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Fluorescent Imaging of *Rhizoctonia solani* Isolate TOM7 Hyphae Anastomosis

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Abstract

Samples of the filamentous fungi *Rhizoctonia solani* isolate strain TOM7 were stained with the nucleic acid-staining fluorescent dye acridine orange in order to image hyphae anastomosis. Images were collected of TOM7 hyphae anastomosing with other TOM7 hyphae, which readily occurred under stressful conditions (e.g., lack of nutrients, etc.). Furthermore, this "self-anastomosis" was not observed when nutrients were prevalent. Additionally, both incompatible and compatible pairs of *R. solani* hyphae were crossed in order to induce anastomosis. The results provided evidence that the hyphae never anastomosed with incompatible pairs (i.e., the hyphae remained parallel or crossed over without merging); and compatible pairs showed limited anastomosis. The images corroborate previous findings that hyphae may release diffusible factors such as viruses or toxins upon contact with hyphae of an incompatible strain.

Keywords: *Rhizoctonia solani*, anastomosis, fluorescent microscopy

1. Introduction

The anastomosis reaction of the plant pathogenic fungus *Rhizoctonia solani* occurs when two separate hyphae come in contact with one another[3]. This results in either hyphal death, which occurs between hyphae of incompatible strains, or fusion followed by no discernable change in growth, which occurs between hyphae of compatible strains or separate hyphae of the same isolate. These interactions have allowed for the categorization of *R. solani* strains into anastomosis groups (AG) by anastomosis compatibility. The presence of double-stranded RNA in *R. solani* strains has been well established, with considerable debate over its influence on pathogenicity[11,8]. It has also been well established that this dsRNA is likely viral in origin[5,7,10]. Furthermore, these dsRNA segments are also known to be transmitted horizontally during the anastomosis reaction between hyphae[4]. The segments, although being ubiquitous to *R. solani*, are different both between and within anastomosis groups, and believed to be the feature that defines compatible and incompatible strains[1,2,9].

The purpose of this experiment was to capture high resolution images of the interactions of *R. solani* hyphae of compatible and incompatible strains. The use of the fluorescence nucleic acid stain acidrine orange allowed for the imaging of nucleic acid location during these interactions. These novel images are an initial step in developing a more complete understanding of cell to cell transmission of fungal viruses.

2. Materials and Methods:

The interactions between compatible and incompatible hyphae of *R. solani* strains were imaged with the nucleic acid stain acridrine orange after their growth on agar slides. One milliliter of liquid potato dextrose agar (PDA) was spread onto each three inch by one inch glass microscope slide and allowed to cool. Slides were inoculated by use

of a 20-200 microliter pipette tip; which, when inverted and pressed into the parent plate's agar, cut away of a small cylinder of agar and isolate. This cylinder of agar and isolate was then removed via scalpel and placed onto the microscope slides. Care was given to ensure that the end of the cylinder with the parent isolate came in contact with the agar of the microscope slide. These samples were allowed to grow in darkness for between three and six days, until the borders of their radial growth could be seen barely touching with the naked eye. At that time the slides were covered in one milliliter of 10 µg/mL acidrine orange / 1.0 M acetate buffer pH 4.5. After thirty minutes incubation, the slides were rinsed thoroughly with autoclaved distilled water and allowed to air dry. Images were taken with the Olympus® FV1000 Confocal Microscope at 10X, 40X and 60X oil immersion magnifications with both the acidrine orange filter and differential interference contrast (DIC) application.

3. Results

Several images were taken showing self-anastomosis, i.e. anastomosis between hyphae of the same strain (Figures 1-4); and were especially prevalent after an extended period of growth, during bacterial contamination (Figures 3, 4) or when the agar had lost a considerable amount of water (Figures 1, 2).

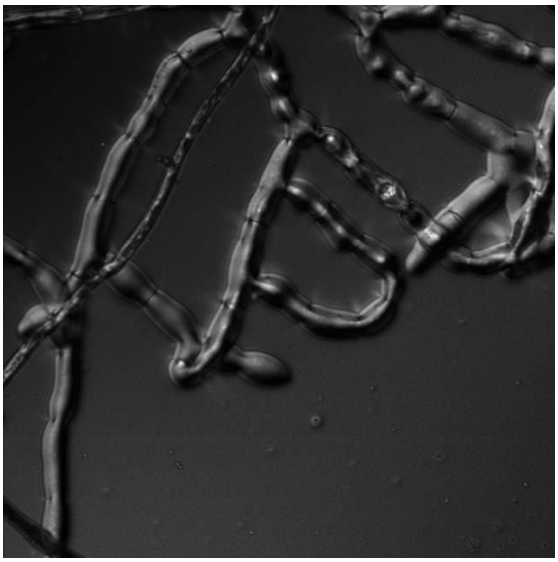

Figure 1. Anastomosis between *R. solani* isolates TOM7 hyphae under stressful conditions (overgrowth and diminished water content), DIC, 60X magnification.

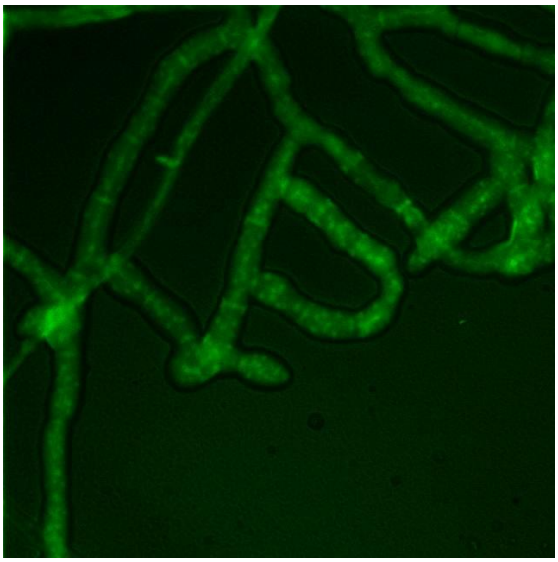

Figure 2. Acidrine orange fluorescence of Figure 1.

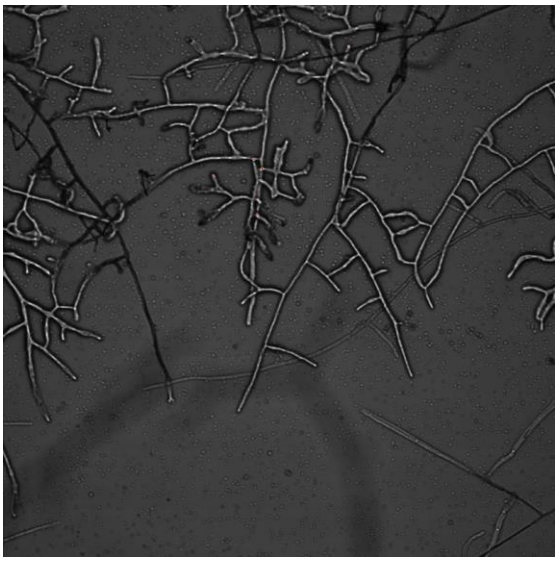

Figure 3. Anastomosis between TOM7 hyphae under stressful conditions (overgrowth and bacterial contamination), DIC, 10X magnificiation.

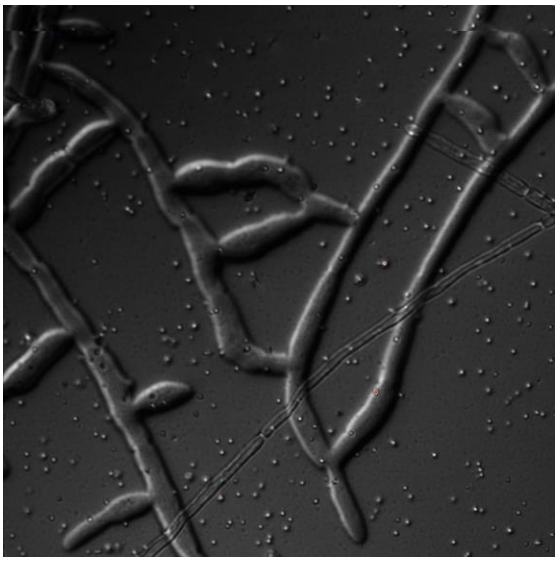

Figure 4. 40X magnification close-up of self-anastomosing region of Figure 3.

Additionally, several images showed the perfectly compatible interactions between compatible strains TOM7 and T2 (Figures 5-8). Although more rare than self-anastomosis, these interactions were found under both stressful conditions (Figures 7-8) and non-stressful conditions (Figures 5-6).

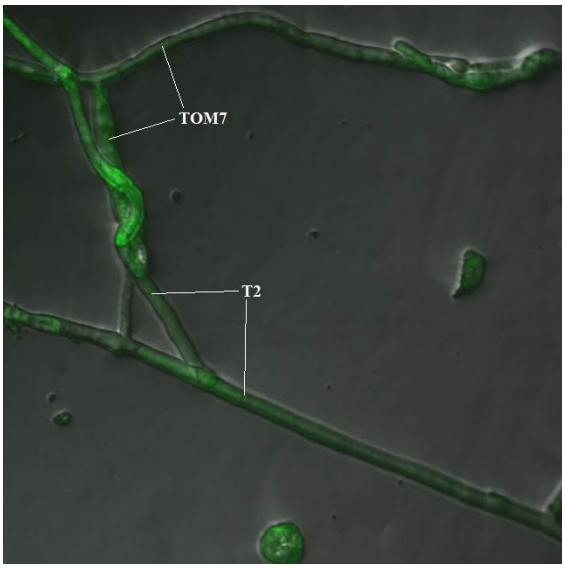

Figure 5. Anastomosis between TOM7 (top) and T2 (bottom) hyphae under non-stressful conditions, DIC and acidrine orange fluoresence, 40X magnification.

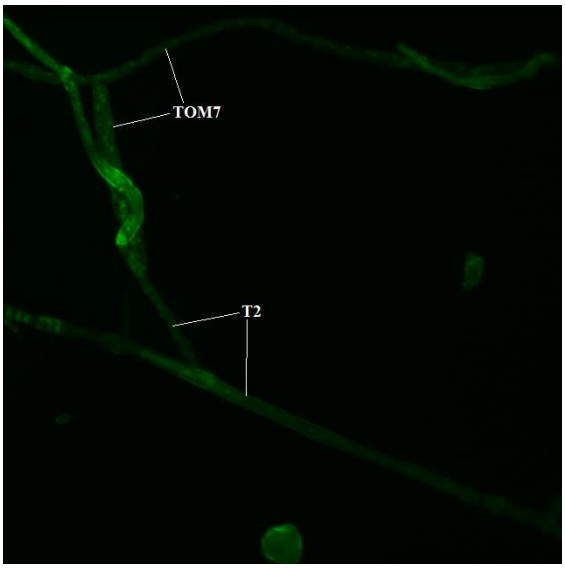

Figure 6. Acidrine orange fluoresence of Figure 5.

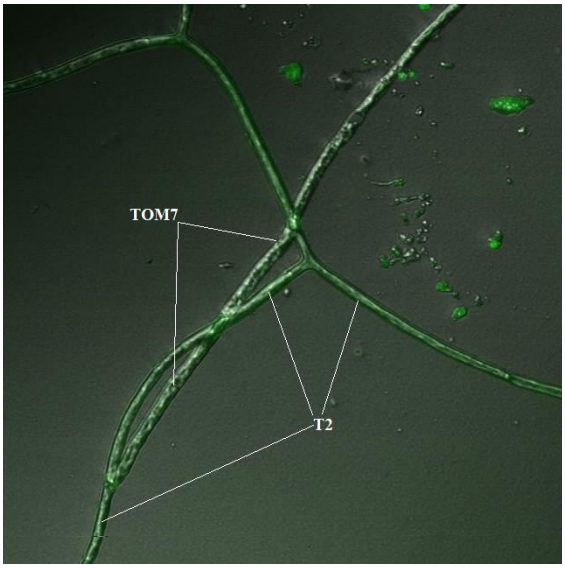

Figure 7. Anastomosis between TOM7 and T2 hyphae under stressful conditions (diminished water content), DIC and acidrine orange fluoresence, 40X magnification.

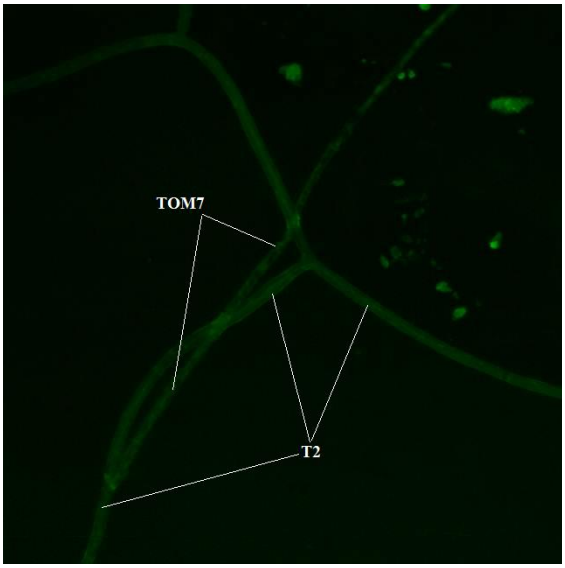

Figure 8. Acidrine orange fluoresence of Figure 7.

The interactions between incompatible strains TOM7 and EGR4 were imaged under both stressful and non-stressful conditions. All of the images of the incompatible interactions presented (Figures 9-18) occurred under non-stressful conditions; and were chosen for presentation due to the clarity of the region of interest. The formation of barrage lines was seen after excess growth of both isolates, and several images showed the interactions between incompatible strains at the initial meeting of their hyphae. The acidrine orange stain successfully arrested further growth of all hyphae after their immersion, and allowed for the imaging of these time-sensitive interactions.

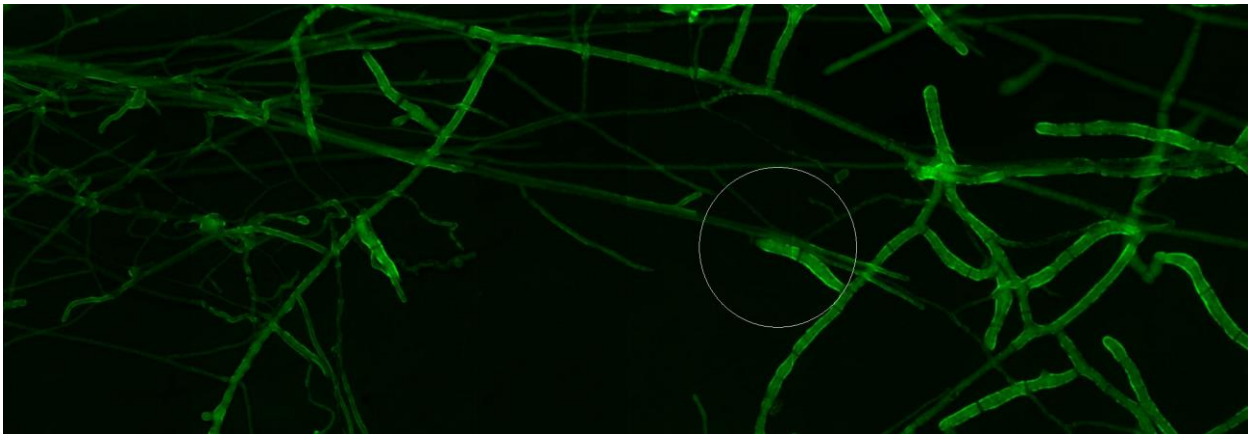

Figure 9. Panorama of initial interactions between isolates TOM7 (left) and EGR4 (right) hyphae. Interaction circled. Composite of five images. Acidrine orange fluoresence, 40X magnification.

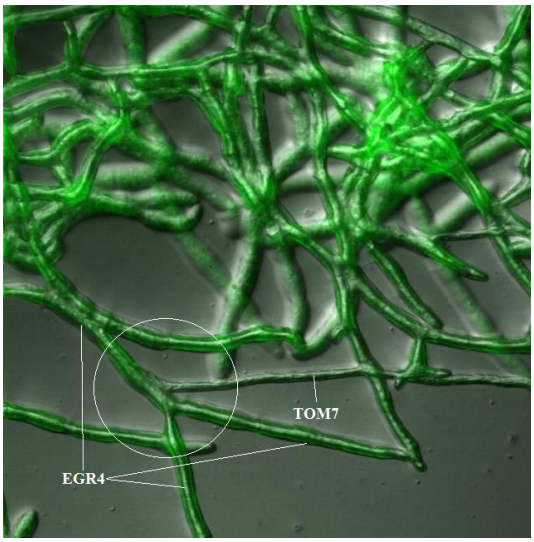

Figure 10. Interaction between incompatible TOM7 and EGR4 hyphae. Interaction circled. DIC and acidrine orange fluoresence, 40X magnification.

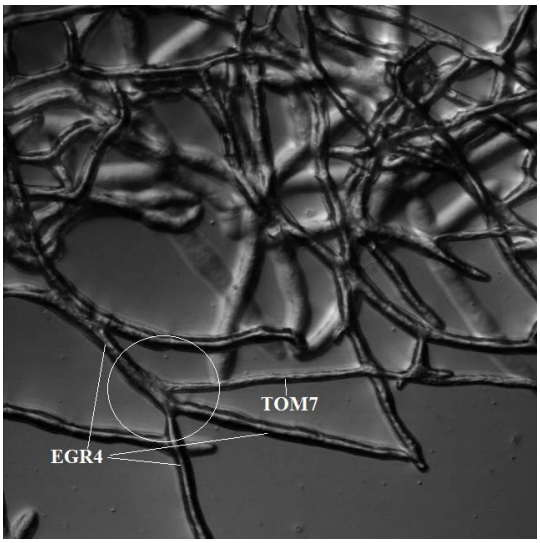

Figure 11. DIC of Figure 10.

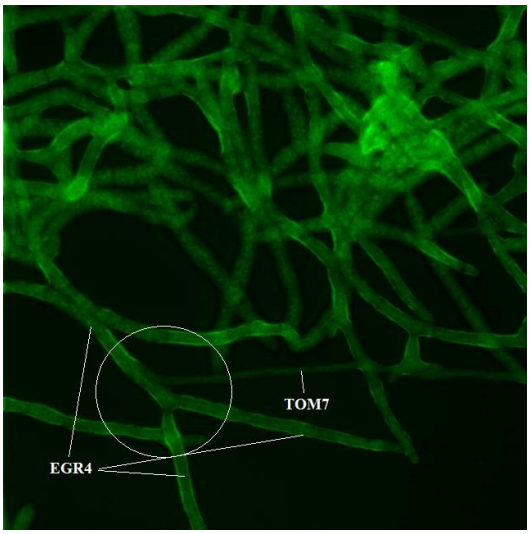

Figure 12. Acidrine orange fluoresence of Figure 10.

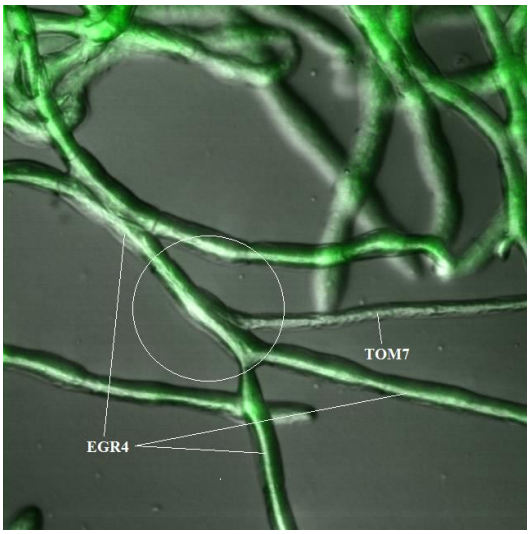

Figure 13. 60X magnification close-up of interaction in Figure 10. DIC and acidrine orange fluoresence.

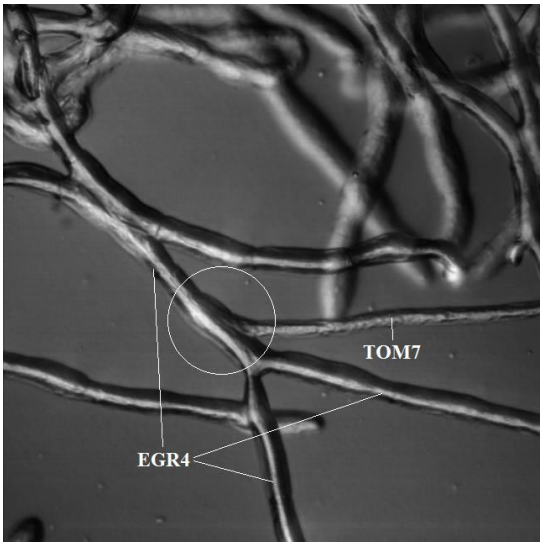

Figure 14. 60X magnification close-up of interaction in Figure 10. DIC.

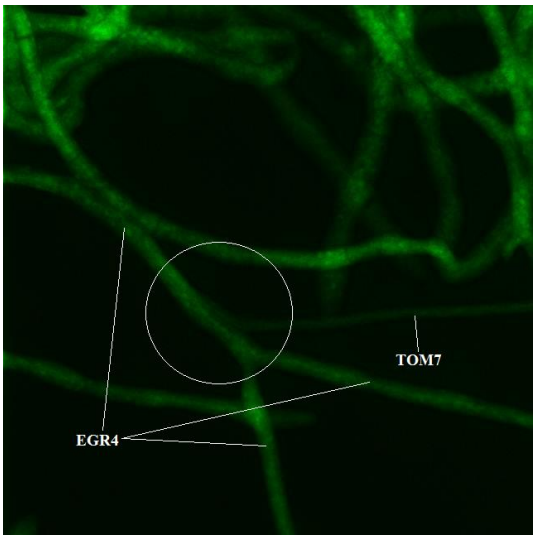

Figure 15. 60X magnification close-up of interaction in Figure 10. Acidrine orange fluoresence.

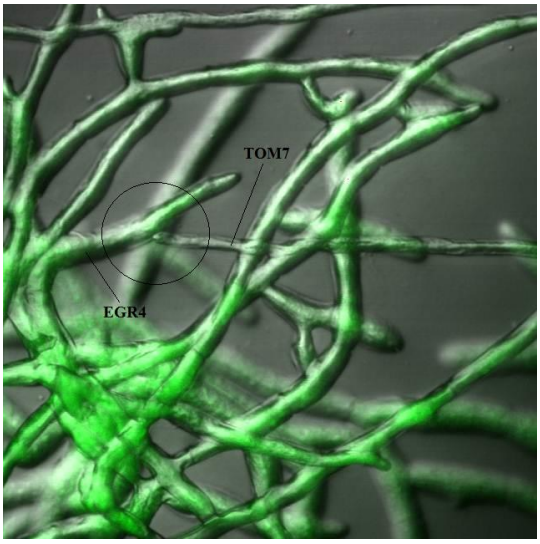

Figure 16. Interaction between incompatible strains TOM7 and EGR4. Interaction circled. DIC and acidrine orange fluorescence, 60X magnification.

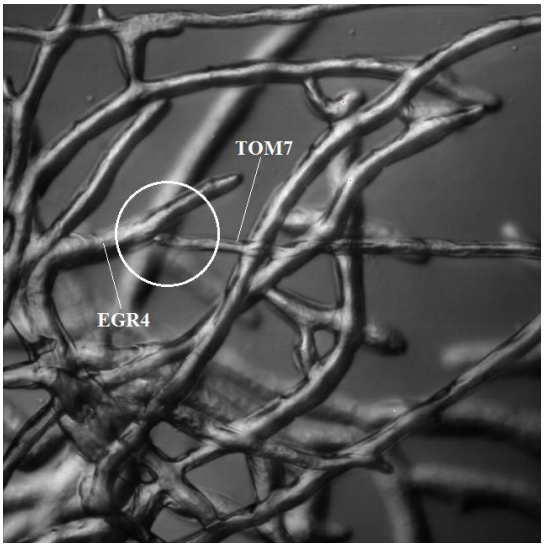

Figure 17. DIC of Figure 16.

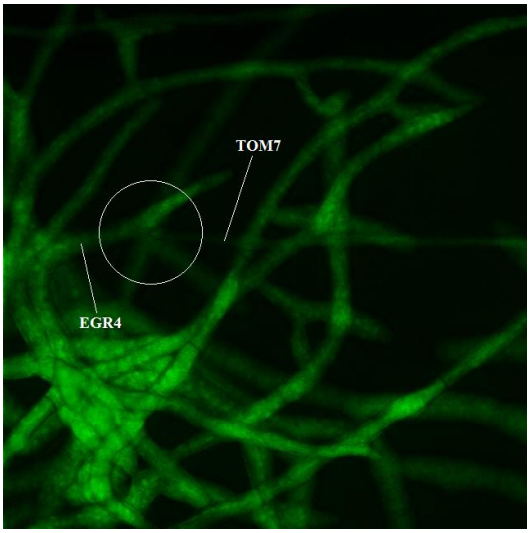

Figure 18. Acidrine orange fluorescence of Figure 16.

4. Discussion

The perfect anastomosis between *R. solani* hyphae of the same isolates (Figures 1-4) was seen during times of low water content in the agar or during overgrowth. This agrees with the current understanding that hyphae anastomosis is required for homeostasis, as a means of mobilizing nutrients when these nutrients are in high demand[6]. More rare, even during times of nutrient depletion, was anastomosis between hyphae of compatible isolates TOM7 and T2 (Figures 5-8). Furthermore, anastomosis between these isolates was found regardless of nutrient content. The visible increase in fluorescing nucleic acid indicates that anastomosis between compatible isolates is a means of transferring genetic material, which also agrees with the prevailing knowledge that dsRNA segments in *R. solani* are transferred during anastomosis[4].

During the anastomosis reaction of incompatible isolates TOM7 and EGR4, the approaching TOM7 hyphae were shown to adopt a parallel growth motif (Figures 9-15). Although hyphae of incompatible strains were found to at least touch (Figures 16-18), no hyphae of incompatible strains were shown to fuse, which would be required for the transmission of virus-like particles and or cytoplasmic dsRNA genetic elements. The parent isolates from which the stained samples were taken were crossed and resulted in the killing reaction characterized by cell death. One reason for this observation may be that the touching hyphae had yet to fuse, but if this were true then it would have been likely to find at least one instance of incompatible hyphae fusion on the multiple slides examined. Another reason for this observation may be that the *R. solani* viruses are transmitted via contact rather than complete hyphae fusion. To further discern the reason for this observation, microscopy could be done in real time with viral dsRNA-specific probes to determine whether or not dsRNA segments are transferred between hyphae upon contact. This remains as a viable avenue of future research.

5. Acknowledgements

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