The Effect of Nitrogen Supplementation by Applying Livestock Waste Compost on the Freezing Tolerance of Japanese Chestnut

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In the context of global warming, freezing injury has tended to occur more frequently in cultivated Japanese chestnuts. To elucidate the cause of such freezing injury, we studied the effect of nitrogen (N) supplementation from livestock waste compost added to gray lowland soil on the freezing tolerance of Japanese chestnut trees in terms of their carbohydrate, water and N statuses. We also investigated the relationship between the endodormancy status and freezing tolerance of trees grown with or without livestock waste compost application. The freezing tolerance of Japanese chestnut trees planted in an excess of livestock waste compost was apparently lower than for trees grown only in gray lowland soil. The current season’s shoots from trees grown in soil only had the highest total sugar content, but the differences in total sugar content were not statistically significant, even though the N supplementation from livestock waste compost reduced the freezing tolerance. By contrast, higher water and N contents were recorded in the current season’s shoots grown in the presence of excessive livestock waste compost than in those grown in gray lowland soil only, all of which led to a reduction in the rate of winter survival among trees treated with livestock waste compost. The date of reaching 70% bud sprouting was delayed by one month or more, but bud break initiation was accelerated by the application of livestock waste compost, possibly due to a disturbance in the normal endodormancy progression. Collectively, these results suggest that normal endodormancy progression can be obstructed by higher water and N contents in trees treated with excessive livestock waste compost; thus, freezing tolerance sufficient to survive winter could not be acquired.

Key Words: endodormancy progression, freezing injury, Japanese chestnut, N content.

Introduction

Recent aberrations in winter temperatures possibly due to global warming have given us new impetus to study cold acclimation in woody plants and perennials. As one of such aberration, Sugiura et al. (2012) reported an increase in the freezing injury of fruit trees ascribed to failed cold acclimation in fall. In chestnut trees, cold hardiness is positively correlated with the sugar content in the current season’s shoots (CSSs) during the winter (Sakamoto et al., In press), as is the case in apple (Kuroda et al., 1985) and pear (Ito et al., 2013). In many deciduous fruit trees, the starch content of the CSSs reaches a maximum in October, and then stored starch is converted into soluble sugars in response to low and freezing temperatures. Accordingly, there is a well-known, positive correlation between freezing tolerance and starch content in fall (Sakai, 1962). In addition, the application of nitrogen (N) fertilizers in late fall reduces cold hardness and increases the potential for freezing injury in apple buds and stems (Kuroda et al., 1984). In general, livestock waste compost is one of the controlled-release fertilizers, unlike readily available fertilizers such as chemical fertilizer, which show lower fertilizer efficiency when applied. In addition to chemical fertilizer, N from livestock waste compost is applied every fall–winter in chestnut orchards (Ishizuka, 1984). More importantly, livestock waste compost was stored in the open air to reduce the readily available N fertilizer until 1999, but Japanese law pro-
hibited the open-air storage of livestock waste compost in 1999. As a result, the total N content of livestock waste compost, including readily available N fertilizer, increased by more than 1% compared with that before the law was enacted (Fujita, 2014). In fact, the intense application of manure might induce freezing injury in young chestnut trees (Mizuta, personal communication). The freezing tolerance at an early age in Japanese chestnut trees was shown to decrease remarkably compared with that of non-fertilized trees (Yasunobu, 1970). Nevertheless, little is known about the relationship between endodormancy progression and cold acclimation under excessive supplemental N conditions. It is generally known that Chinese chestnut has significantly more freezing tolerance than Japanese chestnut used as a rootstock in the Japanese chestnut industry, while Chinese chestnut is not practically used as a rootstock for Japanese chestnut because of the graft incompatibility between Japanese scion cultivars and Chinese rootstocks among others (Kotobuki, 1984). On the other hand, Miyamoto et al. (2011) reported that a new peach cultivar, ‘Hidakokufubeshidare’, was suitable for use as a peach rootstock to prevent young peach trees suffering from death and injury caused by freezing, possibly due to the delayed endodormancy completion of ‘Hidakokufubeshidare’ compared with that of other peach cultivars (Kamio et al., 2003). Additionally, earlier endodormancy completion causes an acceleration of water uptake in roots under warm winter conditions, which results in a rapid reduction in freezing tolerance at an earlier stage (Sakai, 1982). Thus, endodormancy status is a factor potentially hindering cold acclimation.

In this study, we examined the effect of N supplementation from livestock waste compost added to gray lowland soil on the freezing tolerance of Japanese chestnut in terms of the trees’ carbohydrate, water and N statuses in order to identify the cause of freezing injury. We also investigated the relationship between endodormancy status and freezing tolerance with or without the application of livestock waste compost. On the basis of our results, we propose a mechanism for how N affects winter dormancy and freezing tolerance.

Materials and Methods

Plant materials

Experiments were carried out at the NARO Institute of Fruit Tree Science (Tsukuba, Japan), located at 36°3′ N and 140°8′ E. Two-year-old potted trees of Japanese chestnut (Castanea crenata Sieb. et Zucc., ‘Porotan’) were used for all experiments. All of the potted trees were managed according to the ordinary cultural practices used in the chestnut orchards of Tsukuba. Air temperature data were obtained from the nearest weather station (Japan Meteorological Agency) located about 1.5 km west of the experimental orchard. The air temperature data during the experiment are shown in Figure 1.

Soil management of potted trees

Experiments were performed in the 2011/2012 and 2012/2013 seasons. Four types of soil condition were used for the experiments; i) gray lowland soil [soil only], ii) gray lowland soil + livestock waste compost (9:1, v/v; 27:2, w/w) [soil + compost (9:1)], iii) gray lowland soil + livestock waste compost (1:1, v/v; 3:2, w/w) [soil + compost (1:1)] and iv) livestock waste compost [compost only]. In 2011/2012, two-year-old trees were planted in 25-L pots in March 2011, and then 5 g of readily available chemical fertilizer [N-P-K = 10%-10%-10%] was applied to each pot. After potting, the trees were first kept in an open field and later transferred to a rain shelter house on 21 November, 2011. In 2012/2013, two-year-old trees were planted in 7-L pots in March 2012 and moved immediately into a rain shelter house. In mid-April, 5 g of readily available chemical fertilizer [N-P-K = 10%-10%-10%] was applied to each pot. The soil moisture content was calculated based on the volumetric water content in the pots and was maintained at 40% by an automatic irrigation system (DIK-6563-S150; Daiki

Fig. 1. The outdoor maximum/minimum air temperatures from fall 2011 to the spring 2012 (A) and from fall 2012 to spring 2013 (B).
Rika Kogyo Co., Ltd., Saitama, Japan). The soil moisture retention (pF) value was 1.1 on 16 November, 2012. The experimental design is shown in Table 1. In 2011, three types of soil condition [soil only, soil + compost (1:1) and compost only] were prepared using 25-L pots. Twenty-five pots were used for each of soil only and soil + compost (1:1) and ten pots were used for compost only. In 2012, three types of soil condition [soil only, soil + compost (9:1), and soil + compost (1:1)] were prepared using 7-L pots. Twenty-five pots were used for soil + compost (9:1), and five pots were used for soil + compost (1:1). In addition, the total N concentrations of livestock waste compost and gray lowland soil used in this study were 1.5% and 0.1% (w/w), respectively.

Effect of livestock waste compost application on freezing tolerance using whole trees

The freezing tolerance of trees was investigated in trees grown in soil only and soil + compost (1:1) in early February and early March, 2012, and soil + compost (9:1) in mid-February and mid-March, 2013. A programmed freezer (TH-250; OHNISHI NETSU-GAKU CO., LTD., Tokyo, Japan) was used for the freezing treatment. The potted trees were sprayed with water entirely to ensure ice nucleation, and were wrapped with polyvinylchloride film to prevent desiccation. The temperature was first kept at 0°C for 90 min and was gradually lowered by 2°C every 30 min. For the freezing treatments, potted trees were kept at the following temperatures, −4°C, −7°C, −10°C, −13°C, or −16°C, for 1 h in 2012 or 3 h in 2013. After the freezing treatment, pots were maintained at 5°C for 1.5 h using a growth chamber (MIR553; SANYO, Osaka, Japan), and then they were transferred to a greenhouse maintained at 15°C for forcing. The bud-sprouting ratio was determined at 2–4-d intervals to judge freezing injury, the degree of which was indicated by the numbers of withering trees.

Measurement of water, sugar, starch, and N contents in CSSs

In the 2011/2012 and 2012/2013 seasons, water content, sugar content, starch and the total N concentration of CSSs were analyzed. We selected 3 CSSs with 5–10 buds on shoots that were between 30 and 40 cm in length per treatment. The CSSs were collected from each potted tree at about 3-week intervals from early November in the 2011/2012 season and from mid-October in the 2012/2013 season. The water content of the CSSs was measured by the drying method (24 h at 80°C), and the total N concentration was analyzed with a NC analyzer (SUMIGRAPH, NC-220F, Sumica Chemical Analysis, Ltd., Tokyo, Japan). Dried shoot samples of each tree were also used for extraction and analyses of soluble sugars (sorbitol, sucrose, fructose, and glucose) and starch, as described by Ito et al. (2012). Statistical analysis of the water content, sugar content, starch and total N concentration for CSSs was performed by the Tukey-Kramer test.

Effect of livestock waste compost application on endodormancy status using cut shoots in the 2011/2012 season

The CSSs (30–40 cm in length) were collected from each tree at about 3-week intervals beginning in November in order to monitor bud sprouting. They were placed into plastic containers with Rockwool cubes (50 × 50 × 40 mm) submerged in distilled water in a chamber set to 20°C under continuous dark conditions for forcing. The percentage of buds sprouting was determined at 2–4-d intervals. The degree of endodormancy, expressed as the percentage of buds sprouting, was recorded from 5 to 10 buds per shoot (a minimum of 3 shoots per replicate) for up to eight weeks after forcing (at 20°C). Sprout vigor to judge sprout promotion was also measured. In this study, endodormancy was complete when more than 70% of the buds had sprouted, as described by Sugiura and Honjo (1997). Bud sprouting was defined as occurring when green tissue under the bud scales became visible. Sprout vigor was defined as the number of days to initial and 60% bud break relative to the final number of sprouted buds (at 8 weeks) after forcing, as described by Potjanapimon et al. (2008).

Table 1. Experimental design.

| Year     | Treatment               | Freezing tolerance | Water content | Sugar content | Starch content | Nitrogen content | Bud sprouting |
|----------|-------------------------|--------------------|---------------|---------------|----------------|------------------|---------------|
| 2011–2012| Gray lowland soil       | □                  | □             | □             | □              | □                | □             |
|          | Gray lowland soil + compost (1:1) | □                | □             | □             | □              | □                | □             |
|          | Compost                 | □                  | □             | □             | □              | □                | □             |
| 2012–2013| Gray lowland soil       | □                  | □             | □             | □              | □                | □             |
|          | Gray lowland soil + compost (9:1) | □                | □             | □             | □              | □                | □             |
|          | Gray lowland soil + compost (1:1) | □                  | □             | □             | □              | □                | □             |

* Each measurement item was conducted.

* not conducted.
**Results**

*Effect of livestock waste compost on freezing tolerance*

There were no visible changes on the tree just after the freezing treatment, but, when the pots were transferred to the greenhouse, there were two types of morphological features in the potted trees; i) no bud burst was observed and ii) bud burst took place, but growth of the buds was arrested after bud swelling and then the bark turned black (Fig. 2). The effect of soil composition during the cold dehardening period on the freezing tolerance of potted trees is shown in Table 2. Freezing tolerance was defined as the temperature at which half of the trees were damaged or as the temperature that was lethal to 50% of the trees (LT50). The freezing tolerance of Japanese chestnut trees planted in soil + compost (1:1) was \(-10^\circ\text{C}\) in early February and \(-7^\circ\text{C}\) in early March, since the temperature of the amended soil-compost mixture was apparently higher than that in the soil-only treatment. In addition, the freezing tolerance of trees in soil + compost (9:1) was more than \(-16^\circ\text{C}\) in mid-February and more than \(-13^\circ\text{C}\) in late March. In addition, in a previous study (Sakamoto et al., in press), the freezing tolerance of trees in soil only was the same as that in the soil + compost (9:1) treatment results obtained in this study. The total survival rate in soil + compost (1:1) was inferior to those in other treatments at temperatures ranging from \(-4^\circ\text{C}\) to \(-16^\circ\text{C}\).

*Effect of livestock waste compost on the water, sugar, starch, and N contents of CSSs*

The water content of CSSs was the highest in the compost-only treatment and the lowest in the soil-only treatment in 2011/2012 (Fig. 3A). In 2012/2013, the water content of the CSSs decreased gradually towards winter in all treatments, with the highest value in soil + compost (1:1) and the lowest in soil-only treatment (Fig. 3B). In both years, the water content of CSSs tended to increase when an excess amount of compost was applied.

The total sugar content of CSSs increased rapidly with a peak in early February in both seasons. The soil-only treatment resulted in higher total sugar content than that in the other treatments, which subsequently decreased sharply towards early spring in both years regardless of the treatment (Fig. 4). There were significant differences in sugar content on 2 March in 2011/2012 (Fig. 4A) and on 12 November and 1 April in 2012/2013 between the soil-only treatment and the soil + compost (1:1) treatment (Fig. 4B), but there were no obvious differences among treatments during other portions of the experimental period.

The starch content of CSSs decreased rapidly, reached a minimum in January and subsequently increased sharply towards early spring in both years regardless of the treatment (Fig. 5). There were no significant differences among treatments during the experimental period except for 18 April in 2011/2012, when the starch content in soil + compost (1:1) was significantly higher than that in the soil-only treatment.

The effect of livestock waste compost on the total N content of CSSs is shown in Figure 6. The total N contents

### Table 2. The effect of livestock waste compost in the cold dehardening period on the freezing tolerance of potted trees (n = 2–4) in 2012 and 2013.

| Treatment date          | Treatment                          | Temperature (°C) | LT50 (°C)$^z$ | Total |
|-------------------------|-----------------------------------|-----------------|--------------|-------|
|                         |                                   | \(-4\) | \(-7\) | \(-10\) | \(-13\) | \(-16\) |       |
| 6–9 February, 2012     | Gray lowland soil                 | 2/2   | 2/2   | 2/2    | \(-13\) | 6/6    |       |
|                         | Gray lowland soil + compost (1:1) | 2/2   | 1/2   | 0/2    | \(-10\) | 3/6    |       |
| 18–22 February, 2013   | Gray lowland soil + compost (9:1) | 4/4   | 4/4   | 3/3    | \(-16\) | 11/11  |       |
| 6–9 March, 2012        | Gray lowland soil                 | 2/2   | 2/2   | 2/2    | \(-10\) | 6/6    |       |
|                         | Gray lowland soil + compost (1:1) | 2/2   | 1/2   | 1/2    | \(-7\)  | 4/6    |       |
| 17–19 March, 2013      | Gray lowland soil + compost (9:1) | 4/4   | 4/4   | 2/4    | \(-13\) | 10/12  |       |

$^z$ Freezing tolerance was evaluated as the temperature at which half of trees died referred to as lethal temperature 50 (LT50).

$^y$ Number of surviving trees/number of samples.
Fig. 3. The effect of livestock waste compost on the water content of CSSs of Japanese chestnut in 2011/2012 (A) and 2012/2013 (B). Gray lowland soil [soil only] (○); gray lowland soil + livestock waste compost (9:1) [soil + compost (9:1)] (△); gray lowland soil + livestock waste compost (1:1) [soil + compost (1:1)] (◇); livestock waste compost [compost only] (▽). Vertical bars indicate the standard error (SE) (n = 3). Different letters indicate a significant difference at the 5% level by the Tukey-Kramer test.

Fig. 4. The effect of livestock waste compost on the sugar content of CSSs of Japanese chestnut in 2011/2012 (A) and 2012/2013 (B). Gray lowland soil [soil only] (□); gray lowland soil + livestock waste compost (9:1) [soil + compost (9:1)] (△); gray lowland soil + livestock waste compost (1:1) [soil + compost (1:1)] (◇); livestock waste compost [compost only] (▽). Vertical bars indicate the SE (n = 3). Different letters indicate a significant difference at the 5% level by the Tukey-Kramer test.

Fig. 5. The effect of livestock waste compost on the starch content of CSSs of Japanese chestnut in 2011/2012 (A) and 2012/2013 (B). Gray lowland soil [soil only] (□); gray lowland soil + livestock waste compost (9:1) [soil + compost (9:1)] (△); gray lowland soil + livestock waste compost (1:1) [soil + compost (1:1)] (◇); livestock waste compost [compost only] (▽). Vertical bars indicate the SE (n = 3). Different letters indicate a significant difference at the 5% level by the Tukey-Kramer test.
in compost or soil + compost (1:1) were significantly higher than those in soil only on 13 February and 2 March in 2011/2012 (Fig. 6A), and on 10 December in 2012/2013 (Fig. 6B).

**Effect of livestock waste compost on endodormancy status using cut shoots**

The effect of livestock waste compost on bud sprouting is shown in Figure 7. Buds in the soil-only treatment were gradually released from endodormancy as manifested by an increase in bud sprouting, resulting in the completion of endodormancy on 28 December (Fig. 7A). As for the soil + compost (1:1) treatment, the bud-sprouting ratio was about 50% from the end of November to mid-December, but decreased to 20% at the end of December and eventually reached about 90% by the end of January (Fig. 7B). The bud-sprouting ratio in the compost-only treatment did not reach 70% until the end of January (Fig. 7C).

The effect of livestock waste compost on the sprout vigor is shown in Figure 8. The application of livestock waste compost accelerated bud break initiation, except for on 14 December and 28 December in 2011.

**Discussion**

The cultivation of the Japanese chestnut cultivar ‘Porotan’ has gradually increased throughout the chestnut-growing areas in Japan; however, accompanying this increase in cultivation, freezing injury has become more notable. Therefore, prevention measures must be developed. Livestock waste compost has been conventionally applied to chestnut orchards in fall (Fujita, 2014), which may affect freezing injury in the juvenile period. Therefore, to elucidate the cause of freezing injury, we examined the effect of supplemental N from livestock waste compost added to gray lowland soil on the freezing tolerance in terms of the carbohydrate, water and N status. We also investigated the relationship between the endodormancy statuses and freezing tolerance with or without the application of
In many woody plants, there is a positive correlation between sugar content and freezing tolerance (Sakai, 1962). Kuroda et al. (1985) also reported that the freezing tolerance of apple trees during mid-winter to early spring is closely associated with the sugar or starch content in fall; the lower the sugar or starch content, the lower the freezing resistance in mid-winter, which results in a sharp decrease in freezing tolerance in early spring. In both years of this study, the total sugar content of CSSs increased rapidly, reached a maximum toward the middle of winter and subsequently decreased sharply toward early spring in all treatments (Fig. 4). In addition, the sugar content of CSSs in the soil-only treatment was higher than that in the other treatments in both years, but there were no obvious differences in sugar content among treatments, even though freezing tolerance sufficient for survival was not achieved in the

![Fig. 8](image-url)
soil + compost (1:1) treatment. As for the starch content, there were no significant differences among treatments during the experimental period, except for on 18 April, 2012 (Fig. 5). From these results, we propose that the difference in freezing tolerance described above cannot be fully explained in terms of the sugar and starch content of CSSs.

The CSSs grown in the presence of livestock waste compost had a higher water content than the CSSs grown in the soil-only treatment (Fig. 3). Inoue et al. (2014) reported that the rise of soil moisture upon flooding treatment does not promote freezing injury in chestnut trees, suggesting that nitrogen supplementation in winter could be more effective for the occurrence of freezing injury than soil moisture. Kuroda et al. (1984) reported that N fertilization in fall stimulated root activity. It is plausible that N supply via livestock waste compost induced high water content. In addition, several reports have shown that N fertilization in late fall delayed the development of cold acclimation (Matsumoto et al., 2010; Ouzounis and Lang, 2011). In this study, N contents tended to increase in the CSSs grown in the presence of excessive livestock waste compost compared with those grown in gray lowland soil only (Fig. 6). The mobilization and recycling of N during endodormancy release played an important role in flower bud development in cultivars of peach, nectarine and plum (González-Rossia et al., 2008). Sakai (1982) suggested that earlier endodormancy completion accelerates water uptake by the roots under warm winter conditions, which results in a rapid reduction in freezing tolerance. Nevertheless, it is difficult to separate endodormancy and the cold acclimation process for many trees (Rohde et al., 1999). Endodormancy in woody perennials affects both the absolute hardness and the hardness transitions during annual cycles (Kalberer et al., 2007). On the other hand, to the best of our knowledge, little is known about the physiological relationships between nutrients and cold tolerance mechanisms in woody plants.

In the context of global warming, it is possible that the progression of endodormancy could also be a factor hindering cold acclimation. In this study, the date of reaching 70% bud sprouting was delayed by one month or more after applying livestock waste compost (Fig. 7). This delay in endodormancy completion observed in this study is in conflict with the result mentioned above; however, applying livestock waste compost was effective in accelerating bud break initiation, except for from mid-December to the end of December in 2011/2012 (Fig. 8). In general, the vigor and rate of sprouting are indices that show the degree of dormancy (shallow or deep) and the sprouting ability (Kawamura et al., 2004). From these results, we hypothesize that the application of livestock waste compost does not delay the endodormancy completion, but rather accelerates it; that is, the degree of endodormancy may be shallow in the presence of excess N from livestock waste compost. In the Japanese chestnut cultivar used in this study, low bud sprouting in the livestock waste compost treatment may have been caused by buds that lost or lacked the ability for bud sprouting via delayed CSS maturity, including bud development. From the results of this study, we propose that supplementation of N affected the degree of endodormancy, possibly due to the stimulation of root activity. In fact, new fibrous roots appeared on the apple trees with NO3 treatment at low temperature (7.2°C) in apple and peach trees (Nightingale, 1935). It is noteworthy to note that nitrate is not only a major nitrogen source but also a signaling molecule that modulates the expression of a wide range of genes and that regulates growth and development (Konishi and Yanagisawa, 2013). Japanese chestnut trees growing in gray lowland soil + livestock waste compost (1:1, v/v) had a 3°C higher LT50 than trees grown in gray lowland soil in early February and March, but there were no obvious differences between the soil-only and soil + compost (9:1) treatments (Table 2). These results suggest that the depth of endodormancy was shallow upon applying excessive livestock waste compost, and after that, sufficient freezing tolerance could not be obtained for the winter period as a result. Our results may also indicate the possibility that the progression of endodormancy is related to the process of freezing hardiness acquisition process.

In general, when young Japanese chestnut trees are planted, compost is added to about 10%–20% of the total soil volume (Kamio, personal communication). In this study, trees grown under the soil + compost (9:1) treatment had nearly the same freezing tolerance as trees grown under the soil-only treatment. Therefore, we assume that the risk of freezing injury was enhanced by applying significant amounts of compost every fall, in addition to the compost added at the time of planting. In either case, even if using livestock waste compost, which is one of the controlled-release fertilizers, nitrogen fertilizer should be used properly in terms of the timing and quantity applied. Further studies will be necessary to clarify the influence of other soil characteristics on the freezing tolerance of Japanese chestnut trees.

**Literature Cited**

Fujita, Y. 2014. A manuring practice that supports consistent production and environmental preservation of Japanese pear. Kajitsu Nippon 69(5): 54–58 (In Japanese).

González-Rossia, D., C. Reig, V. Dovis, N. Gariglio and M. Agusti. 2008. Changes on carbohydrates and nitrogen content in the bark tissues induced by artificial chilling and its relationship with dormancy bud break in Prunus sp. Sci. Hortic. 118: 275–281.

Inoue, H., S. Kusaba, D. Sakamoto, Y. Mizuta and S. Kamio. 2014. Influence of nitrogen fertilization during winter on freezing injury of chestnut trees. Hort. Res. (Japan) 13 (Suppl. 2): 373 (In Japanese).

Ishizuka, Y. 1984. Sehi. 5. Kachikufunnyonoriyou. p. 72–73. In:
