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The Pharaoh's snakes of the teasel: New insights into Francis Darwin's observations

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Almost 150 years ago, the botanist Francis Darwin, the son of Charles, included *Dipsacus fullonum* (Appendix S1: Section S1), known as the common teasel, in his scientific ventures into plant carnivory (for a historical overview see Schaefer, 2021). At each node on its mainstem, this large biennial herbaceous plant harbors phytotelmata formed by the bases of opposite oblanceolate leaves (Figure 1A,B) (see the video in Lehours et al., 2023). These water-holding cups are capable of retaining up to some 100 ml of water invariably corrupted by dead and putrefying insects and other small invertebrates. To decipher whether the plant was absorbing nitrogen from decaying animals and benefiting from carnivory, Francis Darwin examined the cup-like receptacles. He observed protrusive filaments (Appendix S1: Figure S1A–C) originating from glandular trichomes (i.e., epidermal hairs

specialized for the synthesis of secreted natural products) localized on the inner side of the leaf basins (Darwin, 1877a). In so doing, F. Darwin discovered the astonishing movements made by the filaments, which Ferdinand Cohn, a distinguished Professor of Botany, compared to a “Pharaoh’s snake,” a toy that was a popular Christmas gift in 1865 (Cohn, 1878).

Charles Darwin was enthusiastic about his son’s work and wrote in *Nature’s* “Correspondence” section, “*The observations of my son Francis on the contractile filaments protruded from the glands of Dipsacus, offer so new and remarkable a fact in the physiology of plants, that any confirmation of them is valuable*” (Darwin, 1877b). While some of Darwin’s contemporaries attempted to elucidate the remarkable filament contractions, neither a consensual nor a convincing explanation emerged (e.g., Chodat & Zollikofer, 1892; Cohn, 1878). To our knowledge, these investigations were not pursued by any botanist in the

Antoine Vergne and Eric Giraud have equally contributed to this work.

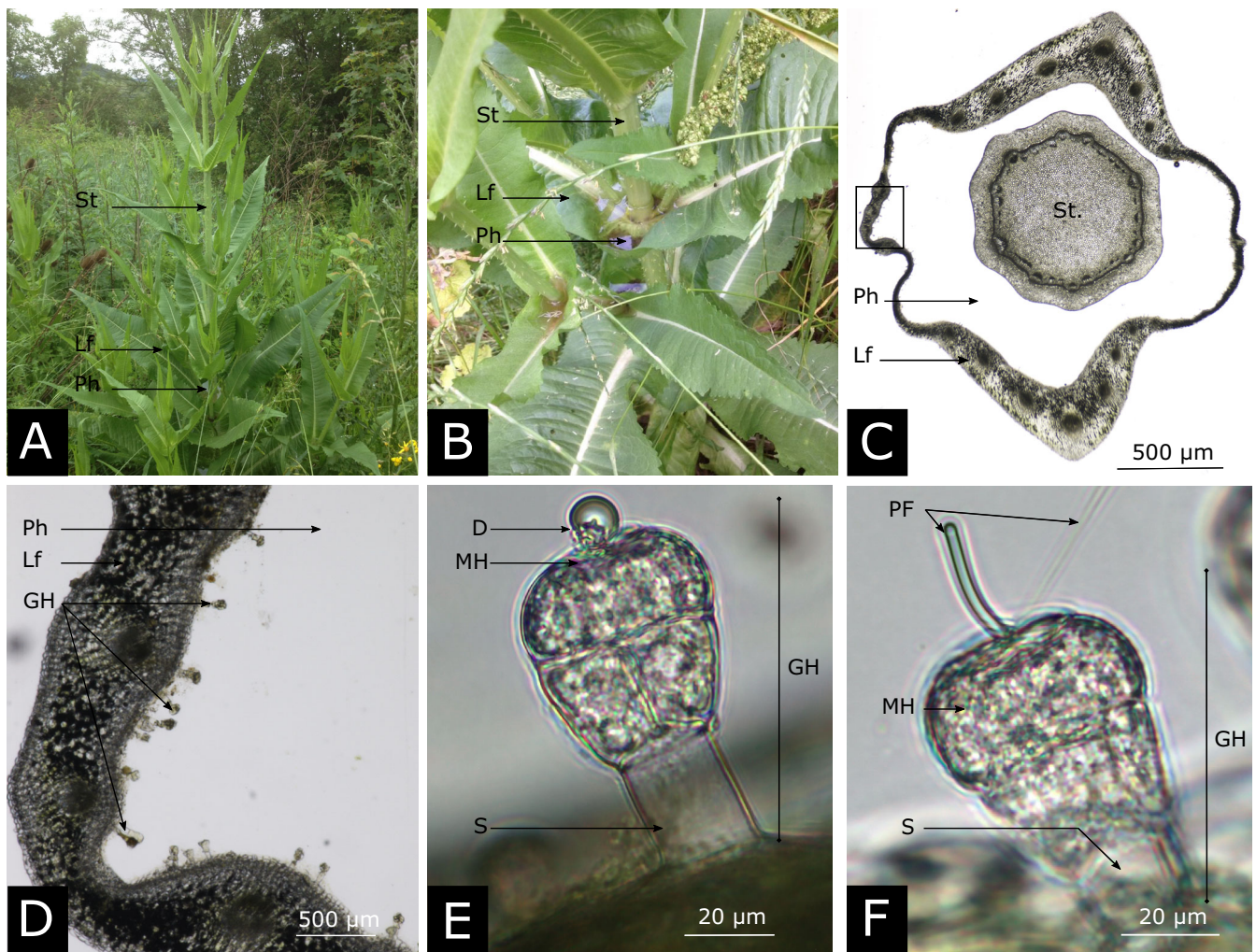


FIGURE 1 Photographs of the biennial herbaceous common teasel, *Dipsacus fullonum* and of the glandular hairs covering the inner side of its leaf basins. (A) The erect plant before flowering showing the phytotelmata formed by the bases of opposite leaves on the mainstem. (B) Close-up of the phytotelmata of the common teasel containing cloudy water. (C) Thin leaf section of a phytotelma observed using an optical stereomicroscope (magnification $\times 5$). (D) Close-up of the framed area in picture (C) showing some projections of epidermal cells around $100\ \mu\text{m}$ in height, the club-shaped glandular hairs, localized on the inner side of the leaf basin. (E) A club-shaped glandular hair whose head is topped by a refractive droplet (magnification $\times 400$). (F) A club-shaped glandular hair whose droplet overhanging the head became filamentous (magnification $\times 400$). D, droplet secretion; GH, glandular hair; Lf, leaf; MH, multicellular head; PF, protrusive filament; Ph, phytotelma; S, unicellular stalk (i.e., the neck cell); St, stem. Photography credit: Antoine Vergne (photographs [A], [B], [E], and [F]) and Eric Giraud (photographs [C] and [D]).

20th or 21st centuries or, in any case, were not reported in the scientific literature, but drifted into oblivion. During our ongoing research on the microbiome of phytotelmata (Lehours et al., 2016; Vergne et al., 2021), we fortuitously “re”-discovered the enigmatic filaments of *Dipsacus* that have no known analog in the plant world. Our primary findings prompted us to further explore past discoveries using modern microscopy and staining methods. Beyond comparing our observations with those of F. Darwin (Appendix S1: Figures S1 and S2) (see the video in Lehours et al., 2023), we demonstrate that the teasel’s Pharaoh’s snakes are part of an orderly process that is likely to be deterministic and under plant control. We also

show that a complex microbial network is associated with glandular hairs and their secretions.

The first step was to explore different aerial regions of fresh leaves and stems (Appendix S1: Sections S2 and S3) collected from emerging vegetative rosettes growing in the first season and from erect plants in the second year. Like F. Darwin (Appendix S1: Figure S2A–C), we observed two morphotypes of trichomes we named “pomegranate” and “club” based on their shape (Appendix S1: Figure S2D–F). The morphotypes may be either two different types of hair or two ontogenetic stages of the same type. The many pomegranate-shaped epidermal outgrowths are ubiquitous on leaves and stems irrespective of the growth stage of the

common teasel. Conversely, the club-shaped hairs are almost only located on the inner side of the leaf basins of erect plants although at high densities (Figure 1C,D; Chodat & Zollikofer, 1892; Darwin, 1877a). The endemic distribution of these club-shaped foliar expansions supports the idea that they play a specific role in the phytotelmata. Their morphology is typical of glandular hairs (i.e., sites of biosynthesis where large quantities of specialized metabolites are stored; Huchelmann et al., 2017; Schuurink & Tissier, 2020), with a basal cell, a cylindrical unicellular stalk, and a multicellular head (Figure 1E,F; Appendix S1: Figure S2E). The bright drops almost continually observed at their top highlight the secretory nature of these glandular cells (Figure 1E; Appendix S1: Figure S2F). The drops can vary in size and number (Appendix S1: Figure S3A), but each consists of a hyaline, uniform and colorless highly refractive mass, which may indicate a dense substance (Figure 1E) (see the video in Lehours et al., 2023). Unexpectedly, when the sections of the leaf basins were immersed in mineral water, the drops were transformed into filaments after a few minutes (Figure 1F; see the video in Lehours et al., 2023). Irrespective of whether there was only one (Figure 1F) or many filaments (Appendix S1: Figure S1E) on each glandular hair, the filaments end in a single point, a sphere, or a bulge, but may also be formed of strings of droplets (Appendix S1: Figure S1F) (video in Lehours et al., 2023). Following their extrusion, the threads increase in length (up to 1 mm) and show vibrations independent of the random motion of particles present in the surrounding water (video in Lehours et al., 2023). After a few minutes to several tens of minutes, the filaments retract spontaneously to become a refractive drop overhanging the head of the trichome (video in Lehours et al., 2023). Occasionally, this retraction phenomenon is accompanied by the formation of a string of drops (video in Lehours et al., 2023).

Although this retraction process can be easily observed by the human eye, it is exceptionally rapid compared with known seismonastic movements in plants (see Mano & Hasebe, 2021 and references therein). As a whole, the filaments mimic the self-propelling and navigational properties of biological cells, such as chemotaxis and durotaxis, although their protean nature (Appendix S1: Figure S1) and the absence of a cell membrane demonstrate they are not alive.

To investigate the biochemical nature of the singular vesicle-shaped or filament-shaped structures, we used specific dyes combined with conventional fluorescence microscopy or confocal laser scanning microscopy (Appendix S1: Sections S4 and S5). Staining of thin leaf sections with Nile Red and BODIPY 493/503 probes revealed the lipid nature of both the filaments and of the drops that cap the gland cells (Appendix S1: Figure S3B,C). Histochemical tests

(Appendix S1: Sections S6), whose staining conditions are not compatible with the protrusion of the filaments, confirmed that the drops contain neutral lipids including triglycerides (Appendix S1: Figure S4A and Table S3). The naphthol and diamine (NADI) reaction showed they also convey terpenes, one of the largest and most structurally diverse families of natural plant products (Appendix S1: Figure S4B and Table S3). These substances can be produced by cells of the capitate glandular trichomes, as similar material was observed in a number of intracellular vesicles of their multicellular head (Appendix S1: Figure S4 and Table S3). According to the biochemical nature of the secretions and to the current knowledge in plant physiology and biophysics, a likely hypothesis to explain the protrusions and undulations of filaments is a two-step process. First, the secretion products accumulate in a subcuticular space creating a pressure that enables the secretion flux to cross some porous area of the cuticle barrier (Muravnik, 2020). Second, following their emission, interfacial tension may play a central role in the phase separation of the binary mixture (aqueous environment and secreted hydrophobic substances) into filament superstructures, which may exhibit vibratile activity in response—for example—to the Marangoni effect (e.g., self-propelled lipid filament) (Abaurrea-Velasco et al., 2019; Peddireddy et al., 2021). The last assumption is consistent with the vitality retained by some filaments even when they are separated from their parent glands (Darwin, 1877a).

However, no conventional mechanism explains filament retraction. This finding rejects the hypothesis of a simple sucking process linked to a depression inside the gland cells, as the retraction of a filament may be almost simultaneous with the protrusion of another filament from the same glandular trichome (video in Lehours et al., 2023). What is more, staining the gland with CellMask Orange Actin Tracking Stain revealed a concentration of filamentous actin around the area where the secretion forms and the filaments protrude (Appendix S1: Sections S4, S5, Figures S3D and S5). Considering that the actin cytoskeleton is known to be involved in organelle movement and in vesicle trafficking in plants (Paez-Garcia et al., 2018), it is possible that the phenomenon of emergence and retraction of these filaments is an active process under the control of the plant.

The role of the filaments in the ecology of the plant is also enigmatic. Francis Darwin and his father shared the conviction that the teasel takes advantage of the organic nitrogen released by the insects caught in the cups and that the protrusive filaments are involved in nutrient acquisition (Darwin, 1877a). To date, there is no clear evidence to support the idea that *D. fullonum* boasts a carnivorous appetite. One study suggested that more seeds are produced when insects are added to teasel cups (Shaw &

Shackleton, 2011) while other studies have concluded that teasel is not carnivorous (Krupa & Thomas, 2019; Rostock, 1904). Nevertheless, following F. Darwin's intuition that interactions exist among the gland cells, the protrusive filaments, and the contents of the water cups, we pursued our investigations by focusing on the peripheral environment of the glandular hairs. Flow cytometry counts enumerated an average of $1.5 \pm 1.2 \times 10^8$ of total cells per milliliter of water in the catchments of *D. fullonum* (Appendix S1: Section S7). Such microbial cell densities, similar in magnitude to those in the water tanks of the Bromeliaceae (Vergne et al., 2021), are a 100 to 1000 times higher than those recorded in freshwater and marine aquatic systems (Whitman et al., 1998). Our microscopic observations showed that microbial cells were highly abundant around the secretory heads of the club-shaped glandular hairs, but were sparsely distributed between the trichomes (Figure 2A; Appendix S1: Figure S6A). These observations collectively suggest that the glandular hairs of the teasel's phytotelmata form microscale resource-specific ecological niches favoring microbial hotspots and confirms their important role in the establishment of epiphytic bacteria in a variety of plants (Baldotto & Olivares, 2008). In addition, confocal imaging identified fungal hyphae and thin fibrils entangled in elaborate knots around the glandular trichomes (Figure 2; Appendix S1: Figure S6) (video in Lehours et al., 2023). These fibrillary elements, whose origin remains unknown, are compositionally distinct from the gland secretions, as shown by their polysaccharide structure (Figure 2A,B). This complex matrix of hyphae and fibrils forms a network to which prokaryotes can attach themselves and probably plays a role in their spatial organization around the gland cells (Figure 2; Appendix S1: Figure S6).

Next, we questioned whether the close interactions among the glands, their secretions, and the teasel's microbiota promote enrichment in beneficial microbial taxa. A recent study revealed that tomato glandular trichomes shape the composition of the surrounding microbial community and recruit a unique and diverse microbiota (Kusstatscher et al., 2020). In this context, the terpenes we identified in the gland secretion products of *D. fullonum* (Appendix S1: Figure S4B and Table S3) could be involved in the microbiome assembly, as this family of natural products is known to play a variety of roles in mediating beneficial interactions between mutualistic partners (Gershenson & Dudareva, 2007). In the same line of thought, Huang et al. (2019) reported that specific terpenes of *Arabidopsis* govern the assembly and long-term maintenance of a specific microbiota, thereby enabling the host plant to tailor the microbial community to its own needs. We recently showed that such specific

microbiota is established in the phytotelmata of tank bromeliads and that a very high proportion of bacteria in these communities have a nitrogen fixation toolkit (Vergne et al., 2021). Biological nitrogen fixation (BNF), the natural process by which atmospheric N_2 is converted into ammonia by the enzyme nitrogenase, is crucial for the biosphere as it provides nitrogen to plants. This knowledge led us to explore whether the teasel's cups promoted a N_2 scavenger microbiome. Using quantitative PCR targeting the BNF marker gene *nifH* (Appendix S1: Section S8), we estimated that the relative contribution of diazotrophs in the teasel's cups accounted for up to 45% of the total prokaryotic community, with an average of $13.3 \pm 11.4\%$ and a median of 9.7% (Appendix S1: Figure S7A). These proportions resemble and even exceed those reported for bulk and rhizosphere soil ecosystems known to host an active and abundant nitrogen-fixing community (Espenberg et al., 2018; Ling et al., 2022). Moreover, the *nifH* gene abundances (1.3×10^6 to 9.1×10^8 copies.mL⁻¹; Appendix S1: Figure S7A) are well above those reported in aquatic systems (Luo et al., 2012). The acetylene reduction activity (ARA) technique, which relies on the reduction of acetylene to ethylene by nitrogenase (Hardy et al., 1968), was also used to assess nitrogenase activity in diazotrophic bacteria occupying the teasel's cups. After incubating bacteria collected from *D. fullonum* water-filled cups in a culture medium without nitrogen, we detected substantial ARA activity (Appendix S1: Figure S7B), highlighting the potential of this microbiota to fix nitrogen under appropriate conditions. Furthermore, ARA activity was also detected directly in the tank fluids taken from several reservoirs, indicating that BNF occurs in the teasel's cups (Appendix S1: Figure S7C). Glandular trichomes may be key players in this BNF process by secreting specific molecules, such as terpenes, to sustain the growth of specific bacteria (Huang et al., 2019) and/or to protect oxygen-sensitive nitrogenases, thanks to their antioxidant activity (Grassmann et al., 2002).

While biological sciences are in the age of multi-omics approaches, we still have a lot to learn from the Natural Sciences of our predecessors who, in the 18th and 19th centuries, observed Nature in infinite detail and knew how to be curious about its peculiarities. The Darwins are among the most illustrious of these naturalists and share a long history with the common teasel, beginning with the poet and naturalist, Erasmus Darwin who, in "*The Loves of the Plants*" (Darwin, 1791), wrote; "*There is a cup around every joint of the stem of this plant, which contains from a spoonful to half a pint of water; and serves both for the nutriment of the plant in dry seasons, and to prevent insects from creeping up to devour its seed.*" Almost a century later, his great-grandson, Francis,

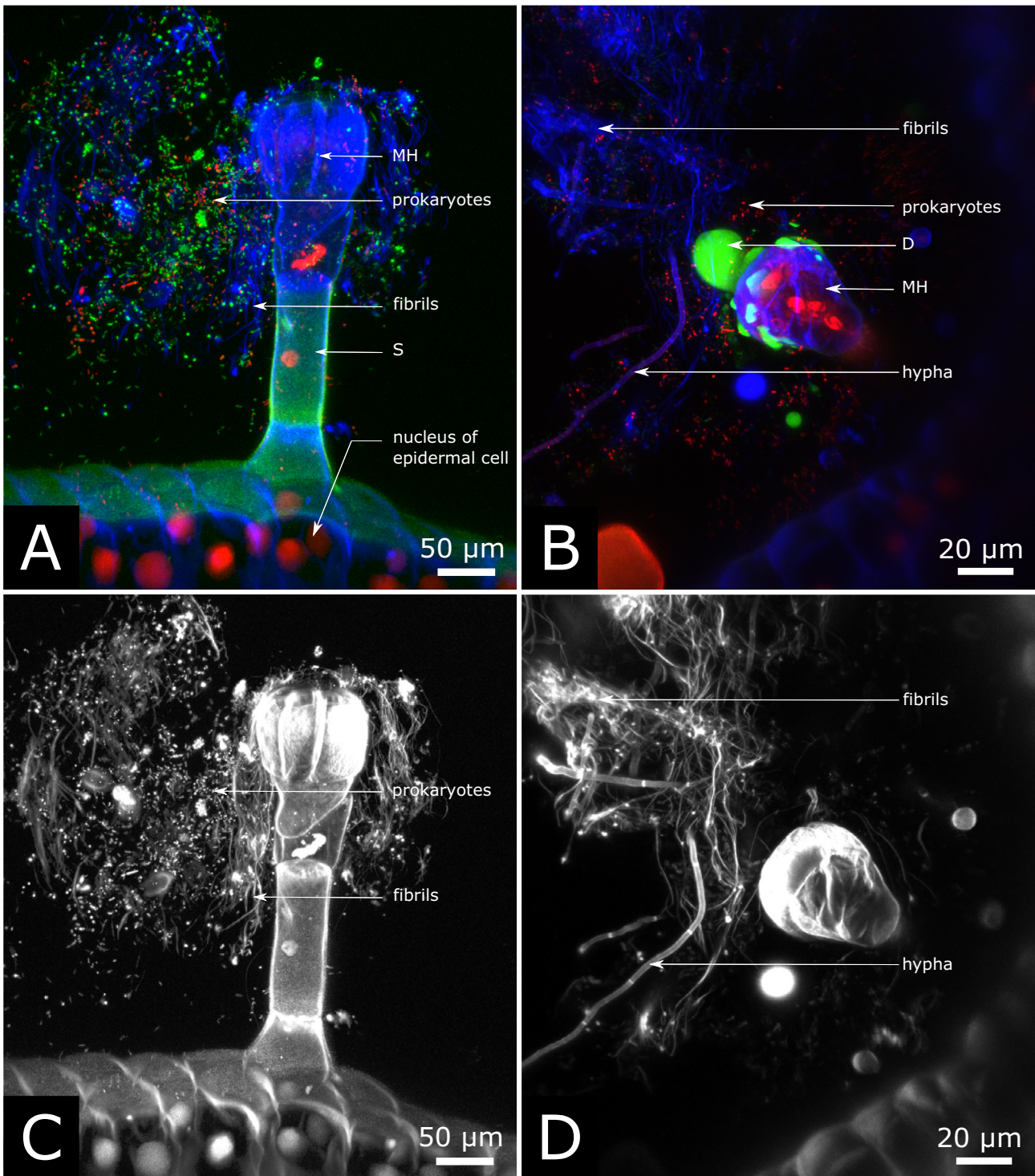


FIGURE 2 Confocal microscope images of the microbial hotspots associated with the club-shaped glandular hairs of the phytotelmata of the common teasel. (A) Prokaryotes that fluoresce after staining with SYTO 9 (green) and propidium iodide (red) embedded in a dense fibrillar matrix (fibrils) that fluoresce blue after staining with calcofluor and that encompass the club-shaped glandular hairs. (B) A club-shaped glandular hair surrounded by fungal hyphae and by a dense fibrillary network (fibrils) thinner than hyphae that fluoresce blue after staining with calcofluor. (C) and (D) are the same photographs as (A) and (B), respectively, but the fluorescence emitted by calcofluor (for C and D) and by SYTO 9 and propidium iodide (for C) are colored gray to make the details more visible, in particular, the fibrils and the hyphae. The bright green fluorescence of the cuticle located on the outer surface of the epidermal cell wall and on the cylindrical unicellular stalk of the glandular hairs probably originates from phenolic acids or flavonoids bound to the cutin backbone. D, droplet secretion; MH, multicellular head; S, unicellular stalk (i.e., the neck cell). Photography credit: Eric Giraud and Antoine Vergne.

expected to find that the plant was absorbing nitrogen from the decaying insects accumulating in these cups using protrusive filaments (Darwin, 1877a), “a grand discovery” as qualified by Charles Darwin (1877b). However, 150 years later, there is no clear evidence that the teasel is a carnivorous plant.

First, and although not proof in itself, there are no other known carnivorous or proto-carnivorous species within the Caprifoliaceae family (The Angiosperm Phylogeny Group et al., 2016). Furthermore, the habitats of the common teasel (Christy, 1923) do not meet the classical harsh abiotic conditions in which carnivorous plants typically occur (Chase et al., 2009; Ellison, 2006). In addition, carnivorous plants have low competitive abilities (Chase et al., 2009; Ellison, 2006), whereas individual teasel plants compete for resources with neighboring plants by spreading large rosette leaves that shade the ground and by extending taproots deeper than the roots of many of its annual and biennial competitors (Rector et al., 2006; Werner, 1975). All of these arguments coupled with the fact that insect supplementation does not increase the teasel fitness (Krupa & Thomas, 2019) challenge the hypothesis of the three generations of Darwins.

However, following the perspective of a link between the water cups and nitrogen inputs, we demonstrated (i) the remarkable capability of the microbiota hosted in the phytotelmata to fix atmospheric nitrogen and that (ii) glandular hairs and their secretions form microbial hotspots. Accordingly, we postulate that the mystery of the unusual and enigmatic protrusive filaments of *Dipsacus* perhaps lies in a close connection between the plant and its microbiota, notably with some N₂-scavenging microbes. These results open up new and exciting avenues in the study of plant–microbiota interactions. Experimental evaluation of our hypothesis is a critical next step to deciphering the fundamental physiological, ecological, and evolutionary mechanisms involved in such plant–bacteria interactions. In this vein, it is crucial to determine whether the common teasel is capable of assimilating nitrogen from its water cups. To this end, isotopic approaches using ¹⁵N-labeled ammonium and/or nitrate would be relevant. Biomanipulation experiments of the microbiota under controlled conditions could decipher the role of nitrogen-fixing microbiota in plant fitness. Finally, the possible role of glandular hairs and of their protrusive filaments as plant/N₂-fixing microbe interfaces, while exciting, will be a complex and time-consuming prospect. It will require very fine-scale approaches such as, for example the, combination of scanning electron microscopy, live imaging, and nanoSIMS, which have been shown as relevant technologies to address similar questions (e.g., Finzi-Hart et al., 2009; Massalha et al., 2017).

AUTHOR CONTRIBUTIONS

Antoine Vergne and Eric Giraud are co-first authors. Alicia Camuel, Corinne Bardot, Hermine Billard, Clémentin Bouquet, Frédéric Mathonat, Djamel Gully, Isabelle Mary, Jean-Claude Caissard, and Anne-Catherine Lehours contributed to the execution of the project. Antoine Vergne, Eric Giraud, Jean-Claude Caissard, Christian Jeanthon, Bruno Corbara, and Anne-Catherine Lehours contributed to dataset analysis and evaluations, and advised on the project. Anne-Catherine Lehours supervised the project.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Video documenting the Pharaoh’s snakes of the Teasel (Lehours et al., 2023) is available in Figshare at <https://doi.org/10.6084/m9.figshare.21842163.v1>. Values of *nifH* gene numbers and of acetylene reduction assays (Lehours, 2023) are available in Figshare at <https://doi.org/10.6084/m9.figshare.21856494.v1>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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