Biocontrol actinomycetes better protects cell membranes in celery (Apium graveolens L.) under freezing stress in the presence of fungal pathogen

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Abstract. Beneficial microbes can mitigate biotic or abiotic stress-induced damage to plant cell membranes. Yet, little is known about the effects of actinomycetes on cell membrane permeability in plants under joint biotic and abiotic stresses. Herein, the effects of three biocontrol actinomycetes (Streptomyces pactum Act12, S. globisporus Act7, and S. globisporus subsp. globisporus C28) on cell membrane permeability in the leaves of celery (Apium graveolens L. cv. “Hanyusiji”) were evaluated under fungal pathogen (biotic) and freezing (abiotic) stresses by using electrical conductivity measurements. Our results showed that, under freezing stress, any of three fungal pathogens alone resulted in increased cell membrane permeability. Under the single stress of freezing, medium and high concentrations of C28 respectively reduced cell membrane permeability by 37.0% and 30.6%; Act7 exerted no significant effects, whereas high concentration of Act12 increased cell membrane permeability. Under the dual stresses of fungal pathogen and freezing, these protective effects of Act12, Act7, and C28 did not differ significantly. Nonetheless, these protective effects depended on the type of pathogen infection involved: the largest reduction in cell membrane permeability occurred in the presence of F. oxysporum f. sp. vasinfectum (46.4-69.2%) followed by A. alternata (17.4-51.8%), with F. sambucinum ranked lowest (8.8-35.5%). In conclusion, inoculating an appropriate concentration of actinomycetes can mitigate freezing-induced cell membrane injury in celery plants. Importantly, the actinomycete strains better protected the cell membrane against freezing injury under fungal pathogen stress, but this benefit depends on the adverse effects of pathogens on cell membrane permeability.

1. Introduction

The cell membrane performs important physiological functions in plants, for example, by selecting and regulating the materials transported into and out of the cells and maintaining a stable intracellular environment [1]. However, many biotic [2,3] or abiotic [4,5] stresses in nature can cause degrade or destroy the structure and function of plant cell membranes. Under stressful conditions, the reactive oxygen species (ROS) metabolism system is disrupted in affected plants, leading to an increase in...
ROS production [6]. This, in turn, causes membrane lipid peroxidation or membrane delipidization, which results in a decreased unsaturated fatty acid content, reduced membrane fluidity, increased membrane permeability, and even membrane phase separation, which damage the cell membrane’s structure and impair its functions. If severe enough, this cell membrane destruction can increase electrolyte leakage from the plant cells and thereby increase their electrical conductivity (EC). Electrolyte leakage from cells is thus an important indicator as it reflects the extent of cell membrane damage and may be used to characterize the stress resistance of plants. Some abiotic [7,8] and biotic [9-12] factors can induce the activation of the antioxidant enzyme system, which leads to a greater content of osmoregulatory substances in plants, thereby mitigating stress-induced damage to their cell membranes.

Beneficial microbes have been found to facilitate plant growth, improve crop yield, and enhance plant resistance to biotic stresses [13], such as fungal [14], bacterial [15], viral [16,17], and nematode [18] diseases. In addition, some of these beneficial microorganisms, most of which are bacteria and fungi, can mitigate plant damage caused by abiotic stresses, such as drought [11,19], salt [20] and low temperature [21,22], or improve the stability of plant cell membranes under stresses [9-12]. For example, rhizosphere growth-promoting bacteria (Pseudomonas spp.) can induce the greater accumulation of osmoregulatory substances (e.g., proline and betaine) in plants, maintain the water content in plant cells, and reduce electrolyte leakage via the cell membrane under conditions of drought stress, thus reducing overall levels of cell membrane injury [11]. Further, some rhizosphere growth-promoting bacteria can also reduce the permeability of the cell membrane and improve its stability in plants under salt stress [23].

Low-temperature stresses in nature are often divided into chilling (i.e., 0-15°C) and freezing (i.e., < 0°C) temperature conditions. It has been reported that after a certain period of chilling acclimation (> 0°C), beneficial bacteria [9,12,22] or fungi [24,25] inoculated into plants can reduce the damage to the latter’s cell membranes caused by chilling, thus increasing the plants’ resistance to cold, with several beneficial bacteria (e.g., Clavibacter and Pseudomonas spp.) found able to attenuate chilling-induced membrane lipid peroxidation, ROS injury, and ion leakage of the membranes [9,12]. Currently, whether or not beneficial microbes can protect the plant cell membrane under freezing conditions remains unclear. Moreover, no studies have yet evaluated the effects of beneficial microbes on plant physio-chemistry (e.g., cell membrane permeability) under joint biotic and abiotic stresses that often co-occur in nature.

Actinomycetes are a large group of bacteria that can promote plant growth and control plant diseases [26-28]. Previous studies have demonstrated the control effects of a key actinomycete strain, Streptomyces pactum Act12, against multiple plant diseases in crops, such as root rot disease in strawberry (Fragaia ananassa Duchesne) caused by Fusarium oxysporum [29], gummy stem blight in melon (Cumis melo L.) caused by Didymella bryoniae [30], and root-knot nematode disease in tomato (Solanum lycopersicum Mill.) [31]. Furthermore, strain Act12 was shown to improve the quality and yield of medicinal plants, such as ginseng (Panax ginseng C. A. Mey) [27] and danshen (Salvia miltiorrhiza Bge) [26]. In addition to S. pactum Act12, two other strains, S. globisporus Act7 and S. globisporus subsp. globisporus C28, are known for their ability to inhibit mycelial growth and sclerotal germination of Sclerotium rolfsii, a pathogen of southern blight in monkshood (Aconitum carmichaeli Debx.) [32]. Presently, however, little is known about how these biocontrol strains of actinomycetes may affect plant cell membrane stability under abiotic stress (e.g., freezing), or under co-occurring biotic (fungal pathogen) and abiotic (freezing) stresses.

In this study, three biocontrol strains of actinomycetes—S. pactum Act12, S. globisporus Act7, and S. globisporus subsp. globisporus C28—were separately inoculated into potting soil at different concentrations. To assess their effects on cell membrane permeability in celery plant leaves, EC measurements were recorded under fungal pathogen (previously isolated from celery fields) and freezing stresses. The aim of this research was to investigate the protective effects provided by actinomycetes to celery plants jointly stressed by abiotic and biotic factors.
2. Materials and methods

2.1. Seeds and strains

Plant: Seeds of the celery (*Apium graveolens* L.) cultivar “Hanyusiji” were provided by the Shanghai Enmao Horticulture Co., Ltd. (Shanghai, China).

Bacterial and fungal strains: Three fungal pathogens, *Fusarium sambucinum* (Fs; accession number: MH588395), *Alternaria alternate* (Al; accession number: MH571759), and *F. oxysporum* f. sp. *vasinfectum* (Fv; accession number: MH571873), were isolated from celery fields affected by celery root rot in Baosheng Village, Taibus Banner, Inner Mongolia. Three biocontrol actinomycete strains, *Streptomyces pactum* Act12 (accession number: MH542148), *S. globisporus* Act7 (accession number: MH819503), and *S. globisporus* subsp. *globisporus* C28 (accession number: MH819504), were isolated from the Ledu County in the Qinghai-Tibet Plateau. The strains were identified using morphological observations combined with a gene sequencing analysis. Pure cultures were cryogenically preserved with sterilized glycerol (fungal strains) or sand and soil (actinomycete strains) in the Laboratory of Microbial Resources, School of Resources and Environmental Sciences, Northwest A&F University (Yangling, Shaanxi Province, China).

2.2. Potting experiment

Preparation of potting soil: Potting soil was obtained from the 0-20 cm topsoil of a Lou soil in Yangling, Shaanxi Province, China. The field soil was passed through a 1-cm sieve after removing any stones and grass roots in it. Manure (Mengda substrate, Inner Mongolia, China; 15 g/kg), urea (0.25 g/kg), and calcium phosphate (0.50 g/kg) were added to the sieved soil and thoroughly mixed in; 5 kg were dispensed into each pot (24-cm diameter × 32-cm height).

Preparation of the actinomycete agents: The biocontrol agents used for our potting experiment were prepared with the actinomycete spores of Act12, Act7, and C28. These spore powder of Act12, Act7, and C28 were prepared by solidstate fermentation, and these three agents contained 4.5 × 10^10, 1.0 × 10^11, and 3.2 × 10^9 colony-forming units (CFU)/g, respectively.

Preparation of the pathogen spores: Three fungal pathogens were grown on potato dextrose agar plates. Their spores were washed separately with 10 mL of sterile water and counted with a hemocytometer. Spore suspensions were respectively mixed with peat powder to obtain a final spore concentration of 10^6 CFU/g of peat powder.

Design of the potting experiment: This experiment was conducted in a greenhouse on the campus of the Northwest A&F University. Celery seeds were sown into each pot on May 20th. Four treatments were used: no pathogen infection or actinomycete inoculation (control), pathogen infection alone, actinomycete inoculation alone, and pathogen infection+actinomycete inoculation. For the actinomycete-inoculated treatments (i.e., actinomycete alone or pathogen+actinomycete), the biocontrol agents of actinomycetes were respectively mixed in with the potting soil at three different concentrations: high (1.0 g/kg), medium (1.5 g/kg), and low (2.0 g/kg). Pathogens were respectively inoculated to potting soil using the same concentration of 10^2 CFU/g. Six pots were prepared per treatment, with three plants per pot, ninety-six pots in total. Pots were then placed in a greenhouse at 28°C, with sunlight for 12 h per day on average.

Stress treatments: Pathogen stress was applied when the celery seeds were sown. Then, after five months of growth, celery plants in all four treatments were subjected to the freezing stress (< 0°C) for 12 h.

2.3. Electrical conductivity measurements

After imposing the freezing stress, six biological samples (replicates) were taken from each treatment group: each sample consisted of the second-most recently expanded leaves (downward from the shoot point) from three plants (growing for five months) in the same pot. Leaf samples used to assay enzyme activity were directly placed into an ice-box after being sampled. All samples were taken to the...
laboratory, where small leaf discs were immediately cut out using a hole puncher (hole diameter = 7 mm. Caution was taken to avoid large veins on each leaf.

The leaf discs were mixed uniformly and immediately weighed to obtain three subsamples (1.0 g each). These subsamples were respectively transferred into three centrifuge tubes, to which 10 mL of distilled water was added. The tubes were then placed in a vacuum desiccator and pumped with an air exhauster for 7-8 min, to extract the air from the intercellular space. The air was then slowly charged; this compressed water into the leaf tissues, allowing the leaf samples to sink. Next, the centrifuged tubes were taken out and placed onto a shaker for extraction duration of 1 h. The EC of the solution was measured with a conductivity meter (DDSJ-319L , Leici, Shanghai, China) at 25°C. After these measurements, the centrifuged tubes were put in a 100°C boiling water bath for 15 min to deactivate cells in the plant tissues. The tubes were then cooled in tap water for 10 min and the EC after boiling was measured with the same conductivity meter at 25°C.

2.4. Calculating the relative permeability and extent of cell membrane injury

The relative permeability (RP) of a plant cell membrane was calculated using formula (1), and the extent of injury (EI) of cell membrane was calculated using formula (2).

\[
\text{RP} / \% = \frac{L_1}{L_2} \times 100
\]

\[
\text{EI} / \% = (1 - \frac{1 - T_1/T_2}{1 - C_1/C_2}) \times 100
\]

where \(L_1\) and \(L_2\) are the EC values of exudate from celery leaves before and after the heat treatment (water boiling), respectively; \(C_1\) and \(C_2\) are the EC values of exudate from the control leaves before and after the heat treatment, respectively; \(T_1\) and \(T_2\) are the EC values of exudate from the treatment leaves, respectively.

2.5. Statistical analysis

Values presented are mean ± standard deviations. All data were analyzed by a one-way ANOVA to test the significance of difference using the SAS software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of pathogen stress on cell membrane permeability in celery leaves under secondary freezing stress

Compared with the control, infection with any of the three pathogens alone resulted in higher cell membrane permeability in the leaves of celery under the freezing stress treatment. The cell membrane RPs in Al- and Fv-infected plants were 25.2% and 33.5%, which were 62.2% and 115.8% higher than those of control plants, respectively (\(P < 0.05\); Figure 1a). The cell membrane EIs were 11.4 and 21.3 times higher in Al- and Fv-infected plants than in the control plants, respectively (Figure 1b). The RP of the cell membrane in Fs-infected plants was 16.4%, which was slightly, but not significantly (5.8%), higher than that of control plants (Figure 1a). These results indicated that after pathogen infection alone, the cell membrane in celery leaves was destroyed and its permeability considerably increased under freezing stress, which exacerbated the extent of freezing injury.

3.2. Effect of biocontrol actinomycetes on cell membrane permeability in celery leaves under the single stress of freezing

Compared with the control treatment, inoculation with the actinomycete strain Act12 alone at 1.0 and 1.5 g/kg reduced the RP of cell membranes in celery leaves by 19.5% and 35.8%, respectively; however, these differences were not statistically significant. By contrast, the cell membrane RP reached 36.7% with Act12 inoculated at 2.0 g/kg, which was 136.2% higher than the control (\(P < 0.01\); Figure 1a). These results indicated that a high concentration of strain Act12 strengthened the
damaging effect of freezing on the cell membrane in celery leaves, whereas a low concentration of Act12 mitigated this impact.

Inoculation with the actinomycete strain Act7 or C28 alone resulted in a 7-37% reduction in cell membrane RP compared with the control, except for the concentration of 1.0 g/kg. This reduction in RP was significant with C28 inoculated at 1.5 and 2.0 g/kg ($P < 0.01$; Figure 1a). Cell membrane EIs were reduced by 1.3-6.8% in Act7- or C28-inoculated plants when compared with control plants (Figure 1b). These results indicated that the Act7 and C28 treatments mitigated freezing injury and improved plant resistance to freezing stress in celery.

![Figure 1. Effects of fungal pathogens or biocontrol actinomycetes on the relative permeability (a) and extent of injury (b) of cell membranes in leaves of celery plants under single stress of freezing. Control, no pathogen infection or biocontrol strain inoculations; Fs, Fusarium sambucinum; Al, Alternaria alternate; Fv, Fusarium oxysporum f. sp. vasinfectum; Act12, Streptomyces pactum; Act7, S. globisporus; and C28, S. globisporus subsp. globisporus C28. Bars are the mean ± standard deviation. * $P < 0.05$ and ** $P < 0.01$ compared with control group.](image)

**Figure 1.** Effects of fungal pathogens or biocontrol actinomycetes on the relative permeability (a) and extent of injury (b) of cell membranes in leaves of celery plants under single stress of freezing. Control, no pathogen infection or biocontrol strain inoculations; Fs, *Fusarium sambucinum*; Al, *Alternaria alternate*; Fv, *Fusarium oxysporum* f. sp. *vasinfectum*; Act12, *Streptomyces pactum*; Act7, *S. globisporus*; and C28, *S. globisporus* subsp. *globisporus* C28. Bars are the mean ± standard deviation. * $P < 0.05$ and ** $P < 0.01$ compared with control group.

3.3. **Effect of biocontrol actinomycetes on cell membrane permeability in celery leaves under dual stresses**

3.3.1. *F. sambucinum*+freezing When the freezing stress occurred immediately after Fs infection, the RPs tended to decreases (by 8.8-35.5%; Figure 2a) and there were reductions in the EIs of cell membranes (by 0.7-5.8%, Figure 2b) in all celery plants inoculated with actinomycete (Act12+Fs, Act7+Fs, and C28+Fs) versus the non-inoculated plants (i.e., Fs alone). Cell membrane RPs increased with a greater concentration of C28, and significant differences were found between the C28+Fs and Fs treatments at 1.0 and 1.5 g/kg ($P < 0.05$; Figure 2a). This indicated that a lower concentration of C28 had a greater protective effect on the cell membrane in celery leaves.

Similar to the results obtained for C28, a low concentration of Act7 resulted in the greatest reduction in cell membrane RP ($P < 0.05$). When Act12 was inoculated, a concentration of 1.5 g/kg resulted in the largest reduction (35.5%) in the RP values ($P < 0.01$), while a significant increase occurred at a concentration of 2.0 g/kg ($P < 0.05$); no significant differences were found for applications of 1.0 g/kg (Figure 2a). Together, these results indicated that after Fs infection, inoculation with any of the three actinomycete strains mitigated freezing injury and improved cold resistance in celery.
3.3.2. *A. alternate*+freezing When the freezing stress occurred right after the Al infection, the cell membrane RPs became markedly reduced in the Act12+Al, Act7+Al, and C28+Al treatments when compared with the Al treatment. The RPs of cell membranes decreased with an increasing concentration of Act12, which were 17.4%, 41.2%, and 51.8% lower for Act12+Al relative to the Al treatment (P < 0.01 or P < 0.05; Figure 3a). The corresponding EIs of cell membranes were reduced by 45.4%, 107.7%, and 134.9% for Act12+Al compared with the Al treatment (Figure 3b).

Similar effects were observed with Act7 and C28, in that the cell membrane permeability increased with an increasing inoculation concentration. The Act7+Al treatment significantly reduced the RPs (by 34-50.6%, P < 0.05) and EIs (by 81.6-11.9%) of cell membranes in comparison with the Al treatment. The C28+Al treatment reduced cell membrane RPs by 34-50.6%, and this decrease was highly significant at a low concentration of C28 compared with the Al treatment (P < 0.01); the corresponding EIs of cell membranes were reduced by 46.4-157.3% (Figure 3). These results indicated that in the presence of Al, all three actinomycete strains reduced the relative permeability and EI of cell membrane in the leaves of celery under freezing stress, which improved this plant’s cold resistance more efficiently than did Fs.

3.3.3. *F. oxysporum*+freezing When the freezing stress suddenly occurred after the Fv infection, both the RPs and EIs of celery’s cell membrane were markedly reduced in the Act12+Fv, Act7+Fv, and C28+Fv treatments compared with the Fv treatment. The reductions in RPs ranged from 46.4% to 69.2%, all of which were highly significant differences (P < 0.01; Figure 4a). The corresponding EIs were reduced by 86.6-129.0% (Figure 4b).

A low concentration (1.0 g/kg) of Act7 resulted in the greatest reduction in cell membrane RP, which correspondingly reduced the EI by 129.0%; conversely, the RP increased with an increasing concentration of this strain. Both Act12 and C28 exerted the highest protective effect on cell membranes at a medium concentration (2.0 g/kg), for which the RPs were lowest, at 64.5% and 63.4%, respectively, while the corresponding EIs were reduced by -120.2% and 118.2%, respectively. Collectively, these results indicated that in the presence of Fv, all three actinomycete strains improved cold resistance in celery, and these improvement effects were superior to those arising from the two other pathogens, Fs and Al.
Figure 3. Effects of biocontrol actinomycetes on the relative permeability (a) and extent of injury (b) of cell membranes in leaves of celery plants under the dual stresses of *Alternaria alternate* and freezing. Al, *Alternaria alternate*; Act12, *Streptomyces pactum*; Act7, *S. globisporus*; and C28, *S. globisporus* subsp. *globisporus C28*. Bars are the mean ± standard deviation. * P < 0.05 and ** P < 0.01 compared with control group.

In summary, all three biocontrol strains of actinomycetes tested here markedly improved the cold resistance of celery plants and mitigated freezing injury to their cell membranes in the presence of different fungal pathogens.

Figure 4. Effects of biocontrol actinomycetes on the relative permeability (a) and extent of injury (b) of cell membranes in leaves of celery plants under dual stresses of infection by *Fusarium oxysporum* f. sp. *vasinfectum* and freezing. Fv, *Fusarium oxysporum* f. sp. *vasinfectum*; Act12, *Streptomyces pactum*; Act7, *S. globisporus*; and C28, *S. globisporus* subsp. *globisporus C28*. Bars are the mean ± standard deviation. * P < 0.05 and ** P < 0.01 compared with control group.

4. Discussion
Actinomycetes comprise a large group of bacteria used for biocontrol, but their effects on the stability of plant cell membranes under low-temperature (chilling or freezing) conditions remain unclear. In this study, we found that inoculation with biocontrol actinomycete strain(s) alone affected cell membrane permeability in the leaves of celery under freezing stress in a concentration-dependent manner. Our findings suggest that *S. globisporus* subsp. *globisporus* can be inoculated at a low or medium concentration to improve cell membrane stability and enhance plant resistance to freezing stress in celery crops.

We also found that compared with the control, prior infection with any of the three fungal pathogens alone resulted in increased cell membrane permeability and electrolyte leakage in celery
leaves under freezing stress. In particular, *Alternaria alternate* and *Fusarium oxysporum* f. sp. *vasinfectum* increased the cell membrane permeability significantly. In the process of infecting plants, pathogens can degrade the host cell membrane, leading to increased membrane permeability which causes electrolyte leakage from the cells [33]. However, under the same freezing condition, the three fungal pathogens affected the cell membrane integrity in celery leaves to varying degrees. This may have been due to the fact that the pathogenicity to celery and the toxins produced by various pathogens are not the same.

*A. alternate* is known to infect celery plants and induce leaf spot disease in celery. The main pathogenic factor of *A. alternate* lies in the toxin it produces, namely tenuazonic acid [34], alternariol, and altenuariol monomethyl ether [35]. When plant cells are exposed to *A. alternate*, these toxins can bind to proteins in the plasma membrane of the host cells, causing damage to the latter’s membrane structure and function, as evidenced by membrane permeability changes, electrolyte leakage, and conductivity increases [36].

In the course of pathogenesis induced by *Fusarium*, fusariotoxins are not only an important causal factor for greater cell membrane permeability in crops but also for plant diseases and severe reductions in crop yield [37]. Fusariotoxins are a variety of secondary metabolites produced by different fungi in the genus *Fusarium*, and mainly included the enniatins A and B, fusaric acid, 10-dehydrofusaric acid, lycomarasmine, and lycomarasamic acid. Fusariotoxins are non-host selective toxins that can cause various plant diseases, such as wilting, root rot, and ear rot [37]. Despite being affiliated with the *Fusarium* genus, both *F. sambucinum* and *F. oxysporum* f. sp. *vasinfectum* caused a markedly different extent of injury to host cell membranes in celery plants in our study. A plausible reason for this disparity is that the toxins produced by these two *Fusarium* species are very different. For example, *F. sambucinum* produces the enniatins A and B, while *F. oxysporum* f. sp. *vasinfectum* produces fusaric acid and dehydrofusaric acid [38].

Under the single stress of freezing, cell membrane permeability in celery leaves was reduced considerably by medium and high concentrations of *S. globisporus* subsp. *globisporus* C28, though apparently unaffected by different concentrations of *S. globisporus* Act7, yet increased by a high concentration of *S. pactum* Act12. However, under the dual stresses of pathogen infection and freezing, all three actinomycete strains were able to reduce cell membrane permeability in celery leaves when inoculated at an appropriate concentration. In particular, the maximum reduction in cell membrane permeability by strain C28 was almost twice that generated under the single freezing stress. This key result indicates a pathogen’s presence stimulated the protective effects of these actinomycete strains on celery’s cell membrane under freezing stress.

The above phenomenon may be attributable to the fact that the biocontrol actinomycetes are capable of inducing systemic resistance in plants [39-41]; for example, strain Act12 can increase the activity of plant defense-related enzymes [30,31]. Once systemic resistance is activated in plants they enter a more sensitive state, one in which only the invasion of pathogens can induce greater expression levels of plant resistance-related genes [42,43]. Subsequently, defense-related antioxidant enzyme systems (e.g., peroxidase) are activated, which are more effective at scavenging ROS, thereby reducing ROS injury to the cell membrane [9,10]. In summary, the protective mechanism of the biocontrol actinomycetes acting upon celery plants under pathogen stress enhances their resistance to secondary freezing stress and thus reducing the risks of freezing injury to its cells. Details of how this mechanism(s) operates are unknown and remain to be studied. We recommend future research focus on biocontrol actinomycetes’ effects on relevant physio-biochemical parameters and gene expression levels in plants solely under freezing stress and jointly under pathogen and freezing stress factors.

5. Conclusions
We found that inoculating an appropriate concentration of biocontrol actinomycetes can reduce cell membrane permeability and mitigate freezing-induced cell membrane injury in celery plants. Any of three fungal pathogens alone resulted in increased cell membrane permeability and electrolyte leakage in celery leaves under freezing stress. Importantly, the actinomycete strains better protected the cell
membrane against freezing injury while under fungal pathogen stress, but this benefit depends on the adverse effects of pathogens on cell membrane permeability.

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