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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2022.137.143

Research Article
Identification of Pathogens Causing Anthracnose on King Oranges (*Citrus nobilis* var. Typica Hassk)

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Abstract
Background and Objective: Anthracnose is one of the main causes seriously affecting the fruit yield and quality of king oranges. This study aimed to identify the fungal pathogen *Colletotrichum gloeosporioides* causing anthracnose on king oranges in Vinh Long. **Materials and Methods:** Fruits with diseases were collected from the king orange garden located in Vinh Long in September, 2020. Firstly, symptoms of infected fruits were observed and recorded. Next, pathogens were isolated from these fruits and then tested using the Koch procedure. Specifically, the isolated strains of fungi were identified based on their morphological traits and ITS sequences on rRNA amplified by a pair of primers ITS1/ITS4. **Results:** A pure culture of the identified fungus was later artificially inoculated into healthy fruits for 1 week to confirm its pathogenicity. The fungal spores were cylindrical, rounded or obtuse at 2 ends, 12.8-16.4 mm in length and 3.47-5.27 mm in width. The ITS sequence of *Colletotrichum* causing anthracnose on king oranges had similar to the following sequences found on the Genbank with percent identities ranging from 97.93-98.29%. In the ITS sequence, the composition of GC was higher than that of AT, with 52.72% against 47.28%, respectively. The results of phylogeny demonstrated that the genetic sequence of the fungus causing anthracnose on king oranges had a close relationship with that of other species of *Colletotrichum* found on NCBI. **Conclusion:** The findings show that *Colletotrichum gloeosporioides*, a type of fungi, was the cause of anthracnose in king orange grown in Vinh Long province.

Key words: *Colletotrichum gloeosporioides*, ITS1/ITS4 primers, anthracnose, king orange, GC

Citation: Huong, B.T.C., H.L.K. Linh, T.T.B. Van, D.T.K. Tien and N.T.T. Nga *et al*., 2022. Identification of pathogens causing anthracnose on king oranges (*Citrus nobilis* var. typica Hassk). Pak. J. Biol. Sci., 25: 137-143.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anthracnose caused by a fungus, *Colletotrichum* spp., is one of the diseases which has serious impacts on fruit yield and quality of citrus trees grown in many countries around the world[1,2]. Due to anthracnose, fruit pedicels become dried and fruits prematurely fall before picking, leading to a decreased yield. In addition, anthracnose is also associated with low-quality fruits after harvest; therefore, fruit producers cannot meet the demand of domestic markets and export[3,4]. There have been many studies around the world, with results indicating that many species of *Colletotrichum* are the cause of anthracnose in citrus trees[2]. It is vital to identify which species of *Colletotrichum* causing the disease to effectively manage and control the disease[5]. It is demonstrated in many studies that *Colletotrichum gloeosporioides* are associated with anthracnose available in Australia, Vietnam, China, Italy, Maroc, Mexico, Pakistan, Ghana, Brazil, Algeria, Greece, New Zealand, Portugal, South Africa, Spain, Tunisia, America and Zimbabwe[6-16]. Research on *Colletotrichum karstii* has been done in South Africa, Portugal, America and Turkey[9,14,17,18]. Research on *Colletotrichum fructicola* has been carried out in China[19] while studies on *C. siamense* have been implemented in Mexico, Vietnam, Bangladesh, Egypt and Pakistan[7,9,13,20].

The Mekong delta is well known as the main area for fruit production in the South of Vietnam. In which, citrus crops including orange, pomelo, lemon and mandarin orange account for 135,000 ha (or 56% of the total fruit area of the South)[21]. King oranges are abundantly grown in Hau Giang and Vinh Long province and their production is expanded to many other locations such as Tra Vinh, Ben Tre and Can Tho city. King oranges of Vinh Long province are famous for the following features: Big size, thick skin, high in water and attractive colour of yellow in their pulp which meets customer tastes. Over the past years, the production of king oranges has brought enormous economic benefits to its producers[22].

In fruit trees, anthracnose is most common in rainy seasons. In Vietnam, however, information on anthracnose-causing pathogens on citrus trees in general and on king oranges, in particular, is still little. Therefore, this research only focused on pathogens causing anthracnose on king oranges grown in Vinh Long province. The study aimed at identifying the fungus *Colletotrichum* responsible for anthracnose on king oranges in Vinh Long, based on the morphological characteristics and genetic sequence of ITS located on rRNA using the ITS1/ITS4 primers.

MATERIALS AND METHODS

Study area: The study was conducted from September, 2020-June, 2021.

Materials

Samples: Fruits of king oranges with typical anthracnose symptoms were collected from Vinh Long province.

Culture media: WA (Water Agar), PDA (Potato Dextrose Agar) and liquid culture medium of PDA (Potato Dextrose Agar).

Chemicals used to extract fungal DNA, perform PCR amplification and electrophoresis included: CTAB buffer, Chloroform: Isoamyl alcohol (24:1), β-mercaptoethanol, SDS 10%, Proteinase K, enzyme RNAase, Isopropanol, Ethanol 70%, TE buffer, PHUSA PCR loading buffer 6X (with SyBr Green I), TAE buffer 1X, a ladder (Gene Ruler 100bp), Master Mix 2X, PCR water.

The 2 primers, namely ITS1/ITS4 were used to amplify ITS region[23,24], in which, the forward primer was ITS1(TCCGTAGGTGAACCTGCGG, while the reverse primer was ITS4 (TCCTCCGCTTATTGATATGC).

Fungal isolation and testing of pathogenicity of the isolated fungus: Pathogens in the infected fruits were isolated by Daoud *et al*.[14] with some modifications made to meet research conditions. Fruits with typical symptoms of anthracnose were collected from the king orange garden in Vinh Long and then taken back to the Plant Pathology Laboratory of the plant protection department, Can Tho University. Next, pathogens were isolated from these fruits and cultured until a pure culture of the fungus was obtained and stored. In detail, fruit tissues were cut into small pieces (about 2×2 mm in size) from the border between healthy and infected tissues and then these pieces were put on medical gauze and disinfected by alcohol 70% for 30 sec to 1 min. After that, these samples were dried by pieces of sterile absorbing paper and transferred to dishes of WA by forceps. The culture dishes with samples were put in lab conditions. Checking of the dishes was daily implemented. After hypha was visible, samples from the WA were transferred to and consecutively cultured on PDA until a pure fungal strain was obtained. The pure fungal strain was then identified based on its morphological characteristics and stored at 4°C in test tubes containing the inclined plane of PDA for further use.

Testing of the pathogenicity of the isolated fungus: The purified fungus was tested under lab conditions. Before the artificial inoculation, king oranges were checked and confirmed that they met the following criteria: Fruits were at 8.5 months old after blooming and even in size and healthy without symptoms of any diseases. Ten healthy fruits were included of which, 2 were selected as the controls. The remainings were processed for later use in inoculation with the fungus, meaning that they were washed under tap water and then disinfected with 70% alcohol to remove all of the possible external pathogens.

After that, 2 artificial wounds at 2 different positions on each fruit were created by pinching a bunch of sewing pins, one at the left cheek and another nearby the pedicel of the fruit. Each wound was 2 mm deep. Meanwhile, a perforated tool (3 mm in diameter) was used to make small round pieces on the fungus dishes (7-10 days of cultivation at 25°C, with 10^6 spores mL^{-1}). Next, 10 µL of sterile distilled water was added to an inoculation site. Finally, each piece of the fungus was placed on the fruit's site of inoculation. The 2 healthy fruits selected as the controls were inoculated with water only. Inoculated oranges were placed inside plastic bags which contained cotton balls soaked in sterile distilled water to create moisture. These bags were placed in a dark area at room temperature of 25°C and checked for the development of anthracnose 7 days upon the day of inoculation[3]. The checking for the disease was also implemented on 10 and 12 days of the inoculation. Finally, pathogens from the artificially infected fruits were re-isolated and then cultured on PDA at 25°C. Following the Koch principle[25], colonies and spores developed from the isolates were compared against those of the initial fungus purified from naturally infected fruits.

Identification of the anthracnose-causing fungus based on its morphological traits: Morphological traits used to identify the fungus include: Colour, development speed and colony structure, appearance and size of conidia, form and size of appressoria, with/without the formation of scleorotium[26].

Identification of the anthracnose-causing fungus based on molecular technologies

DNA extraction: The DNA extraction followed the CTAB based method, with some modifications[24]. About 10 mg of the fungus were cultured on liquid PDA for 3-5 days. After that, 10 mg of the hypha were crushed in 1 mL of CTAB buffer by using a porcelain mortar and pestle and then 10 µL of β-mercaptoethanol, 50 µL of SDS 10% and 5 µL of Proteinase K were added to. Next, the solution was transferred to a 1.5 mL Eppendorf. A mixture of chloroform: Isoamyl alcohol (with a ratio of 24:1) was added to the Eppendorf to create precipitates at the bottom of the Eppendorf. The precipitates were incubated in isopropanol at -20°C for 30 min. DNA precipitates were washed the 2nd time by using 70% alcohol and the collected DNA was dried and stored in 30 µL of TE buffer.

Checking of DNA concentration: The concentration and quality of collected DNA were confirmed by the UV-Vis NanoDrop spectrophotometer (Thermo).

DNA amplification (PCR): The total volume of each PCR reaction was 50 µL, with the following components: PCR water (19 µL), Maxter mix (25 µL), DNA (2 µL), ITS1 (2 µL), ITS4 (2 µL). PCR reactions performed following the thermal cycle[24], with some modifications in thermal cycles: Initial denaturation of DNA: 95°C for 5 min, 35 cycles of 3 stages including (1) 95°C for 30 sec (denaturation), (2) 58°C for 30 sec (annealing), (3) 72°C for 30 sec (extension). Next came 1 cycle of 10°C for 20 min. Finally, PCR products were stored at 4°C.

Sequencing and analysis: Sequencing of PCR products was implemented at Phu Sa Biochemistry Company. The forward and reverse primers were either cut out by Contig or added with various segments by using the BioEdit software. Phylogenetic analysis was performed based on the ITS sequence. All sequences were aligned and compared by the Clustal-W software so that differences in nucleotides among ITS segments of different species from the Genbank could be identified. The MEGA6 software was utilized to establish the phylogenetic tree to investigate the genetic relationship among species[23,27].

RESULTS AND DISCUSSION

Anthracnose symptoms: King oranges collected from the garden in Vinh Long had typical symptoms of anthracnose. The lesions were small round spots that were slightly indented, uneven and light to dark yellow. The skin at and around the lesions was dried and rough and the more severe the disease, the bigger the lesions spread in Fig. 1. The lesions were more likely to appear near or at pedicels and left cheek of fruits. These findings are comparable to those of Daoud *et al.*[14], who described 3 fungal

Fig. 1: Symptoms of anthracnose on king oranges caused by the fungus of *Colletotrichum* spp.

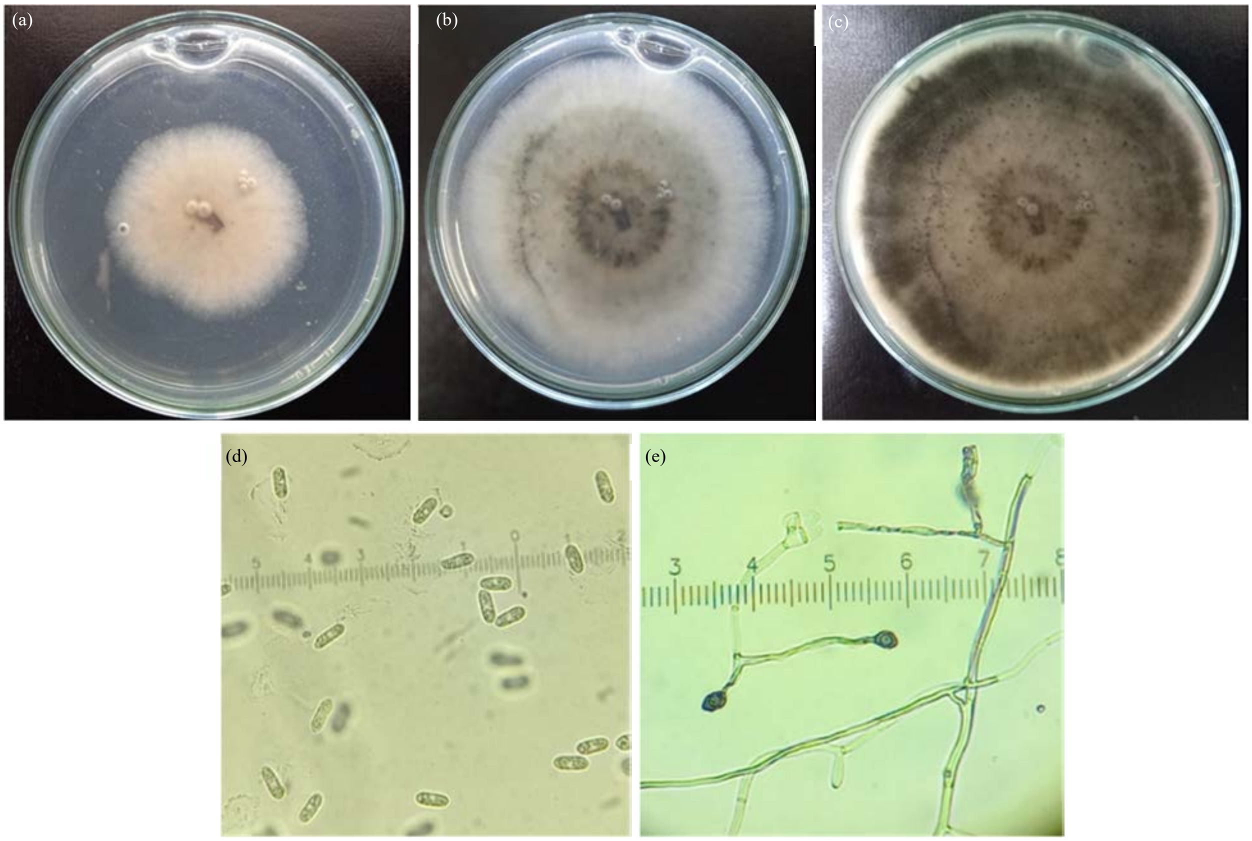

Fig. 2(a-e): Morphological characteristics of *Colletotrichum* spp., cultured on PDA at 25°C, (a) 5 days, (b) 7 days, (c) 9 days, (d) Spores and (e) Appressoria

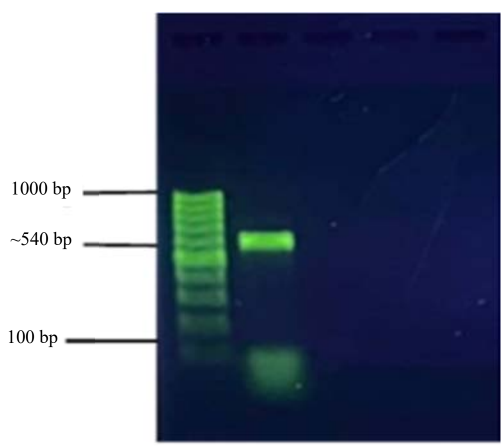

Fig. 3: Agarose gel electrophoresis of a PCR product using the ITS1/ITS4 primer and DNA of anthracnose-causing *Colletotrichum* spp. (well 2) and 100 bp ladder (well 1)

comparable to those of Daoud *et al.*[14], who described 3 fungal stains of *Colletotrichum* spp., as the cause of anthracnose on Malti oranges.

Morphological characteristics of the isolated fungal strain on the culture media: After being isolated, colonies of the isolated fungus were cultured on PDA, with concentric circles of cotton-like mycelia. Over time of cultivation, there were changes in the colour of hypha recorded, from white to light white in Fig. 2a or from pink to orange in Fig. 2b and c.

The fungal spores were cylindrical, rounded or obtuse at 2 ends, 12.8-16.4 mm in length and 3.47-5.27 mm in width in Fig. 2d. The average size of spores of *C. gloeosporioides* causing anthracnose varies depending on which host plants it causes the disease. Especially, the spores are10.0-17.5×2.5-5.0 μm in average size on king oranges, 10.9×3.7 μm on Malti oranges[14] and 5-7.5×15-17.5 μm on citrus trees originated from Iran. Appressoria formed from fungal hypha were grey to black, with fewer wrinkles and oval in shape. They were 6.41-10.6 μm in length and 4.21-6.19 μm in width in Fig. 2e.

Analysis by PCR and sequencing: The concentration of the isolated DNA was measured at 260 and 280 nm, with the concentrations ranging from 57.8-58.6 ng μL^{-1} in Table 1. The average DNA concentration was enough for PCR reactions[28].

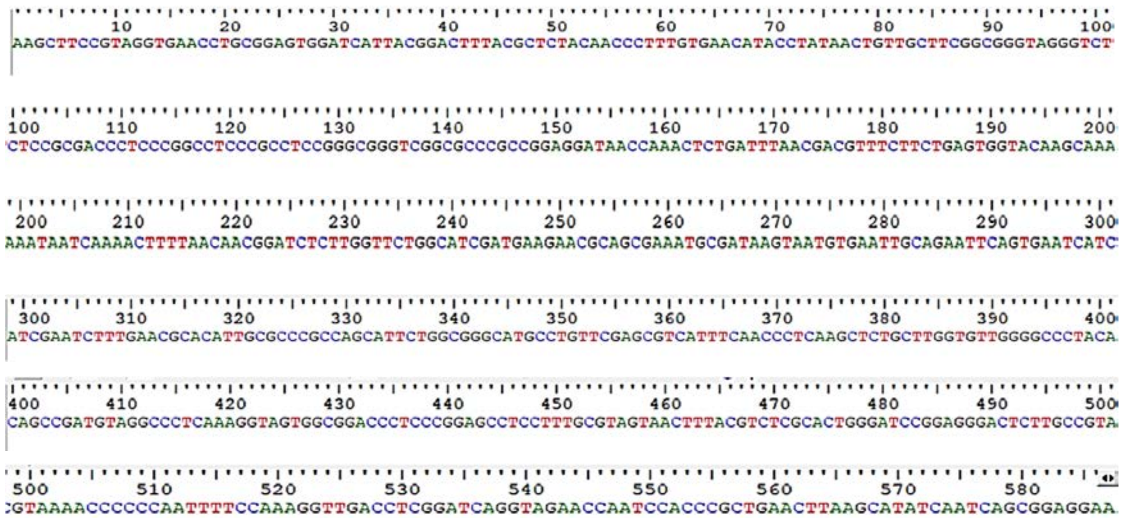

Fig. 4: Sequence of nucleotides in the ITS segment of *Colletotrichum gloeosporioides*

Table 1: DNA concentration of *Colletotrichum* spp. causing anthracnose on king oranges

Repeat	DNA concentration (ng µL^{-1})	260/280 (nm)
1	57.8	1.74
2	58.6	1.73
3	57.9	1.71
Average	58.1	1.73
Minimum	57.8	1.71
Maximum	58.6	1.74

Table 2: Blast results of *Colletotrichum* spp. from NCBI

Gene codes	Species	Size	Coverage (%)	Similarity (%)
MG925324.1	*C. gloeosporioides*	1018	99	98.29
KT192235.1	*C. gloeosporioides*	1014	99	98.28
MK311225.1	*C. gloeosporioides*	1013	98	98.28
FJ459916.1	*C. gloeosporioides*	1013	98	98.28
JN715843.1	*C. fructicola*	1000	98	97.93

A PCR product amplified by the 2 primers ITS1/ITS4 was analyzed by the gel electrophoresis with 1.5% of agarose. Figure 3 displays a single band of 540 bp, which is quite similar to what was found in the research of Moges *et al.*[24], in which their PCR products amplified by ITS1/ITS4 had 570 bp in size. The ITS segment of the fungus causing anthracnose on king oranges, *Colletotrichum gloeosporioides*, had 24.83% of G and 27.89% of C, which was higher than levels of A and T, each with 23.64%. In other words, the composition of GC was higher than that of AT, with 52.72% against 47.28%, respectively in Fig. 4.

The nucleotide sequence of the fungus, *C. gloeosporioides* was compared with the published sequences available in the Genbank of NCBI by BLAST. Table 2 showed that the ITS segment of *Colletotrichum* causing anthracnose on king oranges had similar gene components with those of the following sequences found on the Genbank: MG925324, KT192235, MK311225, FJ459916, JN715843, with the level of similarity ranging from 97.93-98.29% (Table 2).

Only branches with a bootstrap of 50% or above were selected and analyzed. Sequences of rRNA-ITS amplified by the ITS1/ITS4 primers were utilized to create the Neighbor-Joining tree (NJ)[23]. Based on the NJ, the results demonstrated that the genetic sequence of the fungus causing anthracnose on king oranges had a close relationship with that of other species of *Colletotrichum* found on NCBI in Fig. 5. The genetic correlation of *Colletotrichum gloeosporioides* causing anthracnose on king oranges (Contig-0) found with strains/species of *Colletotrichum gloeosporioides.*

The findings in this study were comparable to recordings about *C. gloeosporioides* responsible for anthracnose on citrus trees[7]. In Italy, anthracnose on oranges and leaves of mandarin oranges stemmed from *Colletotrichum gloeosporioides* and

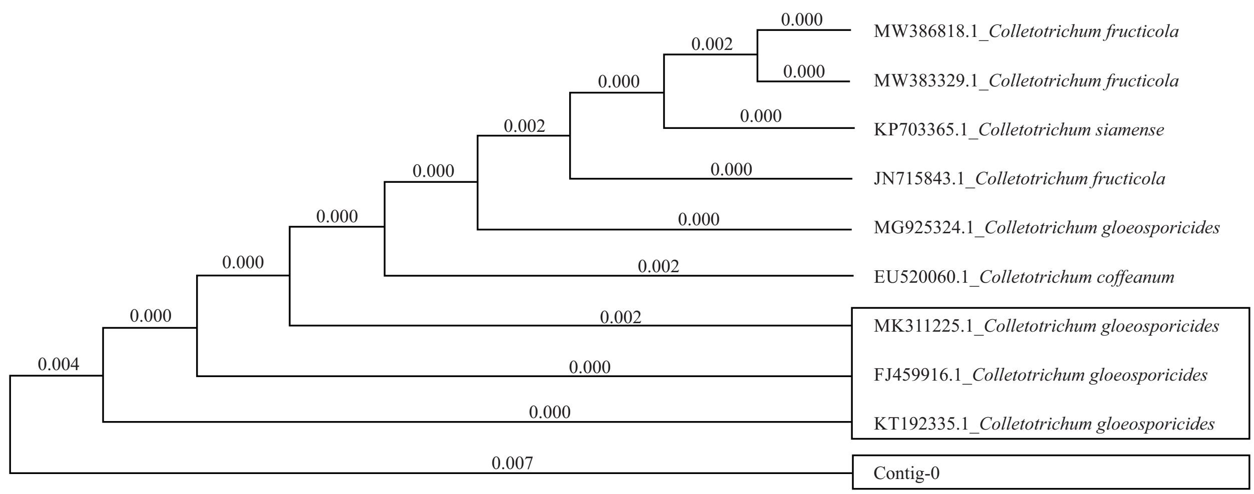

Fig. 5: Genetic correlation of *Colletotrichum gloeosporioides* causing anthracnose on king oranges (Contig-0) with strains/species of *Colletotrichum gloeosporioides* available on NCBI by the MEGA 6 software

C. karstii, respectively[3,14]. In addition, *C. gloeosporioides* is the causal agent of anthracnose on sweet oranges[28] and grapefruits[15].

CONCLUSION

Based on the morphological characteristics and genetic sequence of ITS on rRNA using the ITS1/ITS4 primer, the fungus, *C. gloeosporioides* was identified as the cause of anthracnose on king oranges grown in Vinh Long province. The genetic sequence of the fungus causing anthracnose on king oranges had a close relationship with that of other species of *Colletotrichum* found on NCBI.

SIGNIFICANCE STATEMENT

This study discovered the agent causing anthracnose on king oranges that can be beneficial for agronomists and mycologists. This study will help the researchers to uncover the critical areas of pathology that many researchers were not able to explore. Thus a new theory on prevention of the disease may be arrived at.

ACKNOWLEDGMENT

This study is one of the scientific results of the project at the Ministry level application and development of some technologies for processing and storing some products of king oranges at the Mekong delta (the project code: CT2020.01.TCT.05) of the Program of Science and Technology of Education and Training Department.

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