sagittaria montevidensis* Cham. & Schltdl. (Alismataceae; Miguez and Amela García 2015), *Lantana camara* L. (Verbenaceae; Miguez et al. 2013), and *Jacaranda mimosifolia* D. Don (Bignoniaceae; Rodrigues Alves et al. 2010). The species analyzed without osmophores and/or without evident scent emission are *Aloe arborescens* Mill. (Asphodeloideae; Cousins and Witkowski 2012) and *Canna indica* L. (Cannaceae). The floral parts analyzed were crown, tepals, stamens, and gynoecium in *Narcissus tazetta*; tepals, stamens, staminode, and gynoecium in *Sagittaria montevidensis*; sepals, petals, stamens, and gynoecium in *Lantana camara*; sepals, petals, stamens, staminodes, and gynoecium in *Jacaranda mimosifolia*; tepals, stamens, and gynoecium in *Aloe arborescens*; and tepals, staminode or labelum, and gynoecium in *Canna indica*.

Flowers of living specimens were collected in the field and the corresponding vouchers were deposited in the herbarium LP; the herbarium specimens analyzed are from LP and LPAG (Holmgren et al. 1990). Upon availability, the herbarium specimens were selected to cover a wide range of dates of collection, i.e., ancient specimens (AS) and relatively recently collected specimens (RS), for testing if there are changes in the results with the material aging. Arbitrarily, we distinguished the AS as those collected in the first half of the twentieth century and before, and RS as those collected in the second half of the twentieth century and after. The range of the years of collection of the herbarium specimens analyzed goes from 1897 to 2010. Three to nine specimens per species and two to three flowers per specimen were sampled; mature and immature flowers were analyzed. The Appendix lists these specimens with vouchers.

The samples consisted in entire flower parts (e.g., petals, tepals) or parts of them were cut when the pieces were too large or voluminous. The adaxial and abaxial surface of the laminar parts was observed. We also performed free hand transections of the floral parts analyzed.

The samples were subject to hydration, fixation, and discoloration before the staining step (Table 1). As seen in

Table 1 Hydration, fixation and discoloration processes applied in different types of material. *FAA* formalin: glacial acetic acid: ethanol 70%; *AS* ancient herbarium specimens; *RS* relatively recently collected herbarium specimens

Material	Hydration	Fixation	Discoloration				
			Distilled water	Hydrogen peroxide (H2O2), 10 volume	Ethyl alcohol 96°	Sodium hypochlorite (NaClO)	Ethyl alcohol 96° + NaClO
Fresh, color white, white-yellowish			60 °C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, oven 38°, 24 h	5%, 10%, 15%, 20%, 25%, 30%, 35%, 4–6 h	Column 5 + column 6
Fresh, color white, white-yellowish		FAA	60 °C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, 24 h	5%, 10%, 15%, 20%, 25%, 30%, 35%, 4–6 h	Column 5 + column 6
Fresh, dark colors (e.g., red, green-purplish, blue)			60 °C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, oven 38°, 24 h	5%, 10%, 15%, 20%, 25%, 30%, 35%, 4–6 h	Column 5 + column 6
Fresh, dark colors (e.g., red, green-purplish, blue)		FAA	60 °C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, 24 h	5%, 10%, 15%, 20%, 25%, 30%, 35%, 4–6 h	Column 5 + column 6
Herbarium (RS)	Oven 38°, 24 h		60 °C, 1 min	5%, 10%, 24 h	First step: 5%,10%, 15%, 20%, 25%, 30%, 35%, oven 38°, 24 h + second step: 5%, 10%, 15%, 20%, 25%, 30%, 35%, 24 h	5%, 10%, 15%, 20%, 2–4 h	Column 5 + column 6
Herbarium (RS)	Oven 38°, 24 h	FAA	60 °C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, 24–48 h	5%, 10%, 15%, 20%, 30%, 35%, 2–4 h	Column 5 + column 6
Herbarium (AS)	Oven 38°, 24 h		60 °C, 1 min	5%, 10%, 24 h	First step: 5%,10%, 15%, 20%, 25%, 30%, 35%, 40%, 48–72 h + second step: 5%,10%, 15%, 20%, 25%, 30%, 35%, 40%, 48–72 h	5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 4–8 h	Column 5 + column 6
Herbarium (AS)	Oven 38°, 24 h	FAA	60 ° C, 1 min	5%, 10%, 24 h	5%,10%, 15%, 20%, 25%, 30%, 35%, 40%, 48–72 h	20%, 30%, 40%, 4–8 h	Column 5 + column 6

Table 1, the hydration was only performed in the herbarium material. Some fresh and herbarium material was fixed in FAA (formalin: glacial acetic acid: ethanol 70%); this fixing agent also produces some discoloration of the samples when the material is stored for about 3 weeks. The material stored in FAA must be rinsed 5 min in distilled water before the treatment with the discoloration agents. Each discoloration process shown in Table 1 was tested independently from each other (i.e., they do not represent sequential steps) with the exception of the last column of the table where the samples were immersed in ethyl alcohol, rinsed in distilled water at room temperature for 5 min, and then immersed in sodium hypochlorite.

After hydration, fixation, and discoloration, the samples were rinsed in distilled water at room temperature for 5 min and stained, but some samples of fresh material were stained directly, skipping the previous steps. For histochemical analyses, we used Neutral Red (NR; 5 g in 100 ml of distilled water, dissolved 0.1% when used) which stains vacuoles and cell walls; the dark red color indicates specific content compatible with the presence of osmophores (Vogel 1990; Cosa et al. 2014). Lipophilic substances were tested with Oil Red O (ORO; 0.5 g in 100 ml ethyl alcohol 80°) which stains brilliant orange-red, with Sudan IV (0.5 g in 100 ml ethyl alcohol 80°) which stains red, with Nile Blue A (0.05 g in 100 ml of distilled water) which stains blue, and with Sudan Black B (0.5 g in 100 ml ethyl alcohol 80°) which stains black. Accordingly, the lipophilic components reveal as red, blue, and black colors on cell walls and drops or cytoplasm into the cells (Ruzin 1999; Zarlavsky 2014). We also applied Lugol (iodine-potassium-iodide, IKI) for detecting starch granules and amiloplasts, which effectively expose the activity of the glandular or basal layers of the osmophores (Vogel 1990); small grains dark-blue or black-violet indicates the presence of starch (Ruzin 1999). Flower parts were immersed in each stain for 15 min, washed in tap water, and mounted in glycerin or gelatin-glycerin. We produced a total of 475 slides.

Observations of anatomical features were carried out on a Nikon SMZ 1000 stereomicroscope and on a Nikon Eclipse E200 light microscope. Photographs were taken with a Nikon Coolpix S10.

Results

Most effective discoloration agents

Table 2 shows the discoloration agents with their percentages and the time that led to achieve the best results for each type of material. Effectivity of the agent is considered here as the balance between loss of color and the gentlest treatment of the material that does not damage the tissue. The results indicate that the best discoloration treatment for all types of material was with ethyl alcohol 96° and with sodium hypochlorite (columns five and six of Table 2). The sequential combination of both agents (column seven) produced more discoloration but resulted very aggressive for the tissues and it is not recommended (Fig. 1).

The times and percentages of the agents may vary according to the type and size of the sample. In the not fixed herbarium flowers two steps of discoloration with ethyl alcohol was needed. On the other hand, as the FAA produces some

Table 2 Discoloration agents with the percentage and time that resulted most effective according to each type of material. *FAA* formalin: glacial acetic acid: ethanol 70%; *AS* ancient herbarium specimens; *RS* relatively recently collected herbarium specimens

Material	Fixation	Discoloration				
		Distilled water	Hydrogen peroxide (H2O2), 10 volume	Ethyl alcohol 96°	Sodium hypochlorite (NaClO)	Ethyl alcohol 96° + NaClO
Fresh, color white, white-yellowish				15%, oven 38°C, 24 h	5%, 4–6 h	
Fresh, color white, white-yellowish	FAA			10%, 24 h	5%, 4–6 h	
Fresh, dark colors (e.g., red, green-purplish, blue)				20%, oven 38°C, 24 h	15%, 4–6 h	
Fresh, dark colors (e.g., red, green-purplish, blue)	FAA			15%, 24 h	10%, 4–6 h	
Herbarium (RS)				First step: 20%, oven 38°C, 24 h + second step: 25%, oven 38°, 24–48 h	10%, 2–4 h	
Herbarium (RS)	FAA			25%, oven 38°C , 24–48 h	15%, 2–4 h	
Herbarium (AS)				First step: 30%, oven 38°C, 24 h + second step: 40%, 48–72 h	30%, 4–8 h	
Herbarium (AS)	FAA			20%, 48–72 h	30%, 4–8 h	

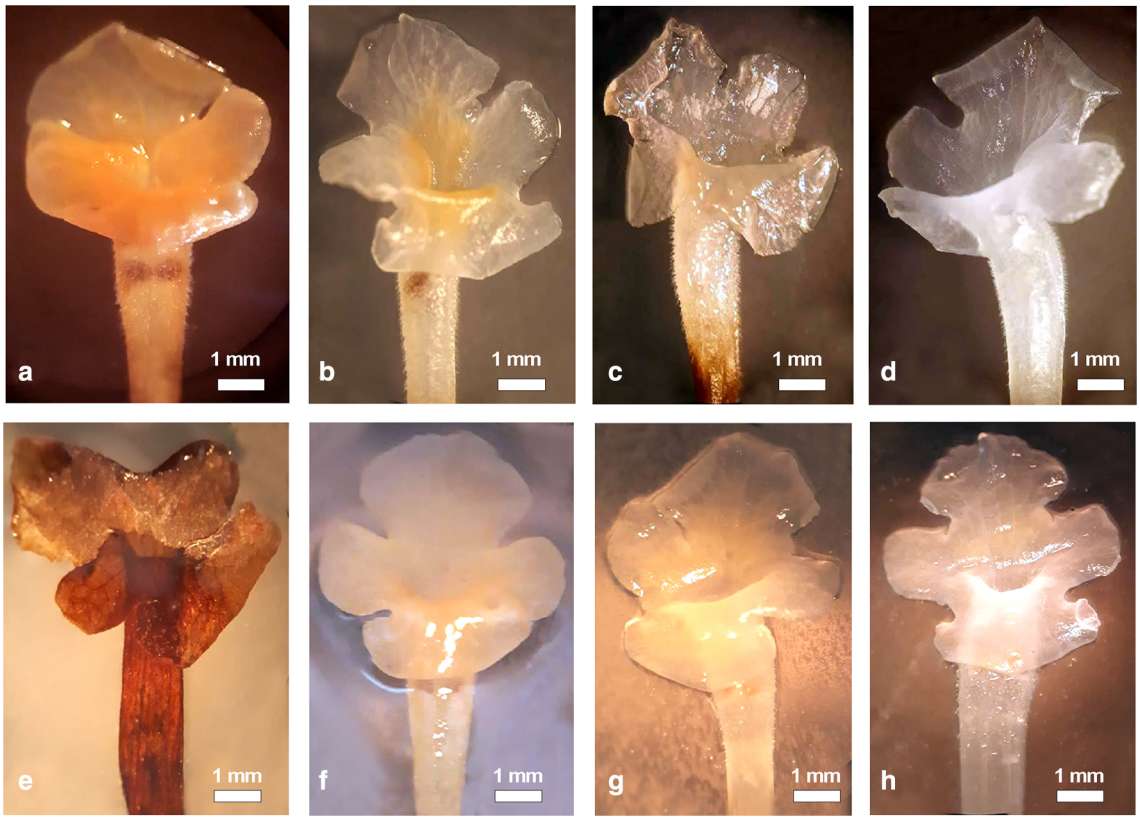

Fig. 1 Different discoloration agents action in flowers of *Lantana camara* (stereomicroscope observation). **a–d** Fresh material. **a** Hydrogen peroxide. **b** Ethyl alcohol 96°. **c** Sodium hypochlorite. **d** Ethyl alcohol 96° + sodium hypochlorite. **e–h** Herbarium material. **e** Hydrogen peroxide. **f** Ethyl alcohol 96°. **g** Sodium hypochlorite. **h** Ethyl alcohol 96° + sodium hypochlorite. **a–d** Hernández 240 (LP). **e–h** Hurrel et al. 1269 (LP)

discoloration, only one step in ethyl alcohol was sufficient in the case of the fixed herbarium samples.

Most effective stains

Table 3 shows the degree of efficiency of the stains for lipophilic compounds employed in this study, i.e., Neutral Red, Nile Blue A, Sudan IV, Sudan Black B, Oil Red O (Fig. 2), and Lugol, in the different types of material. Efficiency is considered here as those stains that give the best staining of the osmophore areas and at the same time are the most differential for osmophores (see "Discussion"). NR shows clearly the potential osmophoric areas (Fig. 2e). ORO tends to stain more intensively the lipophilic substances in cell walls and vacuoles when compared for example with Sudan IV (Fig.

Table 3 Effectivity of the stains employed for osmophore testing in the flowers of different species and types of material. +++ = very efficient, ++ = efficient, + slightly efficient. *FAA* formalin: glacial acetic acid: ethanol 70%; *AS* ancient herbarium specimens; *RS* relatively recently collected herbarium specimens

Material	Stains					
	Neutral red	Nile Blue A	Sudan IV	Sudan Black B	Oil Red O	Lugol
Fresh	+++	++	+	+	+++	+++
Fresh discolored	+++	++	+	+	+++	+++
Fresh in FAA	+++	++	+	+	+++	+++
Herbarium (RS) discolored	+++	++	+	+	+++	+++
Herbarium (RS) in FAA	+++	++	+	+	+++	+++
Herbarium (AS) discolored	+++	++	+	+	+++	+++
Herbarium (AS) in FAA	+++	++	+	+	+++	+++

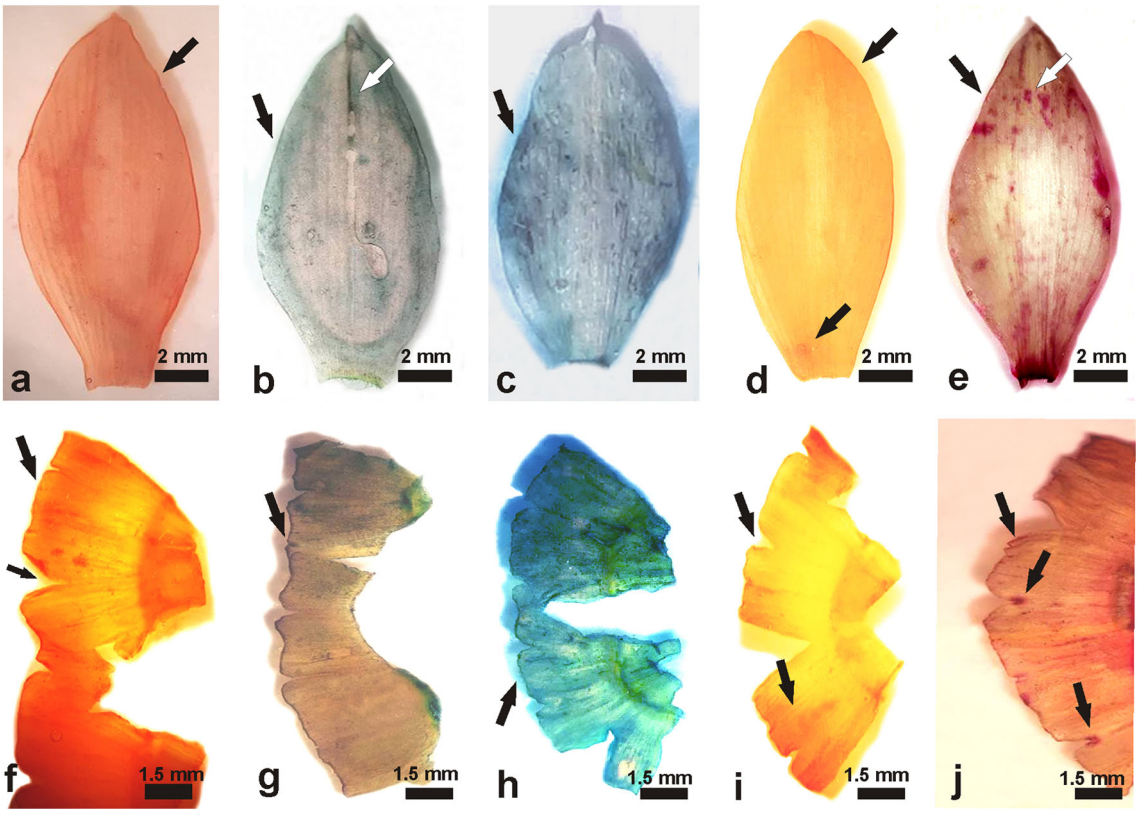

Fig. 2 Different stains action for lipophilic compounds in herbarium flowers of *Narcissus tazetta* (stereomicroscope observation). **a–e** Tepal. **a** Sudan IV. **b** Sudan Black B. **c** Nile Blue A. **d** Oil Red O. **e** Neutral Red. **f–j** Opened crown. **f** Sudan IV. **g** Sudan Black B. **h** Nile Blue A. **i** Oil Red O. **j** Neutral Red. The arrows show the osmophoric areas at the margins, at the sinuses, and on the lamina. **a–e** Hurrel et al. 3600 (LP). **f–h** Delucchi 2529 (LP). **i–j** Delucchi 3279 (LP)

2a), Sudan Black B (Fig. 2b), and Nile Blue (Fig. 2c). For this reason, in surface view, the samples stained with ORO show themselves as more homogeneous (Fig. 2d) regarding other stains, but in transection, ORO is more selective for lipophilic content whereas the preparations with the other stains evidence as a background color making it difficult to distinguish the osmophoric areas from the rest of the tissue. As a result, the most effective stains for osmophore testing are the triple stain with NR, ORO, and Lugol (Fig. 3). We call TIOFH the technique for the identification of osmophores in flowers of herbarium material using these three stains.

All the species that, according to the literature and the smelling test, have osmophores resulted positive for the histochemical techniques applied in this study. In some cases, we also found osmophores in floral parts not mentioned in the literature, for example, in the tepals of *Narcissus tazetta* (the literature mentions only the crown). In summary, we found an osmophore-positive reaction in (1) the tepals and crown of *Narcissus tazetta*; (2) the tepals, stamens, and staminode of *Sagittaria montevidensis*; (3) the corolla of *Lantana camara*; and (4) the corolla, stamens, and staminodes of *Jacaranda mimosifolia*. As expected, the flowers of *Aloe arborescens* reacted negatively in our tests (Fig. 4). In *Canna indica*, with unscented flowers, only the staminode or labelum had a positive reaction.

The different types of material, i.e., fresh, fresh in FAA (Fig. 3a), herbarium (Fig. 3d), and herbarium in FAA (Fig. 4a), reacted equally well to the stains; there were no differences in the degree of staining and quality of the results among samples. Likewise, the treatment applied to herbarium specimens of different dates, i.e., in the old herbarium specimens (AS) and in the more recently collected herbarium specimens (RS), did not yield different results. Even the oldest specimen from 1897, corresponding to *Canna indica*, reacted either positively (the staminode) (Fig. 5) or negatively (the other floral parts) to the osmophore testing.

Protocol for a trichrome technique for the identification of osmophores in flowers of herbarium material (TIOFH3)

Once selected the most effective and appropriate stains, i.e., Neutral Red, Oil Red O, and Lugol, we intended to generate a protocol that combines these three into a single staining procedure. Fresh and herbarium flowers of *Jacaranda*

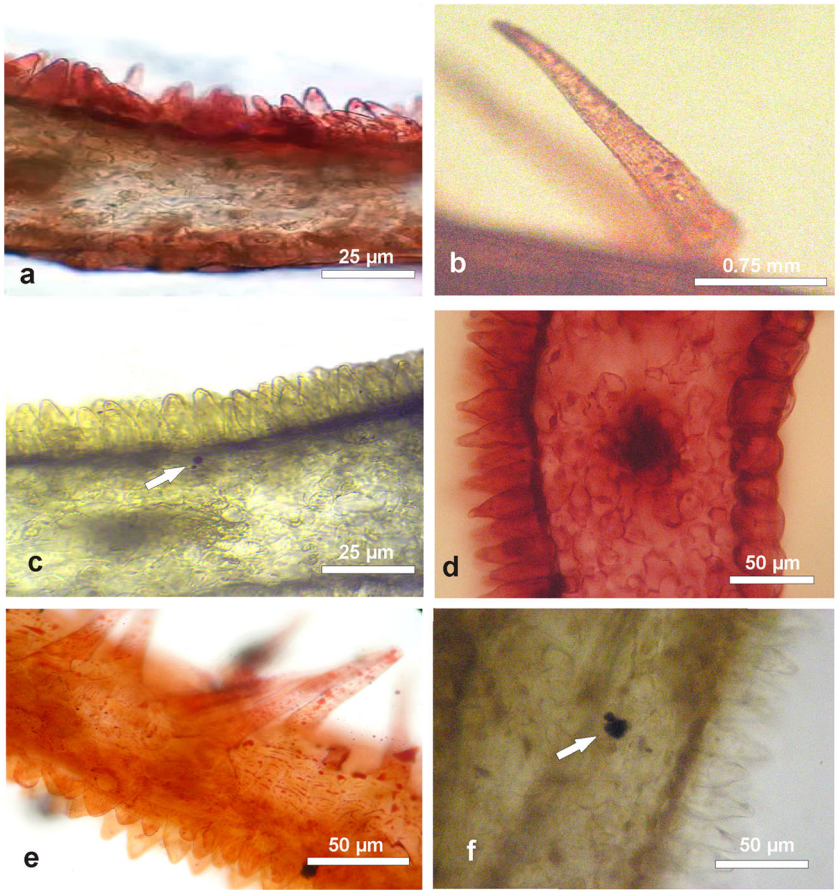

Fig. 3 Positive osmophore-staining (TIOFH) in transections of flowers of *Lantana camara* (microscope observation). **a–c** Fresh material (in FAA). **a** Neutral Red, note the redder epidermis and papillae. **b** Oil Red O, note the droplets of lipophilic content inside the trichome. **c** Lugol, the arrow shows two starch grains below the epidermis. **d–f** Herbarium material. **d** Neutral Red, note the redder epidermis and papillae. **e** Oil Red O, note the droplets of lipophilic content in the epidermis and inside the trichomes. **f** Lugol, the arrow shows starch grains below the epidermis. **a–c** Hernández 246 (LP). **d–f** Delucchi 3198 (LP). Please see the PDF version for color reference

mimosifolia, *Lantana camara*, and *Narcissus tazetta* (three species with osmophores) were employed for testing the protocol.

All possible sequences with the three stains were performed, taking into consideration that when using combinations, the second stain often affects the first and so on. The sequences experimented were (1) NR, ORO, Lugol; (2) NR, Lugol, ORO; (3) ORO, RN, Lugol; (4) ORO, Lugol, RN 4; (5) Lugol, RN, ORO; and (6) Lugol, ORO, RN. The sequence that yielded the best results was Lugol first, secondly the Oil Red O (ORO), and finally the Neutral Red (RN). Transfer from one stain to the other is direct and it is not necessary to wash the samples in each passage.

We propose the following protocol:

1. Hydrate herbarium material in oven at 38 °C, 24 h.

If working with fresh material or with material stored in FAA this step can be skipped, but the samples must be rinsed in distilled water at room temperature, 5 min.

2. Test the presence and location of potential osmophoric areas by submerging the samples (petals, tepals, etc.), 10 min in Neutral Red; they will be evidenced at naked eye or with stereomicroscope as notorious red areas.

It should be noted that any tissue injury that is either already present in the sample or made during the material handling, for example with a needle or because the flower part was cut, may stain with the NR giving false positives for osmophoric areas. Once located the presumptive osmophoric area, perform freehand transversal cuts at that level/s in another sample (i.e., not in that previously stained with NR). Also apply the technique to transversal cuts of a test-plant species to evaluate the results in the target taxon; we suggest using fresh or herbarium petals, stamens, and/or staminodes of *Jacaranda mimosifolia*, corollas of *Lantana camara*, or crown and tepals of *Narcissus tazetta* (Fig. 6).

3. Discolorate the transections according to the type of material (see Table 2).
4. Rinse with distilled water at room temperature, 1 min.
5. Lugol, 10 min.
6. Oil Red O, 10 min.
7. Neutral Red, 10 min.
8. Rinse with distilled water at room temperature, 1 min, shaking gently.
9. Mount in glycerin or gelatine-glycerin.

Results: the cells, papillae, and trichomes of the osmophoric area stain red or orange-red (NR, ORO), either with well-

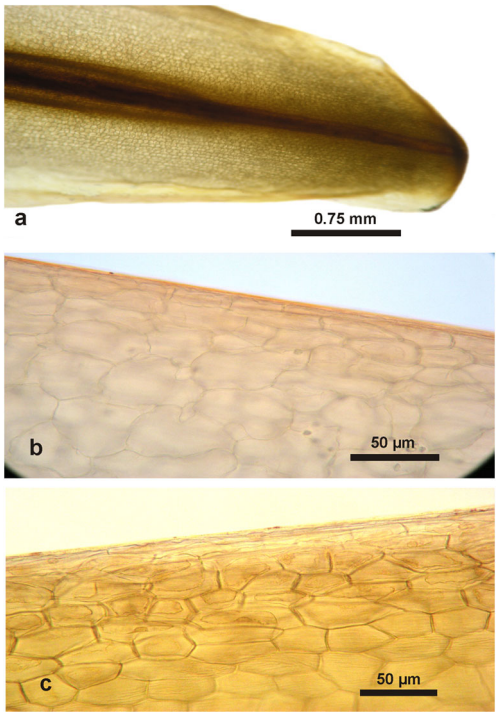

Fig. 4 Negative osmophore-staining of herbarium flowers in surface view of *Aloe arborescens* (microscope observation). **a** Neutral Red, note the lack of areas with red color (in FAA). **b** Oil Red O, note the lack of areas with red color. **c** Lugol, note the lack of starch grains. **a**–**c** Hernández 46 (LPAG). Please see the PDF version for color reference

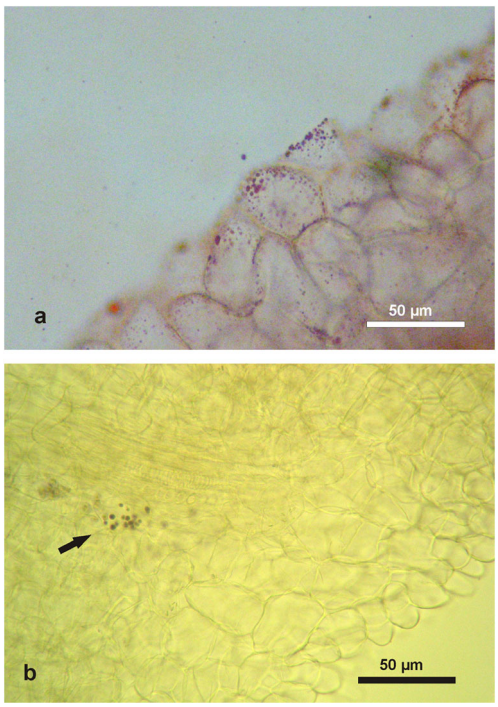

Fig. 5 Osmophore-staining (TIOFH) in staminode transection of herbarium flowers of *Canna indica*, collected in 1897 (microscope observation). **a** Oil Red O, note the droplets of lipophilic content in the papillae. **b**. Lugol, the arrow shows starch grains close to the epidermis. **a**, **b** Spegazzini s.n. (LP)

defined droplets or staining the whole cytoplasm and cell walls. The starch grains nearby the osmophoric area stain violet-black or violet (Fig. 6).

It is recommended to check the results within the next 24 h after the procedure because the stains, mainly Lugol, fade with time. If that happens, as this technique has temporary (glycerin) or semi-permanent (gelatine-glycerin) water mountant media, it is possible to remove slightly the cover with a needle just enough to come to the edge of the cover without oozing out beyond, run some small drops of Lugol (or RN, or ORO), and re-mount the slide.

It is strongly recommended to perform transection cuts of the samples because they show more accurately the structure and location of the osmophores. For example, the transections evidence the eventual droplets of the lipophilic components of the volatile aromatic essences and the nearby placement of the starch to the osmophoric area.

The different test-species selected here reacted well to the trichrome technique and there were no differences in the quality of the results (Fig. 6). The only staining differences were found among mature and immature flowers (the last ones lacked scent when tested in fresh material), and it is probably due to biological reasons and not as a result of the application of the technique. For example, we found that the immature flowers of *Lantana camara* and *Sagittaria montevidensis*, mainly at the bud stage, reacted positively to Lugol but not (or scarcely) to NR and ORO (Fig. 7). Apparently, the osmophore secretion substances were still not present in these samples. Similar results were found by Plachno et al. (2018) in *Genlisea* (Lentibulariaceae) where cell vacuolization of osmophores increases during the flower development.

It should be remarked that we obtained positive results applying this protocol (TIOFH3) in the three tested species but, in the case that the trichrome technique would not reveal clearly the osmophores, there is always the possibility of staining with RN, ORO, and Lugol in three separate preparations (TIOFH).

Discussion

Histochemical techniques for osmophore testing, TIOFH and TIOFH3, are proposed here for first time in herbarium specimens with an ample range of collection dates, giving positive results. This is an important outcome of this study because, according to the literature analyzed, researchers use living plants in their works. Besides, these technique can also be employed in fresh material and material stored in a fixation agent.

On the other hand, the literature proved to be quite heterogeneous in the type and sequence of the stain employed for detecting osmophores, showing that a unified protocol was still lacking. After testing several stains, we arrived to the

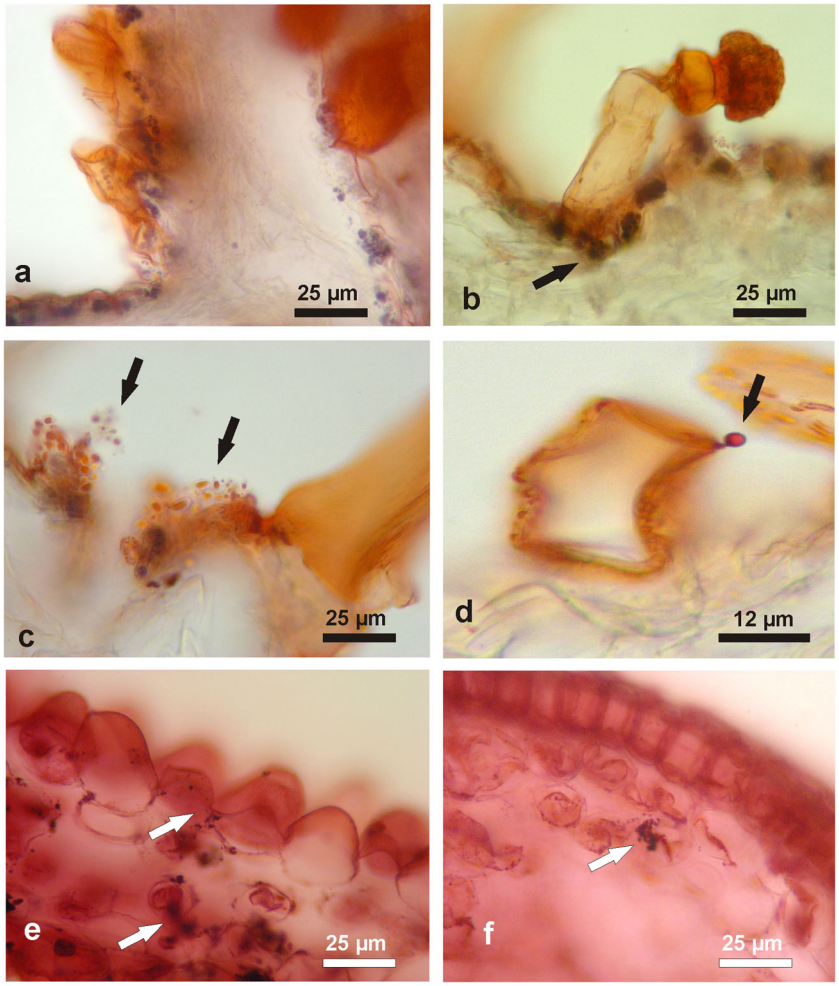

Fig. 6 TIOFH3 in herbarium and fresh material in transection (microscope observation). **a** *Jacaranda mimosifolia* (herbarium material), note the two orange-reddish epidermis and trichomes, and the violet-black starch grains below. **b** *Jacaranda mimosifolia* (herbarium material), note the lipophilic content concentrated at the head of the trichome and the violet-black starch grains in the epidermis (*arrow*). **c** *Jacaranda mimosifolia* (herbarium material), note the droplets with lipophilic content in broken basal cells of the trichomes (*arrows*) and the violet-black starch grains below. **d** *Jacaranda mimosifolia* (herbarium material), basal cell of a broken trichome showing a droplet (*arrow*) of the orange-reddish volatile lipophilic substance, and the orange-reddish cell walls of the trichome. **e** *Narcissus tazetta* (fresh material), note the reddish color of the osmophoric area and the numerous violet-black starch grains below and inside the epidermis (*arrows*). **f** *Lantana camara* (herbarium material), note the redder epidermis and the violet-black starch grains below (*arrow*). **a** Cabrera s.n. (LP 89499). **b–d** Burgueño 166 (LP). **e** Hernandez 239 (LP). **f** Hurrel et al. 1269 (LP). Please see the PDF version for color reference

conclusion that the most appropriate for osmophores are Lugol, Oil Red O, and Neutral Red. It must be noted that the combination of NR, Lugol, and some type of stain specific for lipids and lipophilic substances, such as Sudan III or Sudan IV, was already used in some previous works using fresh material (e.g., Wiemer et al. 2009; Kowalkowska et al. 2018). However, we found that ORO worked better than any other lipid stain, both in fresh and herbarium material, as showed in Table 3. The same results, in living material, were found by Proescher (1927). ORO has an extensive use in animal tissue (e.g., Lillie and Ashburn 1943; Novikoff et al. 1980), but it gives excellent results in plant tissue and principally in herbarium material.

Why Neutral Red, a vital stain, did work perfectly in hydrated herbarium samples goes beyond the scope of this study. Some authors (Plachno et al. 2005), using Scanning Electron Microscope images, found evidence in insectivorous plants of cuticular discontinuities which absorb Neutral Red. Also, the structure and chemistry composition of the cuticule and waxes are very important for the permeability of water and other compounds, such as stains. The cuticle is a permeable layer both for polar as non-polar compounds, with the waxes playing a key role in reducing the permeability to water and other substances which passes slowly through the cuticle (Kerstiens 2006; Lallana et al. 2006). Because the Neutral Red is a lipophilic phenazine dye, it stains and goes through the cuticle due to its affinity for lipophilic structures (Dubrovsky et al. 2006). As the cells die, their ability to uptake NR would decrease. It is possible, however, that despite the desiccation of the herbarium specimens, those relatively

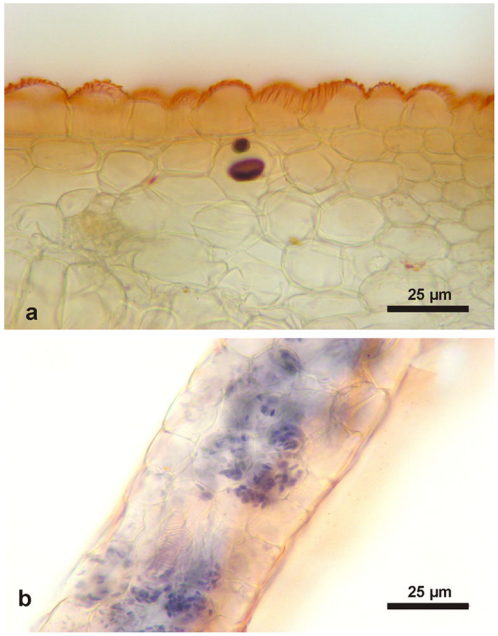

Fig. 7 TIOFH3 in transections of immature flowers of herbarium material (microscope observation). **a** *Lantana camara*, note the reddish cuticle but the scarcely stained epidermis, and the violet-black starch below. **b** *Sagittaria montevidensis*, note the abundant violet-black starch and the scarce red color. **a** Cabrera 2150 (LP). **b** Tur 1562 (LP). Please see the PDF version for color reference

undamaged cells subject to hydration recover enough viability allowing the remnants of lipophilic (hydrophobic) substances to react with NR. As some tonoplasts are probably broken by the manipulation of the material, the NR generally does not accumulate as cell sap but stains the cytoplasm (Stadelmann and Kinzel 1972; our observations).

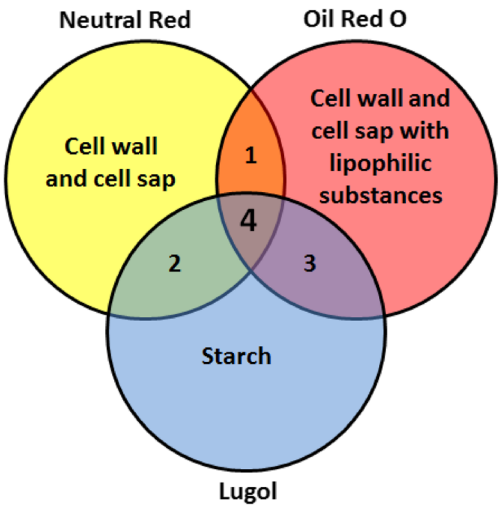

Fig. 8 Diagram showing the staining action of Neutral Red, Oil Red O, and Lugol on plant tissues, both, separately and combined with each other, as displayed in the circles intersections. *1* (orange) vacuoles with liphophilic content, *2* (green) secretion involving vacuoles (e.g., osmophore, nectary), *3* (violet) secretion involving vacuoles with lipophilic substances (e.g., osmophore, stigma secretion), *4* (brown) osmophore. Please see the PDF version for color reference

The three stains used separately bring out some particular chemical substance, but it is the combination of them in one single study what establishes a presumptive evidence for the presence of osmophores (Fig. 8). The NR alone stains all types of vacuole content (Küster 1939; Stadelmann and Kinzel 1972) and the ORO stains lipophilic substances found for example in wax, cuticle, oleosomes, and elaioplasts. Therefore, when combined the NR with ORO, we would evidence vacuoles with a lipophilic component. The combination of Lugol with these stains would imply a secretory activity of the tissue. For example, Lugol with NR could evidence osmophoric activity but also a nectary, an hydatode, a colleter, or any other type of structure with vacuole secretion. The combination of Lugol with ORO could evidence an osmophore, but also a secreting stigma, oil secreting trichomes, cavities, and ducts with lipophilic content, and laticifers. Even when lipids are part of the secretion substances of fragrant compounds, the combination of the three stains still leaves a minimum chance that it could be some secreting structure with vacuoles containing a lipophilic component other than an osmophore, such as an oil gland. Stern et al. (1986) mentioned that the likelihood of any one stain functioning as an absolute indicator of osmophore presence is small. The best scenario would be, besides using the protocol here proposed, to perform the smell registration of flower scent. We are aware that this is not possible to test in herbarium specimens, but generally some information regarding the flowers fragrance can be found in the literature and in the herbarium labels.

The protocol established here (TIOFH3) allows performing the technique in the same slide or sample. This not only simplifies the procedure (ca. 30 min of duration) but also avoids needlessly destroying herbarium and fresh material and using additional quantities of reagents and laboratory supplies.

Another outcome of this work is that the material stored at least during 1 month (and probably more than that time) in fixing agents such as FAA, also resulted positive for osmophore testing. In this way, it offers the possibility to store the samples in FAA during field trips, perform the technique in the laboratory, and expect some results.

Osmophores have been analyzed with different objectives (Gomes da Silva 1990-1992) in taxonomical (e.g., Curry et al. 1991), morphological (e.g., Kowalkowska et al. 2015, 2018), and evolutionary studies (e.g., Bröderbauer et al. 2012). It is also well known that volatile compounds play an important role in floral biology and plant-insect interactions (e.g., Almeida-Soares et al. 2010; Pansarin et al. 2008) and in the perfume industry (Piechulla and Pott 2003). We hope that the techniques for the identification of osmophores in flowers of herbarium material (TIOFH and TIOFH3) proposed here will be helpful for these and other kind of studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix. List of fresh and herbaria material of the analyzed species, with vouchers. Vouchers of the fresh material are marked with an asterisk *.

Aloe arborescens Mill.

Argentina, Provincia Buenos Aires, Partido Berisso, Isla Paulino, 17-V-2009, M. P. Hernández 39 (LPAG). Argentina, Provincia Buenos Aires, Partido Ensenada, Isla Santiago, 17-VIII-2010, M. P. Hernández 46 (LPAG). *Argentina, Provincia Buenos Aires, Partido La Plata, Gonnet, 10-IX-2018, M. P. Hernández 242 (LP).

Canna indica L.

Argentina, Provincia Salta, Pampa Grande, I-1897, C. Spegazzini s.n. (LPS 19001 in LP).

Argentina, Provincia Salta, Departamento Rosario de Lerma, Río Blanco, 26-III-1945, Abbiatti & Claps 937 (LP). Argentina, Provincia Salta, Salta, ruta 34, 4 km al S de Pichanal, III-1970, H. Fabris & F. Zuloaga 7889 (LP) (2 sheets). Argentina, Provincia Buenos Aires, Partido Berisso, Isla Paulino, 29-III-2009, M. P. Hernández 23 (LPAG). *Argentina, Provincia Buenos Aires, Partido La Plata, Jardín Bótánico y Arboretum Carlos Spegazzini, 15-IX-2018, M. P. Hernández 243 (LP). *Argentina, Provincia Buenos Aires, Partido La Plata, Jardín Bótánico y Arboretum Carlos Spegazzini, 15-IX-2018, M. P. Hernández 244 (LP).

Jacaranda mimosifolia D. Don

Argentina, Provincia Buenos Aires, Partido La Plata, La Plata, vivero del bosque, XII-1939, A. Cabrera s.n. (LP 89499). Argentina, Provincia Buenos Aires, Capital Federal, 3-XII-1994, G. Burgueño 166 (LP). Argentina, Provincia Buenos Aires, Partido La Plata, La Plata, 4-XII-1996, G. Delucchi 1478 (LPAG). Argentina, Provincia Buenos Aires, Capital Federal, 23-XI-2007, G. Delucchi & J. Hurrell 3179 (LP). *Argentina, Provincia de Buenos Aires, La Plata, calle 51 esquina 16, 11-XI-2018, M. P. Hernández 245 (LP).

Lantana camara L.

Argentina, Provincia Misiones, Santa Ana, I-1907, sin leg. (LP). Argentina, Provincia Buenos Aires, Partido La Plata, ribera del Río de la Plata, Isla Santiago, 24-IV-1932, A. Cabrera 2150 (LP). Argentina, Provincia Buenos Aires, Isla Martín García, 23-X-1992, J. Hurrell et al.1269 (LP). Argentina, Provincia Buenos Aires, Isla Martín García, camino a Barrio Chino, 21-I-1998, J. Hurrell et al. 3857 (LP). Argentina, Capital Federal, Costanera Sur, 8-XII-2007, G. Delucchi 3198 (LP). Argentina, Provincia Buenos Aires, Partido Berisso, Isla Paulino, 29-III-2009, M. P. Hernández 19 (LPAG). Argentina, Provincia Buenos Aires, Partido Ensenada, Isla Santiago, 18-III-2011, M. P. Hernández 74 (LPAG). *Argentina, Provincia Buenos Aires, Partido La Plata, Jardín Bótánico y Arboretum Carlos Spegazzini, 20-VIII- 2018, M. P. Hernández 240 (LP). *Argentina, Provincia Buenos Aires, Partido La Plata, Jardín Bótánico y Arboretum Carlos Spegazzini, 20-VIII- 2018, M. P. Hernández 241 (LP).

Narcissus tazetta L.

Argentina, Provincia Buenos Aires, Isla Martín García, área urbana cerca del monumento a los Héroes Comunes, 20-VIII-1997, J. Hurrell et al. 3600 (LP). Argentina, Provincia Buenos Aires, Partido La Plata, Gonnet, Bordenave y 15 bis, 25-VI-2001, G. Delucchi 2529 (LP). Argentina, Provincia Buenos Aires, Partido Avellaneda, Villa Dominico, 10-VII-2008, G. Delucchi 3279 (LP). Argentina, Provincia Buenos Aires, Partido La Plata, Gonnet, 9-VIII-2009, G. Delucchi 3445 (LP). *Argentina, Provincia Buenos Aires, Partido La Plata, Jardín Bótánico y Arboretum Carlos Spegazzini, 20-VIII-2018, M. P. Hernández 239 (LP).

Sagittaria montevidensis Cham. & Schltdl.

Argentina, Provincia Buenos Aires, Partido Ensenada, Isla Santiago, cerca de La Plata, 18-XI-1928, A. Cabrera 520 (LP). Argentina, Provincia Buenos Aires, Partido Magdalena, Atalaya, 14-XI-1973, N. Tur 1562 (LP). *Argentina, Provincia Buenos Aires, Partido La Plata, Gonnet, calle Centenario frente al Club Rugby La Plata, 17-XI-2018, M. P. Hernández 246 (LP).

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