Seed transmission of pathogens associated with roselle diseases in Mexico

Teolincacihuatl Romero-Rosales[1], Rocío Toledo-Aguilar[2*], Jovany Feliciano-Morales[2], Abraham Monteon-Ojeda[1], Moisés Camacho-Tapia[3], and Elías Hernández-Castro[1]

[1]Universidad Autónoma de Guerrero (UAGro), Facultad de Ciencias Agropecuarias y Ambientales, Iguala de la Independencia, México
[2]Instituto de Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), campus Iguala, Iguala de la Independencia, México
[3]Universidad Autónoma Chapingo (UACh), Texcoco, México.

**Corresponding author email:* rociotoag@gmail.com

Calyx spot and vascular wilting are diseases that considerably limit hibiscus production in Mexico. However, the mechanisms of inoculum dissemination are unclear. Seeds are the main method of propagation in hibiscus cultivation. This study aimed to evaluate the seed transmission of fungi associated with calyx spot and vascular wilting in roselle. The study was conducted in Guerrero, Mexico in 2020. Material collected in producing areas of the entity was used. Roselle seeds were collected, disinfected, and cultivated in Petri dishes with potato dextrose agar (PDA) medium until colonies were developed. Isolates were then purified and identified by conventional and molecular methods. Based on foliar symptoms, fungal colony and spore morphology, pathogenicity tests, and ITS and TEF sequence analysis of the isolates, *Coniella diplodiella*, *Curvularia lunanta* and *Fusarium languescens* were the primary pathogens associated with leaf and calyx spot and vascular wilt. *Rhizoctonia solani* was also identified, but with severity was less than 10%, which resulted in low pathogenic capacity. The sequences of three isolates showed 99 - 100% similarity with *C. diplodiella*, *C. lunanta*, *R. solani* and *F. languescens* sequences from GenBank by BLAST analysis. The latter is the first report for this crop in Mexico. The study contributes to the understanding of the dynamics of the disease, which is crucial for effectively managing the spread of the pathogens to other regions.

Keywords: Disease, infection, pathogenicity, symptoms

In Mexico, roselle calyces (*Hibiscus sabdariffa*) are used to prepare a nutritious and refreshing drink that is part of the national culture (Reyes-Luengas et al. 2015). The crop can be found in all tropical and subtropical regions of the world, it is very adaptable and can grow in different types of soil (Bobadilla-Carrillo et al. 2016). Mexico produces more than 7,000 t of roselle every year (SIAP 2024). Production is concentrated in Guerrero, in the municipalities of Ayutla de los Libres, Tecoanapa, and San Marcos. These locations are responsible for more than 75% of the production of dehydrated calyces in the country (SIAP 2024). Roselle production is a source of income, employment, and family subsistence in this region. Conventional tillage (use of harrows and ploughs) is common on plots cultivated with roselle; sowing is done manually and directly to the ground, placing 3 to 5 seeds per position with a distance between plants of 130 cm. "Criolla" type seed (local free-pollinated materials) from the previous cropping cycle is used (Babatunde et al. 2002; Terán 2004). It is sown either after the first rains in the month of June or a late sowing in September (Alarcón-Cruz and Legaria-Solano 2013). The crop cycle from sowing to harvest is 5 - 6 months (Bobadilla-Carrillo et al. 2016). The harvest is done manually with baskets; the calyxes of each plant are removed, leaving the fruit or capsule to dry and open; approximately 10 days later this will allow the harvest of mature seeds, an activity that requires a lot of time and labour (Bobadilla-Carrillo et al. 2016).

Two major diseases of roselle crops are calyx spot and vascular wilt. Calyx spot is associated with *Coniella diplodiella, Corynespora cassiicola, Curvularia lunata,* and *Phoma* spp. (Noriega-Cantú et al. 2019; Ortega-Acosta et al. 2015; Romero-Rosales et al. 2021; Trujillo-Tapia and Ramírez-Fuentes 2015). Lesions are first observed in the leaves

0041–3216/2024/040421–430

and then in the calyces during flowering, decreasing quality and yield. These circular lesions can necrotise the entire calyx (Hernández-Morales et al. 2021; Romero-Rosales et al. 2021; Trujillo-Tapia and Ramírez-Fuentes 2015). This disease is widely distributed in Guerrero (Noriega-Cantú et al. 2021). Vascular wilt is observed in both young and mature plants and is attributed to *Fusarium oxysporum* and *Phytophthora parasitica* (Hernández-Morales et al. 2021; Michel-Aceves et al. 2019; Romero-Rosales et al. 2021). The inoculum usually comes from infected plant remains and infested soil. Infected plants show wilting, weakening, discoloration, basal necrosis, and eventual death (Ansari et al. 2013).

The losses caused by both diseases can range between 70 - 100% (Michel-Aceves et al. 2019; Noriega-Cantú et al. 2021; Romero-Rosales et al. 2021). In Guerrero, leaf and calyx spots have been reported with incidences greater than 93% in Tecoanapa, Ayutla, and Acapulco (Noriega-Cantú et al. 2021). Despite the significance of this disease, phytosanitary management consists solely of applying chemical fungicides. These products lead to negative environmental effects, development of resistance, residual fungicide in food, and risks to human health (Albert and Loera-Gallardo 2005; Puerto et al. 2014).

The mechanism by which the inoculum of both diseases is disseminated is unclear. However, the spore dispersion in the environment has been related to the incidence of calyx spot (Noriega-Cantú et al. 2019. In Mexico, González et al. 2012 found contamination from the movement of infested soil from other plots (by tractor wheels, tools, or work shoes) favouring the incidence of these diseases. Genotype, altitude, environmental conditions, and spore fluctuations are important factors in the severity and incidence of roselle calyx spot and vascular wilt (Noriega-Cantú et al. 2019).

Transmission through seeds is a common route in different pathosystems and has been reported in pathogens from the genus *Curvularia* or *Fusarium* in forages (*Brachiaria* spp.), sorghum, wheat, and corn (Al-Juboory and Juber 2013; Girish et al. 2011; Majumder et al. 2013; dos Santos et al. 2014). However, no previous study has reported the dispersion of the inoculum via seeds in roselle. Therefore, this study aimed to identify and verify the seed transmission of phytopathogenic fungi associated with calyx spot and vascular wilt in roselle crops in Guerrero, Mexico.

Materials and methods

This study was conducted at Universidad Autónoma de Guerrero in 2020. Roselle seeds of the Ayutla genotype ("criolla" type) were collected from commercial roselle plots in Ayutla de los Libres, Guerrero with a history of high incidence of calyx spot and vascular wilt. Seeds were stored at 10 ± 2°C in paper bags and dried at ambient temperature (30 ± 2°C) for 7 days.

Pathogens were isolated following the methodology reported by Piedragil-Ocampo et al. (2018) with a few modifications. A total of 100 healthy-looking seeds were selected. Seeds were disinfected by immersion in 5% sodium hypochlorite (NaClO) for 5 min and 70% methanol for 30 sec. Then, the seeds were washed thrice with sterile distilled water and dried at room temperature (23 ± 2°C). Groups of ten disinfected seeds were placed in Petri dishes with potato dextrose agar (PDA) and incubated at 23 ± 2°C with a photoperiod of 12 h (light: dark) for 5 days. Using the hyphal tip method, the mycelial growth was transferred to new Petri dishes with PDA and incubated under the same conditions until pure cultures were obtained.

Pure cultures were then identified by the macro- and -microscopic morphologic characteristics of their colonies, mycelium, and spores using the taxonomic keys proposed by Sneh et al. (1991), Barnett and Hunter (1998) and Leslie and Summerell (2006). DNA was extracted from the isolates following the method described by Sambrook and Russell (2001) for molecular identification. For all isolates, the polymerase chain reaction (PCR) was performed according to the protocol by White et al. (1990). The ITS region was amplified using the ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAA CAAGG-3') primers. The TEF1 gene was amplified with the TEF1-728F (5'-CATC GAGAAGTTCGAGAA GG-3') and TEF1-986R (5'-TACTT GAAGGAACCCTTACC-3') primers (Carbone and Kohn 1999). The amplification was conducted in a C-1000 thermocycler according to the conditions described by Rojas-Martínez et al. (2016). The amplified products were separated by electrophoresis at 90 volts per minute on a 1% agarose gel using TAE 1X (Tris-Acetate-EDTA) as a running buffer. Fragments were observed in an M-26X transilluminator equipped with a GelDoc-It™ 300 imaging system. The amplified fragments were then subjected to paired-end sequencing. The obtained sequences were assembled and edited with the Contig Assembly Program (CAP) option of BioEdit v7.0.9.1 (Hall 1999). Sequences were deposited and compared in the GenBank database of the National Center for Biotechnology Information (NCBI, USA) using the Basic Local Alignment Search Tool (BLAST) with pre-established parameters.

Controlled inoculation of the fungal isolates was conducted to demonstrate the seed transmission of the fungi associated with calyx spot and vascular wilt and corroborate their pathogenic ability. Each isolate was inoculated in 15 healthy roselle plants of the Ayutla genotype; 25-day-old plants were established in pots with sterile peat as substrate and maintained under controlled conditions (28 ± 2°C, 60 - 70% relative humidity) in protective agriculture (greenhouse). A control without inoculation (only sterile water) was included.

Inoculations were carried out with 10-day-old isolates by combining two methods, foliar spraying and substrate application of 20 mL of a solution of $1x10^6$ conidia mL^{-1}. Disease symptoms were monitored; these included rotting and drowning of stems and roots for vascular wilt and necrotic spots in leaves for calyx spot disease. Vegetative material (leaves and stems) with characteristic symptoms were collected, disinfected, and the pathogenic agents re-isolated. The colonies and reproductive structures obtained were morphologically identified and compared with the original isolates to complete Koch's postulates. The bioassay was replicated twice.

Results

During microscopic observation, some isolates with septate whitish mycelium that turned grayish 10 days after culture showed the formation of globose pycnidia with central circular and smooth ostioles, which were initially hyaline and then turned dark brown. Additionally, dark masses of central conidia were detected. Conidia were unicellular, 10 - 16.2 x 5 - 7 µm in size, and smooth, initially hyaline, then turned dark brown, ellipsoidal, and slightly curved. These characteristics correspond to *Coniella* sp. (*Phoma diplodiella*) (Figures 1a and b).

Curvularia sp. isolates were also identified. These isolates were characterised by white or grey cottony colonies that turned olive green and dark brown. These colonies had dematiaceous septate hyphae and brown straight conidiophores. Conidiophores were multicellular, simple, or branched, bent at the points where the conidia originate, with sympodial growth. Conidia of 8.14 x 21 - 35 µm were dark, multicellular, ellipsoidal, curved in the central cell, and with three to five transversal septa (Figures 1c and d).

Fusarium sp. isolates were characterised by whitish-pinkish to purple mycelial growth. Conidiophores were short, simple, and branched with canoe-shaped, hyaline, and multiseptated macroconidia (usually three septa) (Figure 1e). Isolates with hyaline mycelium turned dark, long cells with septa that usually started from main hyphae and branches, and slightly coloured and poorly formed sclerotia were identified as *Rhizoctonia* sp. (Figure 1f).

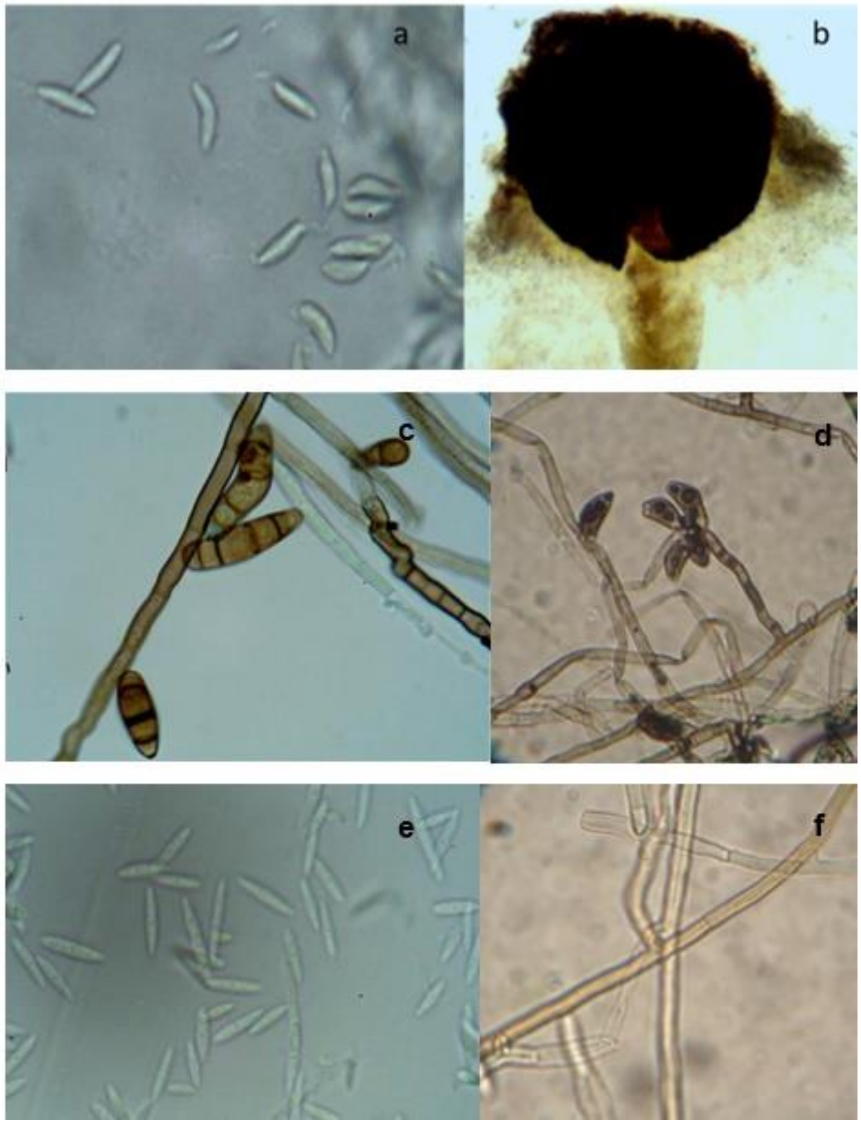

Figure 1: Morphological characteristics of conidia (a) and pycnidia (b) of *Coniella diplodiella*, conidia (c) and conidiophores (d) of *Curvularia lunata*, conidia (e) of *Fusarium* sp., and *Rhizoctonia solani* hyphae (f) from isolates obtained from roselle seeds collected in Guerrero, Mexico. Lactophenol staining with methylene blue. 100X. Laboratory of phytopathology - School of Agricultural and Environmental Sciences, Universidad Autónoma de Guerrero, Iguala de la Independencia, Guerrero, Mexico. 2021.

The molecular analysis resulted in the ITS4 and ITS5 primers producing sequences of 634, 589, and 704 bp, in the BLASTn analysis, a sequence similarity of 99% was found with *Coniella diplodiella*, *Curvularia lunata*, and *Rhizoctonia solani*, these were deposited at GenBank with the accession numbers: MK532913, MK532912 and MK532911, respectively.

The amplification of the TEF1-728F (5’-CATCGAGAAGTTCGAGAAGG-3’) and TEF1-986R regions of the TEF1 gene resulted in a 276 bp sequence, in the BLASTn analysis a sequence similarity of 99% was found with *Fusarium languescens* and were deposited at the GenBank with the accession number: MK600382. This study is the first to report *F. languescens* as an etiologic agent of roselle vascular wilt in Mexico, contributing to understanding this disease's etiology and epidemiology.

The proportion of the incidence of pathogens in the 100 roselle seeds was: 30% *C. lunata*, 20 % *F. languescens*, 12 % *R. solani,* and 6% *C. diplodiella*.

After 4 - 8 days, all seedlings inoculated with *C. diplodiella* and *C. lunata* showed leaf spot symptoms, these symptoms were characterised by reddish circular spots of 1 - 2 mm (Figures 2a and b); The inoculated plants with *C. lunata*, presented wounds and reddish spots on mature leaves, general chlorosis and loss of vigor (Figures 2c and d). After 6 - 10 days those inoculated with *F. languescens* developed symptoms of vascular wilt. The inoculation of *F. languescens* in healthy roselle plants led to vascular wilt and damage. These symptoms are associated with tender stems and shoots, general decay and discoloration, and necrotic lesions at the base of the stem that extend to branches and leaves (leaf blight) (Figures 2e and f). Although *Rhizoctonia solani* induced typical symptoms (small dark brown sunken lesions on stems and roots and reduced growth), the severity was less than 10%, which resulted in low pathogenic capacity.

The control plants (without inoculation) did not present symptoms associated with any of the pathologies described, they remained healthy.

Figure 2: Calyx spot and vascular wilt symptoms in roselle plants inoculated with *Coniella diplodiella* (a and b), *Curvularia lunata* (c and d), and *Fusarium languescens* (e and f). Laboratory of phytopathology - School of Agricultural and Environmental Sciences, Universidad Autónoma de Guerrero, Iguala de la Independencia, Guerrero, Mexico. 2021.

Discussion

To design a successful disease management strategy, it is essential to determine the causality and elucidate the life cycle, development conditions, and mechanisms of dissemination, transmission, and infection of the associated pathogens (etiology and epidemiology) (Ojiambo et al. 2017). This study identified *Coniella diplodiella, Curvularia lunata, Fusarium languescens,* and *Rhizoctonia solani* as the pathogens associated with calyx spot and vascular wilt in roselle. Identification was made via observation of morphological characteristics and using molecular techniques.

These results are similar to those reported in other studies. In Oaxaca, Mexico (Pacific coast), Trujillo-Tapia and Ramírez-Fuentes (2015) isolated and characterised *Fusarium oxysporum*, *Coniella* sp., *Corynespora* sp., *Curvularia* sp., and *Phoma* sp. from roselle leaves and calyces showing symptoms of calyx spot. In Tabasco, Mexico (Atlantic coast), Correa et al. (2011) reported *Coniella* and *Corynespora* as etiologic agents of leaf spot in roselle. In a study also conducted in Guerrero (Pacific coast), Ortega-Acosta et al. (2015) identified *Corynespora cassiicola* as the etiologic agent of leaf and calyx spot in roselle. In a recent study *Rhizoctonia solani* was identified as a cause of root and basal stem rot on roselle in Guerrero, results that are similar with the findings of this research (Palemón-Alberto et al. 2024). In this study, we did not identify any *Corynespora* sp. or *Phoma* sp., suggesting differences in the proportion of inoculum of different species according to the sampling areas also associated with pathogen dissemination methods.

Epidemiological studies have a crucial role in understanding the origin of the pathogenic elements due to the introduction of different inoculum sources at susceptible phenological stages (dos Santos et al. 2014). Noriega-Cantú et al. (2019), in a study on roselle also conducted in Guerrero, used volumetric traps to capture the spores in the environment. They captured and identified spores from *Coniella* sp., *Corynespora* sp., *Curvularia* sp., *Alternaria* sp., and *Lasiodiplodia* sp., pathogens associated with the roselle calyx spot. They also reported a significant positive correlation between *Coniella* sp. and *Curvularia* sp. population fluctuations, high temperatures (31 - 33°C), and disease severity. This could indicate different sources of inoculum with potential for infection and occurrence of epidemics in the field.

As observed in this study, seeds can harbour and transport microorganisms or phytopathogenic agents in many taxonomic groups (dos Santos et al. 2014). Seed contamination with pathogenic fungi can occur in various phases of the crop cycle, during harvest, post-harvest, or storage. In this case, it could not be determined in which phase of the crop cycle the seed was contaminated, but Koch's postulates in our study indicated that the seed generated in the previous cycle was indeed infected.

The importance of the above is that demonstrating seed transmission represents a window of opportunity to establish strategies (seed treatments, for example) that decrease the risk of contamination between plots, reduce the incidence of these diseases, and increase the yield and quality of the products (Maeso and Walasek 2012; Velásquez-Valle et al. 2007).

The pathogenicity tests in our study demonstrate the pathogenic and seed transmission capacity of *Coniella* sp., *Corynespora* sp., *Curvularia* sp., *Alternaria* sp., and *Lasiodiplodia* sp. in vascular wilt and calyx spot in roselle. Knowing that the seed can be a primary source of inoculum for the incidence of roselle diseases enhances the ability to design management strategies.

Several studies have focused on demonstrating the seed transmission of phytopathogens as a starting point for understanding and managing diseases. Mandour et al. (2013) verified the distribution and transmission of bean common mosaic virus (BCMV) via seeds in three cultivars. In another study, Vallad et al. (2005) verified the transmission via seed of *Verticillium dahliae* in lettuce, they found up to 80% incidence of wilting symptoms in plants obtained from seeds infected with the fungus.

The evidence of seed transmission of the phytopathogenic fungi associated with vascular wilt and calyx spot in roselle contributes to understanding the etiology and epidemiology of these diseases.

It is important to know the percentage of incidence associated with the different possible sources of inoculum (spores in the environment, water, soil, seed), determine what the effects are on the viability of the seed, characterise the conditions of inoculum survival associated with seed storage for sowing in subsequent cycles. There is also the need to develop and evaluate the effectiveness of multiple seed disinfection techniques and implement them as routine activities in the sanitary management of the crop. There are various cases of the use of seed treatments based on chemical and organic fungicides, thermal treatments and biological control agents to prevent the incidence of diseases in the field, such as smut (*Ustilago hordei*) and common root rot in barley, smut (*Ustilago tritici* and *Ustilago segetum*) in wheat, damage by *Pythium* sp. in wheat, damage and damping off by *Pythium* sp. and *Phythophthora* sp. in alfalfa and small-seeded legumes or *Rhizoctonia* sp. problems in soybeans (McMullen and Lamey 2000). However, all these strategies must be based on experimentation, taking into account factors such as genotype, pathogen, environmental conditions, and crop management (time and type of sowing, irrigation, nutrition).

Conclusions

The seed transmission of pathogenic fungi in roselle represents a latent risk to the crop's health. *Fusarium languescens,* associated with vascular wilt, and *Coniella diplodiella* and *Curvularia lunata*, associated with calyx spot, were found in the seeds collected from the field. Seeds are a source of inoculum for the development of these diseases. This knowledge allows for the formulation of comprehensive management strategies that include using healthy seeds and developing and applying prophylactic treatments.

Acknowledgement

This study was funded by **Consejo Nacional de Humanidades, Ciencias y Tecnologías** (Conahcyt); project 317237: "Towards self-managed food sovereignty: strengthening the milpa system through agroecological management and the conservation of native genetic resources in the regions of Acapulco and the Costa Chica of Guerrero.

Author contributions

Conceptualisation: T.R.R., M.C.T. and R.T.A. Methodology: T.R.R. Research: J.F.M. Writing: original draft preparation: A.M.O. and T.R.R. review and editing: T.R.R. and E.H.C.

Conflict of interest

The authors declare that they have no competing interests.

References

Alarcón-Cruz, N., and J.P. Legaria-Solano. 2013. "Caracterización Morfológica de una Muestra Etnográfica de Jamaica (*Hibiscus sabdariffa* L.)." *Revista Chapingo. Serie Horticultura* **19 (1)**: 85−98. https://doi.org/10.5154/r.rchsh. 2010. 03.011

Albert, L., and R. Loera Gallardo. 2005. "Química y Ecotoxicología de los Fungicidas." In *Golfo de México Contaminación e Impacto Ambiental,* edited by A.V. Botello, J. Rendón von Osten, J.A. Benítez, and G. Gold-Bouchot, 213−234. *Diagnóstico y Tendencias,* Universidad Autónoma de Campeche.

Al-Juboory, H.H., and K.S. Juber. 2013. "Efficiency of Some Inoculation Methods of *Fusarium proliferatum* and *F. verticilloides* on the Systemic Infection and Seed Transmission on Maize Under Field Conditions." *Agriculture Biology Journal of North America.* https://www.scihub.org/ABJNA/PDF/2013/6/ABJNA-4-6-583-589.pdf.

Ansari, M., T. Eslaminejad, Z. Sarhadynejad, and T. Eslaminejad. 2013. "An Overview of the Roselle Plant with Particular Reference to its Cultivation, Diseases and Usages." *European Journal of Medicinal Plants* **3 (1)** Article 135. https://doi.org/ 10.9734/EJMP/2013/1889.

Babatunde, F.E., T.O. Oseni, B.M. Auwalu, and G.N. Udom. 2002. "Effect of Sowing Dates, Intra-Row Spacings and Nitrogen Fertilizers of the Productivity of Red Variant Roselle (*Hibiscus sabdarifa* L)." *Pertanika Journal of Tropical Agricultural Science* **25 (2)**: 99−106. http://pertanika2.upm.edu.my/resources/files/Pertanika%20PAPERS/JTAS%20Vol.%2025%20(2)%20Sep.%202002/04%20JTAS%20Vol.25%20(2)%202002%20(Pg%2099-106).pdf

Barnett, H.L., and B.B. Hunter. 1998. *Illustrated Genera of Imperfect Fungi* (4th ed.). APS Press. https://doi.org/10.2307 /3755678.

Bobadilla-Carrillo, G.I., R. Balois-Morales, M.G. Valdivia-Reynoso, M.L. Machuca-Sánchez, and L. González-Torres. 2016. "Preharvest, Harvest and Postharvest Factors Inherent to Roselle (*Hibiscus sabdariffa* L.) Crop: A Review." *Revista Bio Ciencias* **3 (4)**: 256−268. https://revistabiociencias.uan.edu.mx/index.php/BIOCIENCIAS/article/download/158/221/1289.

Carbone, I., and L.M. Kohn. 1999. "A Method for Designing Primer Sets for Speciation Studies in *Filamentous ascomycetes*." *Mycologia,* **91**:553−556. https://doi.org/10. 1080/00275514.1999.12061051

Correa, S.E., G.C.F. Ortiz, C.M. Torres, M.C.C. Bautista, C.M.C. Rivera, E.L.C. Lagunes, and S.J.H. Hernández. 2011. "Etiología de la Mancha Acuosa de la Jamaica (*Hibiscus sabdariffa*) en Tabasco, México." *Revista Mexicana de Fitopatología* **29**:165−167. http://www.scielo.org.mx/scielo.php?pid=S0185-33092011000200008&script=sci_arttext

dos Santos, G.R., P.H. Tschoeke, L. de Godoi Silva, M.C.A. Carnerio de Silveira, H.B. Reis, D.R. Brito, and D. de Souza Carlos. 2014. "Sanitary Analysis, Transmission and Pathogenicity of Fungi Associated with Forage Plant Seeds in Tropical Regions of Brazil." *Journal of Seed Science* **36**:54−62. https://doi.org/10.1590/s2317-15372014000100007.

Girish, G.A., M.M.V. Baig, K. Anitha, and S.K. Chakrabarty. 2011. "Curvularia Species Detected in Sorghum Seeds Collected from Marathwada Region of Maharashtra." *Indian journal of Plant Protection* **39 (4)**: 299−303. https://www.researchgate.net/profile/Baig-Mmv/publication/269819363_Curvularia_Species_Detected_in_Sorghum_Seeds_Collected_From_Marathwada_Region_of_Maharashtra/links/549e681a0cf202801ea6bdf2/Curvularia-Species-Detected-in-Sorghum-Seeds-Collected-From-Marathwada-Region-of-Maharashtra.pdf.

González, I., Y. Arias, and B. Peteira. 2012. "Aspectos Generales de la Interacción *Fusarium oxysporum* f. sp. Lycopersici-Tomate." *Revista de Protección Vegetal* **27(1)**: 1−7. http://scielo.sld.cu/scielo.php?pid=S1010-27522012000100001&script=sci_arttext&tlng=en.

Hall, T.A. 1999. "BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT." *Nucleic Acids Symposium Series* **41**:95−98. http://brownlab.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf.

Hernández-Morales, J., T. Romero-Rosales, A.C. Michel-Aceves, M. Vargas-Hernández, A. Monteón-Ojeda, and J.L. Valenzuela-Lagarda. 2021. "Antagonismo Enzimático de *Trichoderma* spp. sobre *Fusarium oxysporum* y *Phytophthora parasitica* en *Hibiscus sabdariffa* L." *Bioagro* **33 (3)**: 203−214. https://doi.org/10.51372/bioagro333.6.

Leslie, J.F., and B.A. Summerell. 2006. *The Fusarium Laboratory Manual*. Blackwell Publishing. Ames, Iowa, 230−239. https://doi.org/10.1002/9780470278376.

Maeso, D., and W. Walasek. 2012. "Evaluación de Métodos Para Desinfectar Semillas de Tomate Contra Cancro Bacteriano (*Clavibacter michiganensis* subsp. michiganensis)." *Agrociencia (Uruguay)* **16 (1)**: 134−142. http://www.scielo.edu.uy/scielo.php?pid=S2301-15482012000100016&script=sci_arttext.

Majumder, D., T. Rajesh, E.G. Suting, and A. Debbarma. 2013. "Detection of Seed Borne Pathogens in Wheat: Recent Trends." *Australian Journal of Crop Science* **7 (4)**: 500−507. https://www.cabidigitallibrary.org/doi/pdf/10.5555/20133173059.

Mandour, A.M., H.M. Abdelmaksoud, and M.A. El-Tahlawey. 2013. "Seed Transmission of Bean Common Mosaic Virus." *Egyptian Journal of Agricultural Research* **91 (2)**: 403−410. https://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1987Articles/PlantDisease71n01_51.pdf

McMullen, M.P., and H.A. Lamey. 2000. "Seed Treatment for Disease Control." https://library.ndsu.edu/ir/bitstream/handle/10365/9120/PP447_2000.pdf?sequence=1.

Michel-Aceves, A.C., J. Hernández-Morales, R. Toledo-Aguilar, L.J.E. Sabino, and T. Romero-Rosales. 2019. "Capacidad Antagónica de *Trichoderma* spp. Nativa Contra *Phytophthora parasitica* y *Fusarium oxysporum* Aislados de Cultivos de Jamaica." *Revista Fitotecnia Mexicana* **42 (3)**: 235−241. https://doi.org/10.35196/rfm.2019.3.235-241.

Noriega-Cantú, D.H., E.R. Garrido-Ramírez, J. Pereyda-Hernández, R. Toledo-Aguilar, R. Vásquez-Ortiz, A. Alejo-Jaimes, and R. González-Mateos. 2021. "Incidencia y Distribución Geográfica del Manchado del Cáliz de Jamaica en Guerrero, México. *Tropical and Subtropical Agrosystems* **24**: Artículo 83. https://www.revista.ccba.uady.mx/ojs/index.php/TSA/article/view/2998/1638.

Noriega-Cantú, D.H., R. Toledo-Aguilar, R. Vásquez-Ortiz, A. Alejo-Jaimes, E.R. Garrido-Ramírez, J. Pereyda-Hernández, and R. González-Mateos. 2019. "Relationship between Spore Fluctuations, Environmental Conditions and Severity of Calyx Spot on Roselle (*Hibiscus sabdariffa*). *Revista Mexicana de Fitopatología* **38 (1)**: 1−24. https://doi.org/10.18781/r.mex.fit.1909-1.

Ojiambo, P.S., J. Yuen, F. Van den Bosch, and L.V. Madden. 2017. "Epidemiology: Past, Present, and Future Impacts on Understanding Disease Dynamics and Improving Plant Disease Management — A Summary of Focus Issue Articles." *Phytopathology* **107 (10)**: 1092−1094. https://doi.org/10.1094/PHYTO-07-17-0248-FI.

Ortega-Acosta, S.A., J. Hernández-Morales, D.L. Ochoa-Martínez, and V. Ayala-Escobar. 2015. "First Report of *Corynespora cassiicola* Causing Leaf and Calyx Spot on Roselle in Mexico." *Plant Disease* **99 (7)**: Article 1041. https://doi.org/10.1094/pdis-04-14-0438-pdn.

Palemón-Alberto, F., G. Reyes-Garcia, S.A. Ortega-Acosta, E. Toledo-Hernández, Y. Romero-Ramírez, J. Toribio-Jimenez, J. Terrones-Salgado, A.J. Gonzaga-Segura, and J.L. Evaristo-Ruiz. 2024. "First Report of *Rhizoctonia solani* AG-4 HG-I Causing Root and Basal Stem Rot on Roselle (*Hibiscus sabdariffa*) in Mexico." *Plant Disease* **108 (3)**: 803. https://doi.org/10.1094/pdis-09-23-1830-pdn.

Piedragil-Ocampo, B., A. Monteon, L. Torres-Rueda, J. San-Juan Lara, J.M. Alarcón García, and J. Martinez Delgado. 2018. "Evaluación de la Actividad Fungicida de Cuatro Extractos Vegetales Sobre Control de Mancha Chocolate *Botrytis fabae* en Haba *Vicia faba* L. en Puebla, México." *Revista Mexicana de Fitosanidad,* **2 (2)**: 13−24.

Puerto, R.A.M., S.S. Tamayo, and E.D.E. Palacio. 2014. "Efectos de los Plaguicidas sobre el Ambiente y la Salud." *Revista Cubana de Higiene y Epidemiología* **52 (3)**: 372−387. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1561-30032014000300010.

Reyes-Luengas, A., Y. Salinas-Moreno, M.E. Ovando-Cruz, R.I. Arteaga-Garibay, and M.D. Martínez-Peña. 2015. "Análisis de Ácidos Fenólicos y Actividad Antioxidante de Extractos Acuosos de Variedades de Jamaica (*Hibiscus sabdariffa* L.) con Cálices de Colores Diversos." *Agrociencia* **49**:277−290. https://agrociencia-colpos.mx/index.php/agrociencia/article/ view/1146.

Rojas-Martínez, R.I., M. Camacho-Tapia, E. Zavaleta-Mejia, and J. Levy. 2016. "First Report of the Presence of Haplotypes A and B of *Candidatus Liberibacter* solanacearum in Chili (*Capsicum annuum* L.) in the Central Region of Mexico." *Journal of Plant Pathology* **98 (1)**: 111−115.

Romero-Rosales, T., J. Acuña-Soto, A. Azuara-Domínguez, M.O. Lázaro-Dzul, A. Monteon-Ojeda, H. Vargas-Madriz, and Y. Secundino-Eusebio. 2021. "Identificación y Evaluación Antagónica In Vitro de Cepas Nativas de *Trichoderma* spp. Sobre Hongos Fitopatógenos Asociados Al Manchado del Cáliz en Jamaica (*Hibiscus sabdariffa* L.)." *Tropical and Subtropical Agroecosystems* Artículo 24. https://doi.org/10.56369/tsaes.3518

Sambrook, J., and D.W. Russell. 2001. *Molecular Cloning. A Laboratory Manual,* 3rd ed. Cold Spring Harbor Laboratory. https://doi.org/10.1086/394015.

SIAP (Servicio de Información Agroalimentaria y Pesquera). 2024. "Cierre de la Producción Agrícola 2023." https://www.gob.mx/siap/acciones-y-programas/produccion-agricola-33119

Sneh, B., L. Burpee, and A. Ogoshi. 1991. *Identification of Rhizoctonia Species*. APS Press. https://doi.org/10.2307/2806936.

Terán, Z. 2004. "Evaluación de Densidades de Plantación en el Cultivo de la Jamaica (*Hibiscus sabdariffa* L.)." *Cultivos Tropicales* **25 (1)**: 67−69. https://ediciones.inca.edu.cu/index.php/ediciones/article/download/537/pdf.

Trujillo-Tapia, M.A., and E. Ramírez-Fuentes. 2015. "Fitopatógenos Asociados al Manchado del Cáliz de la Jamaica (*Hibiscus sabdariffa* L.)." *Revista Iberoamericana de Ciencias* **2 (4)**: 58−62. http://www.reibci.org/publicados/2015/julio/0600115.pdf.

Vallad, G.E., R.G. Bhat, S.T. Koike, E.J. Ryder, and K.V. Subbarao. 2005. "Weedborne Reservoirs and Seed Transmission of *Verticillium dahliae* in Lettuce." *Plant Disease* **89 (3)**: 317−324. https://apsjournals.apsnet.org/doi/pdf/10.1094/PD-89-0317.

Velásquez-Valle, R., M.D. Amador-Ramírez, M.M. Medina-Aguilar, and F. Lara-Victoriano. 2007. "Presencia de Patógenos en Almácigos y Semilla de Chile (*Capsicum annuum* L.) en Aguascalientes y Zacatecas, México." *Revista Mexicana de Fitopatología* **25 (1)** 75−79. https://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0185-33092007000100010

White, T.J., T.B. Bruns, S.B. Lee, J.W. Taylor, M.A. Innis, D.H. Gelfand, and J. Sninsky. 1990. "Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics." In *PCR Protocols: A Guide to Methods and Applications*, edited by A.V. Botello, J. Rendón von Osten, J.A. Benítez, and G. Gold-Bouchot, 315−322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1