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**Influence of the Freeze-thaw Process under Acid Conditions on the Viability of Wintering Plants**

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Acid precipitates in snow may be a stress factor that affects the growth of wintering plants. The objective of this study was to determine the influence of acid-snow stress on leaves of wintering plants by *in vitro* experiments. Three different experiments, equilibrium freezing, prolonged freezing and repeated freeze-thawing, were carried out in order to simulate freeze-thawing of acid snow during winter. The results showed that acidification in the process of freeze-thawing caused enhancement of freezing injury of wintering plants.

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* **英語の要旨（200 words以内）**
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INTRODUCTION

It has been observed in the past decade that pH of meltwater of snow has become more acidic than pH of pure water (pH 5.6), which dissolved saturated carbon dioxide, as well as pH of acid rain or mist1-3), indicating precipitation of acid snow during winter. Acid snow is thought to be completely different from acid rain or mist since acid substances in snow or ice crystals may be partly concentrated, especially in the surface and central part of granular snow during winter4-6). At the early and/or late stages of snowmelt, meltwater with relatively strong acidity due to acid substances on ice crystals may be released from acid snow layers into soil, streams and lakes1,7). Wintering plants covered with acid snow may be subjected to freezing stresses, caused by reduction in freezing temperature or exposure to a prolonged subzero temperature during mid-winter, and freezing stresses caused by repeated freeze-thawing in early winter and/or early spring, in the presence of concentrated acid substances in snow or ice crystals.

研究報告

[Key words: Winter wheat leaf, Acid-snow stress, Freeze-thawing, Injury]

* **脚注はテキストボックスとして挿入**

We have recently started to study the responses of plants to freezing stress under acid conditions as simulated acid snow stress *in vitro*. Previous study suggested that acid snow stress might affect the growth of wintering plants8). In this study, the effects of acid-snow stress on leaves of winter wheat were analyzed by carrying out three different experiments, equilibrium freezing, prolonged freezing and repeated freeze-thawing, under acid conditions.

MATERIALS AND METHODS

1. Plant materials

Seeds of winter wheat (*Triticum aestivum* L. cv Chihokukomugi) were germinated for 2 days on wet paper at 18°C in the dark. Seedlings were planted in cultivating soil and grown at 18°C (12-h light) / 16°C (12-h dark) for 1 week in a growth chamber. One-week-old seedlings were cold-acclimated at 4°C (12-h light) / 2°C (12-h dark) for 4 weeks in a growth chamber.

1. Freezing tests under acid conditions

Leaf segments of cold-acclimated winter wheat (50 ± 2 mg fresh weight) were put into each test tube. Three hundred µl of Milli Q water (pH 5.6) as a control or sulfuric acid solution (pH 2.0) was added to each test tube since sulfuric acid is known to be a typical acid pollutant. Freezing was initiated by ice-seeding at -1°C in a programmable freezer (EP40-MV, Julabo, Germany). In an equilibrium freezing test, samples were cooled at 2.4°C/h to desired subzero temperatures. In a prolonged freezing test, the samples were kept at the desired subzero temperature for 7 days after they had been cooled at 2.4°C/h to the desired subzero temperatures. In a repeated freeze-thawing test, samples were cooled at 2.4°C/h to -2°C and kept at -2°C for 8 h and then they were warmed at 2.4°C /h to 2°C and kept at 2°C for 8 h. After completion of each freeze-thaw cycle, ice-seeding was done to start the next cycle. The freeze-thaw cycle was repeated 7 times. After all samples had been taken from the freezer, they were thawed at 4°C overnight in the dark. In all freezing tests, values for 100% and 0% injuries were obtained from samples treated with liquid nitrogen for 10 min and kept at 4°C, respectively.



* **図と図の説明をグループ化する**

Measurement of electrolyte leakage is usually used for estimating survival rates of samples after freeze-thawing. However, this method was not suitable for estimation of survival rates under acid conditions since the conductivity of cell extracts in the presence of sulfuric acid was not related linearly to the concentration of the extracts (Fig. 1A). Measurement of amino acid leakage by ninhydrin reaction9) was found to be suitable since the existence of sulfuric acid did not interfere with measurement of amino acid content (Fig. 1B). Therefore, measurement of amino acid leakage by ninhydrin reaction was used for estimation of survival rates in all freezing tests.

RESULTS AND DISCUSSION

1. Simulation of reduction in freezing temperature under acid conditions

The equilibrium freezing test under acid conditions was done in order to estimate the effect of exposure to reduction in freezing temperature under acid conditions on the survival of leaves of winter wheat. Although survival rates of leaf samples treated with pH 4.0 and 3.0 were similar to the survival rate of leaf samples treated with water (data not shown), the survival rate of leaf

samples treated with pH 2.0 was markedly　decreased (Fig. 2). This result showed that the addition of sulfuric acid solution (pH 2.0) before freezing caused extensive damage to the leaf samples. In this assay system, acidification before freezing caused damage to the tissues, but acidification after freeze-thawing with water caused little damage (Fig. 3). Acidification in the process of extracellular freezing and/or thawing may be harmful to the leaf samples of winter wheat. In an extracellular freezing process, plant cells were dehydrated and deformed by the growth of extracellular ice10,11). Severe extracellular freezing induced irreversible structural changes in cell membranes, especially plasma membranes10,12,13). Therefore, it is possible that an acid condition may enhance the irreversible structural changes of cell membranes in a process of extracellular freezing of plant cells. Alternatively, an acid condition may promote injury of membranes and/or cell structure in a different way from freeze-induced structural changes.



1. Simulation of prolonged freezing with acid snow

The prolonged freezing test under acid conditions was done in order to estimate the effect of exposure to a subzero temperature for a long time under acid conditions on the survival of leaves of winter wheat.　In this test, the samples were frozen under an acid condition (pH 2.0) at various constant temperatures for 7 days (Fig. 4A-F). Damage caused by acidification was related to lowering of the freezing temperature. The survival rates of leaf tissues frozen with water and sulfuric acid (pH 2.0) gradually decreased during the freezing period. It has been reported that prolonged exposure to subzero temperatures induced additional injury of cells mainly due to the solution effect, which is a harmful effect of the concentration of intracellular and/or extracellular ions caused by　extracellular freezing14).According to this hypothesis, further acidification due to freezing-induced concentration of protons for a long time may be toxic for leaf samples at subzero temperature.

1. Simulation of repeated freeze-thawing with acid meltwater

The repeated freeze-thawing test under acid conditions was done in order to estimate the effect of repeated freeze-thawing in acid meltwater in early winter and/or early spring on the survival of leaves of winter wheat (Fig. 5). Survival rates of leaf samples were gradually decreased by repeated freeze-thawing. Treatment with sulfuric acid solution resulted in more damage than did treatment with pure water. In this system, pH of the aqueous solution in the test tube to which leaf samples were exposed changed from 2.0 before freezing to about 4.0 after thawing since pH of the solution was neutralized by intracellular contents that had leaked from damaged cells. Thus, the aqueous solution in a test tube was re-acidified to pH 2.0 after freeze-thawing four times and then leaf samples were put through repeated freeze-thawing three times. Damage of the samples caused by repeated freeze-thawing with re-acidification (pH 2.0) was greater than that of samples without pH re-acidification.

The results from the three *in vitro* experiments suggested that acidification in the processes of freeze-thawing caused enhancement of freezing injury of leaves. Acid snow may intensify the freezing injury of wintering plants during winter.



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REFERENCES

1. Fushimi H, Kawamura T, Iida H, Ochiai M, Nakajima T, Azuma Y: Internal distribution of acid materials within snow crystals, *In* "Acid Rain 2000", Satake K, eds, Kluwer Academic Publishers, Netherlands, p1709-1714 (2001)



1. Noguchi I, Katoh T, Sakai S, Iwata R, Akiyama M, Ohtsuka H, Sakata K, Aga H, Matsumoto Y: Snowcover components in northern Japan, *In* "Acid Rain 2000", Satake K, eds, Kluwer Academic Publishers, Netherlands, p421-426 (2001)
2. Tsukahara H, Iida T, Ueki K, Yamaya M, Fukushima J, Taketa Y, Ikai H, Yasukuni K, Shimoida R, Netsu A: Local concentation of acidic ions in snowfall at the heavy snow area in the Japan Sea side of northern Honshu, J Jpn Soc Snow Ice, **62**, 254-264 (2000) (in Japanese)
3. Bale RC, Davis RE, Stanley DA: Ion elution through shallow homogeneous snow, Water Resour Res, **25**, 1869-1877 (1989)
4. Fushimi H: Acid snow in Lake Biwa catchment area, Japan (1), J Jpn Soc Snow Ice, **56**, 19-29 (1994)
5. Suzuki K: Review on snow chemistry in Japan, J Jpn Soc Snow Ice, **63**, 85-196 (2000) (in Japanese)
6. Johannessen M, Henriksen A: Chemistry of snow meltwater, Changes in concentration during melting, Water Resour Res, **14**, 615-619 (1978)
7. Inada H, Arakawa K: Influence of acidic condition on freezing injury of winter wheat, J Plant Res, **116**, suppl S, p93 (2003)
8. Siminovitch D, Therrien H, Gfeller F, Rheaume B: The quantitative estimation of frost injury and resistance in balck locust, alfalfa, and wheat tissues by determination of amino acids and other ninhydrin-reacting substances released after thawing, Can J Bot, **42**, 637-649 (1964)
9. Fujikawa S, Jitsuyama Y, Kuroda K: Determination of the role of cold acclimation-induced diverse changes in plant cells from the viewpoint of avoidance of freezing injury, J Plant Res, **112**, 237-244 (1999)
10. Steponkus PL: Role of plasma membrane in freezing injury and cold acclimation, Annu Rev Plant Physiol, **35**, 543-584 (1984)
11. Fujikawa S, Miura K: Plasma membrane ultrastructural changes caused by mechanical stress in the formation of extracellular ice as a primary cause of slow freezing injury in fruit-bodies of Basidiomycetes (*Lyophyllum ulmarium* (Fr.) Kuhner), Cryobiology, **23**, 371-382 (1986)
12. Uemura M, Steponkus PL: Cold acclimation in plants: relationship between the lipid composition and the cryostability of the plasma membrane, J Plant Res, **112**, 245-254 (1999)
13. Mazur P: Physical and chemical basis of injury in single-celled microorganisms subjected to freezing and thawing, *In* "Cryobiology", Meryman HT, eds, Academic Press, New York, p213-315 (1966)

**凍結融解過程における酸性条件が越冬性植物の生存に及ぼす影響 :** 稲田　秀俊，藤川　清三，荒川　圭太 （北海道大学大学院農学研究科環境資源学専攻）［キーワード：冬小麦緑葉, 酸性雪ストレス, 凍結融解, 傷害］

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