Isolation and Identification of Ice Nucleation Active *Fusarium* Strains from Rapid Apple Declined Trees in Korea

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In biological particles such as *Fusarium* species, ice nucleation activity (INA) has been observed. *Fusarium* strains isolated from apple declined trees in Korea were identified with a multilocus sequence analysis using the *tef1* and *rpb1* genes. Droplet-freezing and tube-freezing assays were used to determine the INA of the strains, using *Pseudomonas syringae* pv. *syringae* KACC 21200 as a positive control and resulting in seven INA+ fungal strains that were identified as *F. tricinctum* (KNUF-21-F17, KNUF-21-F18, KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, KNUF-21-F43, and KNUF-21-F44). The effect of *Fusarium* INA+ KNUF-21-F29 was compared to that of INA– strains on *Chrysanthemum morifolium* cv. Shinma explants. A higher callus formation and no-shoot formation were observed, suggesting that fungal INA could play a role in cold injuries and be a factor to consider in rapid apple decline. To the best of our knowledge, this is the first report of INA fungal strains isolated in Korea.

Keywords: cold injury, *Fusarium*, ice nucleation activity

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but the frequency, distribution, and mechanisms of this INA within the genus are still not well known (Kunert et al., 2019). In Korea, only *Pseudomonas syringae* pv. *syringae* has been reported as an INA-positive microorganism (Kim et al., 1987; Lim et al., 2019), however, no fungal strains have been identified as INA-positive. Therefore, it is important to evaluate this characteristic in fungal isolates and further study its potential ecological role. This research aims to evaluate INA in fungal strains related to RAD in hopes of further understanding the role of this characteristic in RAD, cold injuries, and plant growth.

In 2021, during a survey of RAD trees in apple orchards in Chungbuk, Jeonbuk, Gyeongbuk, and Gyeonggi provinces, Korea, fungal strains were isolated from branches of RAD trees (*Malus domestica*) and cultured in potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25°C. *Fusarium* isolates were chosen based on cultural characteristics and further used in this study, in addition to collected pathogens such as *Botryosphaeria* sp., *Diaporthe* sp., *Rosellinia* sp., and *Valsa* sp. that were used as control strains for the experiment. The HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) was used to extract total genomic DNA from the strains according to the manufacturer’s instructions. A polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region using the ITS1F/ITS4 (Gardes and Bruns, 1993; White et al., 1990) primer pair. The amplified PCR ITS region products were purified and sequenced (Bioneer Co., Daejeon, Korea) and a total of 29 fungal strains; 21 *Fusarium* spp., 3 *Diaporthe* spp., 3 *Botryosphaeria* spp., 1 *Rosellinia* sp., and 1 *Valsa* sp. were identified at the genus level using the National Center for Biotechnology Information’s (NCBI) Basic Local Alignment Tool (BLAST) (data not shown).

Based on previous research, a droplet-freezing assay was used to assess the strains’ INA (Lindow et al., 1978; Pouleur et al., 1992). All the isolated fungal strains were grown on PDA and potato dextrose broth (PDB; Difco) at 25°C for 7 days. PDA-grown mycelium was collected and suspended in 500 µl of sterile double distilled water (DDW) and vigorously homogenized. Five 10-µl droplets per sample were placed on a working surface maintained at −8°C to −7°C in a styrofoam container with an ethanolic mixture. The working surface was prepared by coating aluminum foil with mineral oil and folding it into a tray shape. Frozen droplets were observed visually, and a strain was considered as INA positive if 3 to 5 droplets froze within 1 min. DDW was used as a negative control and *Pseudomonas syringae* pv. *syringae* strain KACC 21200 was used as a positive control. Other RAD tree isolated strains; *Botryosphaeria* spp., *Diaporthe* spp., *Rosellinia* sp., and *Valsa* sp. were also tested. As a result of the mycelial suspension droplet-freezing assay, 7 *Fusarium* sp. isolates out of 21 revealed high INA (5/5 frozen droplets), the same outcome as *P. syringae* pv. *syringae* KACC 21200. The remaining 14 *Fusarium* sp. isolates, *Botryosphaeria* spp., *Diaporthe* sp., *Valsa* sp., and *Rosellinia* sp. showed negative INA (0/5-2/5 frozen droplets) (Table 1). PDB liquid cultures were filtered with ADVANTEC 0.45 µm disposable membrane filters to remove the mycelium, and the same process was repeated with these mycelium-free suspensions. *P. syringae* pv. *syringae* KACC 21200 was also cultured on liquid medium (Luria-Bertani broth, Difco) and filtrated using ADVANTEC 0.22 µm disposable membrane filters. As a result, 4 of the previously 7 *Fusarium* sp. INA-positive isolates; KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, and KNUF-21-F44 continued to show positive INA (3/5-5/5 frozen droplets) (Table 1). INA was not observed in the control culture filtrate of *P. syringae* pv. *syringae* KACC 21200. INA was confirmed again through a tube-freezing assay, in which 50 µl of culture filtrate were added in 0.2 ml tubes and the tubes were placed for 2 min on a refrigerated circulator bath (JEIO Tech Co., Daejeon, Korea) maintained at −8°C, obtaining the same results as the droplet-freezing assay (Supplementary Fig. 1). Although *Fusarium* is one of the best studied INA fungi, little is known about its INA mechanism (Kunert et al., 2019). Ice-nucleating proteins anchored to the outer cell membrane of INA+ bacteria have been reported to induce the formation of ice (Pandey et al., 2016), which could explain why INA was lost in the cell-free filtrate of *P. syringae* pv. *syringae* KACC 21200. In the case of fungi, INA may also be due to proteinaceous compounds produced by them (Failor et al., 2021). High activity has been reported in ice nucleation particles found in *F. avenaceum*. Additionally, it was discovered that these proteinaceous particles function independently from the fungal cells and are nanometers in scale (O’Sullivan et al., 2015). Fungal ice-nucleating proteins, unlike bacterial ones, are not anchored to the cells and therefore can be separated from the fungus. Because of these characteristics, even after filtration, the four above-mentioned *Fusarium* strains still showed INA. The absence of INA after filtration for KNUF-21-F17, KNUF-21-F18, and KNUF-21-F43 indicates the presence of fungal structures or ice-nucleating macromolecules with a more complex nature. Many *Fusarium* species produce a wide range of metabolites that belong to several classes of compounds; however, the biological activity of all these compounds has not been researched (Vesonder and Golinski, 2014). INA in *Fusarium* strains is not due to one single...
compound; therefore, further systematic studies are needed to better understand the INA phenomenon at a molecular level. INA-positive *Fusarium* sp. KNUF-21-F29, INA-negative *Fusarium* sp. KNUF-21-F37, and INA-negative *Botryosphaeria* sp. KNUF-21-B1 were selected to test the effect of *Fusarium* sp. INA on plant growth. *Chrysanthemum morifolium* cv. Shinma leaves were cut into 0.5-1.0 cm long segments and cultured on Murashige and Skoog

Table 1. Sample collecting location, strain identification and ice nucleation activity results

| Strain code  | Location                        | Species complex | Species       | Ice nucleation activity           |
|--------------|---------------------------------|-----------------|---------------|-----------------------------------|
|              |                                 |                 |               | From solid culture                | 0.45 μm filtrate | Final classification |
|              |                                 |                 |               | Classification                     | Classification |                          |
| KNUF-21-F15  | Boeun-gun, Chungbuk province    | FTSC            | *Fusarium tricinctum* | 0/5 – 0/5 – Negative |
| KNUF-21-F16  | Boeun-gun, Chungbuk province    | FTSC            | *F. tricinctum* | 0/5 – 0/5 – Negative |
| KNUF-21-F17  | Boeun-gun, Chungbuk province    | FTSC            | *F. tricinctum* | 5/5 + 0/5 – Positive |
| KNUF-21-F18  | Boeun-gun, Chungbuk province    | FTSC            | *F. tricinctum* | 5/5 + 1/5 – Positive |
| KNUF-21-F20  | Goesan-gun, Chunbuk province    | FOSC            | *Fusarium oxysporum* | 1/5 – 0/5 – Negative |
| KNUF-21-F23  | Boeun-gun, Chungbuk province    | FNSC            | *Fusarium commune* | 2/5 – 0/5 – Negative |
| KNUF-21-F24  | Boeun-gun, Chungbuk province    | FOSC            | *F. oxysporum* | 0/5 – 0/5 – Negative |
| KNUF-21-F25  | Boeun-gun, Chungbuk province    | FSSC            | *Fusarium solani* | 0/5 – 0/5 – Negative |
| KNUF-21-F26  | Boeun-gun, Chungbuk province    | FSSC            | *F. solani* | 0/5 – 0/5 – Negative |
| KNUF-21-F27  | Boeun-gun, Chungbuk province    | FSSC            | *F. solani* | 0/5 – 0/5 – Negative |
| KNUF-21-F28  | Boeun-gun, Chungbuk province    | FNSC            | *Fusarium commune* | 0/5 – 0/5 – Negative |
| KNUF-21-F29  | Gunwi-gun, Gyeongbuk province   | FTSC            | *Fusarium tricinctum* | 5/5 + 4/5 + Positive |
| KNUF-21-F32  | Muju-gun, Jeonbuk province      | FTSC            | *Fusarium tricinctum* | 5/5 + 4/5 + Positive |
| KNUF-21-F33  | Cheongsong-gun, Gyeongbuk province | FOSC         | *Fusarium oxysporum* | 0/5 – 0/5 – Negative |
| KNUF-21-F34  | Muju-gun, Jeonbuk province      | FTSC            | *Fusarium tricinctum* | 0/5 – 0/5 – Negative |
| KNUF-21-F36  | Cheongsong-gun, Gyeongbuk province | FTSC         | *Fusarium acuminatum* | 2/5 – 0/5 – Negative |
| KNUF-21-F37  | Cheongsong-gun, Gyeongbuk province | FTSC         | *Fusarium acuminatum* | 2/5 – 0/5 – Negative |
| KNUF-21-F38  | Boeun-gun, Chungbuk province    | FTSC            | *Fusarium tricinctum* | 5/5 + 5/5 + Positive |
| KNUF-21-F42  | Yeoju-si, Gyeonggi province     | FTSC            | *Fusarium acuminatum* | 1/5 – 0/5 – Negative |
| KNUF-21-F43  | Muju-gun, Jeonbuk province      | FTSC            | *Fusarium tricinctum* | 5/5 + 3/5 + Positive |
| KNUF-21-F44  | Muju-gun, Jeonbuk province      | FTSC            | *Fusarium tricinctum* | 5/5 + 3/5 + Positive |
| KNUF-21-D1   | Boeun-gun, Chungbuk province    | n/a             | *Diaporthe*   | 2/5 – 0/5 – Negative |
| KNUF-21-D2   | Gunwi-gun, Gyeongbuk province   | n/a             | *Diaporthe*   | 2/5 – 0/5 – Negative |
| KNUF-21-D3   | Muju-gun, Jeonbuk province      | n/a             | *Diaporthe*   | 1/5 – 0/5 – Negative |
| KNUF-21-Ros  | Boeun-gun, Chungbuk province    | n/a             | *Rosellinia*  | 0/5 – 0/5 – Negative |
| KNUF-21-B1   | Boeun-gun, Chungbuk province    | n/a             | *Botryosphaeria* | 0/5 – 0/5 – Negative |
| KNUF-21-B2   | Boeun-gun, Chungbuk province    | n/a             | *Botryosphaeria* | 0/5 – 0/5 – Negative |
| KNUF-21-B3   | Uiseong-gun, Gyeongbuk province | n/a             | *Botryosphaeria* | 0/5 – 0/5 – Negative |
| KNUF-21-Val  | Muju-gun, Jeonbuk province      | n/a             | *Valsa*       | 0/5 – 0/5 – Negative |

FTSC, *Fusarium tricinctum* species complex; FOSC, *Fusarium oxysporum* species complex; FNSC, *Fusarium nisikadoi* species complex; FSSC, *Fusarium solani* species complex; +, (positive) presents ice nucleation activity; –, (negative) does not present ice nucleation activity; n/a, does not apply.
(MS) medium containing 3 g/l of Gelrite and supplemented with 6-benzyladenine and α-naphthaleneacetic acid. Explants were cultured with 16 h photoperiod (Naing et al., 2016). Furthermore, 1.5 ml tubes were prepared with 1 ml of 0.45 µm filtrates of the previously mentioned strains and MS liquid medium as control. In sterile conditions, three to four explants were placed per treatment tube and tubes were placed on a refrigerated circulator bath (JEIO Tech Co.) set at −8°C for 1, 2, or 3 min. Explants were removed from the tubes after the cold treatment and excess treatment solution was dried out with sterile tissue paper. The explants were placed again in the plates containing MS medium and cultured as previously mentioned. After 7, 14, 24, and 30 days, the plates were photographed to assess explant development, shoot regeneration percentage, and the number of shoots per explant. Additional explants were left without any intervention. When comparing the treatments to the controls after 30 days, there was no significant difference in the regeneration percentage and the number of shoots per explant (data not shown). However, three types of tissue were overall observed in the Chrysanthemum explants: callus, shoot buds, and shoots (Fig. 1). For the explants with no intervention, shoots were mostly present (over 60%), while for the MS control and the INA negative strains KNUF-21-F37 and KNUF-21-B1, shoot buds were predominant. This shows that even without an INA effect, the explants suffered from cold stress. Both Fusarium strains KNUF-21-F37 and KNUF-21-F29 presented callus formation, with the INA-positive strain KNUF-21-F29 having the highest percentage of callus formation among treatments (50%). Callus consists of unspecialized and unorganized cells that can undergo differentiation when in the right conditions (Bhatia, 2015), and, in this case, the presence of this tissue can be related to the effect of the strain’s INA on the explants.

To identify the isolated Fusarium species, EF1/EF2 and RPB1-Fa/RPB1-R8 (or RPB1-F5/RPB1-G2r) were used to amplify the translation elongation factor 1-α (tef1) and the RNA polymerase largest subunit (rpb1), respectively (O’Donnell et al., 1998, 2010). Sequences from 21 samples for tef1 and rpb1 were obtained, registered in NCBI (tef1: LC702293-LC702313, rpb1: LC701711-LC701731), and identified through NCBI’s BLAST. A neighbor-joining phylogenetic tree constructed using MEGA version X (Kumar et al., 2016) based on the tef1 partial sequences showed that the fungal strains clustered together with their expected species complex (Supplementary Fig. 2), including species from F. tricinctum species complex (FTSC), F. oxysporum species complex, F. nisikadoi species complex, and F. solani species complex. In particular, INA-positive Fusarium strains were identified as members of the FTSC. A further phylogenetic analysis was done to get a better resolution on the phylogeny of the FTSC strains, combining tef1 and rpb1 partial sequences. The INA-positive strains KNUF-21-F17, KNUF-21-F18, KNUF-21-F29, KNUF-21-F32, KNUF-21-F38, KNUF-21-F43, and KNUF-21-F44 clustered together with F. tricinctum isolate 24E with high bootstrap values (Fig. 2).

Fungal pathogens such as Fusarium have been associated with apple decline syndrome (Villani, 2018). This genus comprises several species with reported INA, such as Fusarium acuminatum, F. avenaceum, F. tricinctum (FTSC), F. armenium, F. langsethiae (F. sambucinum species complex), F. begoniae, F. concentricum, and F. langsethiae (F. fujikuroi species complex) (Crous et al., 2021; Kunert et al., 2019). As mentioned above, the seven positive INA strains belonged to the FTSC and were identified as F. tricinctum, matching previously reported data. It is important to highlight that other INA negative strains such as KNUF-21-F16 and KNUF-21-F34 were also clustered together within the FTSC with F. tricinctum 24E (Supplementary Fig. 2). These findings are in good agreement with what was suggested by Kunert et al. (2019), indicating that not all strains

Fig. 1. Effect of different treatments and fungal ice nucleation activity (INA) on Chrysanthemum morifolium cv. Shinma explants and regeneration percentage after 30 days. Control, no intervention; MS, Murashige and Skoog medium; B1, Botrysphaeria KNUF-21-B1 (INA−); F37, F. acuminatum KNUF-21-F37 (INA−); F29, F. tricinctum KNUF-21-F29 (INA+). Data belongs to the 2-min treatment.
within one *Fusarium* species exhibit ice nucleation activity. The production of ice nucleation proteins requires energy; hence, it could be a trait that won’t be expressed all the time. Environmental conditions are thought to have an effect on the gene expression of this characteristic; however, the specific trigger is still not identified. Moreover, after a series of subcultures, some *Fusarium* strains can show a reduction of their INA, with some of them even losing this ability (Kunert et al., 2019).

In bacteria, INA has been extensively studied, and it has been suggested that bacterial ice nuclei play an important role in cold damage in many cold or frost-sensitive plants (Kishimoto et al., 2014; Lindow, 1983; Lindow et al., 1978). Furthermore, cold injury has been proposed to be a predisposing factor in terms of plant infection, making the infection more severe if the pathogen is INA-positive (Kennelly et al., 2007; Lindow, 1983). Cold extremes are expected to decrease because of climate change (IPCC, 2021). With temperatures getting continuously warmer and ice-nucleating microorganisms present in apple orchards, cold injuries can become more frequent and so does other related problems like RAD. With the present study, we look forward to contributing to a better understanding of the ecological role of ice nuclei active fungi. However, more research on fungal INA, its mechanisms, and its effect on different crops, like apple, both *in vitro* and under on-field conditions is necessary to eventually identify the role of INA fungi on cold injury and RAD.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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**Electronic Supplementary Material**

Supplementary materials are available at The Plant Pathol-
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