

How to Visualize the Spider Mite Silk?

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ABSTRACT *Tetranychus urticae* (Acari: Tetranychidae) is a phytophagous mite that forms colonies of several thousand individuals. Like spiders, every individual produces abundant silk strands and is able to construct a common web for the entire colony. Despite the importance of this silk for the biology of this worldwide species, only one previous study suggested how to visualize it. To analyze the web structuration, we developed a simple technique to dye *T. urticae*'silk on both inert and living substrates. Fluorescent brightener 28 (FB) (Sigma F3543) diluted in different solvents at different concentrations regarding the substrate was used to observe single strands of silk. On glass lenses, a 0.5% dimethyl sulfoxide solution was used and on bean leaves, a 0.1% aqueous solution. A difference of silk deposit was observed depending the substrate: rectilinear threads on glass lenses and more sinuous ones on bean leaves. This visualizing technique will help to carry out future studies about the web architecture and silk used by *T. urticae*. It might also be useful for the study of other silk-spinning arthropods. *Microsc. Res. Tech.* 72:659–664, 2009. © 2009 Wiley-Liss, Inc.

INTRODUCTION

Tetranychus urticae is a ubiquitous phytophagous mite and is known as a major pest of many cultures; it can be found on various types of plants (more than 900 plants including commercial crops such as vegetables, cotton, or strawberries) (Van Impe, 1985). One of the particularities of *T. urticae* is its abundant silk production. This spider mite seems to deposit silk continuously while walking as reported by Hazan et al. (1971).

In silk-spinning arthropods, silk displays a diversity of material properties and chemical constituents (Craig, 1997). Silks are often described as fibrous biopolymer filaments or threads with diameters ranging from 50 µm to a few hundred nanometers. Silks can be derived from several glands (colleterial, salivary, dermal glands, Malpighian tubules) with different evolutionary origins (Craig, 1997). The whole podocephalic complex of *T. urticae* seems to be involved in silk production (Mills, 1973). Amino acids composition of spider silk (mainly Glycine, Alanine, and Serine) was previously described (Andersen, 1970; Bradfeild, 1951; Craig, 1997; Works, 1981) and shows a considerable variability between individuals even for the same individual along its lifetime (Work and Young, 1987).

The majority of studies dealing with silk have been made about spiders and lepidopterous insects (Bernard and Krafft, 2002; Cassem et al., 1999; Craig et al., 1999, 2000; Craig and Riekel, 2002; Eberhard, 1990; Fitzgerald, 2003; Johnson et al., 2006; Saffre et al., 1999).

Functions of silk strands in arthropods are numerous: protective shelter (e.g., Coleoptera), structural support (e.g., Neuroptera), reproduction (e.g., Thysanoptera), foraging (e.g., Trichoptera), or dispersal (e.g., Lepidoptera) (Craig, 1997). Moreover, some insects and spiders use their silks for life lines (e.g., Lepidoptera) or as a support for chemical communication among individuals (caterpillars, spiders) (Anderson and

Morse, 2001; Clark et al., 1999; Fitzgerald, 1993). In the case of *T. urticae*, silk was reported to play four main purposes: (1) protection against biotic agents like mites predators (Helle and Sabelis, 1985; Hoy and Smilanick, 1981) or competitors (Van Impe, 1985), (2) protection against abiotic agents, such as eggs protection against extreme humidity conditions (Hazan et al., 1975) or rain (Linke, 1953), wind (Davis, 1952), and acaricides (Davis, 1952; Linke, 1953), (3) sex pheromone substrate or carrier (Cone et al., 1971; Penman and Cone, 1974), which stimulates male searching behavior for quiescent deutonymphs (Penman and Cone, 1972), and (4) locomotion and dispersion (Saitô, 1977, 1979; Yano, 2008).

Previous works have allowed a better understanding of *T. urticae* silk roles (Cone et al., 1971; Davis, 1952; Hazan et al., 1975; Helle and Sabelis, 1985; Hoy and Smilanick, 1981; Linke, 1953; Penman and Cone, 1972, 1974; Saitô, 1977, 1979; Van Impe, 1985; Yano, 2008), but *T. urticae* silk itself remains poorly studied and only one work has been published on its chemical composition (Hazan et al., 1975). In spite of the silk value for *T. urticae* survival, web architecture is still unexplored. The first step to study *T. urticae* silk networks is to visualize the silk. The ability to visualize *T. urticae* silk using fluorescent stain could be a useful tool for

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exploring different domains like the link between mite movement and silk deposition, difference between individuals, variation in architecture, role in social organization, and influence of the substrate. A *T. urticae* silk visualizing method is thus developed in this study. After several attempts to reveal silk strands (powders, dew, colorants), the use of fluorescent products seemed the most suitable. Johnson et al. (2006) have already developed a method to reveal the silk produced by *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) neonates on a plant surface using a fluorescent dye, the Fluorescent Brightener 28 (FB). This water-soluble product is a fluorescent whitening agent that has been used in the early-1940s in textile and paper industries (Zollinger, 1991). It has a specific binding affinity to both cellulose and chitin and emits fluorescence under UV light, enabling microscopic detection (Perry and Miller, 1989). FBs chitin-binding specificity makes it a good laboratory stain to study cell walls of plants and fungi (Hoch et al., 2005) and to bind to arthropod silk in a simple staining reaction. Johnson et al. (2006) specified that their protocol could stain all types of arthropod silk including tetranychid mites, but they did not get optimal results for silk networks from infested plants and they did not try for silk on glass lenses. Therefore, we developed here new protocols by using the same fluorescent dye in order to optimize silk visualization on inert and living substrates, including web under natural *T. urticae* environment.

MATERIALS AND METHODS

Rearing of Mites

Mites were provided by the "Institut National Agronomique," Tunis, Dr. Lebdi Grissa Kaouthar (50°40'). The two spotted mites were reared on bean (*Phaseolus vulgaris*). Leaves were placed on damp cotton in Petri dishes (85 mm in diameter, 13 mm deep). Stocks breeding were maintained in climate room under 26°C, with a relative humidity of 50–70% and a photoperiod of L16:D8.

Silk Collection

Silk was collected on two different substrates: on glass lenses and on bean leaves.

(A) *On glass lenses*: one individual was deposited onto the lens (L1) (14 mm diameter, 0.13–0.16 mm thickness) during a period of 30 min. The mite was then removed from the cell with great care thanks to a brush with one hair.

(B) *On bean leaves*: one individual was deposited for 48 h on a bean leaf circle (B1) (14 mm diameter). Because it was more difficult to visualize the silk on leaf, a greater quantity of silk threads and therefore of time were required than on glass lens. After 2 days, the mite was removed of the bean leaf.

(C) *For the web located between two leaves*: samples were collected directly on 12-days infested beans (silk networks between two distinct leaves).

Staining of Silk

The dye used was the Calcofluor White M2R (Sigma-Aldrich NV/SA, Belgium) also known as Fluorescent Brightener 28. Two solvents were tested: water (Hoch et al., 2005; Johnson et al., 2006) and dimethyl sulfoxide (DMSO) (Millard et al., 1997). Two elutions were

tested by solvent: 0.1% (Hoch et al., 2005; Johnson et al., 2006) and 0.2% in alkaline-distilled water; 0.5% and 1% in DMSO. To facilitate the solubilization process in water, 39 μ L of 10 M KOH was added to 100-mL stock solution while stirring. The DMSO solution needed to be mixed until the complete dissolution of the FB powder. All four dyeing solutions were then filtered twice with 11- μ m cellulose filters.

The two substrates, glass lenses and bean leaves, required two different staining methodologies.

(A) *For the glass lenses*: *T. urticae* silk was sandwiched between two glass lenses. Five microliters of staining solution was deposited on a clean glass slide (L2) (76 mm \times 26 mm). The lens with the face where the mite laid its silk (L1) was deposited downward on the solution (5 μ L). The glass lens (L1) remained on the glass slide (L2) for 5 min. To speed up the drying processes, the set-up (L1 + L2) was then placed in an oven (100°C) during 5 min for the water solution and 90 min for the DMSO solution. The set-up was finally observed under fluorescent microscope.

(B) *For the leaves*: the leaf was dipped into water (0.1, 0.2%) and DMSO (0.5, 1%) solutions for 20 min (Johnson et al., 2006). As *T. urticae* silk is very fragile, it was impossible to remove the excess of dye without destroying the web. Therefore, the leaf was then dried out on a glass lens at 26°C and leaving it for 1 h for the water fluorescent brightener 28 and 24 h for DMSO solution.

(C) *For the web located between two leaves*: the web was carefully sandwiched between a glass lens and a glass slide with 5 μ L solution on it. Then, the lenses were placed in an oven (5 min for W and 90 min for DMSO). Table 1 summarizes the methods used to visualize *T. urticae* silk on glass lenses, bean leaves, and on infested bean plants.

Observation of the Silk

Silk images were captured using a Konica FT-1 camera placed on a Polyvar Reichert-Jung microscope. Photos were taken using a 10 \times ocular and a 10 \times magnification, with a size of 1.56 mm by 1.04 mm (Fig. 1), under a mercury vapor lamp OSRAM (330–380 nm wavelengths).

RESULTS

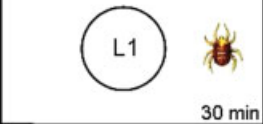

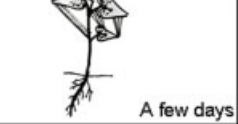


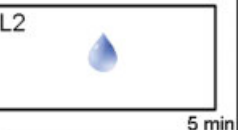
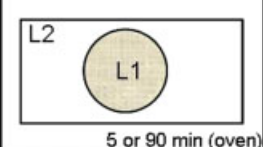
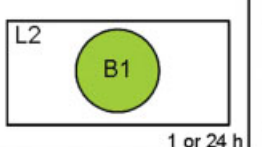
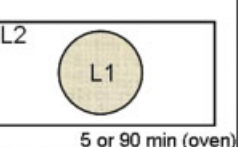
Methodology

For both glass lenses and bean leaves, solutions obtained with elutions of FB in water 0.2% and in DMSO 1% were too concentrated and produced crystalline deposit of dye. Fluorescent Brightener 28 allowed the visualization of the spider mite silk. Treatment of silk-covered surfaces with the fluorescent brightener solution strongly stained the silk, allowing threads to be viewed along their whole length under fluorescent illumination and a 100 \times magnification.

(A) *Silk visualization on the glass lenses*: the elutions of FB in water 0.1% and in DMSO 0.5% allowed correct observations of the silk, without crystalline deposit. This dyeing solution (DMSO, 0.5%) gave better results than the water one: every silk thread was stained uniformly and did not produce drying traces on glass lens.

(B) *Silk visualization on the bean leaves*: the DMSO dyeing solution damaged the structures of the leaf and thus could not be used in this case.

TABLE 1. Different steps needed to visualize *T. urticae* silk on glass lens, bean leaf, and on infested bean plant

Substrate/step	On glass lens	On bean leaf	On bean plant
1. Silk collection	 30 min	 48h	 A few days
2. Silk coloration	 5 min	 20 min	 5 min
3. Drying (W or DMSO)	 5 or 90 min (oven)	 1 or 24 h	 5 or 90 min (oven)

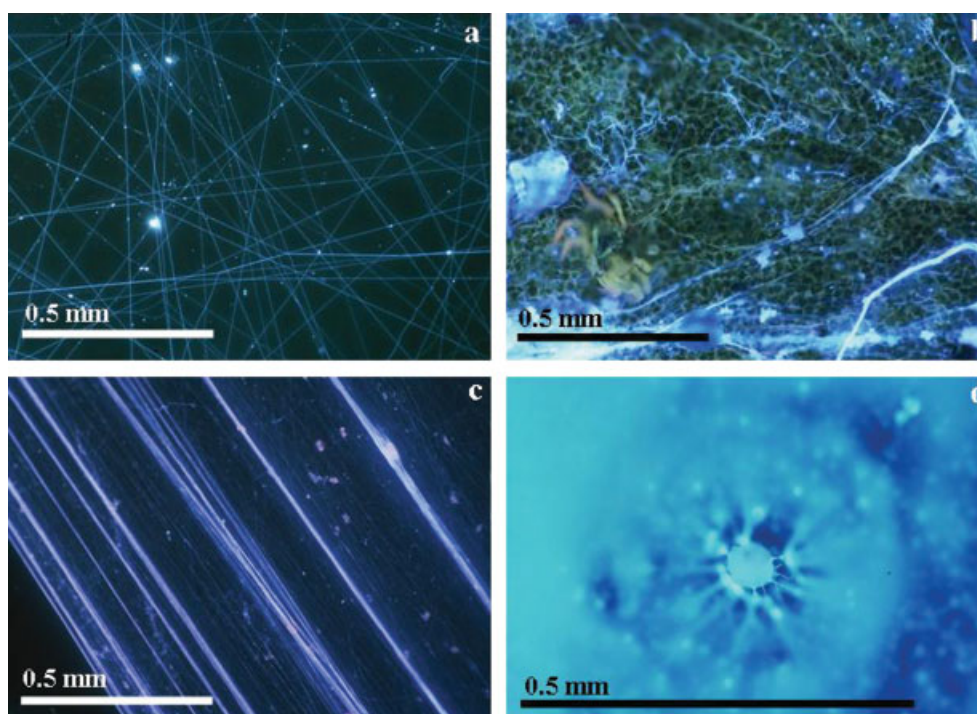


Fig. 1. All figures were taken thanks to a fluorescence microscope with a 10 \times magnification except for the figure d (25 \times). (a) Silk laid by spider mite on glass lens (FB-DMSO, 0.5%). (b) Silk laid by spider mites on bean leaf (FB-Water, 0.1%). (c) Silk removed between two

leaves of an infested bean (FB-DMSO, 0.5%). (d) Feces surrounded with silk threads. Silk came from an infested bean (FB-DMSO, 0.5%).

(C) For the web located between two leaves: both water and DMSO solutions colored silk networks. However, DMSO allowed a stronger and more homogeneous coloration of silk. Silk networks were more fluorescent what allowed a better detection of the web architecture.

Influence of the Substrate on the Spider Mite Silk Deposit

The comparison between the two substrates revealed great differences in silk deposit. The first action that the mite did on the glass lens was to explore the sub-

strate. The glass lens is a smooth surface and the silk threads were rectilinear (Fig. 1a). Thirty minutes were enough time for the mite to deposit a large quantity of silk on a glass lens. On the leaf, the majority of threads was not rectilinear but sinuous and deposited in clusters (Fig. 1b). Eggs and feces were covered with silk. Several observations were made from dyed silk removed from bean plant (Fig. 1c). First, the silk threads were organized; threads were deposited following a direction. Second, we noticed different thread sizes. Webs contained feces, dead individuals, and exuvies. Those elements were not simply deposited on the web but included inside the web structure and fixed with many threads as showed for the feces in the Figure 1d.

DISCUSSION

Thanks to this method, it will become possible to explore a new domain of research for *T. urticae*. Silk visualization will help to better understand silk strands organization and how silk strands are used by *T. urticae* population.

Fluorescent dyes have been useful in morphological and developmental studies of bacteria (Amann and Fuchs, 2008), fungi (Hoch et al., 2005), plant (Friker et al., 2006), and animals (Vignal et al., 2002). Fluorescent brightener 28 has been particularly useful and has been used as a fluorescent dye to view cell walls of many genera of fungi, bacteria, algae and higher plants (Albani, 2001; Apoga and Jansson, 2000; Butt et al., 1989; Hughes and McCully, 1975; Prigione and Marchisio, 2004; Pringle, 1991; Ruchel et al., 2000). Such brighteners have also been used in insect gut content analysis (Hugo et al., 2003; Schlein and Muller, 1995) and in visualizing silk produced by *Helicoverpa armigera* on plant surface (Johnson et al., 2006).

Silks are produced solely by arthropods and only by animals in the classes Insecta, Arachnida, and Myriapoda (Craig, 1997). They are fibrous proteins containing highly repetitive sequences of amino acids (Craig, 1997). In *T. urticae* silk, glycine, glutamic acid, serine, aspartic acid, and alanine are the more abundant amino acid present, comprising altogether about 60% of the total (Hazan et al., 1975). According to Hazan et al. (1975) spider mite silk most resembles prekeratin, which is a fibrous protein. Fluorescent Brightener 28 is known to stain tissue elements such as keratin, collagen, and elastin (Monheit et al., 1984). This fluorochrome binds also to cellulose, chitin, carboxylated polysaccharides, a variety of other β -linked polymers (Herth, 1890; Maeda and Ishida, 1967) and finally the spider mite silk in a simple staining reaction.

On the basis of the seminal work of Johnson et al. (2006), we tested two solvents water and DMSO. Our aim was to develop a method to visualize *T. urticae* silk on living substrate (bean leaves) as well as on inert one (glass lenses). The use of two different solvents gave different advantages and disadvantages. DMSO solution gave a more intense coloration and reduced traces presence but it damaged the leaf structures. Observations of webs on natural environments are feasible thanks to water solution. Therefore, the use of the DMSO solution on inert substrates and the use of water solution on living substrates such as leaf are recommended in future experiments. Also, DMSO, which

is a clear, colorless to yellowish solvent (Pope and Oliver, 1966) has a very low toxicity to humans and the environment (Vignes, 2000). Moreover, it is recyclable after most uses (Vignes, 2000). DMSO shows qualities that makes it a good solvent for our purpose.

The glass lens is a smooth surface where the mite attached silk threads. Those are stuck to the glass lens all along their lengths. Without irregularities, silks were straight and reflected mite's movements. On glass lens, the silk visualization was very easy, because FB was fixed only on the silk threads making them very conspicuous. Silk visualization in bean leaves was interfered with irregularities and coloration of some parts of leaves. Beside, on the leaf, the mite used any irregularity (leaf nerves, hairs, feces, eggs, exuvies, dead mites, and others organisms, debris, pupae) to deposit its silk. Therefore, threads were not rectilinear but mainly sinuous. The presence of irregularities on the leaf served probably to structure the web architecture. Mites are depositing silk continuously while walking as already reported Hazan et al. (1971). When *T. urticae* individuals have infested a plant, webs are quickly produced all around it. To produce efficient webs, *T. urticae* individuals have to "work" together. Each individual has to spend time and energy to produce silk threads made of proteins. This silk production may represent a large component of the organism's energy budget. Despite this silk production cost, *T. urticae* individuals have gained a distinctive selective advantage by evolving a life style based on webbing.

T. urticae spin three-dimensional webs that may seem chaotic in organization. However, silk networks visualization demonstrated that silk threads inside webs had a certain organization. The building process of *T. urticae* webs may be more organized than suspected. It will be interesting to observe how the silk threads are deposited over time and finally how the architecture is created as studied for *Steatoda triangulosa* (Araneae: Theridiidae) by Benjamin and Zschokke (2002, 2003). Saito (1983) characterized already the spider mite life with respect to the patterns of webbing on the leaf surface. According to his study (Saito, 1983), *T. urticae* belong to the CW (complicated web type) "life types" that means that *T. urticae* construct highly complicated and irregular web on the leaf surface. A more detailed study of the structure and the density of web could be possible with the technique described in our work. Some comparative studies of web construction behavior could be done. Finally, it will be possible to explore how *T. urticae* alters its web construction behaviors in response to their environment like already shown for some orb-weaving spiders (Heiling and Herberstein, 1999; Higgins, 1990).

In conclusion, the technique reported in this study represents a rapid and efficient method to visualize *T. urticae* silk. The method which differs according to substrates is easy to carry out and needs only a minimum amount of material. The mites used their environment to deposit their silk strands. This fluorescent method will help to improve current knowledge of *T. urticae*: threads deposit and difference between age and sex, spider mite web architecture and difference in organization, influence of the substrate, or crowding and social organization. This technique will allow a better understanding of *T. urticae* at the individual

and collective levels. Silk removed between leaves on infested beans gave many open doors on future studies. Studies are ongoing in our lab to understand the silk threads organization within the web and the influence of the environment in the silk architecture. This simple dying technique might be an interesting tool to further studies of silk related to its different purposes and in other species of arthropods like other spider mites producing silk or spiders.

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