BRUNIFICAREA FLORILOR CA SURSA DE INFECŢIE CU ANTRACNOZĂ LA CURMALUL CHINEZESC

FLOWER BROWNING AS INFECTION SOURCE WITH ANTHRACNOSE ON JUJUBE

Seung-Han Kim, Seok-Hee Park, Min-ki Kim and Sook-Hee Lee
Gyeongsangbuk-Do Agricultural Research and Extension Services, Daegu 702-708, Korea

Abstract

In the main producing province of jujube *Zizyphus jujuba*, Gyeongsnagbukdo, disorder of jujube flowers was found. The symptoms were browning of petal and dropping of flowers without fruit setting. The damaged flowers were collected and investigated, and confirmed that disorder of flower was caused on infection by *Colletotrichum gloeosporioides*. *C. gloeosporioides* was isolated in 95.4% of discolored flower collected in Gyeongsan area, Aerial mycelium was white in early growth stage but turn to grayish with age. Orange color spore masses were produced outward from the center of the culture. The conidia were cylindrical with both apices rounded or with one apex rounded and the other end pointed. The conidial sizes were 9.2-13.2 x 3.2-5.8 µm. *Apressoria* were dark grey, ovate to clavate, and size was 5.9-7.2x4.2-5.1 µm. Bark and fruiting twig were surveyed to know overwintering place of *C. gloeosporioides* and the results are following that *C. gloeosporioides* was abundantly isolated from fruiting twigs, especially including fruiting twig appeared previous year, but not isolated from bark. This means that the reservoir of infection of anthracnose was considered as fruiting twig appeared previous year.

Cuvinte cheie: *Colletotrichum gloeosporioides*, curmalul chinezesc, floare, sursa de infecţie
Key words: *Colletotrichum gloeosporioides*, Jujube, flower, infection source

1. Introduction

Jujube fruit has been used as food or medicine in Korea for a long time. Also, it has been traditionally used in ancestor memorial ceremony in Korea. In the past, therefore, few jujube trees were planted in backyard of almost every house for food and medicinal use. Recently, with increase of processed food market, demand of jujube fruits has also been increased. The increased requirement of fruit demand induced increase of jujube orchards, mainly concentrated around Gyeongsangbukdo province. The most serious problem in the cultivation of jujube is anthracnose occurring on the fruits, and fungicides have been sprayed 8 - 12 times a year, to control this disease. Recently, the new problem of flower was reported. The flowers were changed to brown color and dropped. This phenomenon occurred over a wide area and big economic loss occurred because of pure fruiting by flower drop. Most of the jujube farms suffered damages, but we did not know the exact cause. Jujube flower browning was surveyed in accordance with the requirements of farmer asking to discover the cause of browning.

The result of survey was the phenomenon that flowers were infected with *Colletotrichum gloeosporioides*. The disease appearing by infection with *C. gloeosporioides* is generally called anthracnose in almost plant as well as jujube. But anthracnose occurring in flower has not been reported yet, although source of fruit anthracnose has been not known yet. The purpose of this paper is to report the results about our investigation on the cause of flower browning and experiment to find out a source of anthracnose occurrence.

2. Material and methods

2.1. Occurrence and **Pathogen identification of flower browning.** Three orchards were selected in this area, respectively, to investigate occurrence rate of flower browning as well as collection of damaged flower sample. The collected sample was carried to laboratory to use in the further study. Infected flowers were collected and sterilized with 70% ethanol and NaClO and rinsed in sterilized water. These samples were placed on plates of potato-dextrose agar (PDA) and incubated at 25^{o}C. The collected flowers were also sterilized with 70% ethanol and placed inside plastic box humidified with sterile water to induce sporulation of pathogen. PDA with mycelium cultured from flowers was cut and moved to new PDA. This procedure was repeated several times to confirm pure culture.

2.2. Identification and polymerase chain reaction (PCR). The pure cultured isolated were identified using light microscope (LM Nikon) and scanning electron microscope (SEM, Carl Zeiss). Spores produced on PDA media were used by both electron microscopes. For SEM observation, specimens

were dehydrated and dried in a critical-point dryer with liquid CO_2, and sputter coated with gold palladium. For PCR identification, isolate was grown in 100 ml of potato dextrose broth (PDB) at 25°C for 7 days. Thereafter, the mycelium was collected by filtration through Kimtech paper (Yuhan Kimberly) and lyophilized until dry. DNA was extracted and purified with Maglisto 5M plant genomic extraction kit (Bioneer). The purified DNA was dissolved in 0.5 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 30 to 100 µg/ml and diluted to a final concentration of 10 ng/µl for PCR reactions. For PCR reactions, species-specific primers were synthesized for *C. gloeosporioides* (CgInt) 5′-GGCCTCCCGCCTCCGGGCGG-3′, for *C. acutatum* (CaInt) 5′-GGCGCCGGCCCCCACCACGGGGA-3′ from ITS1 of the ribosomal DNA (rDNA) gene based on published DNA sequences (Sreenivasaprasad et al. 1996). Target primers were used with the conserved primer ITS 4. Primers were synthesized by Bioneer Ltd., (Daegeon, Korea). PCR amplification was performed using Accupower PCR premix (Bioneer). A thermocycler (C1000 touch thermal cycler, Biorad) was programmed for 35 cycles of denaturation (94°C for 1 min), annealing (59°C for 2 min), and DNA extension (72°C for 2 min). Following amplification, PCR products were separated using electrophoresis in 1.2% agarose gels.

2.3. Pathogenicity on flowers and fruits. Isolates was cultured at 25°C for 7 days and spores formed on surface were collected with the following method: 20 ml of sterile water was poured into prepared media and the surface of mycelium was rubbed with mess to separate spores from medium. Water inside medium including spores was collected in 50 ml corn tube and hyphae were filtrated with kimtech paper (Yuhan Kimberly). This spore suspension was stored and used for artificial inoculation.

Healthy jujube flowers were collected and sterilized in 70% ethanol for 10 seconds. These flowers were dried inside clean bench and placed in humidified plastic box. Previously prepared spore suspension was sprayed several times over jujube flower and tightly sealed before incubated at 25°C. Disease occurrence was surveyed 7 days after inoculation. Various growth stage fruits, from young fruits to mature fruits, were collected to survey sensitivity to anthracnose according to maturity level. The prepared fruits were inoculated with isolate by the same method as above and infection was surveyed 7 days after inoculation.

2.4. Inoculum source survey. *Colletotrichum* spp. is known as to be transmitted by rain drop dispersion. Fruit infection occurred over the tree without any relationship with tree height. This means that some infection source should be in tree, but there is no information about infection source. For survey, bark and fruiting twig of jujube were collected and moved to laboratory for inspection. The surface of samples was observed with a light microscope to find out some sign related to anthracnose and fungi were isolated with previous method.

3. Results and discussions

3.1. Occurrence of flowers browning and fruits anthracnose. Results of surveying the flowers and fruit infected with *C. gloeosporioides* is as follows. The browning of flowers started from tip of petal and spread to other side (Fig. 1). Infected flowers finally turned to black color and dried to drop. Infection rate of both flowers and fruits were higher in Gyeongsan area that jujube orchards are concentrated than Daegu area. It is well known that single crop cultivation on broad area sometimes leads to pest outburst (Jabłońska-Sabuka et al., 2015). Therefore, it was guessed that this area showed more occurrences because the density of the pathogen was presumably much higher by long time and mass cultivation of jujube. The environmental factor must also be considered for understanding disease epidemics (van Maanen & Xu. 2003), but this experiment did not consider because flower browning was not regarded as one of disease at that time. The infection began in early July and increased gradually. Occurrence showed a peak at August and since then gradually decreased. Anthracnose infection is closely related to the rain. Three *Colletotrichum* species infecting strawberry were studied related to rain effect (Ntahimpera et al. 1999). Monsoon season in Korea starts normally from late June. This environment, humid and hot weather, would lead to outbreak of anthracnose in jujube because that condition is favorable for disease occurrence.

3.2. Identification of pathogen. The characteristics of pathogen isolated from flower were following. Developing colonies on PDA media were greyish white, circular, wooly and black spots, scattered over surface of mycelium (Fig. 2). Microscopic observation of spores revealed that conidia were straight with rounded ends, hyaline, and single cell. The size of conidia was 9.2-13.2x3.2–5.8 µm (average 11.0x4.3 µm) (Table 1). This results were consistent with the description of *C. gloeosporioides* (Kwon et al., 2008). As the results of PCR, Cgint primers specific to *C. gloeosporioides* produced PCR products of approximately 0.5kb size, however, CaInt primer has no product of PCR (Fig 3). These results indicated that this isolate was *C. gloeosporioides* and the same conclusion was drawn with microscopic observation.

3.3. Pathogenicity on fruits and flowers. When isolated fungus was inoculated to flowers, the same symptom, browning, appeared. Over time, the spores mass was formed on the surface of petal. These spores were observed under light microscope and identified as *C. gloeosporioides*. When

sensitivity of jujube fruits was surveyed by fruit size, almost all fruits less than 10 mm length were infected, but a few fruits over 10 mm length were infected. These results means that mature fruits presumably have resistance to anthracnose. There are many researches about physiological and chemical resistance of plant against disease (Yang et al., 1992; Shao et al., 2015). Physiological and chemical change of jujube fruit related to disease resistance has not been known, but resistance expression in mature fruits will be possible because physiological barrier like cuticle changes. Therefore, proper time for control of jujube anthracnose would be from flowering time to just before maturing of fruits.

3.4. Inoculum reservoir survey. *C. gloeosporioides* was not detected in bark of jujube tree, but was detected in many fruiting twigs. Especially, old twigs that appeared the previous year were highly infected with this pathogen (Table 2). This means that *C. gloeosporioides* overwinter inside fruiting twigs and plays a role as source of infection. Spores of plant fungal pathogen are disseminated to new host by rain splashes (Huber et al. 1998). Also, the association of rain with diseases caused by *Colletotrichum* spp. has been well established (Madden et al. 1992). Therefore, the spores formed on the surface of overwintering twigs splashed by rain in the growing season leading to infection of fruits. According to these results, remove of old fruiting twig is required for successful control jujube anthracnose.

4. Conclusions

In the main producing province of jujube, Gyeongsnagbukdo, disorder of jujube flowers, browning and drop, was found. When the damaged flowers were collected and investigated, it was confirmed that disorder of flower was caused on infection by *Colletotrichum gloeosporioides*. Infection rate of browning flower was 95.4% for the ones collected in Gyeongsan area. In survey of source, bark and fruiting twigs of jujube tree was surveyed and confirmed that *C. gloeosporioides* overwinter at fruiting twigs. This means that the source of infection of anthracnose was considered the fruiting twigs that appeared the previous year. Therefore, removing of twig is important tool for control of anthracnose.

5. Acknowledgements

This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01082303)” Rural Development Administration, Korea.

References

1. Sreenivasaprasad, S., Sharada, K., Brown, A.E., Mills, P.R., 1996. PCR-based detection of *Colletotrichum acutatum* on strawberry. Plant Pathology 45:650-5.
2. Jabłońska-Sabuka, M., Kalaria, R., Kauranne, T., 2015. A dynamical model for epidemic outbursts by begomovirus population clusters. Ecological Modelling 297:60-8.
3. Van Maanen, A., Xu, X.M., 2003. Modelling Plant Disease Epidemics. European Journal of Plant Pathology 109:669-82.
4. Ntahimpera, N., Wilson, L.L., Ellis, M.A., Madden, L.V., 1999. Comparison of rain effects on splash dispersal of three colletotrichum species infecting strawberry. Phytopathology 89:555-63.
5. Kwon, J.H., Cheon, M.G., Park, C.S., 2008. Anthracnose of blueberry (*Vaccinium* spp.) of Caused by *Colletotrichum gloeosporioides* in Korea. Res. Plant Dis. 14:61-3.
6. Yang, J., Verma, P.R., Lees, G.L., 1992. The role of cuticle and epidermal cell wall in resistance of rapeseed and mustard to *Rhizoctonia solani*. Plant and Soil 142:315-21.
7. Shao, W., Zhang, Y., Ren, W., Chen, C., 2015. Physiological and biochemical characteristics of laboratory induced mutants of Botrytis cinerea with resistance to fluazinam. Pesticide Biochemistry and Physiology 117:19-23.
8. Huber, L., Madden, L.V., Fitt, B.D.L., 1998. Rain-splash and spore dispersal: a physical perspective. In The Epidemiology of Plant Diseases, ed. DG Jones:348-70. Dordrecht: Springer Netherlands. Number of 348-70 pp.
9. Madden, L.V., Wilson, L.L., Yang, X., Ellis, M.A., 1992. Splash dispersal of *Colletotrichum acutatum* and *Phytophthora cactorum* by short-duration simulated rains. Plant Pathology 41:427-36.

Tables and Figures

Table 1. Morphological characteristics of *C. gloeosporioides* isolated from jujube flower

Chacrateristics		Present isolate	*C. gloeosporioides*
Colony	color	white to grey	-
Conidia	color	colorless	colorless
	shape	straight, cylindrical apex obtuse	straight, cylindrical apex obtuse
	size	9.2-13.2x3.2-5.8 µm	10-20x4-12 µm
Appressoria	shape	clavate, ovate	clavate, ovate
	size	5.9-7.2x4.2-5.1 µm	6-18x4-12 µm

Table 2. Infection rate of fruiting twigs of jujube by *C. gloeosporioides* according to surveyed time

Year of twigs appearance	surveyed time	Infection rate (%)			
		Cg[1]	Bd	UN	NI
2014	Jun. 2015	89.5	3.2	7.1	0
2014	Sep. 2015	13.0	0	60.8	0
2015	Nov. 2015	83.0	0	4.0	1.3

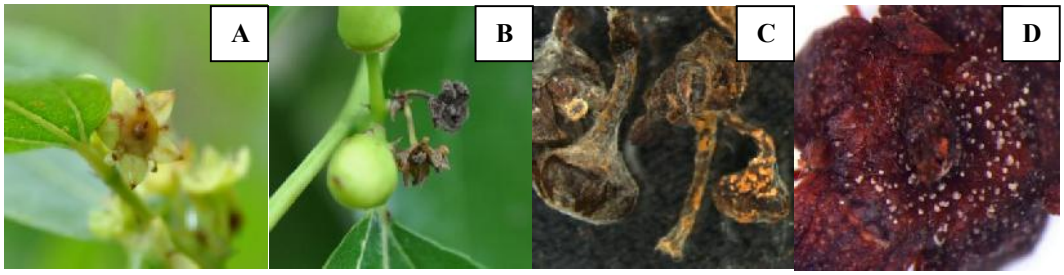

Fig. 1. Early symptom (A) and late symptom (B) of flower infected in *Colletotrichum gloeosporioides* in field, spores produced on calyx (C), symptom induced by artificial inoculation (D).

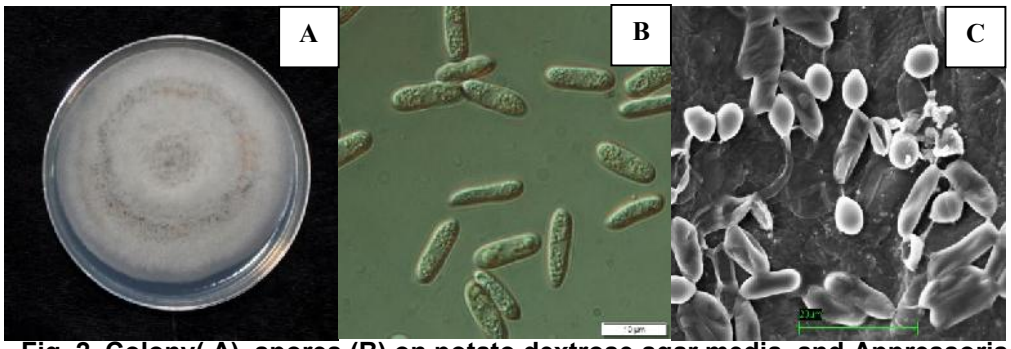

Fig. 2. Colony(A), spores (B) on potato dextrose agar media, and Appressoria formed on jujube leaf by artificial inoculation (C).

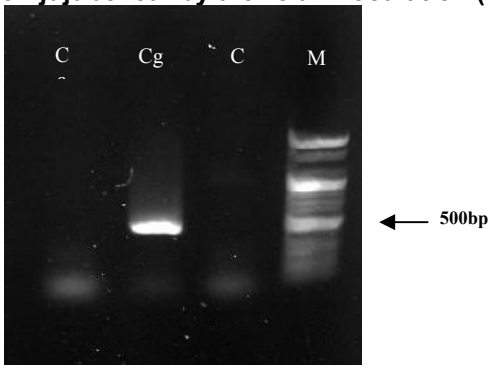

Fig. 3. Amplification products with the primer pairs CaInt/ITS4 and CgInt/ITS4 for detection of *Colletotrichum gloeosporioides*. Lane M, DNA marker (100bp DNA ladder, Bioneer); lane C, control; lane Cg, primer pairs CgInt/ITS4; lane Ca, primer pairs CaInt/ITS4