Growth of endophyte, Neotyphodium, and its host plant, tall fescue (Festuca arundinacea), under 3D-clinorotation

Kaori Tomita-Yokotani1* and Satoshi Shinozaki2

1Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8572 Japan.
2Maekawa MFG.Co.Ltd, Moriya, Ibaraki 302-0018 Japan.

Abstract Growth of a filamentous fungus endophyte, Neotyphodium, and its host plant, tall fescue, Festuca arundinacea, was examined during the seed germination process under pseudo-micrgravity generated by three dimensional (3D-) clinorotation. The shoot growth of tall fescue infected with the endophyte was remarkably suppressed on a 3D-clinostat compared with that of the ground control. Without being infected, shoot growth of tall fescue was not strongly affected by the 3D-clinorotation. Many aggregated hyphae were observed in the plant seed incubated for 1-day on the 3D-clinostat than in those kept on the ground. These results indicate that the clinorotation induces responses in the endophyte and its host plant different from those under normal gravity.

Key words: endophyte, tall fescue, Neotyphodium, 3D-clinostat

Materials and Methods

Plant materials

Tall fescue, Festuca arundinacea, both infected and uninfected with the endophyte (Neotyphodium, Maekawa line), were used as plant materials in this study. Tall fescue, Festuca arundinacea, and Neotyphodium, Maekawa line were provided from Maekawa Co. Ltd. Japan. The rate of germination in both seeds was over 80% on average.

Incubation and growth measurement of tall fescue

The layout for incubation of tall fescue is shown in Fig. 1. The surface of tall fescue seeds were sterilized with 0.1% sodium hypochlorite solution for 10 min, and washed three to four times with distilled water. They were sown in Agripot® (Kilin, Tokyo) that contained 80 ml of agar medium (Nacalise, Kyoto), and incubated at 25°C in the dark for six days. The embryo of the tall fescue seeds was aligned upwards in the agar. After six days, shoot length was measured as an index of plant growth.

Detection of endophyte

Existence of tissue endophyte was determined by microscopic observation with modified procedure of Vazquez de Aldana et al. (2001). Seeds were soaked overnight in a 5% aqueous solution of sodium hydroxide at room temperature, and rinsed with distilled water. The seeds were stained by a solution (0.6 g aniline blue par 100 ml of lactic acid and 200 ml of water) and boiled for 3-
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5 minutes. Stained seeds were placed on a slide glass, and pressed by finger over a cover glass. Stained specimen was observed by an optical microscope, and its photoimages were taken.

**Counting aggregated hyphae**

We counted “Aggregated hyphae” in the seed tissue incubated for one day. Degree of aggregation was used as an index to analyze morphology of fungus hyphae that responded to different gravitational environment. Aggregated hyphae were counted under microscope as shown in Fig. 2. Loop of hypha was defined as the characteristic diameter of loop, $a$, being longer than the gap of opening, $b$. When hypha showed $a$ shorter than $b$, it was not judged as loop. Degree of aggregation of hyphae of endophyte in plant seed was evaluated by counting looped hypha in an area of 20 by 20 $\mu$m square.

All the experiments were repeated twice to confirm reproducibility of the results.

**Results**

In this study, tall fescue seedlings were divided into four groups as shown in Table 1. They were characterized with two indexes; infected or non-infected with the endophyte, and loaded with 3D-clinorotation or normal gravity.

**Incubation and growth of tall fescue.**

The growth of tall fescue was compared among the four groups. Figure 3 shows the effects of two factors on the growth of the plant after the incubation in Agripot®. Shoot growth of tall fescue was not strongly affected by the infection with the endophyte under normal gravity, as seen in the comparison of B against A. A significant growth suppression by the infection was observed under the 3D-clinorotation, D against C ($t$-test, $p<0.05$). The shoot growth of uninfected tall fescue was weakly suppressed by 3D-clinorotation compared to normal gravity, C against A. However, the degree of suppression in C versus A, among the pair of non-infection, was lower than D versus B, in the case of infection.

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**Table 1 Indexes in this study**

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<thead>
<tr>
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<th>Non-infected plant</th>
<th>Infected Plant</th>
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<tr>
<td>Ground control</td>
<td>A</td>
<td>B</td>
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<tr>
<td>3D-clinostat</td>
<td>C</td>
<td>D</td>
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**Fig. 2.** Definition of loop and aggregate of hyphae.

**Fig. 3.** Effect of shoot growth in tall fescue seedlings of the four groups A to D. Bars indicate S.E. ($n=10$)

**Fig. 4.** Endophyte in tall fescue seed incubated for one day.

**Fig. 5.** Two types in morphology of endophyte hypha.
Morphology of endophyte.

Microscopic images of endophyte in seeds of the group B and D are shown in Fig. 4. They were stained by aniline blue after 1-day incubation of tall fescue infected with endophyte. Figure 5 shows an image of a smashed seed layer after 1-day incubation of ground control, group B, at the higher magnification. A long hypha was folded in the seed tissue, type A, and it was gradually straightened, type B, during the growth. In the initial state of germination, most of hypha was found in type A. As the germination process proceeded, the ratio of type B was increased. There were many hyphae of type A was observed in the group D under 3D-clinorotation.

Aggregated hyphae

Degree of aggregated hyphae in seed under ground control (B) and 3D-clinorotation (D) is shown in Fig. 6. The average number of the loop in initial state of germination in the 20 mm square on the day-0 was 2.3. This number in the group D under 3D-clinorotation decreased to 1.8 after the seven days incubation, but higher than that of the group B kept on ground, 1.2 (t-test, p<0.05) as shown in Fig. 6.

Discussion

In recent years, endophyte has been well studied by many groups in the world, scoping to develop new protective systems using the symbiotic combination of endophyte and its host plants (Spiering et al. 2002, Vazque de Aldana et al. 2001). We have not much information on how endophyte, Neotyphodium, first infected to the host plant, tall fescue. It remains still an important question. Endophyte lives inside the seeds, growing together with the host plant once it germinates. Endophyte transfers into the aerial part of the host plant when it is matured. It immigrates back into seeds of the host plant when the plant matured to full-grown. This life cycle has been repeated since the symbiosis of plant and endophyte started. Because Neotyphodium endophyte in tall fescue does not cause any damages to its host plant, it is difficult to find whether the plant infected with endophyte or not. We found both endophyte and host plant respond to altered gravitational environment. Interestingly, Hasenstein (1999) and Kem (1999) suggested that the gravisensing in fungi is different from that in plants. Gravisensing in fungi has received relatively little attention in contrast to higher plants. Fungal hyphae lack dense organelles such as amyloplasts known to be responsible for gravisensing in higher plants. On this basis, it would be possible to have different response between endophyte and host plant at exposing to clinorotation.

This study showed that shoot growth of tall fescue is suppressed by the 3D-clinoration, compared to that of the ground control. Furthermore, the infection with endophyte induced higher suppression of shoot growth. Dynamic gravitational stimulus generated by 3D-clinorotation is known to affect some physiological functions and development of organisms (Hoson et al. 2002, 2003). Fungus endophyte in tall fescue showed different response against gravitational stimuli (Figs. 4 and 6). There are many species of fungus endophyte, similar to Neotyphodium. They express their own character differing each others (Christensen 1995, 2000). The filamentous fungus that we used in this study shows usually increase of straight hypha during the germination of seed of its host plant.

These results indicate that the physiology and development of both endophyte and tall fescue are under the influence of gravity. Rice growth was reported to be promoted under microgrvity in space (Levine, et al. 2001, Hoson et al., 2002) and under the gravity unloaded condition on ground. Hoson et al., (2003) elucidated that the cell wall extensibility is increased in space. Such changes in the cell wall properties might be responsible to the observed response of the endophyte hyphal morphology and growth under the 3D-clinorotation found in this study (Hoson, pers. comm.). It also suggests that tall fescue infected with endophyte produces some growth inhibiting substances because the shoot growth was suppressed even under the 3D-clinorotation. The seed of tall fescue infected with endophyte release some growth inhibiting substances to their surroundings (Tomita-Yokotani et al. 2003). There is a possibility that the producuction of these substances, such as allelochemicals, are affected by the 3D-clinorotation. Study on the endophyte and its host plant growth under the exotic gravitational environment may lead new findings of phenomena closely related to the symbiosis of organisms.

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