Isolation and Molecular Identification of *Epicoccum nigrum* and *Cladosporium cladosporioides* from Exotic Vegetables in Aberdeen

Aigbe, S. O[*,#]

[*]*Department of Crop Science, Ambrose Alli University, Ekpoma, Nigeria*

[#] *School of Biological Sciences, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen, UK*

***Abstract*- In an experiment involving the isolation and molecular identification of fungi associated with exotic vegetables in two Aberdeen shops, *Epicoccum nigrum*, and *Cladosporium cladosporioides* were isolated and identified from snowpea (*Pisum sativum var. saccharatum*), mango (*Mangifera indica*) and pawpaw (*Carica papaya*) imported from Kenya and Brazil. Isolates were identified using colony and morphological characters on Potato Dextrose Agar and PCR analysis. The DNA of fungi were extracted using a QiagenDNeasy Plant Mini Kit and PCR products were purified with a QIAquick PCR Purification Kit after electrophoresis. PCR amplifications were run using the primer pair ITS1/ITS4. DNA sequences were compared to published sequences in GenBank using BLASTn. *Epicoccum nigrum*, and *Cladosporium cladosporioides* have both been reported as pathogens of economically important crops plants. They have also both been reported as biological contro agents of some pathogens of certain crops. The pathogenicity and the biological control potentials of this isolates are currently being planned.**

***Keywords*- *Epicoccum nigrum*, *Cladosporium cladosporioides*, vegetables, Aberdeen, PCR**

I. INTRODUCTION

Epicoccum nigrum is a species of fungus, in the phylum Ascomycota. A plant pathogen and endophyte, it is a widespread fungus which produces coloured pigments that can be used as antifungal agents against other pathogenic fungi (Anderson *et al*., 1981). *Cladosporium* is one of the most common genera of fungi occurring on various substrates and includes species with diverse lifestyles. Besides saprophytic behavior, antagonism to pathogenic fungal species has been described for the genus (Singh and Singh, 1994).

In an experiment in Poland, to identify the pathogens present in the pea seeds of different cultivars, *Epicoccum nigrum* was one of the fungus isolated along with other fungal pathogens such as *Alternaria* and *Fusarium* species Wilman *et al*. (2014). Pathogecity of *E. nigrum* were similarly reported in *Cucumis melo* by Bruton *et al*. (1 993) and in *Lablab purpureus* and loquat by Mahadevakumar *et al*. (2014) and Wu *et al*. (2017) respectively. In the later study, isolates having 98% identity with *E. nigrum* (KC568289 and KY303832) caused leaf spot in *Lablab purpureus* and brown leaf spot in loquat, respectively. Recently and also for the first time, Colavolpe *el al*. (2018) reported leaf spot in *L. corniculatus* caused by *E. nigrum* in Argentina.

Like *E. nigrum*, *C. cladosporioides* has also been reported to be pathogenic to plants. For instance, *C. cladosporioides* was reported to cause papaya scab in Iran (Baharvandi, H. A.; Zafari, D. 2015), leaf spot on *Dendrobium officinale* in China (Sun *et al*., 2017), leaf spot of pecan and *Alstroemeria aurea* plants in Brazil (Walker *et al*., 2018; Meneses *et al*., 2018), blossom blight in strawberries in Korea (Nam *et al*., 2015) and fruit blotch of zapote mante (*Pouteria campechiana*) in Mexico (Nabor-Romero, 2018).

Despites the pathogenicity report of *E. nigrum*, most isolates have been reported to control many impotant plant pathogens. It is commonly found growing on cereals and seeds, as well as other crops including corn, beans, potatoes, peas and peaches (Anderson *et al*., 1981; Flannigan *et al*., 2011). It produces a variety of pigmented and non-pigmented antifungal and antibacterial compounds (Brown *et al*., 1987; Gribanovski-Sassu and Foppen, 1967). These antimicrobial compounds are effective against other fungi and bacteria present in soil (Brown *et al*., 1987). In Brazil, *E. nigrum* is used to support root growth and control sugarcane pathogens (Fávaro *et al*., 2012). Hashem and Ali in 2004 observed that *E. nigrum* could be used successfully as an environmentally safe and economic biological control agent to protect cotton (cv. Giza 83) from damping-off and root-rot diseases caused by *P. debaryanum*. Effective disease suppression by *E. nigrum* has similarly been demonstrated against a diverse range of pathogens including: *Monilinia laxa* (Larena *et al*. 2005; De Cal *et a*l. 2009; Larena and Melgarejo 2009), Sclerotinia sclerotiorum (Hoyte *et al*. 2007; Huang and Erickson 2008), Diplodia corticola (Campanile *et al*. 2007), *Botrytis cinerea* (Elmer and Reglinski 2006; Walter *et al*. 2006; Card *et al*. 2009), *Fusarium oxysporum* f. sp. *conglutinans* (Park et al. 2002), *Magnaporthe grisea* (Kawamata *et al*. 2004), *Phytophthora* spp. and *Pythium* spp. (Brown et al. 1987; Hashem and Ali, 2004), *Macrophomina phaseolina* (Hashem, 2002) and *Rhizoctonia solani* (Lahlali and Hijri 2010; Hashem, 2002). Recent study by Alcock *et* al. (2015) showed flavipin and epirodin produced by saprophytic

isolates of *E. nigrum* had antimicrobial properties. Preliminary studies by Alcock *et al*. (2015) on 280 New Zealand isolates of *E. nigrum* confirmed that all but two produced a yellow, intensely pigmented substance in sufficient amounts to inhibit the germination of *Botrytis cinerea* conidia. Prior to this in 2015, F avaro *et al*. also observed that an *E. nigrum* strain, isolated from sugarcane leaves, showed *in vitro* antagonistic activity against the sugarcane phytopathogens *Fusarium verticillioides*, *Colletotrichum falcatum*, *Ceratocystis paradoxa*, and *Xanthomonas albilineans*.

Some of the most common examples of the antagonism in *Cladosporium* come from the relationship between *Cladosporium* spp. and rust pathogens (Pusz *et al*., 2015; Wilman *et al*., 2014), such as *C. cladosporioides* parasitizing *Venturia inequalis* and *Puccinia striiformis* f.sp. tritici (Stupar *et al*., 2014). *Cladosporium cladosporioides* was observed to significantly promotes host seed germination of coastal plant *Suaeda salsa* and other plant growth. *Cladosporium cladosporioides* was also present in the phyllosphere, rhizosphere and root endosphere of *S. salsa*, supported the evidence of its primary soil-borne origin and both epiphytic and endophytic infection of host tissues (Qin., *et al*., 2016). This work reports another detection of E. nigrum and C. cladosporioides from exotic snow pea, mango and pawpaw in Aberdeen.

II. MATERIALS AND METHODS

A. Sterilization of Equipment

The glassware used were washed in detergent, rinsed with tap water and dried with paper towel. They were then either rapped or covered aluminum foil paper before being autoclaved. Scalpels were sterilised by dipping in 70% alcohol for 5 minutes and then passing through a flame. Inoculating chamber were sterilised by scrubbing with 70% alcohol. All isolations and inoculations were carried out in the sterile inoculating chamber. The working surfaces of laminar flow cabinets were disinfected with 70% ethanol before working on them.

B. Isolation of Fungi

The isolation of fungi were carried out from from snowpea, mango and pawpaw purchased from two shops in Aberdeen. The labels on the vegetables indicated that the snowpea was imported from Kenya while the mango and pawpaw were both imported from Brazil. Isolation from tissues were done by surface sterilizing 5 pieces of tissue in 3% NaOCl for 3 minutes and rinsing in 2 changes of sterile distilled water before placing on 1/4 strength Potato Dextrose Agar (PDA) medium. They were then incubated at 25°C for two to three days after which they were examined for colony growth. Subcultures were made on new 1/4 strength PDA to obtain pure isolates. Single spore/hyphal tip isolation on water Agar were carried out to further purified cultures. The spores/hyphal tips were transfered to new full strength PDA plates and incubated at 25°C in the incubator for 7 days. The purified cultures were then stored in the cold room for subsequent colony and molecular analyses.

C. Identification of Isolates

Morphological identification was carried out by observing colony features on full strength PDA. Mycelia structures were examined and photographed with a research light microscope by interference contrast microscopy. Morphological features with reference to standard literatures were used for the preliminary identification of the isolates. Molecular identification was carried out by DNA exraction and PCR analysis.

1) DNA Extractions / PCR Analysis

Isolates were grown on a sterile cellophanes placed on full strength PDA for 7 days after which fungal mycelia were scraped washed into a sterile conical flasks with sterile distilled water The DNA of fungal isolates were extracted using a QiagenDNeasy Plant Mini Kit and PCR products were purified with a QIAquick PCR Purification Kit after electrophoresis. The PCR amplification was done using the ITS1 and ITS4 primers designed to amplify the internal transcribed spacer (ITS) region. DNA sequences were compared to published sequences in GenBank by blasting the sequences at NCBI database using BLASTn.

Quick DNA extraction was carried out with a modified method from Modified method from Collado-Romero *et al.*, 2006. The samples were sent to 'Source BioScience' company, Scotland, for sequencing. The software used to analyse them was CLC Main Workbench.

III. RESULTS AND DISCUSSION

Macroscopic features of *Epicoccum nigrum* colony on PDA and mycelia characteristic were characterised under a microscope and conformed morphologically most closely to the genus *Epicoccum*. *Epicoccum nigrum* produced pigment in PDA whic was dark orange to green-yellow color. Colonies were fast growing, suede-like to downy, with a strong yellow to orange-brown diffusible pigment (Fig. 1). *Epicoccum nigrum* did not produce any spore on PDA. *Cladosporium cladosporioides* colony on PDA had a velvet-like appearance, and their color ranged between olivaceous-brown and smoky-grey to olive and almost black. Conidia were numerous and limoniform, ovoid, obovoid to subglobose, aseptate (Fig. 1).

Morphological and molecular analyses identified isolates as *Epicoccum nigrum* and *Cladosporium cladosporioides*. The BLAST search revealed 100% identity to *E. nigrum* and *Cladosporium cladosporioides* in GenBank (Fig. 2). *Epicoccum nigrum* had 5% frequency of isolation from snowpea imported from Kenya, while *Cladosporium cladosporioides* had isolation frequencies of 60% on same

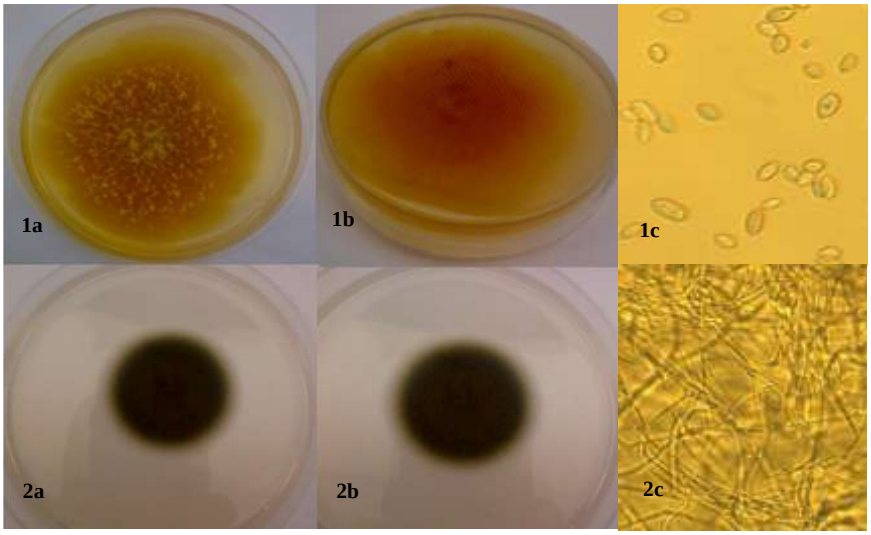

Fig. 1 Colony morphology (Front (a) and back (b) on PDA), *Epicoccum nigrum* (1a and 1b), (1c) mycelium; *Cladosporium cladosporioides* (2a and 2b), (2c) aseptate conidia

snowpea from Kenya and 30% from both mango and pawpaw from Brazil. As observed in this experiment for *E. nigrum* colony color on PDA, Adelaide (2016) also reported *E. nigrum* to have yellow colony which appear reddish toward center at back side of plate. Adelaide also reported *E. nigrum* to produce pigment in PDA whic was dark orange to green-yellow color. Similarly according Adelaide, no spore was produced. As observed for *C. cladosporioides* in this experiment, Torres *et al*. (2017). observed *C. cladosporioides* colony on PDA medium to be olivaceous with diffuse aerial mycelia, floccose-felty, reverse olive-black color (Torres *et al*., 2017). Conidia were also numerous, limoniform, ovoid, obovoid to subglobose, aseptate and light brown (Torres *et al*., 2017).

Increasing restrictions on pre- and postharvest pesticide treatments (Jacometti *et al*. 2009; Hillocks 2012) combined with an ever greater demand for organic food (Karabulut *et al*. 2010) have stimulated interest in biological control of plant pathogens as an alternative to the use of synthetic fungicides. In some countries, entire regions have converted their pest and disease control strategies to biologically based methods (Maxwell 2008). Many microorganisms, including the filamentous fungus, *Epicoccum nigrum* (Link) (syn. *E. purpurascens* Ehrenb. Ex Schlecht), have previously been evaluated as potential biological control agents against a wide range of economically important plant diseases (Elmer and Reglinski 2006). Researchers in biological control have been attracted to *E. nigrum* for a variety of reasons including the following: antimicrobial metabolite production (Brown *et al*. 1987), ease of culturing on simple substrates (Larena *et al*. 2004), tolerance to environmental extremes (Hannusch and Boland 1996), persistence on host tissues (Boyd-Wilson et al. 1998) and good efficacy in field experiments (Elmer and Reglinski 2006).

Recently, Hulikere *et al*. (2016) identified *Cladosporium cladosporioides*, isolated from seaweed (*Sargassum wightii*), as an endophytic fungus containing ethyl acetate extract with significant antioxidant and angiosuppressive activity. This extract confer on the fungus an antiangiogenic, wound healing and antioxidant property. Also, in an apple field trials carried out by Köhl *et al*. (2015), the overall results of the field trials consistently showed—for the first time—that stand alone applications of the antagonist *C. cladosporiides* H39 can reduce apple scab in leaves and fruit. Results from a study carried out by Torres *et al*. (2017) on chrysanthemum white rust, indicate that *Cladosporium* species had potential as biological control agents According to Fernando (2009), integrated pest management (IPM) involves the use of resistant varieties and healthy seeds, crop rotation, frequent crop monitoring, early detection and identification of the pest or disease etc. Therefore chemical use for pest and disease control can be drastically reduced if integrated disease management that is based on the use of biological control agents is practised. The widespread use of the chemical fungicides has become a subject of the research concern due to their harmful effect on non-target organisms as well as their possible carcinogenicity. The use of fungal biocontrol agents is becoming an increasingly important alternative to chemicals in crop protection against many diseases (Tyler *et al*., 2001). This work reports another detection of *E. nigrum* and *C. cladosporioides* from exotic snow pea, mango and pawpaw in Aberdeen. Further studies should be carried out to established their pathogenicity or biological control potentials.

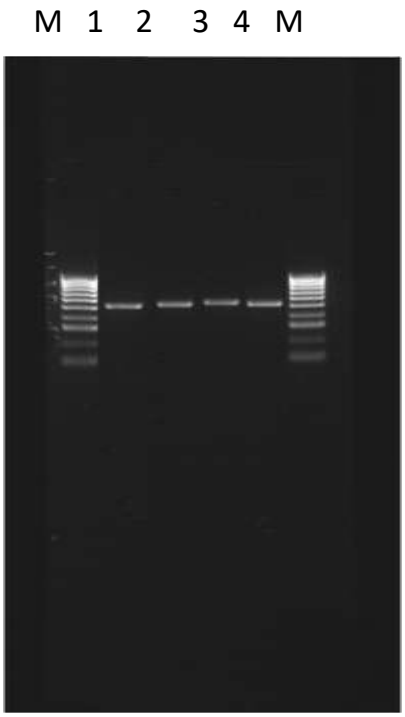

Fig. 2 PCR products amplified with primer pair ITS1 and ITS4 (fungal isolates 1 (*Epicoccum nigrum*), 2-4 (*Cladosporium cladosporioides*))

ACKNOWLEDGEMENTS

The work was supported by TETFund National Research Grant, Nigeria, the Ambrose Alli University, Ekpoma, Nigeria and the University of Aberdeen, Aberdeen, UK.

REFERENCES

[1]. Adelaide (2016). *Epicoccum nigrum.* Mycology Online. University of Adelaide, South Australia, Australia. Website: https://mycology.adelaide.edu.au/descriptions/hyphomycetes/epicoccum/

[2]. Alcock, A., Elmer, P., Marsden, R., and Parry, F. (2015). Inhibition of *Botrytis cinerea* by Epirodin: A Secondary Metabolite from New Zealand Isolates. Journal of Phytopathology, Volume 163: 841-852

[3]. Anderson, K.H. Domsch, W. Gams, Traute-Heidi. (1981). Compendium of soil fungi. London: Academic Press. ISBN 978-0-12-220401-2.

[4]. Baharvandi, H. A.; Zafari, D. (2015). First report of IRST REPORT OF CLADOSPORIUM CLADOSPORIOIDES CAUSING SCAB DISEASE ON PAPAYA IN IRAN. Journal of Plant Pathology . 2015, Vol. 97 Issue 3, p549-549. 1/2p.

[5]. Brown, Averil E.; Finlay, Ruth; Ward, J.S. (1987). "Antifungal compounds produced by Epicoccum purpurascens against soil-borne plant pathogenic fungi". Soil Biology and Biochemistry. 19 (6): 657–664.

[6]. Bruton BD, Redlin SC, Collins JK, Sams CE. (1 993). Post-harvest decay of cantaloupe caused by *Epicoccum nigrum*. Plant Dis. 77:1060–1062.

[7]. Colavolpe B, Ezquiaga J, Maiale S, Ruiz O, 2018. First report of *Epicoccum nigrum* causing disease in *Lotus corniculatus* in Argentina. *New Disease Reports* 38, 6. [http://dx.doi.org/10.5197/j.2044-0588.2018.038.006]

[8]. Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A., & Jiménez-Díaz, R. M. (2006). Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology, 96(5), 485-495.

[9]. Elmer PAG, Reglinski T. (2006) Biosuppression of Botrytis cinerea in grapes. Plant Pathol 55:155–177.

[10]. Fávaro, Léia Cecilia de Lima; Sebastianes, Fernanda Luiza de Souza; Araújo, Welington Luiz; Liles, Mark R. (4 June 2012). "Epicoccum nigrum P16, a Sugarcane Endophyte, Produces Antifungal Compounds and Induces Root Growth". PLoS ONE. 7 (6): 36826.

[11]. Fernando, N. (2009). Snow pea and Sugar snap pea. Farm Services Victoria, Department of Environment and Primary Industries, Melbourne, Victoria. Website: agriculture.vic.gov.au/agriculture/horticulture/vegetables/vegetables-a-z/snow-pea-and-sugar-snap-pea

[12]. Flannigan, B., Samson, R. A., Miller, J. D. (2011). Microorganisms in home and indoor work environments : diversity, health impacts, investigation and control (2nd ed.). Boca Raton, FL: CRC Press. ISBN 978-1-4200-9334-6.

[13]. Gribanovski-Sassu, Olga; Foppen, Fredrik H. (January 1967). "The carotenoids of the fungus Epicoccum nigrum link". Phytochemistry. 6 (6): 907–909. doi:10.1016/S0031-9422(00)86041-0.

[14]. Hashem, M. (2002) Biological control fungal of two phytopathogenic species isolated from rhizoplane of soybean(Glycine max (L.) Merr.). International Conference on Biological Sciences (ICBS), Fac. Sci. Tanta Univ.,Egypt, 27– 28 April 2002.

[15]. Hashem, M. and Ali, E. 2004. *Epicoccum nigrum* as biocontrol agent of *Pythium* damping-off and root-rot of cotton seedlings, Archives of Phytopathology and Plant Protection, 37:4, 283-297, DOI: 10.1080/03235400310001612955

[16]. Hillocks RJ. (2012) Farming with pesticides: pesticide review and resulting challenges for UK agriculture. Crop Prot 31:85–93.

[17]. Hulikere, M. M., Joshi, C. G., Ananda, D., Poyya, J., and Nivya, T. 2016). Antiangiogenic, wound healing and antioxidant activity of Cladosporium cladosporioides (Endophytic Fungus) isolated from seaweed (Sargassumwightii), Mycology, 7:4, 203-211, DOI: 10.1080/21501203.2016.1263688

[18]. Jacometti MA, Wratten SD, Walter M. (2009) Review: alternatives to synthetic fungicides for Botrytis cinerea management in vineyards. Aust J Grape Wine Res. 16:154–172.

[19]. Karabulut OA, Smilanick JL, Crisosto CH, Palou L. (2010)Control of brown rot of stone fruits by brief heated water immersion treatments. Crop Prot 29:903–906.

[20]. Lahlali R, Hijri M. 2010. Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. FEMS Microbiol Lett. 311:152–159.

[21]. Mahadevakumar S, Jayaramaiah KM, Janardhana GR, 2014. First report of leaf spot disease caused by Epicoccum nigrum on Lablab purpureus in India. Plant Disease 98, 284. http://dx.doi.org/10.1094/PDIS-07-13-0798

[22]. Maxwell S. (2008) Almeria and Murchia target clean future. Eurofruit 407:108–109.

[23]. Meneses, P. R., Dorneles, K. R., Belle, C., Moreira-Nuñez, V. L., Gonçalves, V., and de Farias, C. R. J. 2018. First Report of Cladosporium cladosporioides Causing Leaf Spot on Alstroemeria aurea in Brazil. Plant Disease Vol. 102: 1849

[24]. Nabor-Romero O, Silva-Valenzuela M, Rojas-Martínez RI, Garza-García R. (2018). First report of Cladosporium cladosporioides, a fungus that causes rot in zapote mante fruits in Mexico. Revista Mexicana de Fitopatología 36(2): 356-362.

[25]. Nam, M. H., Park, .M. S., Kim, H. S., Kim, T., and Kim, H. G. 2015. *Cladosporium* cladosporioides and *C.tenuissimum* Cause Blossom Blight in Strawberry in Korea, Mycobiology, 43:3, 354-359, DOI: 10.5941/MYCO.2015.43.3.354

[26]. Pusz, W.; Plaskowska E.; Yildirim, İ.; Weber, R. (2015). Fungi occurring on the plants of the genus Amaranthus L.. Turkish Journal of Botany. 39: 147–161.

[27]. Qin, Y., Pan, X., and Yuan, Z. (2016). Seed endophytic microbiota in a coastal plant and phytobeneficial properties of the fungus *Cladosporium cladosporioides*. Fungal Ecology, Volume 24: 53-60

[28]. Singh, J., and Singh, J. (1994). Building Mycology Management of Decay and Health in Buildings (1st ed.). Hoboken: Taylor & Francis Ltd. ISBN 978-0-203-97473-5.

[29]. Stupar, M.; Grbić, M. Lj.; Džamić, A.; Unković, N.; Ristić, M.; Jelikić, A.; Vukojević, J. (2014). "Antifungal activity of selected essential oils and biocide benzalkonium chloride against the fungi isolated from cultural heritage objects". South African Journal of Botany. 93: 118–124

[30]. Sun, C., Wang, T., Shen, X L., Wang, G. R., Gao, Q. K., Lou, B. G., and Shao, Y. Q. (2017). "First Report of Leaf Spot Caused by Cladosporium cladosporioides on Dendrobium officinale in China." Plant Disease, 101(6), p. 1055

[31]. Torres DE, Rojas-Martínez RI, Zavaleta-Mejía E, Guevara-Fefer P, Márquez-Guzmán GJ, Pérez-Martínez C (2017) *Cladosporium cladosporioides* and *Cladosporium pseudocladosporioides* as potential new fungal antagonists of *Puccinia horiana* Henn., the causal agent of chrysanthemum white rust. PLoS ONE 12(1): e0170782.https://doi.org/10.1371/journal.pone.0170782

[32]. Tyler, J.A., Richard, C.H. and Richard, R.B. (2001) Approaches to molecular characterization of fungal biocontrol agents: some case studies. Can. J. Plant Pathol., 23, 8–12.

[33]. Walker C, Muniz M, Martins RO, Rabuske J, Santos AF. (2018). Susceptibility of Pecan Cultivars to Cladosporium cladosporioides Species Complex. Floresta e Ambiente 2018; 25(4): e20170267. https://doi.org/10.1590/2179-8087.026717

[34]. Wilman, K.; Stępień, Ł.; Fabiańska, I.; Kachlicki, P. (2014). "Plant-Pathogenic Fungi in Seeds of Different Pea Cultivars in Poland". Archives of Industrial Hygiene and Toxicology. 65 (3): 329–338.

[35]. Wu D, Zhang DH, Timko MP, Li MY, Liang JL. (2017). First report of Epicoccum nigrum causing brown leaf spot of loquat in southwestern China. Plant Disease, 1553. http://dx.doi.org/10.1094/PDIS-12-16-1840