

# Colonization of Rice Roots by a Green Fluorescent Protein-Tagged Isolate of *Ustilagoidea virens*

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Received 7 August 2015; accepted 7 September 2015; published 10 September 2015

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## Abstract

The fungus *U. virens* is the causal agent of rice false smut disease. The green fluorescent protein (GFP) was used to mark this fungus in order to visualize and analyze the colonization and infection processes *in vivo*. Using epifluorescence microscopy colonization and infection on rice roots were visualized *in vivo*. After inoculation for 2 to 15 d, it was observed that the conidia and their germ-tubes had penetrated into epidermis of young roots. The hyphae were found inside the root xylem 18 d after inoculation. Generally, the transformed fungus colonized the rhizosphere, the cortex as well as the vascular tissues with symptoms of root necrosis observed. The results of this work show that *U. virens* colonize not only rice panicles but also the roots.

## Keywords

Endophytes, Plant-Fungal Interactions, Rhizosphere, Root Colonization, *Ustilagoidea virens*

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## 1. Introduction

Rice (*Oryza sativa* L.) is an important cereal crop and staple food for a large number of populations all over the world. Its demand is increasing day by day with the increase in human population. Smut disease which is caused by the ascomycete fungus *Ustilagoidea virens* (Cooke) Takah (Anamorph) is one of the potential threats to rice cultivation and causes severe yield losses of rice around the world [1]. The yield loss estimates ranged from

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2.8% to 81% in different rice-producing areas depending on the rice variety and disease intensity [2]-[4]. In addition to yield loss, *U. virens* also contaminates rice seed and straw by producing antimetabolic cyclic peptides that are poisonous to both humans and animals [5] [6]. It was reported that *U. virens* which was able to reproduce both sexually and asexually with multiple propagules could be able to invade rice coleoptiles and roots at the young seedling stage and the stamen filaments at the earlier booting stage [7]-[9]. The disease cycle of *Ustilagoideae virens*, which begins with the infection of plant tissues and culminates in new sporulation, has not been fully characterized [10]. In false smut, spore balls replace one or more kernels on mature heads as a result of infection of the plant. From one to more than 20 smut balls can be found on a single infected rice panicle. Spore balls can survive up to four months in soil and it is presumed that the sclerotia can survive much longer [10]. In addition, spores (sometimes called chlamydospores) found in the spore balls can produce one or more secondary spores after germination which are capable of infection.

A certain pathogen can be considered as either a “foliar/root pathogen” basically if it has the ability to colonize these tissues and also based on its ability to incite symptoms on leaves or roots rather than where infection occurs [11]. Characterizing the diverse interactions between the fungus and plant roots could help define the differences between pathogenic and nonpathogenic strains. Observing fungi in soil was very difficult and most of the studies describing the colonization of root surfaces by a fungus were carried out using hydroponic systems [12]-[14] or with a substrate such as sand or vermiculite [15]. Like most other pathogenic fungi, *U. virens* is able to survive in the soil and is extremely difficult to control. More detailed knowledge on the *in vivo* interactions between the pathogenic fungus and the plant could lead to the discovery of more efficient ways to control the disease. Details of these interactions can be essential in studies of the biological control of the fungus by beneficial antagonistic microorganisms that colonize the rice rhizosphere. The conventional methodologies used to study the interactions between pathogens and their host plants are not able to realize real-time and a live view of the fungal behaviors and the host reactions. The discovery of the fluorescent protein marker system offers a direct and non-destructive way to analyze the systemic infection of fungi (especially for those soil-borne pathogens that are difficult to be observed) in their hosts in a dynamic mode [16]-[18].

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* [19] [20] is a widely used reporter protein to facilitate the observation of hyphae on the root surface [21]. It was used to label *U. virens* in order to visualize by confocal laser scanning microscopy (CLSM) and epifluorescence microscopy the mode and path of infection in rice floral organs [22] [23]. GFP's fluorescence is stable and species independent and does not require any cofactors or substrates. In addition, visualization of GFP labeled organisms with epifluorescence microscopy and CLSM is an effective, fast, and noninvasive tool that allows the spatiotemporal analysis of interactions while preserving the integrity of the organisms under study or without destruction of the target tissues.

In a previous study, we generated *U. virens* strains expressing green fluorescence by introducing the GFP gene via *Agrobacterium tumefaciens*-mediated transformation (ATMT) [23]. Using a GFP-labeled fungal strain, this study was initiated to investigate the systemic infection and colonization of this fungus on/in rice roots.

## 2. Materials and Methods

### 2.1. Fungal Strains and Inoculum Preparation

The pathogenic strain *Ustilagoideae virens* isolated from a diseased rice plant was transformed with the GFP gene as described previously by [23]. The transformed fungus strain was cultivated on a potato sucrose agar (PSA) at 28°C on a rotary shaker at 180 rpm. After 7 days of growth, fungal cultures were filtered through a sterile number 2 sintered-glass funnel (40- to 100- $\mu$ m-pore-size mesh) to retain the mycelia. The microconidia remaining in the filtrate were harvested by centrifugation (7500  $\times$  g; 5 min at 24°C) and washed three times in sterile distilled water. The concentration of the conidial suspension was estimated under the microscope with a hemocytometer and adjusted as necessary with sterile distilled water (10<sup>6</sup> microconidia ml<sup>-1</sup>).

### 2.2. Plant Inoculation and Cultivation

The rice cultivar Huangxiuzan, “HXZ” which is susceptible to false smut was grown in the experimental station of Plant Protection Institute, Guangdong Academy of Agricultural Science, Guangdong Province, China and used in this experiment. Before plant inoculation, the conidial viability of the fungus was checked according to the suggestion of [24]. Seeds were surface sterilized in 75% ethanol for 1 min and 1% sodium hypochlorite

(NaClO) for 5 min, and thoroughly rinsed in distilled water five times. Seeds were germinated on sterile Whatman filter paper in petri dishes and incubated in the dark at 28°C for 6 days. When the third seedling leaves were unfolded, roots were dipped in a microconidial suspension for 1 and 1/2 h. The density of the microconidial suspension was determined with a hemocytometer and adjusted by dilution to the desired concentration for root inoculation ( $10^6$  microconidia  $\text{ml}^{-1}$ ). Seedlings were aseptically transferred into tubes containing a sterile nutrient solution. The nutrient solution contained 0.09 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.05 mM  $\text{KH}_2\text{PO}_4$ , 0.05 mM  $\text{KNO}_3$ , 0.03 mM  $\text{K}_2\text{SO}_4$ , 0.06 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.07 mM  $\text{MgSO}_4$ , 0.11 mM Fe-EDTA, 4.6  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 1.8  $\mu\text{M}$   $\text{MnSO}_4$ , 0.3  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.3  $\mu\text{M}$   $\text{CuSO}_4$  (pH = 5.5 - 6.0). The plants were cultivated in a growth chamber at 27°C during the day and 24°C at night. Control plants were dipped in sterile distilled water and then cultivated, as were the inoculated plants. Radicles were sampled after 24 hpi (hours post-inoculation) and a close observation of the different parts of the roots continued for six weeks. For this fungal strain which was under study, 16 inoculated plants and 8 control plants were processed for microscopic observations of root sections.

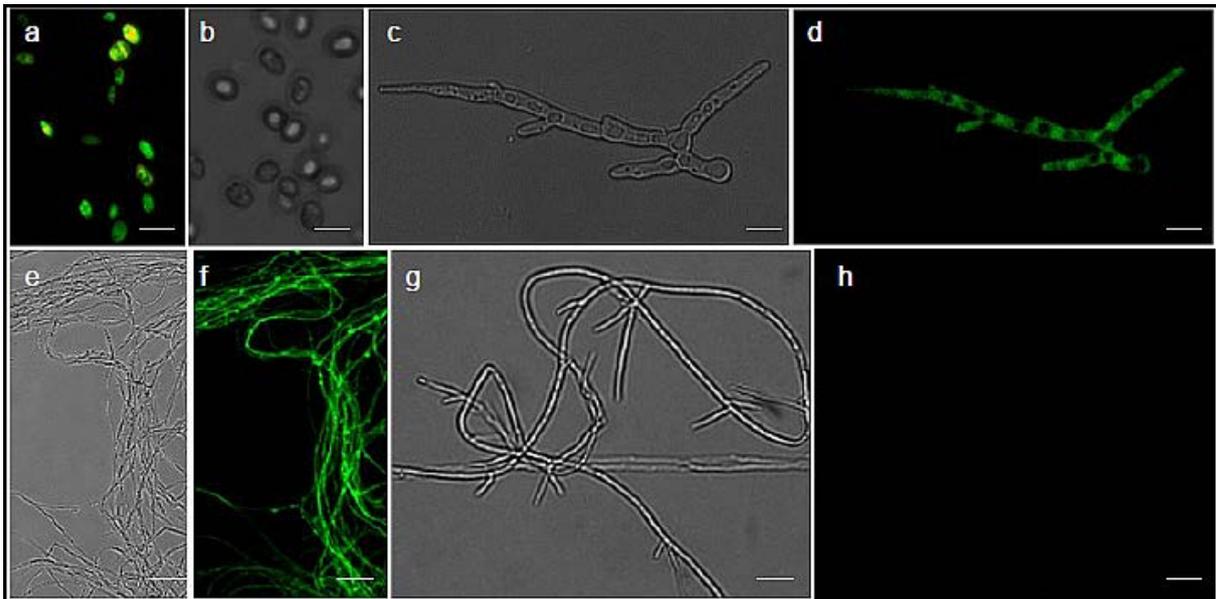
### 2.3. Microscopic Observations

Systemic colonization by *U. virens* in rice root tissues was determined at 1, 2, 3, 4, 5, 10, 15, 18, 21, 24, 27 days and 4 weeks post-inoculation (dpi). Cross sections and longitudinal sections of tap and lateral roots were hand-sectioned and each placed directly on a glass slide in a water drop with a cover slip. Observation of the green fluorescence was conducted using an epifluorescence microscopy (LeicaDM6000 B) with excitation at 455 to 490 nm and emission at 515 to 560 nm.

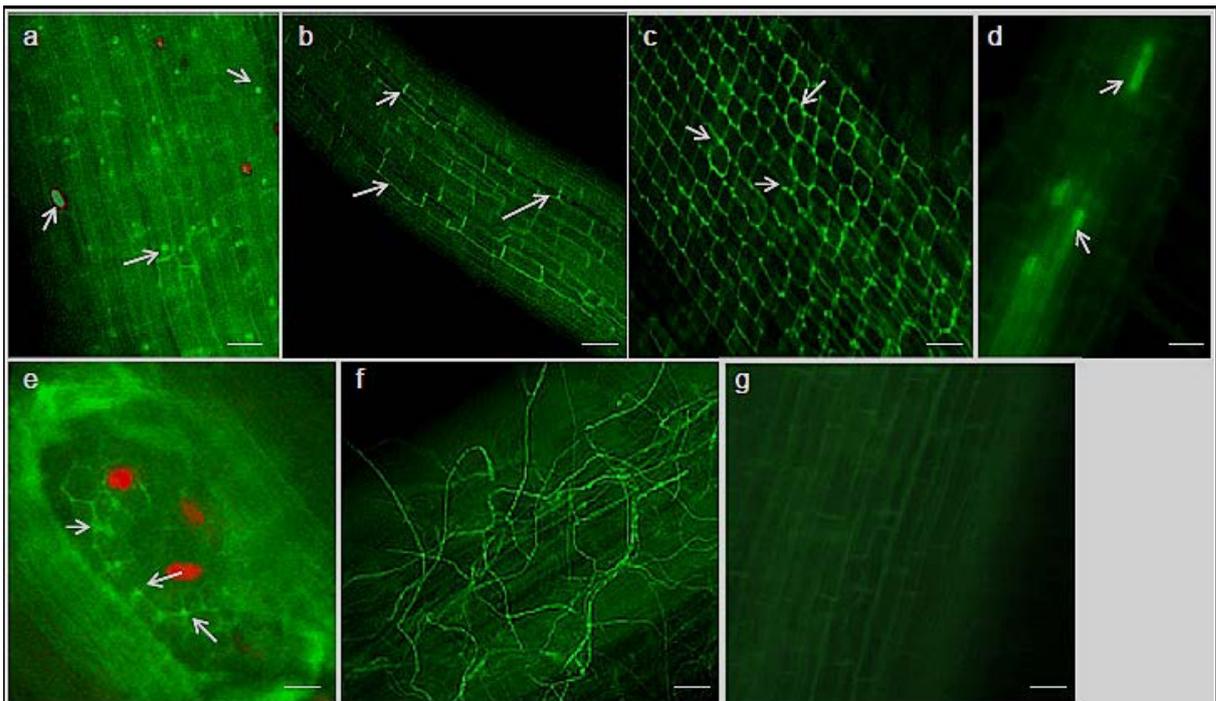
## 3. Results and Discussion

Plants are involved in mutualistic or parasitic interactions with a variety of micro-organisms, which has a strong impact on ecosystems, agriculture and forestry [25]-[28]. Generally, GFP-transformed pathogens can be easily visualized in living tissues without any further processing or manipulation of the samples and have a paramount importance in order to study the interaction of plants with different pathogenic organisms. In this study, a GFP-marked strain of *U. virens* was used to follow colonization of root tissues in HXZ, a susceptible rice variety. The use of a transformed isolate of *U. virens*, which is capable of expressing a GFP construct in conjunction with the appropriate illumination and optical filters allowed for easy discrimination of the fungus in planta, while requiring only minimal preparation of plant tissues. This property makes GFP-expressing strains extremely useful for analysis of in planta fungal development [29]. Fungal strains expressing fluorescent proteins have been used mainly to characterize their interactions with a plant [30]. Clone 40 was selected for the uniformity and strength of the resulting fluorescent signal in all fungal structures (Figure 1). Similar to other pathogenic fungi which were transformed with GFP [11] [31] [32], this clone was re-isolated from inoculated rice plants with no observable loss of GFP fluorescence or hygromycin B resistance, which confirmed the stability and robustness of the transformant.

Infection assay followed by a time course of microscopic observations was performed in order to determine whether *U. virens* could be able to infect and colonize itself on rice roots or not. The infection assays were performed with the HXZ-susceptible rice cultivar. Twenty-four hours after inoculation via the root-dipping method, roots were covered with conidia, of which only a small fraction exhibited signs of germination (Figure 2(a)). By 48 h, at least half of the spores on root surfaces had germinated (Figure 2(b)). Germinating hyphae continued elongating parallel with the longitudinal axis of the root along the junctions of epidermal cells, which were evident by 72 h post-inoculation (Figure 2(c)). At this point, lateral, swellings could be seen at the junction between plant cells and on the root surface (Figure 2(c) and Figure 2(d)). More complex hyphal networks that form a net-like structure along the root epidermis grow within the grooves between epidermal cells after 5 dpi (Figure 2(d)). Conidia germinated to form hyphae instead of forming an appressoria like structure on the root surface even though spore suspensions were used as inoculum. A lot of hyphae colonized on and around the small lesions formed on rice roots, when the root tissues with lesions were observed under fluorescence microscope (Figure 2(e)). Generally, the hyphae always covered the regions beyond the lesions. Although the colonization of hyphae was observed on the large and greenish lesions, the extension of hyphae was limited inside the lesions (Figure 2(e)). Some lesions on rice roots were developed from the sites on the main roots where the root hairs were located (data not shown).



**Figure 1.** The GFP-tagged *Ustilaginoidea virens* strain. (a) Spore of the GFP-tagged fungus under epifluorescence microscopy; (b) Spore of the GFP-tagged fungus under bright field microscopy; (c) Germinating spore of the transformed fungus under bright field; (d) Germinating spore of the GFP-tagged fungus under epifluorescence microscopy; (e) Hypha of the transformed fungus under bright field microscopy; (f) Hypha of the GFP-tagged under epifluorescence microscopy; (g) Hypha of the wild fungus under bright field microscopy (h) Hypha of the wild fungus under epifluorescence microscopy. Each bar represents 100  $\mu\text{m}$ .

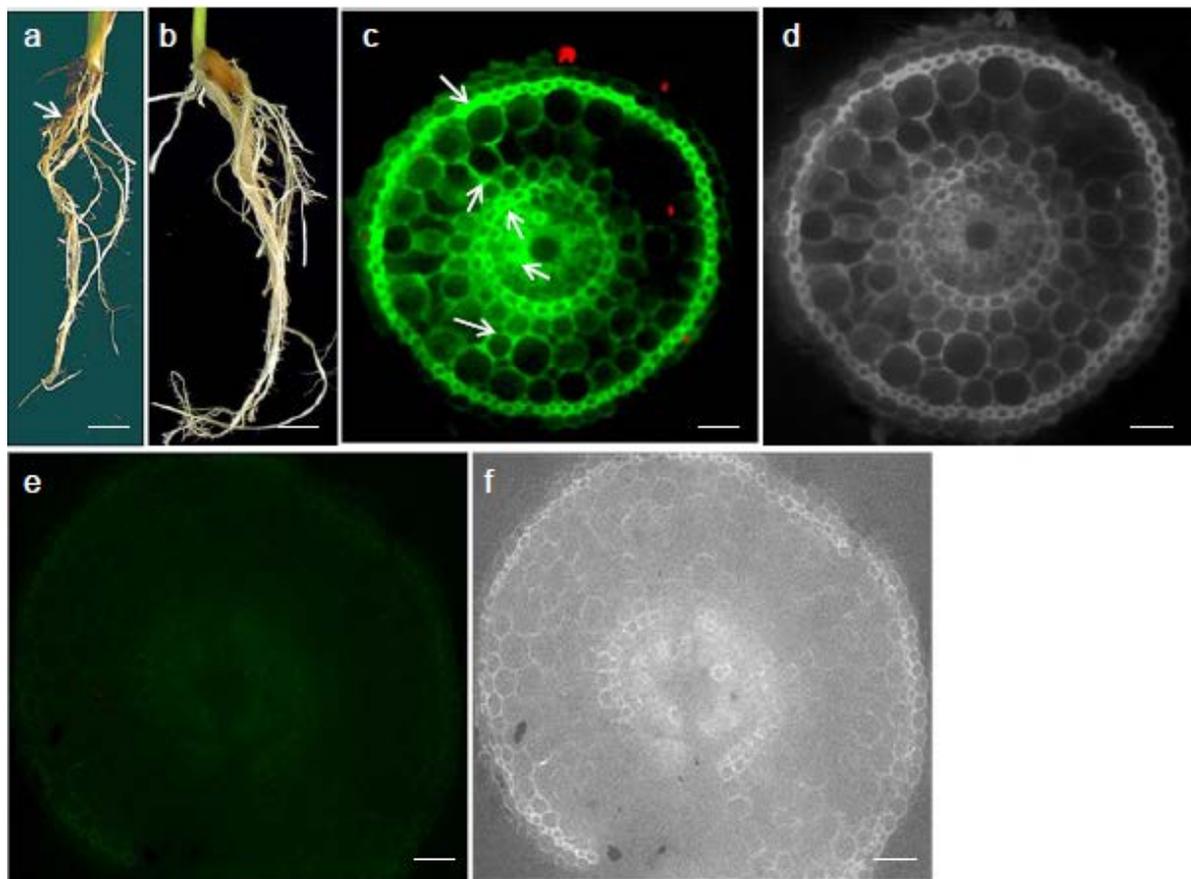


**Figure 2.** Early and late colonization stages of susceptible rice roots (HXZ) by GFP-tagged strains of *U. virens*. (a) spores of the GFP-tagged strains of *U. virens* after 1 dpi; (b) Fungal colonization on the rice root tended to form runner hyphae (arrow) (Bar = 100  $\mu\text{m}$ ); (c) Growing hyphae forming a net-like structure on the root surface 4 dpi (Bar = 100  $\mu\text{m}$ ); (d) Lesion formed on the adjacent region between main root and the fibrous root (Bar = 100  $\mu\text{m}$ ); (e) Lesion formed on the region of the main root and the fungi located on the lesion (Bar = 200  $\mu\text{m}$ ); (f) Mycelium densely covering the main root after 15 dpi (Bar = 200  $\mu\text{m}$ ); (g) Control (Bar = 100  $\mu\text{m}$ ).

The areas attacked formed brown lesions and the light brown color of lesions became dark brown (**Figure 3(b)**) and even black at later stages of infection. Finally, the root systems were full of lesions. Similar behavior of hyphae has been observed and reported for other root endophytes [33]-[35]. Once inside the root, the hyphae swelled and spread intracellularly into the cytosol of the cell through specific points of contact with other epidermal and cortical cells. At 15 dpi, hyphae were also observed invading the intercellular spaces of epidermal roots cells (**Figure 2(f)**). At 3 dpi, attachment had occurred along the root epidermis and after a successful penetration into the epidermal cells, the hyphae reached the vascular tissue intercellularly through the cortical cells then it continued to grow and ramify and lastly a hyphal net within the xylem vessels was observed by 18 dpi; this implies that penetration of the epidermis, cortex and the xylem took place between days 2 and 18. The rapid proliferation of hyphae within the xylem vessel cells mainly occurred longitudinally.

The hyphae appeared to be confined to the infected cell until the intracellular space was completely filled with hyphae. Only certain epidermal and cortical cells appeared to become colonized in this manner, while the surrounding cells remained uninfected, resulting in a mosaic pattern of plant cell colonization (**Figure 2(d)**). This pattern of plant cell colonization continued for 10 to 25 dpi. *U. virens* hyphae could be seen colonizing the surfaces of mature roots, lateral roots, root caps, root elongation zones, and root hairs (**Figure 2**), indicating that there was no specific root site preference.

Transverse sections from infected and uninfected roots were examined at various time points to determine the rates and extents of colonization. At 18 dpi, the pathogen colonized the epidermis and the cortical cells, both intercellularly and intracellularly (**Figure 3(c)**). Then later, the whole root cortex was heavily colonized, until the



**Figure 3.** Systemic infection and colonization of GFP-tagged *U. virens* strain on the susceptible rice root (HXZ). (a) Symptoms on the root systems of rice following inoculation with GFP-tagged strain of *U. virens* on the elongation region (arrow) (Bar = 2  $\mu\text{m}$ ); (b) Uninfected 20-day-old rice root (Bar = 2  $\mu\text{m}$ ); (c) Cortical cells are colonized by hyphae and oval conidia (arrow) after 18 dpi on a rice root cross section (Bar = 100  $\mu\text{m}$ ); (d) Cross section of the root observed in bright field (Bar = 100  $\mu\text{m}$ ); (e) Cross section of an uninfected 18-day-old rice root under epifluorescence microscopy (Bar = 100  $\mu\text{m}$ ); (f) Cross section of the uninfected root observed in bright field (Bar = 100  $\mu\text{m}$ ).

fungus reached the endodermis (**Figure 3(c)**). The endodermis appeared to delay the progress of the hyphae, and it was not until 28 dpi that the fungus was observed colonizing the vascular cylinder (**Figure 3(c)**), where it could be observed colonizing the endodermis, sieve cells (phloem) and xylem. Even though xylem vessels were colonized by hyphae neither blockage of the vascular system nor did the plants exhibit wilting symptoms, typically found with vascular diseases from our observation. In the uninfected roots, light green auto-fluorescence was observed in the rice epidermis, phloem and xylem (**Figure 3(e)**).

Although the colonies of the transformed fungus in the cortex of rice were sparse, the hyphae was able to grow towards the vascular tissues with little hindrance. As colonization progressed in the lateral roots, two colonization fronts were clearly observed; one front enveloped the epidermis and cortical tissues and the other within the vascular tissues. The former brings the eventual collapse of the infected root tip whereas, the latter one continued its progression through infected xylem vessels of the infected lateral root from base to apex. There were no symptoms observed except the collapse of the root tip beyond the infection site as the mycelia advanced systemically through xylem vessels towards the taproot. Generally, from our observation, host penetration in most cases was through the intercellular space and it may also be through the junction that is formed between the root hairs and the lateral roots can be considered as another entry point for the fungus. Similar results were observed in rice, tomato and brassica on root infection and colonization by GFP-labeled *Klebsiella*, *Fusarium* and *Verticillium* respectively [15] [36] [37]. This could possibly be explained by the existence of a common mode of infection which is usually used by pathogens that attack vascular tissues to enter root hair zones. So the pathogens first attach and then penetrate directly into the epidermal cells, due to a stronger chemical attraction of the fungus to the root hairs than the root surface [30]. In this study, the hyphae grow irregularly on the root surfaces. The hyphae of most endophytes deviated from the direction along the root axes when they grew inside cells [38]. It is normal to see hyphae grew in irregular directions when they colonized living cells.

#### 4. Conclusion

The present study, which is the first report of root colonization by the GFP-transformant of *U. virens* provides a new tool for studies of early endophyte root colonization stages and localization in the host tissue. *Ustilaginoidea virens* attacked rice roots by the initial infection of the root hairs, and then colonizing without killing them. In the susceptible rice cultivar HXZ, *U. virens* tended to form mosaic patterns of infection by filling individual cells with hyphae.

#### Acknowledgements

This work was supported by grants from the National Natural Sciences Foundation of China (911161001) and the Important Direction Research on Knowledge Innovation Project (KSCX2-EW-N-06) and the fund from Department of Agriculture of Guangdong Province awarded to J. X. Li. M. Andargie was a recipient of the “Visiting Fellowship for Researchers from Developing Countries” Award from Chinese Academy of Sciences.

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