PLANT ROOT DAMAGED BY SOIL FREEZING IN LOW SNOW WINTER: ESTABLISHMENT OF MONITORING SYSTEM BASED ON PHYSIOLOGICAL OBSERVATION

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Global warming is one of the pressing issues in the world. For several decades, decreasing snowfall in a high latitude region in the northern hemisphere has been reported. Snow coverage on the ground protects soil from freezing during the winter season. The effect of heat insulation of snow is necessary to keep the root of trees or crops underground warm in winter. Lack of snow coverage leads to deep freezing of soil and irreversible injury to root tissue when the temperature drops in the nighttime. Many recent reports show that plant death caused by soil freezing influences a local ecosystem and crop cultivation. Hokkaido, located in the most northern prefecture of Japan is also facing the issue in recent years. To tackle it urgently, we designed a forecasting system that could be used for rescuing and mitigating root freezing damage. Online monitoring consisted of a thermocouple logger, and Wi-Fi was installed in a test field and monitored the transition of ground temperature. The logger alerts when the temperature is below -5°C. On the following day, the root samples were collected and stained histochemically with 2,3,5-triphenyl tetrazolium chloride (TTC) to visualize the location of injury at the tissue or cellular level.

Key Words: *plant root damage, soil freezing, global warming, environmental monitoring*

1. INTRODUCTION

Snow covering the ground plays a critical role in insulating air temperature. In the region with high latitude, the temperature drops typically below zero during nighttime. In the current global warming trend, low snowfall has been one of the pressing issues during the winter season. It causes a lack of snow coverage which leads to enhance the depth of soil freezing. Severe soil freezing harms underground plant tissues, i.e., root part and microorganisms[1-4]. Damaging their living organisms causes irreversible influence and strongly prevents them from recovering in the upcoming springtime. Recently, many reports have warned about this issue because it will be a significant threat to an ecological system that is directly connected to agriculture and the economy soon. In Hokkaido, Japan, low snowfall is becoming problematic for local crops, especially pasture plants, which are an essential source for feeding farm animals. Although pasture plants are perennial, the recent soil freezing causes the plants' reduction in spring[5)].

It was shown that soil freezing caused root damage by the artificial snow reduction in Canada[6)]. The previous report confirmed that the root was injured by the freezing temperature rather than physical damage caused by frost heaving. Although the entire root system was not significantly damaged, first and second-order roots were affected by the freezing[6)]. It is urgently required to reveal how roots are injured in the freezing condition in Hokkaido, Japan.

In this work, we conducted a combination of outdoor and indoor experiments to monitor the process of root freezing. Real-time monitoring with an alert system was also designed to prevent plant damage caused by soil freezing.

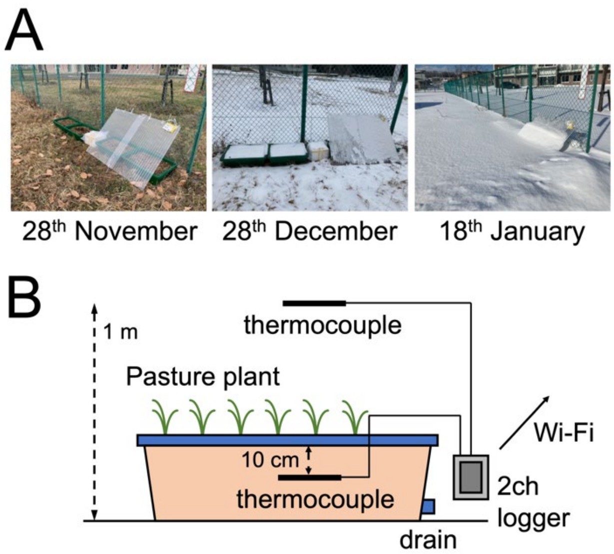

Fig.1 The set up of the outdoor experiment. **A.** The images of the snow situation from November 2020 to January 2021. **B.** The diagram of the components.

2. MATERIALS & METHODS

(1) Plant materials and growth condition

For an outdoor experiment, twenty-eight liters of the soil (commercial cultivating soil, FOREX, Japan) was mixed with 25 g of chemical fertilizer (NPK=8:8:8). One gram of seed of a pasture plant, timothy (cv. Kunpuu), was sowed on the soil in a plastic container (610 mm x 468 mm x 185 mm). The seeds were first germinated inside a laboratory at 23°C with 50% r.h. (relative humidity). The plastic container was placed in a test field when the shoot was 5 cm in length, and the experiment was started.

A model plant, *Arabidopsis thaliana* L., was selected for the indoor experiment to confirm root damage caused by freezing conditions under a controlled environment. The surface of the seed of Col-0, ecotype of A. thaliana, was sterilized with 10% (v/v/) of sodium hypochlorite with 0.1 % of Triton-X for 10 min. The seeds were then rinsed with autoclaved distilled water three times inside a clean bench. The seeds were sowed on a phytagel-solidified half strength of Murashige-Skoog basal media with 1 % (w/v) sucrose in a 9 cm Petri-dish and stratified at four °C for 24 hours. The plates were placed in an incubator (NK, Japan) at 23°C, 70 % r.h. for seven days for growth. Seven-day after germination of seedlings were used for the indoor experiment.

(2) Setup of outdoor experimentation and snow manipulation

Four of the plastic container were set at the position of 43°49'29.0"N 143°53'57.7"E, as shown in fig.1B. Two containers were placed on the ground with a canopy of transparent polycarbonate corrugated plate that protects the container from snowfall (The right two in the picture in fig.1A). Others without the canopy were exposed. The component of the container is shown in fig.1B. Two thermocouples were connected to a two-channel logger to record air and ground temperature every 30 min. The first thermocouple was placed 1 m above the ground, and the second one was buried 10 cm depth of the soil in the container. The temperature of the container, both with the canopy and without the canopy were recorded. The logger was connected to a Wi-Fi network in a campus of Kitami Institute of Technology, and the data were constantly uploaded to a server. The monitoring server was set to alert when the soil temperature was lower than -5 °C that was lasting longer than 1 hour. Following a morning of the alert, the plant materials were sampled and assessed inside the laboratory.

(2) Indoor experimentation

The Petri-dish growing seedling of seven-day-after germination was placed on a metal plate equipped with a Peltier cooling system. The temperature of the surface of the phytagel was measured with a non-contact thermometer. The plant tissues on the phytagel in the dishes were chilled gradually down to -4 °C and kept for 1 hour.

(3) Visualization of root damage

TTC histochemical staining solution[7] was prepared as follows; 0.5% (w/v) of 2,3,5-triphenyl tetrazolium chloride (Wako, Japan) and 0.01% (w/v) Tween-20 (Wako, Japan) were dissolved in 50 mM potassium phosphate buffer (pH 7.4). The low temperature-treated roots were submerged in the staining solution and infiltrated by vacuum for 5 min. The samples were then incubated at 30°C for 1 hour in the darkness. The staining pattern was observed and imaged with a microscope (BX51, Olympus, Japan).

3. RESULTS & DISCUSSION

(1) Outdoor temperature monitoring with snow manipulation

On November 28, 2020, the plastic containers were set up in the test field when it was no snow coverage. In the middle of December 2020, it started snowing gradually, and all area of the test filed was entirely covered in January 2021 (fig.1A).

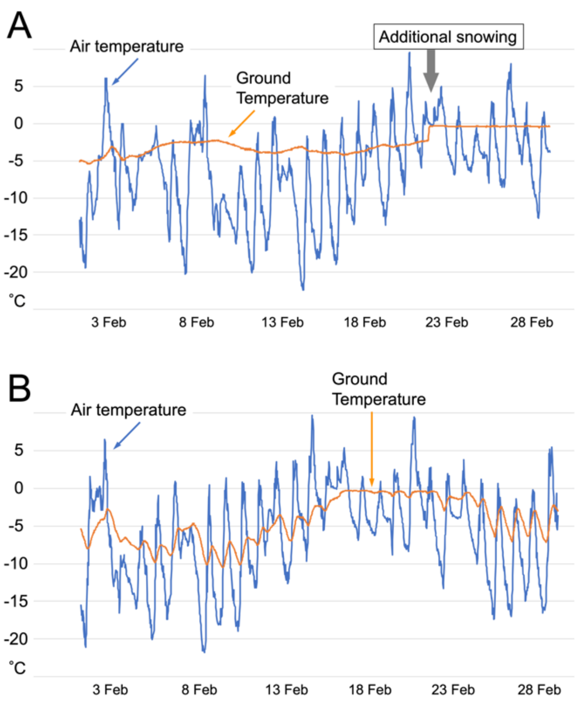

Fig.2 The recording of air and ground temperature in Feburuary 2021 in Kitami, Hokkaido. **A.** The transition of the temperature of the container without the canopy. **B.** The temperature with snow manipulation by using the canopy.

We confirmed that there was no snow coverage beneath the canopy. In the beginning, we planned to harvest the pasture plant to assess the root injury caused by soil freezing. However, all plants were dead soon after the container was placed outside. It was probably because the young seedlings of the plants were not grown enough to resist the outer cold environment. Therefore, we decided to change the plan that the plant experiments were conducted inside the laboratory. The recording of temperature was kept with the designed setup, as shown in fig.1B.

The air temperature ranged from below to around zero during night and daytime in both the container and without the canopy. The snow coverage on the exposed container stabilizes the ground temperature around zero (fig.2A). On the other hand, the ground temperature in the container with the canopy synchronously fluctuated in response to the air temperature (fig.2B). No snow coverage resulted in severe soil freezing in the nighttime with a temperature lower than -5°C for a week at the beginning and the end of February 2021. The monitoring server correctly sent the alert during this period. The result proves that the experimental setup can be used for the upcoming years to assess the plant root damage caused by nighttime freezing in the soil.

(2) Root membrane damage caused by freezing

The soil freezing environment at -5°C that was recorded by the outdoor experiment was reproduced in the laboratory. The root damage caused by the freezing was histochemically visualized by using the TTC reduction staining. The principle of TTC-based visualization of injury of tissues is as follows; TTC is a white-colored chemical, and it changes into red-colored 1,3,5-triphenyl formazan (TPF) by reduction (fig.3A). TTC in the cytosol is reduced by impaired mitochondria, which have excess electrons, and turned into colored TPF.

In the control A. thaliana root grown and incubated at 23°C, no staining of TTC was observed (fig.3A). In contrast, the reddish staining pattern in the root apex region in the -4°C treated root was observed (fig.3B). Interestingly, the root cap and root transition zone (located between elongation and meristem zone) showed the most robust staining. These regions are well known to be susceptible to the external environment. This finding suggests that roots undergo the freezing temperature perceives the damage first in the area and modulate the further gene expressions and physiological conditions to overcome the impending danger of freezing.

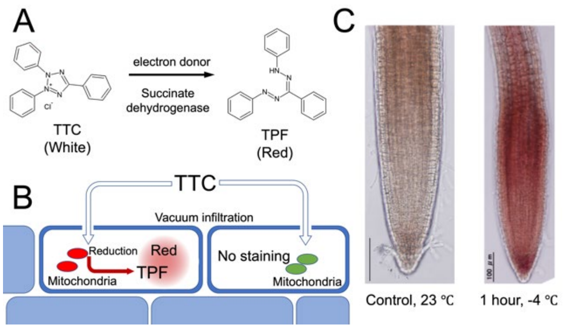

Fig.3 Visualization of root damage caused by freezing. **A.** TTC is reduced by a dehydrogenase into red-colored TPF. **B.** The reduction of TTC is catalized by impared mitochondria (red: impaired, green: normal) in the cytosol. **C.** Freezing treatment for 1 hour caused root injury. Bars indicate 100 µm. The representative images are shown.

4. CONCLUSION

In conclusion, the on-site temperature recording revealed the importance of snow coverage to prevent soil freezing. It suggests that the reduction of snowfall during the winter season may harm plant underground tissues. We have confirmed the usability of the experimental setups with snow manipulation and the temperature monitoring system. The TTC histochemical staining effectively visualized the root damage caused by the sub-zero temperature.

Based on the results reported here, the outdoor growth experiments in the test field will be planned for the next winter season. Furthermore, additional biological experiments that connect between laboratory-based and field experiments are required.

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