(Reference) Endophyte hypha

[Staining of endophyte hypha]

1 Rose bengal staining and aniline blue staining

Scope of application: hay (perennial ryegrass, Italian ryegrass and tall fesque etc.)

A. Analysis sample preparation

Crush the whole plant of hay to be 1-4 mm in length, or otherwise cut the stem of hay to be 3-4 cm and further cut into four lengthwise to be a sample.[1]

B. Reagent preparation

1) Rose bengal staining solution. Weigh 0.25 g of rose bengal, put it in a glass container, and dissolve by the addition of 100 mL of water to be the rose bengal staining solution (store in a refrigerator).
2) Aniline blue staining solution. Weigh 1.0 g of aniline blue, put it in a glass container, dissolve by the addition of 100 mL of water, and add 50 mL of 85 v/v% acetic acid solution to be the aniline blue staining solution (store in a refrigerator).

C. Staining

Alkali treatment. Weigh about 1 g of the sample, put it in an 100-mL beaker, add about 20 mL of 2.5 w/v% sodium hydroxide, cover with a watch glass, boil by an electric heater for about 1 minute [2] then filter with a stainless mesh, wash with water until the pH of washing becomes 6.0-6.5, and subject to staining.

Staining/ fixation

1) Rose bengal staining solution. Transfer the sample to another 100-mL beaker, add about 20 mL of the rose bengal staining solution, cover with a watch glass, and boil for 20 minutes. Wash the inside of the beaker and the watch glass with a small amount of water, and leave at rest for 1 hour or more. Wash with a sufficient amount of water for about 30 minutes until the washing becomes clear.
2) Aniline blue staining solution. Transfer the sample to another 100-mL beaker, add about 20 mL of the aniline blue staining solution, cover with a watch glass, and boil for 20 minutes. Wash the inside of the beaker and the watch glass with a small amount of water, and leave at rest for 40 minutes or more. Wash with a sufficient amount of water for about 10 minutes until the washing becomes clear.

D. Identification of hypha

Place a suitable amount of the precipitated sample on a glass slide, and cover with a coverslip and lightly press the tissue to crush[3] to be a preparation. Examine at 100x to 400x under a microscope with a blue filter on a light source.

Hypha that meets the following three criteria is judged as endophyte hypha.

I	Hypha that does not invade into the plant cell.
II	Hypha without a branch.
III	Hypha stained red by rose bengal staining or dark blue by aniline blue staining.

<<Summary of analysis method>>

While endophyte hypha extends in the intercellular space of most parts in the plant body as well as on the surface of the medullary cavity of culms and panicle bases, it does not intrude into the host cells or go outside the plant body to exhibit signs. Therefore, usually the plant infected with *Neotyphodium* endophytes does not show symptoms and thus it is impossible to confirm visually the presence or absence of infection. Hypha is detected by observation by optical or electron microscopy in the plant tissue such as the seed or the leaf sheath, ELISA, liquid chromatography, or isolation and incubation of endophytes on culture media.

In this method, hay is treated with alkali to soften the plant tissue, and infiltrated with the staining solution to the inside of the tissue to stain endophyte hypha.

The flow sheet of the staining method is shown in Figure 5.2.3-1.

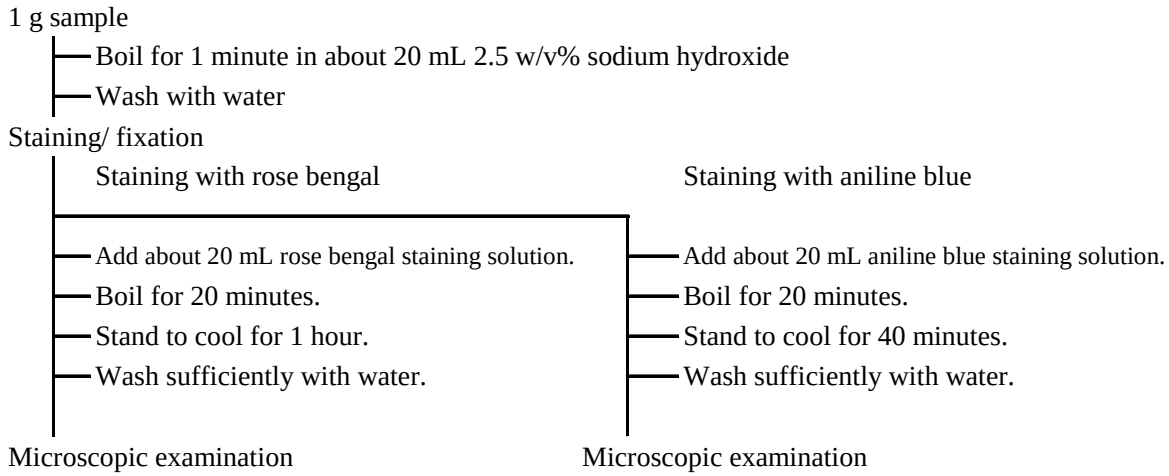

Figure 5.2.3-1 Flow sheet of the staining methods for endophyte hypha

References: Takayuki Koyama and Kyoko Akimoto: Research Report of Animal Feed, **24**, 131 (1999)

<<Notes and precautions>>

[1] Cut a sample to an appropriate size for sufficient infiltration of the staining solution into the tissue because the hay tissue is hard.

[2] When boiled for 2 minutes or more, the plant tissue becomes soft but endophyte hypha may be damaged and sometimes cannot be observed. On the other hand, the tissue may be hard and the staining solution may not be infiltrated by boiling for less than 1 minute.

[3] Hypha can be better observed by disintegrating the plant tissue/ cells by lightly

slipping the sample. Endophyte hypha that extends in the intercellular space in the plant body as well as on the surface of the medullary cavity of culms and panicle bases can be observed. Photos of endophyte hypha of perennial ryegrass that extends in the leaf sheath are shown in Figure 5.2.3-2.

(A) (B)

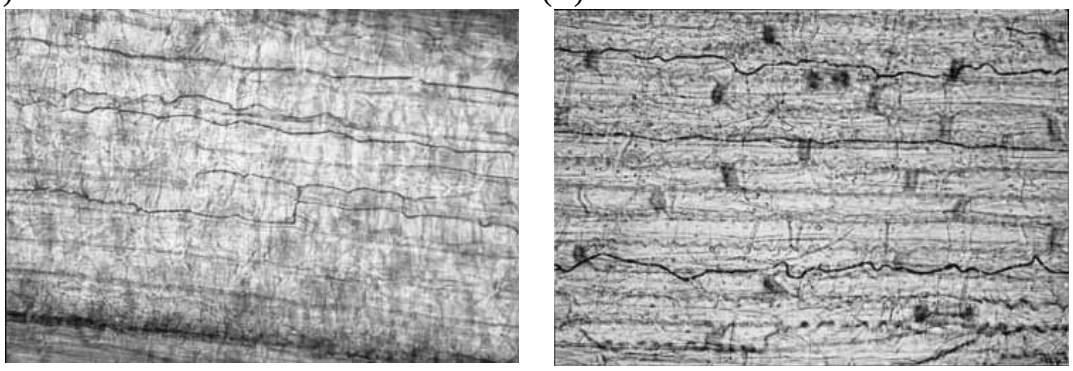

Figure 5.2.3-2 Microscopic images of endophyte hypha of perennial ryegrass that extends in the leaf sheath.
(A) Staining with rose Bengal.
(B) Staining with aniline blue.