Reaction of some citrus cultivars to *Phaeoramularia* fruit and leaf spot in Kenya

Abdurabi Abdurabi Seif[a]*
Rory Hillocks[b]

[a] GTZ IPM
Horticulture Project,
PO Box
41607 Nairobi, Kenya

[b] Natural Resources Institute,
Chatham Maritime,
Kent ME4 4TB, UK

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Abstract — Introduction. *Phaeoramularia* fruit and leaf spot of citrus was first reported in Kenya in 1984. It affects 20% of total citrus hectarage in Kenya and causes 20 to 100% yield loss. Reaction of commonly grown citrus cultivars to *Phaeoramularia angolensis* was studied. **Materials and methods**. The study was carried out for a period of 2 years (four seasons). Cultivars were assessed for susceptibility to *P. angolensis* using leaf assays under laboratory conditions and by field observations on disease incidence and severity on fruits and leaves. **Results**. Susceptibility varied among citrus species and cultivars within a species; also in a same cultivar, fruit and leaves were not equally susceptible. Marsh seedless grapefruit was most susceptible while Tahiti lime was least. Incubation and latent periods ranged from 9 to 16 d and 16 to 32 d in Marsh seedless grapefruit and Tahiti lime, respectively. No significant differences were observed on relative sporulation of the fungus on the various species/cultivars. Spore germination and stomatal penetration percentages were not significantly different on Marsh seedless grapefruit and Tahiti lime but there was a marked difference in disease severity. **Discussion**. Since all citrus types grown in Kenya are affected by the disease, susceptibility or resistance alone does not offer a solution to the problem, and, therefore, incorporation of chemical intervention in disease management is necessary. The leaf assays used in this study could be useful in in vitro screening of citrus for resistance to *P. angolensis*. © Éditions scientifiques et médicales Elsevier SAS

Kenya / *Citrus* / plant diseases / fungi / *Phaeoramularia angolensis* / variety trials / disease resistance

Comportement de quelques cultivars d'agrumes vis-à-vis de la maladie des taches sur feuilles et fruits, due à *Phaeoramularia* au Kenya.

Résumé — Introduction. Cette maladie, signalée pour la première fois au Kenya en 1984, affecte 20 % des superficies d'agrumes du pays et cause 20 à 100 % de perte parmi les récoltes. La réaction à *P. angolensis* des cultivars d'agrumes les plus communs a été étudiée. **Matériel et méthodes**. L'étude a été menée pendant 2 ans (quatre saisons). Les cultivars ont été évalués quant à leur sensibilité à *P. angolensis* en utilisant des tests sur feuilles en laboratoire et en effectuant des observations en champ sur l'incidence et la gravité de la maladie sur feuilles et fruits. **Résultats**. La sensibilité au pathogène a varié d'une espèce à l'autre et selon les cultivars d'une même espèce ; pour un cultivar donné, les feuilles et les fruits sont affectés différemment. Le pomelo « Marsh seedless » a été le plus sensible, alors que le limettier Tahiti a été le moins affecté. Les périodes d'incubation et de latence ont varié respectivement de 9 à 16 d et de 16 à 32 d pour le pomelo et pour le limettier. La sporulation relative du champignon a été semblable quelles que soient l'espèce ou la variété considérées. Les taux de germination des spores et de pénétration stomatale n'ont pas été significativement différents sur pomelo ou sur limettier, mais la gravité de la maladie a varié. **Discussion**. Puisque tous les types d'agrumes exploités au Kenya sont affectés par la maladie, l'attaque par *P. angolensis* ne peut être contrôlée par le seul caractère de sensibilité ou de résistance ; dès lors, il est nécessaire de faire intervenir la lutte chimique. Les tests sur feuilles utilisés au cours de cette étude pourrait être utiles pour cribler les agrumes vis-à-vis de leur résistance à *P. angolensis* par des techniques in vitro. © Éditions scientifiques et médicales Elsevier SAS

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* *Correspondence and reprints*

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1. introduction

Citrus is an important cash crop in Kenya with an annual return of about $US 6.6 million. The total area under the crop is about 19,000 ha and it is grown mainly by smallholders from the coastal belt of Kenya through to the highlands [1]. Size of the orchards ranges from 0.3 to 0.5 ha with mixed plantings of different citrus cultivars. Commonly grown citrus cultivars include Kara mandarin, Marsh seedless grapefruit, rough lemon, sweet orange (cvs. Pineapple, Valencia Late and Washington Navel) and Tahiti lime.

Fruit and leaf spot [*Phaeoramularia angolensis* De Carvalho and Mendes, P.M. Kirk (syn.: *Cercospora angolensis*)] is the main disease of the crop in the highlands west of the Great Rift Valley. It was first observed in western Kenya in 1984 on cv. Washington Navel [2] and it is now widespread in production areas in the highlands where it affects all commonly grown citrus cultivars [3]. Young leaves and fruit are particularly susceptible to the disease. Leaf and fruit inocula are present in citrus fields throughout the year because of overlap of crops. Wet periods and moderately cool temperatures (24 °C–26 °C) favour disease development [4].

The disease affects about 20% of total citrus hectarage in Kenya and is very severe in areas of western Kenya where total loss of marketable fruit is common in unsprayed orchards [5]. It is estimated that 71% of citrus growers in the affected areas of Kenya use contact and systemic fungicides for the control of *Phaeoramularia*. Losses in marketable yield in sprayed orchards range from 20% to 50% [6]. The cost effectiveness of chemical control is doubtful, especially in small citrus orchards typical of Kenya. Also, continuous use of systemic fungicides may lead to the development of resistance in *P. angolensis*.

Reaction to infection by *P. angolensis* has been shown to vary between and within *Citrus* spp. [7–10]. However, these findings were based on field observations or on inoculation of nursery plantlets. In this study, attempts were made to determine under laboratory conditions 1) reaction to *P. angolensis* of young detached leaflets of citrus cultivars commonly grown in Kenya, 2) spore germination and germ tube penetration of *P. angolensis* on leaf disks of Marsh seedless grapefruit (very susceptible) and Tahiti lime (less susceptible). In addition, 14 cultivars at a field site in western Kenya were assessed for susceptibility to the disease on basis of incidence and severity on fruit and leaves.

2. materials and methods

2.1. reaction of citrus detached leaflets to inoculation with *P. angolensis*

This work was carried out under laboratory conditions using detached young circa 2 week-old leaves of Kara mandarin, Marsh seedless grapefruit, rough lemon, sweet orange (cvs. Pineapple, Valencia Late and Washington Navel) and Tahiti lime.

Young leaves of similar age of the various cultivars were picked on the same day early in the morning from actively growing seedlings in a screenhouse. They were washed in running tap water, surface sterilized with 5% sodium hypochlorite, rinsed three times in sterile distilled water and blotted dry. The leaflets were floated on sterile distilled water, lower surface up, in 9 cm petri dishes. The abaxial side of the leaflets was inoculated by finely spraying with a spore suspension (105 conidia·mL^{-1}) of *P. angolensis* cultured on V8-juice agar at 25 °C–26 °C. Petri dishes were sealed with Parafilm and incubated at 25 °C–26 °C (Gallenkamp; Model: IR211 GA) under a 12 h photoperiod.

Each treatment (cultivar) was replicated five times and each replicate comprised of one petri dish with four leaflets.

Observations made were: 1) incubation period (no. of days from inoculation to the day when 50% of the leaflets have developed lesions); 2) latent period (no. of days from inoculation to when 50% of the lesions were sporulating); 3) relative sporulation (conidia·mL^{-1} from a pooled sample when 50% of the lesions were found active and determined using a haemocytometer);

and 4) total number of lesions per leaflet per cultivar 40 d of inoculation.

Square root transformation was applied to the sporulation and disease severity data prior to analysis of variance. Data on incubation and latent periods were not transformed. Standard error of mean differences (SEM) was used to separate the means.

2.2. spore germination and germ tube penetration of *P. angolensis*

Leaf disks of Marsh seedless grapefruit (very susceptible) and Tahiti lime (less susceptible) were cut from 2 week-old leaflets using a 15 mm cork borer. Then they were washed in running tap water, surface sterilized with 5% sodium hypochlorite, rinsed three times in sterile distilled water and floated on sterile distilled water in 9 cm petri dishes.

The disks were inoculated by placing on the abaxial surface a droplet (2.5 µl) of a spore suspension of *P. angolensis* (104 conidia·mL^{-1}) using a micropipette (Labsystems, Finnpipette, Finland). A low concentration of conidia was used to facilitate counting of the conidia with a light microscope. The petri dishes containing leaf disks were individually sealed with Parafilm and incubated at 25 °C–26 °C under a 12 h photoperiod.

Each treatment was replicated five times, and each replicate consisted of a petri dish with 10 leaf disks. A total of random 50 conidia per replicate were counted under 20× magnification. A conidia was considered germinated if the length of germ tube was at least twice its diameter.

Germ tube penetration was determined by Murray and Huazhi's method [11]: a leaf disk from each replicate was picked every 24 h for a period of 7 d and fixed in circa 99% glacial acetic acid and 50% ethyl alcohol (1:17 v/v) for at least 12 h. The samples were then placed in test tubes containing 0.01% trypan blue in lactophenol, heated in a water bath at 85 °C for 10 min to stain and clear the tissues, and mounted in lactophenol on glass slides.

Twenty conidia per replicate were observed for germ tube penetration with a light microscope at 100× magnification.

2.3. disease severity assessment

The number of lesions per leaf disk for the remaining 30 disks in the two treatments was counted 40 d after inoculation to assess disease severity. Data obtained were subjected to Student's *t* test for comparison. Percent spore germination and germ tube penetration were transformed into arcine values, and square root transformation was applied to number of lesions prior to analysis.

2.4. incidence and severity of fruit and leaf spot disease

Observation on incidence and severity of fruit and leaf spot disease was carried out on 14 citrus cultivars for a period of 2 years at a government prison orchard in Trans-Nzoia District in western Kenya where fruit and leaf spot disease is endemic. Altitude of the site is 1,600 m. Annual mean minimum and maximum temperatures at the site were 12.0 °C and 26.0 °C respectively. Average annual rainfall was 1,600 mm. Size of the orchard is about 25 ha comprising various 10 to 16 year old citrus species. The species included citrange, grapefruit, lemon, lime, sweet orange and tangelo on rough lemon stock. No fungicides nor oil-based insecticides were applied to trees under observation for the 2 years.

Disease incidence on fruit and leaves was assessed on five random trees in the middle row of each cultivar. Destructive sampling was carried out for disease assessment on fruit and leaves. Incidence on leaves was determined on eight random terminal shoots from lower and upper halves of a tree, total number of leaves counted and the disease incidence expressed as a proportion of leaves with at least one lesion. Incidence on fruit was determined on the same five trees by picking random 40 fruits per tree (10 from each of four quarters of the tree; five from lower and five from upper canopy), recorded as + or – diseased and expressed as a proportion of the total number of fruits.

Assessment of severity was done on the same samples used for disease incidence. Severity on leaves was based on the fol-

Table I.
Reaction of detached leaflets of seven citrus cultivars to inoculation with *Phaeoramularia angolensis* under laboratory conditions floated on sterile distilled water in petri dishes; 12 h photoperiod; 25 °C).

Cultivar	Incubation period[1] (d)	Latent period[2] (d)	Relative sporulation[3] (conidia·mL^{-1})	Disease severity (spots/leaf)
Marsh seedless grapefruit	9.4	16.2	20,180 (142.1)	11.5 (3.4)[4]
Pineapple sweet orange	10.4	18.2	20,920 (144.6)	10.3 (3.2)
Washington Navel	10.6	23.0	20,600 (135.5)	8.3 (3.0)
Valencia Late	10.8	24.2	20,720 (143.9)	8.0 (2.8)
Kara mandarin	10.8	24.4	20,460 (143.0)	7.7 (2.8)
Rough lemon	12.6	28.6	20,920 (144.6)	5.4 (2.3)
Tahiti lime	15.8	32.4	21,080 (145.2)	2.1 (1.4)
Standard error of mean differences (p = 0.05)	0.3	0.4	561 (23.7)	0.3 (0.5)
Coefficient of variation (%)	6.1	3.7	6.1	4.4

[1] No. of days from inoculation to when 50% of leaflets developed lesions.
[2] No of days from inoculation to when 50% of lesions sporulated.
[3] Mean no. of conidia in 1 mL aliquots from a pooled sample.
[4] Square root transformed values of the means in parentheses.

lowing score system derived from graphic representation of lesions on the leaf surface area: 1 = 0%; 2 = <1%; 3 = 1–3%; 4 = 3–5% and 5 = >5% of leaf area affected. A similarly devised rating system was used for fruit severity: 1 = 0%; 2 = <5%; 3 = 5–20%; 4 = 20–50% and 5 = >50% of fruit surface. Out of 100 random field samples of diseased leaves and fruits used in devising the rating system, severity scales did not exceed 5% and 50% for leaves and fruits, respectively.

Biweekly data on leaves were collected during the rainy seasons (April-June / October-November) and fruit was assessed in January-February and September-October every year. The two-year data was pooled and means calculated.

3. results

3.1. reaction of citrus detached leaflets to inoculation with *P. angolensis*

Incubation period for the cultivars inoculated with *P. angolensis* ranged from 9.4 d to 15.8 d representing Marsh seedless grapefruit and Tahiti lime, respectively. Latent period between the cultivars ranged from 16.2 d to 32.4 d with Marsh seedless grapefruit and Tahiti lime at two extremes, respectively. Disease severity scores for the cultivars followed a similar trend as incubation and latent periods. However, there was no significant difference (p = 0.05) between cultivars in sporulation capacity of *P. angolensis* (*table I*).

3.2. spore germination and germ tube penetration of *P. angolensis*, and disease severity

About 78% of the conidia germinated 24 h post-inoculation on leaf surfaces of susceptible Marsh seedless grapefruit and resistant Tahiti lime, initiating one or two polar germ tubes per conidium.

Germ tubes grew randomly and elongated without branching, in some cases passed near or over stomata without penetrating. The germ tubes were 40 µm and more in length. Germ tubes initiated infection hyphae, which effected penetration through both closed and open stomata. Penetration was observed 48 h post-inoculation. Neither direct penetration nor formation of appressoria were discerned.

Table II.
Conidial germination and penetration of *Phaeoramularia angolensis*, and disease severity on leaf disks of Marsh seedless grapefruit and Tahiti lime (floated on sterile distilled water; 24 h photoperiod; 25 °C).

Citrus cultivar	Germination[1] (%)	Penetration[1] (%)	Disease severity[2] (lesions/leaf disk)
Marsh seedless grapefruit (very susceptible)	79.4 (63.0)	90.2 (71.8)	6.7 (2.6) a
Tahiti lime (less susceptible)	78.7 (62.5)	89.8 (71.4)	1.2 (1.1) b
Significance	No significant	No significant	Significant

[1] Arcine transformed values in parentheses.
[2] Square root transformed values in parentheses.
a, b: Means significantly different at $p = 0.05$ (Student's t test).

There was no significant difference ($p = 0.05$) in percent germination between the two treatments 24 h post-inoculation. Neither was the difference significant in percent penetration between the treatments (*table II*). However, there was a marked difference in disease severity between Marsh seedless grapefruit and Tahiti lime.

3.3 incidence and severity of citrus fruit and leaf spot disease

Incidence and severity of fruit and leaf spot disease at a field site in western Kenya varied among the citrus species and cultivars within a species. Grapefruit manifested most disease followed in descending order by sweet oranges, Minneola tangelo, tangerines, lemons, Troyer citrange and Tahiti lime (*table III*). Incidence was generally higher on fruit than foliage, except for Cleopatra tangerine, Satsuma tangerine and Troyer citrange. Nine out of 14 cultivars reflected higher severity scores on foliage than fruit, and this trend was particularly expressed in sweet oranges.

4. discussion

Detached leaf assays and field observations yielded similar results on host susceptibility to *P. angolensis*: susceptibility varied among citrus species and cultivars within a species, and also in a same cultivar, fruits and leaves were not equally susceptible. It may be useful to compare the detached leaf assay with the plantlets inoculation technique described by Kuate et al. [9]. Under the conditions of western Kenya, Marsh seedless grapefruit, Pineapple orange, Valencia late, Washington Navel, Cleopatra tangerine, Kara tangerine, Minneola tangelo, Villa Franca lemon, and Lisbon lemon were very susceptible. Eureka lemon, Satsuma tangerine and Troyer citrange were less susceptible, while Tahiti lime was least susceptible. These findings are generally in agreement with the observations in West Africa [7–10]. Considering differences in climate between East and West Africa, it may appear that resistance to the disease could be an inherently stable trait in citrus, and presumably, therefore, the relative virulence of the pathogen may not be location specific. However, more investigations are needed to validate the hypothesis.

Results of this study indicated that spore germination of *P. angolensis* and stomatal penetration were not species dependent. There were no significant differences in percent spore germination and stomatal penetration between Marsh seedless grapefruit and Tahiti lime. Furthermore, germ tube penetration was apparently independent of size of stomatal aperture: although stomatal density of grapefruit and lime is the same, stomatal opening of the former is larger [12]. However, variation in severity of infection on Marsh seedless grapefruit and

Table III.
Incidence and severity of *Phaeoramularia* fruit and leaf spot on 14 citrus cultivars at a prison orchard, Kitale, Kenya (altitude: 1,600 m; mean annual rainfall: 1,600 mm; mean annual min–max temperature: 12 °C–26 °C). Neither fungicides nor oil-based insecticides were applied to study trees during work period; data are means of 2 years (four seasons) observation.

Citrus cultivars	Disease incidence (%)		Disease severity[1]	
	Foliage	Fruit	Foliage	Fruit
Troyer citrange	22.0	20.0	2.0	1.6
Marsh seedless grapefruit	76.0	95.0	3.6	4.2
Lisbon lemon	16.0	55.0	2.2	1.5
Eureka lemon	18.0	40.0	2.0	2.4
Villa Franca lemon	18.0	95.0	2.0	3.0
Tahiti lime	8.0	14.0	2.0	1.3
Valencia Late orange	64.0	75.0	2.7	2.2
Washington Navel orange	68.0	75.0	2.9	2.6
Pineapple orange	72.0	90.0	3.4	3.2
Temple tangerine	18.0	35.0	2.4	2.0
Satsuma tangerine	36.0	20.0	2.6	2.2
Cleopatra tangerine	64.0	45.0	2.0	1.8
Kara tangerine	46.0	95.0	2.4	2.9
Minneola tangelo	54.0	70.0	2.0	3.0

[1] Disease severity on leaves was based on the following score system: 1 = 0%; 2 = <1%; 3 = 1–3%; 4 = 3–5%; 5 = >5% of leaf area affected. A similarly system was used for fruit severity: 1 = 0%; 2 = <5%; 3 = 5–20%; 4 = 20–50% and 5 = >50% of fruit surface.

Tahiti lime could be due to differences in the ability of the species to overcome colonization by the pathogen. This type of resistance could be considered species dependent and post-infectional. The question of cultivar differences within a species in susceptibility to *P. angolensis* needs to be addressed.

The leaf assays could be useful in screening for resistance of citrus to *P. angolensis* under laboratory conditions and, if adequately standardized, would facilitate comparison of results from different countries thus permitting a greater understanding of this destructive disease. Lastly, susceptibility or resistance to *P. angolensis* alone does not offer a solution to the disease problem in Kenya as all commonly grown citrus cultivars are affected, and, therefore, incorporation of chemical intervention in disease management is still necessary.

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Comportamiento de algunos cultivares de agrios frente a la enfermedad de las manchas sobre hojas y frutas, causada por *Phaeoramularia* en Kenia.

Resumen — Introducción. Esta enfermedad, señalada por primera vez en Kenia en 1984, afecta el 20% de las superficies de agrios del país y ocasiona un 20 a un 100% de pérdida en las cosechas. Se estudió la reacción a *P. angolensis* de los cultivares de agrios más comunes. **Material y métodos**. El estudio fue llevado a cabo durante 2 año (cuatro temporadas). Se evaluaron los cultivares en cuanto a su sensibilidad a *P. angolensis* al utilizar tests sobre hojas en laboratorio y al efectuar observaciones en campo sobre la incidencia y la gravedad de la enfermedad sobre hojas y frutas. **Resultados**. La sensibilidad al patógeno varió de una especie a otra y según los cultivares de una misma especie; para un cultivar dado, las hojas y las frutas se hallan distintamente afectadas. El pomelo "Marsh seedless" fue el más sensible, mientras que el limero Tahiti fue el menos afectado. Los periodos de incubación y de latencia variaron respectivamente de 9 a 16 d y de 16 a 32 d para el pomelo y para el limero. La esporulación relativa del hongo fue parecida cualesquiera que sean la especie o la variedad examinada. Las tasas de germinación de los esporos y de penetración estomatal no fueron significativamente distintas sobre pomelo o sobre limero, pero la gravedad de la enfermedad varió. **Discusión**. Dado que todos los tipos de agrios explotados en Kenia se hallan afectados por la enfermedad, el ataque por *P. angolensis* no puede ser vigilado por el único carácter de sensibilidad o de resistencia; a partir de entonces, resulta necesario mandar intervenir el control químico. Los tests sobre hojas utilizados durante este estudio podrían ser utiles para cribar los agrios frente a su resistencia a *P. angolensis* mediante técnicas in vitro.

Kenia / *Citrus* / enfermedades de las plantas / hongos / *Phaeoramularia angolensis* / ensayos de variedades / resistencia a la enfermedad