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SHORT NOTE

Uvitex2B: a rapid and efficient stain for detection of arbuscular mycorrhizal fungi within plant roots

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Abstract The study of arbuscular mycorrhiza often requires the staining of fungal structures using specific dyes. Fluorescent dyes such as acid fuchsin and wheat germ agglutinin conjugates give excellent results, but these compounds are either hazardous or very expensive. Here, we show that a safer and inexpensive dye, Uvitex2B, can be efficiently used to stain intraradical fungal structures formed by the arbuscular mycorrhizal fungus *Glomus intraradices* in three plant species: carrot, *Casuarina equisetifolia*, and *Medicago truncatula*. The intensity and stability of Uvitex2B allow the acquisition of high-quality images using not only confocal laser scanning microscopy but also epifluorescence microscopy coupled with image deconvolution. Furthermore, we demonstrate that Uvitex2B and β-glucuronidase staining are compatible and can thus be used to reveal arbuscular mycorrhizal structures in the context of promoter activation analysis.

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Introduction

Arbuscular mycorrhiza (AM) is a symbiotic association involving the vast majority of land plants and fungi from the phylum Glomeromycota. The development of the symbiosis leads to the colonization of the root cortex and, most of the time, to the formation of branched structures, the arbuscules, within the cortical cells. This extensive branching allows the development of a huge exchange surface between the plant and the fungal partners, allowing the transfer of minerals and water to the plant partner in exchange of sugars (Smith and Read 2008). Because the number, longevity, and morphology of arbuscules are linked to the functionality of the symbiosis (Smith and Read 2008), the evaluation of these parameters is an essential tool in AM research. Although AM can be detected in intact unstained roots in certain cases, the vast majority of studies performed on AM are based on the observation and quantification of stained material (Vierheilig et al. 2005). A variety of staining techniques are used to detect and quantify arbuscular mycorrhizal fungi (AMF) in roots (Vierheilig et al. 2005), the most widespread being trypan blue (Phillips and Hayman 1970), based on root clearing and subsequent staining with a trypan blue solution. A similar method using chlorazol black E is also often used to stain intraradical structures and allows the visualization of arbuscules with great detail (Brundrett et al. 1984). However, trypan blue and chlorazol black E are hazardous; both stains have been listed as carcinogens (IARC 1987; IARC 1999). A cheaper and nontoxic staining method giving excellent results is the use of ink/vinegar staining developed by Vierheilig et al. (1998). More recently, the observation of mycorrhizal structures was improved by the use of fluorescent dyes in combination with confocal laser scanning microscopy (CLSM), allowing the visualization of mycorrhizal structures at higher resolution without mechanical sectioning (Czymmek et al. 1994). With the exception of *Gigaspora gigantea*, which is strongly autofluorescent (Genre et al. 2008), a staining procedure is required to visualize most AM fungi. A commonly used technique is based on acid fuchsin, a red fluorescent dye, which binds to fungal structures. However, acid fuchsin also binds to root tissues, the staining procedure is time consuming, and in addition, it is a hazardous dye (Combes and Haveland-Smith 1982). A more specific method to detect AMF is based on wheat germ agglutinin (WGA), conjugated with a fluorophore. WGA is a lectin that specifically binds to chitin molecules present in the fungal cell wall (Vierheilig et al. 2005). Because it yields high-contrast images of intraradical AM structures, this technique is particularly suited for the analysis of plant mutants that show alterations in AM development (Javot et al. 2007). However, WGA conjugates are relatively expensive and unstable dyes.

Here, we report the development of a cheaper and less toxic technique to visualize AM structures inside roots, based on Uvitex2B. Uvitex2B is a fluorescent brightener similar to calcofluor white; it is extensively used in the paper and textile industries to whiten and prevent "yellowing" of papers and fabrics. Uvitex2B binds to β-1-4-polysaccharides as those found in chitin and cellulose and produces an intense white–blue fluorescence when excited with UV light. These properties allow the rapid detection of a wide variety parasitic fungi, making Uvitex2B staining a widely used technique in human clinical mycology and parasitology (Wachsmuth 1988). Uvitex2B has also been used with plants, mostly to detect pathogenic fungi (Fu et al. 2009; Moldenhauer et al. 2006) and, more recently, to study the colonization of poplar roots by the ectomycorrhizal fungus *Laccaria bicolor* (Felten et al. 2009). In all these studies, high-quality images were obtained in combination with CLSM, allowing the observation of fungal structures with great detail. Uvitex has already been used to stain extraradical AM fungi such as *Glomus coronatum* (Giovannetti et al. 1991), *Glomus mosseae* (Giovannetti et al. 1993), and *Glomus viscosum* (Walker et al. 1995), but the possibility to use this staining technique to visualize fungal structures inside the root is not mentioned. Here, we tested Uvitex2B on three plant species colonized by *Glomus intraradices*: two species frequently used on AM studies–the model legume *Medicago truncatula* and carrot (*Daucus carota*)–and, in addition, an actinorhizal tree on which we are currently working, *Casuarina equisetifolia*. We show that the Uvitex2B staining can be used in combination with CLSM and that good-quality images can also be obtained with epifluorescence microscopy if a deconvolution algorithm is applied. Using transgenic *M. truncatula* plants expressing a β-glucuronidase (*gus*) gene in mycorrhiza, we also demonstrate that Uvitex2B and GUS staining are compatible.

Materials and methods

Plant material and AM inoculation

The starting cultures of *D. carota*/*G. intraradices* DAOM 197198 were provided by G. Bécard (Laboratory of Cell Surfaces and Plant Signalling, UMR CNRS-Paul Sabatier University, Toulouse, France). Carrot roots inoculated with *Glomus clarum* DAOM 234281 were kindly provided by Y.

Table 1 Assessment of optimal staining time of *C. equisetifolia*, *M. truncatula*, and carrot roots with Uvitex2B

Uvitex2B concentration (%)	Staining time (min)	AM staining	Background
0.005	5	–	–
	15	+	–
	30[a]	++	–
	60	++	+
	120	++	+++
0.01	5	+	+
	15	++	+
	30	++	+
	60	++	+
	120	++	+++
0.05	5	++	++
	15	++	++
	30	++	++
	60	++	+++
	120	++	+++

[a] indicates the best combination

Prin and Caroline Bournaud (Laboratoire des Symbioses Tropicales et Méditerranéennes, Baillarguet, France). Seeds of *C. equisetifolia* were collected in Notto Gouye Diama (Senegal). The *M. truncatula* transgenic line L416, containing the 2.3-kb 5' upstream region of the *MtEnod11* gene, fused to the β-glucuronidase (=*ProMtEnod11*::*gus*), described in Charron et al. (2004), was provided by D. Barker (Laboratory of Plant–Microbe Interactions, UMR INRA/CNRS (441/2594), Castanet Tolosan, France). *M. truncatula* and *C. equisetifolia* plantlets were grown from seeds and inoculated with *G. intraradices*, as described (Gherbi et al. 2008); roots were harvested at least 5 weeks after inoculation. Plants were grown in a culture chamber at 25°C, with an 18/6-h photoperiod. Carrot roots were grown in two-compartment plates and kept in the dark at 25°C, as described (Douds 2002). Roots were harvested from 3-month-old plates.

Staining procedures

Roots were gently washed with water and cleared with 10% KOH (*w*/*v*) at 90°C for 2 h (carrot and *M. truncatula*) or 12 h (*C. equisetifolia*). Uvitex2B was purchased from Polysciences Europe (Eppelheim, Germany). Staining with Uvitex2B was carried out at 90°C for 5 to 120 min, with aqueous solutions of Uvitex2B ranging from 0.005% to 0.05% (*w*/*v*). Roots were rinsed with water and incubated for 12 h in water to eliminate the excess Uvitex2B. Staining with trypan blue (Sigma–Aldrich, St. Louis, MO, USA) was performed in parallel, as described (Gherbi et al. 2008). Histochemical GUS assays using X-gluc (5-bromo-4-chloro-3-indoxyl-b-D-glucuronic acid cyclohexylammonium salt; Duchefa Biochemie BV, The Netherlands) were performed for 24 h at 37°C on *M. truncatula* roots, as described (Svistoonoff et al. 2004). Roots were fixed in an ethanol–acetic acid solution (1:1) for 12 h, then cleared and stained with Uvitex2B, as described above.

Microscopy

Roots were mounted in Hoyer's solution (chloral hydrate/gum arabic/glycerol/water [100:7.5:5:30]). For the establishment of the staining procedure, samples were observed using a DMRB microscope (Leica) using an A filter (excitation, 340–380 nm; stop, 425 nm; Leica). Images of roots stained for GUS activity were acquired with an MP5 cooled camera (Qimaging, Surrey, BC, Canada) and QcapturePro software (Qimaging). Confocal imaging was performed using a 510 META confocal microscope (Zeiss) and LSM 510 software with a Plan Apochromat ×63/1.4 oil objective. Two independent acquisitions were performed, one at 405/420–480 (excitation/emission) for Uvitex2B and one at 488/530 LP for autofluorecence, with pinhole sizes of 102 and 100 µm, respectively. High-quality epifluorescence imaging was carried out on a DM6000B microscope (Leica) equipped with a C4742-80-12AG ORCA–ER camera (Hamamatsu) with a ×63 oil objective using Volocity 5 (Perkin–Elmer, Waltham, MA) software. Acquisitions were performed with an A4 filter (Leica) for DAPI (excitation BP, 360/40 nm/stop, 400 nm/emission BP, 470/40 nm) or I3 filter (Leica) for FITC (excitation BP, 470/40 nm/stop, 510 nm/emission LP, 515 nm). Image deconvolution was carried out with Volocity 5 software. An iterative restoration was performed (confidence limit, 99%; iteration limit, 25), using a calculated point spread function. Images were assembled using Photoshop 7.0 (Adobe Systems, Mountain View, CA).

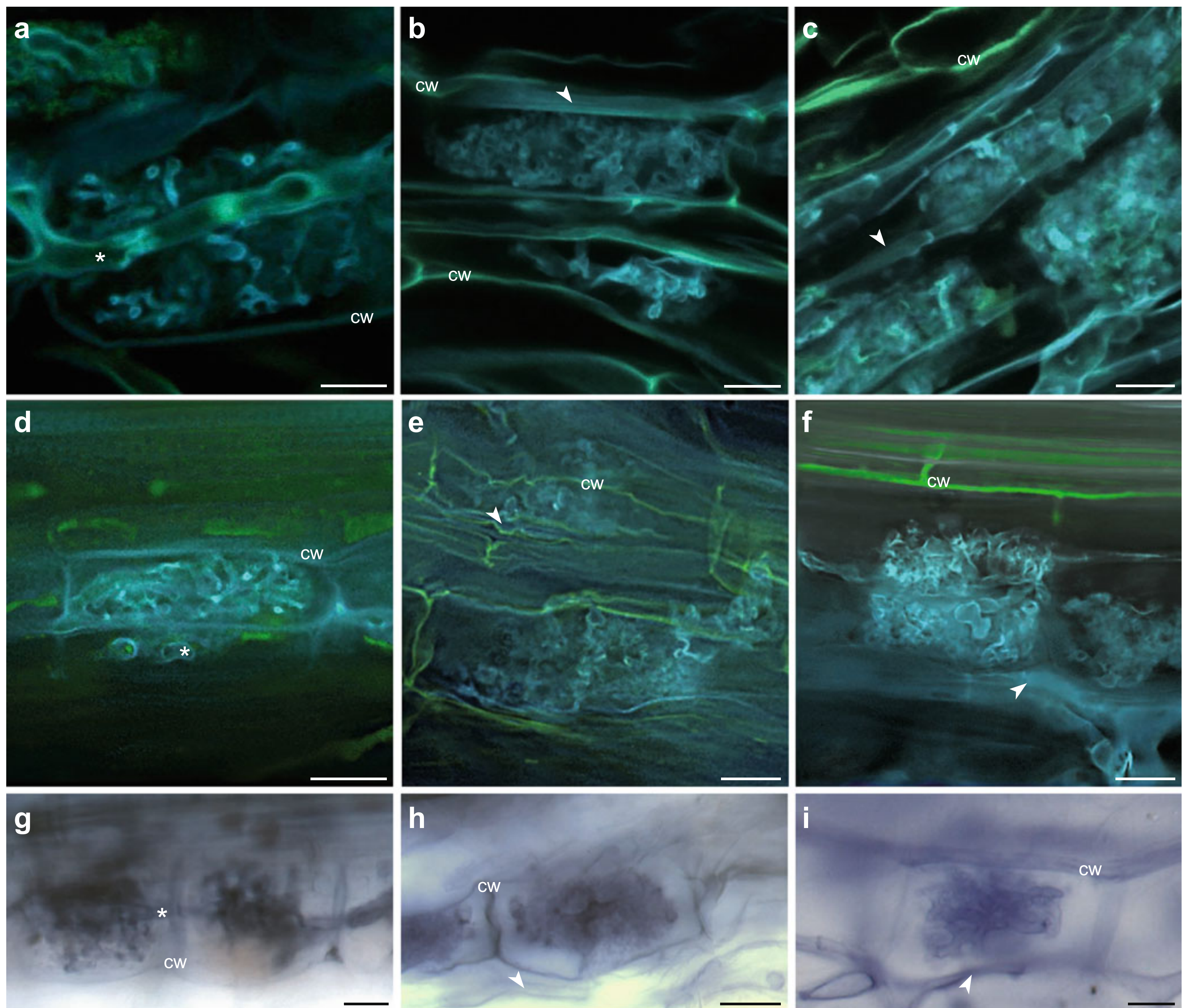

Fig. 1 Carrot, *C. equisetifolia*, and *M. truncatula* roots (*first*, *second*, and *third columns*, respectively) colonized by *G. intraradices*. **a**–**f** Images of roots stained with Uvitex2B, acquired using CLSM (**a**–**c**) or epifluorescence microscopy (**d**–**f**). Uvitex2B fluorescence acquired with the DAPI filter is shown in *blue*, and cell wall autofluorescence acquired with the FITC filter is shown in *green*. Epifluorescence images were reconstructed from z series using a deconvolution algorithm. **g**–**i** Images of roots stained with trypan blue acquired with brightfield microscopy. *cw* cell wall, *asterisk* intracellular hyphae, *arrows* intercellular hyphae. *Bar*=10 µm

Results

Optimization of AM staining using Uvitex2B

In order to evaluate the concentration and incubation time needed to obtain the best staining with Uvitex2B, cleared samples of carrot, *C. equisetifolia*, and *M. truncatula* were incubated for 5–120 min with different concentrations of Uvitex2B (Table 1). Although AM structures could be stained at all concentrations if samples were left for more than 15 min, increased concentrations or longer incubation times resulted in higher levels of background. The best compromise was obtained with 0.005% Uvitex2B for 30 min (arrow).

Imaging AM structures with CLSM

Carrot, *C. equisetifolia*, and *M. truncatula* roots inoculated with *G. intraradices* were stained with Uvitex2B, and CLSM images were acquired using a 405 diode to excite Uvitex2B (Fig. 1a–c). As shown in Fig. 1a–c, the combination of Uvitex2B staining with CLSM allowed the visualization of fungal structures with great detail and higher resolution, compared to the classic trypan blue staining (Fig. 1g–i). To visualize plant structures, a second image was acquired sequentially with a 488-nm argon laser. However, only in *C. equisetifolia* were the levels of autofluorescence high enough to allow a clear distinction

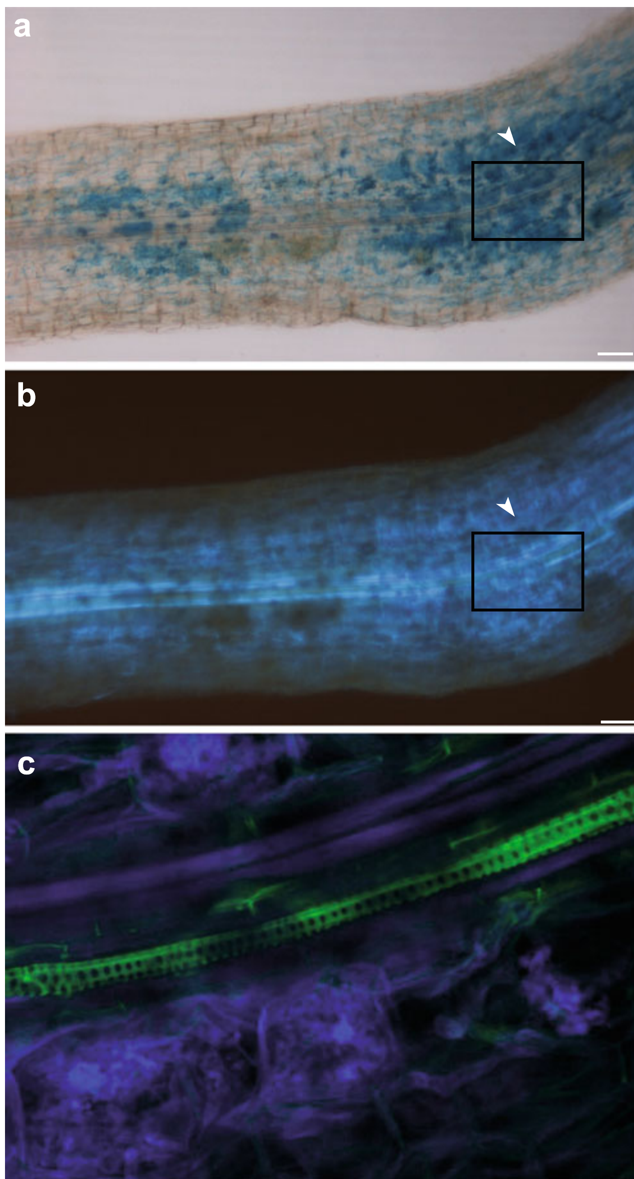

Fig. 2 Histochemical localization of β-glucuronidase activity in roots of *M. truncatula* transformed with a *MtEnod11::gus* fusion stained with Uvitex2B. **a** GUS activity observed with brightfield microscopy. **b** The same root observed with epifluorescence microscopy (DAPI filter). Fungal structures can be observed within the root in cortical cells. Autofluorescence is also detected in vascular tissues. A zone with strong GUS activity is indicated (*arrow*). **c** Image acquired using CLSM, showing a zone with high levels of GUS activity. **a**, **b** *bar*= 100 µm, **c** *bar*=10 µm

of the plant cell walls (Fig 1a–c). In all three species, a low level of blue autofluorescence was detected in plant cell walls. Similar results were obtained with carrot roots inoculated with *G. clarum* (data not shown).

In addition to high resolution, CLSM offers the possibility to explore fluorescent objects in three dimensions. This feature is particularly useful to analyze the colonization of the root cortex by AMF: depending on the plant and fungal species, the colonization is mainly intercellular, and only arbuscules develop inside the cells (=Arum-type), or intracellular, with almost no intercellular filaments and coiled hyphae or small arbuscules arising from the intracellular hyphae (=Paris-type). In the carrot inoculated with *G. intraradices*, the sequential observation of the z series (Fig. S1a) allows the distinction of an intracellular pattern of colonization and the development of small arbuscules from the central intracellular hyphae, the features of a Paris-type mycorrhiza (Smith and Read 2008). In contrast, for *C. equisetifolia*, intercellular hyphae are very common; arbuscules fill the whole cell and are connected to the extracellular hyphae at their bases; those are typical features of an Arum-type mycorrhiza (Fig. S1b; Smith and Read 2008).

High-quality images can be obtained with epifluorescence

In order to evaluate an alternative method for obtaining good-quality images, z series of images were acquired with a motorized epifluorescence microscope and processed with a deconvolution algorithm. Deconvolution is a mathematical operation used in image restoration to recover an object from an image that is degraded by blurring and noise, using a point spread function (PSF). The PSF is a model of how one point is imaged by the microscope and is obtained through a theoretical calculation based on optical parameters such as the medium refraction index, the numerical aperture, and the wavelength of the emitted light. As shown in Fig. 1d–f, this technique yielded very good results, allowing the visualization of arbuscules with great detail, compared with nondeconvoluted images (Fig. S2).

Uvitex2B staining is compatible with GUS staining

A widely used technique to study gene expression patterns is based on the analysis of plants containing a promoter::*gus* fusion. To evaluate whether Uvitex2B staining is compatible with GUS staining, we used transgenic *M. truncatula* plants containing a *ProMtEnod11::gus* fusion. MtEnod11 is a cell wall repetitive proline-rich protein from *M. truncatula* widely used as a marker of symbiotic interaction since it is highly expressed in the context of the symbiotic interaction with *Sinorhizobium meliloti* but also with AMF, during all stages of AM colonization (Chabaud et al. 2002). Roots colonized with *G. intraradices* were first stained with X-gluc to reveal GUS activity and, subsequently, with Uvitex2B. As shown in Fig. 2, GUS activity and intraradical fungal structures can be detected in the same root. The accumulation of X-gluc crystals is particularly high in the zone of the root where arbuscules are present. First staining with X-gluc does not seem to reduce the fluorescence of the following staining with Uvitex2B.

Discussion

Uvitex has already been used to stain several species of AMF, in particular, to visualize anastomosis of fungal filaments (*G. mosseae*; Giovannetti et al. 1993) and the cell wall of fungal spores in *G. viscosum* (Walker et al. 1995) and *G. coronatum* (Giovannetti et al. 1991). Here, we show that it can also be used to stain intraradical structures.

The main disadvantage of the Uvitex2B staining is the need to clear the root tissues to visualize intraradical AMF structures. Here, the clearing process was performed with KOH at 90°C, and incubation times ranging from 2 to 12 h were needed in order to obtain satisfactory results. As a consequence, the Uvitex2B technique can only be used to visualize fixed dead material. When long incubation times are required, another consequence is a deformation of root cells such as the one obtained for *C. equisetifolia* samples. Like for other AM-staining techniques, the best results are obtained when fine, relatively transparent roots are analyzed. Despite the above inconvenience, Uvitex2B offers a series of advantages compared to other staining methods:

1. It is a fluorophore, and can thus be used in combination with CLSM to obtain high-resolution images of internal structures, and its blue fluorescence offers an increased theoretical resolution compared to red fluorophores. In addition, it can be excited with the 405 diode, available in most modern CLSMs. Compared to WGA-based methods, Uvitex2B is less expensive but has the disadvantage of being less specific because Uvitex2B also binds to the plant cell wall. However, in roots of woody plants such as *C. equisetifolia*, it is usually very difficult to distinguish between the fluorescence of a specific dye such as WGA-488 and the strong autofluorescence of plant cell walls (N. Diagne, unpublished). The Uvitex2B method may therefore be particularly well suited for the observation of AM in species showing high levels of autofluorescence.
2. Uvitex2B fluorescence is very intense and fades less than other fluorochromes of the same family (Wachsmuth 1988). Very weak excitation is therefore needed, allowing the acquisition of multiple images of the same object and the construction of z series. A deconvolution algorithm is then sufficient to reduce noise considerably and obtain images of high resolution, comparable with CLSM.
3. Provided the samples are protected from light, Uvitex2B staining is stable; in our hands, it has not faded in over 12 months of storage at 25°C.
4. No carcinogenic effects or reproductive toxicity has been shown to date, according to the manufacturer's material safety data sheet. Diluted Uvitex2B solutions employed to stain AMF are therefore relatively safe, in comparison with other dyes such as acid fuschin or trypan blue, two possible carcinogens (Combes and Haveland-Smith 1982; IARC, 1987).
5. Uvitex2B is compatible with GUS staining; it can be used instead of WGA conjugates when high-quality images are required in combination with gene expression studies.

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