Endophytic hyphal compartmentalization is required for successful mycobiont-wheat interaction as revealed by confocal laser microscopy

Vladimir Vujanovic[1], Lobna Abdellatif[1], and Susan Kaminskyj [2]
[1]Department of Food and Bioproduct Sciences and [2]Department of Biology,
University of Saskatchewan, Saskatoon, SK, S7N 5A8

Key words: *Triticum turgidum*, root, fungal endophytes, cell compartmentalization, symbiosis, cell morphology

Abstract

Root endophytic fungi are seen as promising alternatives to replace chemical fertilizers and pesticides in sustainable and organic agriculture systems. Fungal endophytes structure formations play key roles in symbiotic intracellular association with plant-roots. To compare the morphologies of Ascomycete endophytic fungi in wheat, we analyzed growth morphologies during endophytic development of hyphae within the cortex of living *vs* dead root cells. Confocal laser scanning microscopy (CLSM) was used to characterize fungal cell morphology within lactofuchsin-stained roots. Cell form regularity *Ireg* and cell growth direction *Idir,* indexes were used to quantify changes in fungal morphology. Endophyte fungi in living roots had a variable *Ireg* and *Idir* values, low colonization abundance and patchy colonization patterns, whereas the same endophyte species in dead (γ-irradiated) roots had consistent form of cells and mostly grew parallel to the root axis. Knot, coil and vesicle structures dominated in living roots, as putative symbiotic functional organs. Finally, an increased hypha septation in living roots might indicate local specialization within endophytic Ascomycota. Our results suggested that the applied method could be expanded to other septate fungal symbionts.

Introduction

Endophytic fungi are natural intracellular inhabitants of plant tissues that ensure essential function in plant, like mycovitality and mycoheterotrophy (Vujanovic & Vujanovic 2007) including enhanced growth, defense and stress tolerance (Waller *et al.* 2005). Also, it is proved that the endophytes can colonize living and dead host tissues and suppress fungal pathogens during growth and reproduction. However, little data exist that describe ascomycote endophytic structures in colonized root of domesticated cereals. Waller *et al.* (2005) suggested the different functional structures of an endophyte , including those associated to the reproduction cycle, were consequences of the life stages of colonized plant organs, that is, they were affected by association with living *vs* dead tissue. Biological significance of the root-cortex cell death in wheat on proliferation of ascomycetous weakly pathogenic *Cochlioholus* and avirulent *Phialophora* isolates has also been suggested. In either case, establishment of the parasitic or mutualistic interaction is the result of a highly sophisticated cross-talk between the partners. Hadacek & Kraus (2002) speculated that fungal morphology changes may possibly be related to chemical variation specifically in the type of carbohydrates present in the host cell. Whether root cell structural changes (volume) or

carbohydrate changes in dead cells (associated with decomposition) are involved in fungal morphogenesis is still unclear.

In this study, we hypothesized that the mutualistic pressure, two way fungus plant interactions, may differently affect the endophytic structures formed in living roots compared to those in dead roots. If so, the same endophyte might have different cell morphologies before and after root senescence. Similarly, endophyte fungi might adopt different colonization patterns depending on the metabolic activity of the host plant cell tissue. The aim of this study was therefore to compare Ascomycete endophyte coloniziation patterns and morphologies in living and killed roots. To prevent major changes in killed roots, *i. e.* cell membrane or volume modifications and arrangement deficiency, we used low-dose γ-irradiation to ensure no shifting in root-inactive cell structural forms. According to Natarayan & Kesayan (2005), γ-irradiated barley meristemic cells remain biochemically unchanged, so their influence on endophytic structural formation changes should be minimal. Here, we describe fungus-root interactions in living and killed roots.

SMCD* - Saskatchewan Microbial Collection and Database, College of Agriculture & Bioresources, University of Saskatchewan, SK, Canada.

Materials and Methods

Two endophytic Ascomycota mitosporic strains SMCD 2204 (class Dothideomycetes) and SMCD 2213 (class Eurotiomycetes) isolated from root of durum wheat *Triticum turgidum* L. (Saskatchewan, Canada) were used in this study.

Endophyte growth experiments:
Seeds were surface-sterilized with 95 % ethanol for 10 s, rinsed in sterile distilled water (SDW) for 10 s, then submerged for 3 min in 5 % sodium hypochlorite, rinsed 3 times in SDW, and placed on potato dextrose agar (PDA) for germination. Ten seeds were spread over 9 cm Petri plates. After 3 d of incubation (Precision Fisher Scientific Inc., Incubator MDL3EG) at 21 ºC in darkness, half of the young seedlings were co-cultured in association with fungal mycelia (5 mm2) for 4 d. Half of the 3 d old seedlings were removed from the medium, their roots carefully washed, killed by γ-irradiation [9.37 Gy per min, 12 h, modified Natarajan & Kesavan (2005)], then returned to co-culture as before for 4 d. Roots placed on PDA without fungal partners were used as a control. All treatments were repeated twice with three replicates per treatment. After 7 d, all roots were prepared for microscopic analyses, described below.

Microscopy
Observation under a Zeiss Axioskop 2 light microscope with 100x magnification showed that the irradiation dose used did not reduce cell-wall thickness or destroy the cell-wall, and there was no leakage of cytoplasm. Root segments were fixed in formalin, cut into 2 cm segments, and stained with lactofuchsin (www.sigmaaldrich.com) as described by Kaminskyj (2008). Stained roots were examined with a Zeiss META 510 confocal laser scanning microscope with 514 nm (argon) excitation and LP585 emission filters. Images were collected using a Plan-Neofluar 25 x N.A.0.8 DIC multi-immersion objective or a C-Apochromat 63x N.A.1.2 phase-contrast water immersion objective. Fluorescence and transmitted images were collected simultaneously.

Cell Morphometry

All experiments were performed in wheat roots. Fungal cell morphometry was described quantitatively to compare colonization pattern between living and dead root cells. All of the hyphae in ten 100 µm x 100 µm areas in randomly-selected confocal optical sections were assessed for each type of quantification. Each host fungus/cell status combination was repeated twice with five replicates. All values are presented as the averages ± standard error. Statistical analyses were performed using Student's *t*-test ($p<0.05$).

Two indexes were created to asses the shift in fungal strain colonization pattern between living and dead root cells. These indexes are:

(1) *Index of fungal cell regularity* (*Ireg*) was employed to discriminate a shift in fungal cell form. According to Ainsworth *et al.* (1971), cell form can be characterized combining three-dimensional cell structure (rotation about the central axis) and cell shape distinguished by a length (L)-width (W) aspect ratio. In this study, *Ireg* (L/W) index values ranged from 1 to 4 – meaning that a cell 3 µm wide would an aspect ratio of 4 has adjacent septa 12 µm apart. Length was measured parallel to the hyphal axis, between adjacent septa. Width was perpendicular to length and was the greatest cell diameter between each pair of septa. Within calculated *Ireg* scale (1 to 4), two distinct shape groups were distinguished based on variability measured between minimal ($Ireg_{min}$) and maximal ($Ireg_{max)}$ aspect ratio. Type I with cylindrical or regular cell form was characterized by an $Ireg_{max} > 2$ (Fig. 1b), and Type II with round (globose to subglobose) or irregular cell form was characterized by an $Ireg_{max} < 2$ (Fig. 1a). In both types, no differences were observed in $Ireg_{min} = 1.2\text{-}1.6$; thus, it was chosen for the determining the range of *Ireg* variability in the two types (I and II).

(2) *Index of fungal cell directionality* (*Idir*) describes fungus cell direction changes for individual hyphae inside a root (Fig. 1c, d, e). Straight hyphae that grew aligned with the root axis were defined as the baseline pattern (0) in which the cell growth parallel to the root axis indicated not shifts in direction (Fig. 1c). Fungus cells within an individual hypha whose growth axes deviated up (+) or down (–) from baseline (0) were scored for each change in growth direction. Thus, *Idir* = number of baseline cells/number of deviated cells x 100, which resulted in a frequency scale of 5 - 85 %. Based upon this scale, fungal cell direction were categorized in two types: Type A with $Idir > 45$ has cells aligned with the root axis, and Type B with $Idir < 45$ has deviated cell growth from the central root axis.

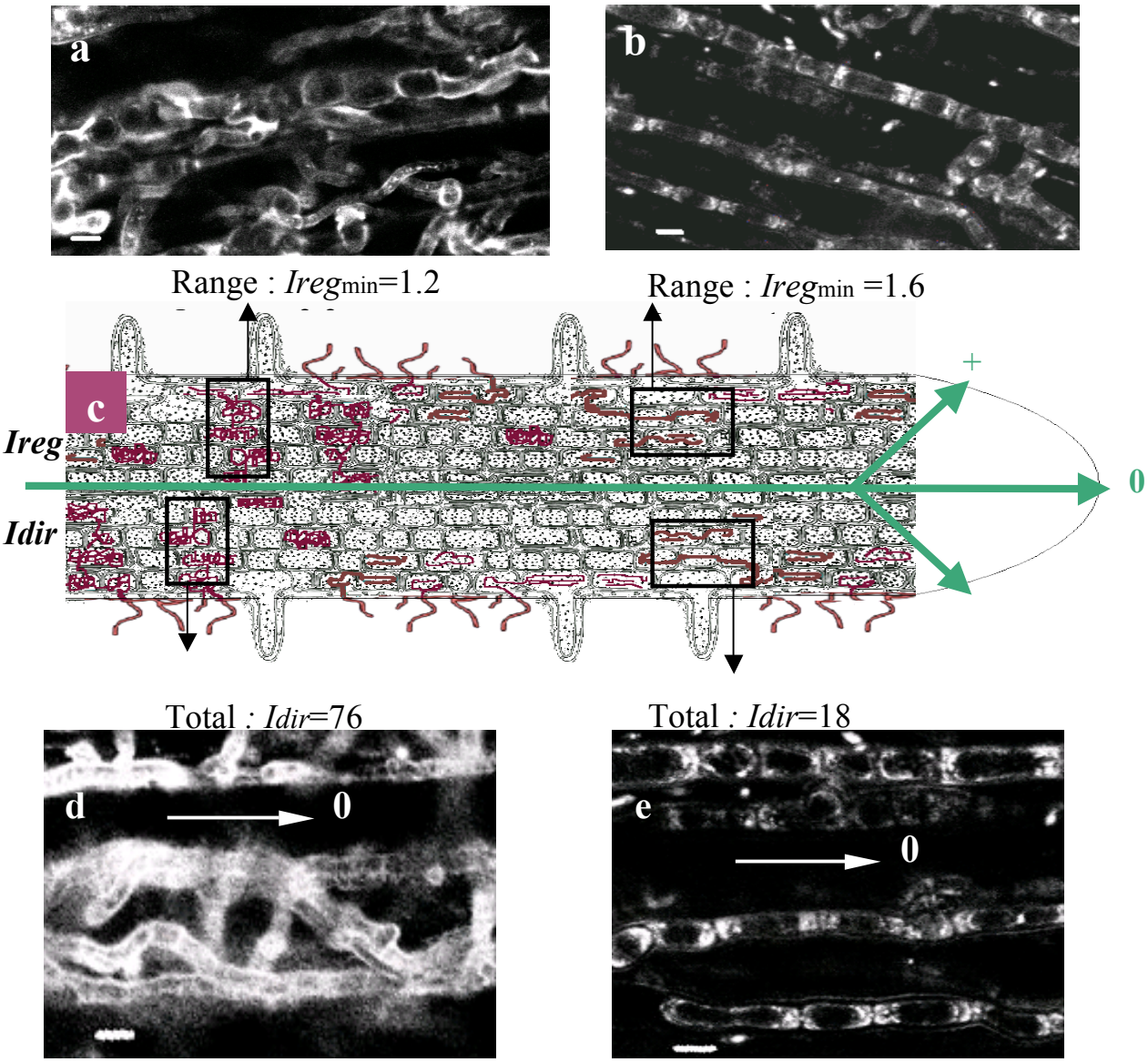

Figure 1. Endophytic fungal hyphae showing formation of: (a) Type I - irregular cell shapes and (b) Type II - regular cell shapes in wheat root. Scale length depicted in red and width in green. (c) showing different root-colonization pattern and deviation in fungus cell directions compared with root-cell membrane horizontal direction: d) Type A - pronounced deviations in cells direction and e) Type B - without considerable deviation in cells direction (+ up and — down) compared with root axe (0) (arrows in green). Bar – 10µm.

Results & Discussions

In this study, we used quantitative descriptors to compare and contrast the cell form based on hyphae septation pattern, cell form (*Ireg*) and cell growth direction *(Idir)* in living versus killed roots. Fungus cell irregularity and direction showed considerably higher mean *Ireg* range and *Idir* values (Fig.2 a, b) in the living cells compared with the dead cells. $Ireg_{min}$ values were similar in living and killed cells, however there were substantial differences in $Ireg_{max}$ and *Idir* values. Beside the hyphal colonization pattern changes, it seems important to better understand the cellular morphological changes within intracellular growing hyphae.

Although conducted *in vitro*, our observation suggests that it is possible assess the gradient of the continuum along the mutualism in leaving host cells to the saprotrophism in dead host cells - depending on plant physiological active vs. inactive environments with different availability of cell nutrition resources (Violi *et al.* 2007). Thus, the assessment of Ascomycota hyphal compartmentalization through changing in cell shape and direction (Fig. 1) is seen as cornerstone for avoiding sometimes confusing ecological interpretation based on anatomical/morphological microscopic structure overlaps, usually reported in the context of superior Ascomycota and Basidiomycota related with nutrient exchanges (Peterson *et al.* 2008) within active and inactive host cells.

Each fungal strain showed Type I-irregularity (symbiotic cell form) and Type A or three-dimensional direction (pattern of cell growth) within living cells (Fig. 1a, d, 3a). In addition, Type II - regular cell forms and Type B - linear growth patterns were exclusively associated with inactive/dead host cells (Fig. 1b, e, 3b). We can suppose that the linear hyphal growth gives to fungi the ability to move relatively longer distances to explore for available food sources instead of waiting for the food coming through close mutualistic exchange found in interaction with the active plant cells. Under field conditions, saprotrophs must explore to acquire nutrients, whereas symbionts or parasite exploit a living interaction that presumably can supply ongoing nutrients.

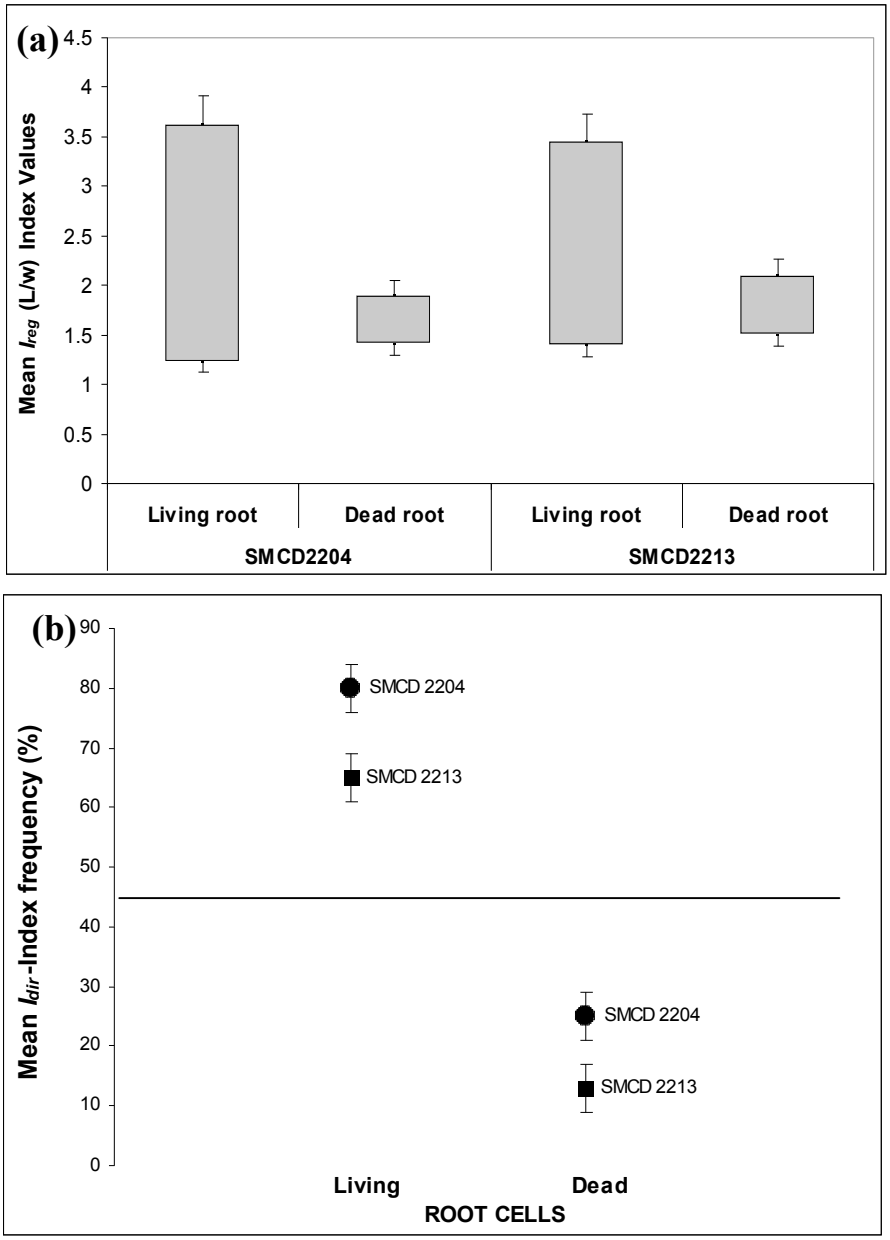

Figure 2. Fungal endophyte – (a) cell form alteration based on measured L/W and calculated *Ireg* -cell irregularity index. (b) Cell direction alteration based on measured frequency of direction changes: 0, + and – and calculated *Idir* -cell deviation index. Vertical bars represent standard errors of the means ($p < 0.05$)

In conclusion: Root endophytic fungi in wheat production may be used as alternatives for chemical fertilizers and pesticides (Abdellatif *et al.* 2007). They can induce beneficial morphological, physiological and molecular changes in cereal hosts, resulting in reprogrammed host-cell tolerance to abiotic stresses, diseases resistance, and higher yield (Waller *et al.* 2005). To elucidate the lifestyle of Ascomycota endophytic fungi in wheat, we analyzed the symbiotic interactions trough an endophytic development of hyphae within root cortex of active vs. inactive host cells. In this study, we assessed the colonization pattern of SMCD2204 - class Dothideomycetes and SMCD2213 - class Eurotiomycetes proposing a quantitative approach to assess endophyte morphology in Ascomycota looking at the cellular level determinable changes by using *Ireg* and *Idir* indexes.

It is proposed to move from traditional descriptive approach to a more objective microscopic approach in order to advance our comprehension on the formation of functionally "complex structure" found in ascomycetous endophytic and mycorrhizal root-symbionts (Massicotte, 2005). This approach could be also expanded on Basidiomycota as another multicellular fungal endophytic phylum, but unlike Glomeromycota mycorrhiza.

Acknowledgements

This research was financially supported by Natural Sciences and Engineering Research Council of Canada Discovery Grant and Saskatchewan Agri-Food Innovation Found Chair (AFIF) in Microbial Biotechnology & Bioproducts.

References

Abdellatif, L., Bouzid S., and Vujanovic, V. (2007), *Plant Canada - Growth for the Future* (Proceedings), pp. 137–139

Ainsworth, G.C., Hawksworth, D.L., and James P.W. (1971) *Ainsworth & Bisby's Dictionary of the Fungi*, CAB, UK

Hadacek, F., and Kraus, G.F. (2002). *FEMS Microbiology Ecology* **41**: 161–170

Kaminskyj, S.G.W. (2008). Sustainable Agriculture and Forestry (ZA Siddiqui, MS Akhtar, K Futai, Editors). Springer-Verlag, Dordrecht, The Netherlands

Massicotte, H.B., Melville, L.H., and Peterson, R.L. (2005). *Canadian Journal of Botany* **83**: 1057–1064

Natarajan, A.T., and Kesavan, P.C. (2005). *Current Science India* **89**: 360–365

Peterson, R.L., Wagg, C., and Pautler M. (2008). *Canadian Journal of Botany* **86**: 445–456

Violi, H.A., Menge, J.A., and Beaver, R.J. (2007). *American Journal of Botany* **94**, 690–700

Vujanovic, V., and Vujanovic J. (2007). *Symbiosis* **44**: 93–99

Waller, F., Achatz, B., Baltruschat, H., Fodor J. et al. (2005) *Proceedings of the National Academy of Sciences USA* **102**: 13386–13391

For more information on this work, contact Dr. Vladimir Vujanovic at vladimir.vujanovic@usask.ca