GSC Biological and Pharmaceutical Sciences
eISSN: 2581-3250 CODEN (USA): GBPSC2
Cross Ref DOI: 10.30574/gscbps
Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Detection and management of soil-borne pathogens in citrus using non-inorganic control practices under greenhouse conditions

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GSC Biological and Pharmaceutical Sciences, 2023, 24(03), 247–265

Publication history: Received on 10 August 2023; revised on 16 September 2023; accepted on 19 September 2023

Article DOI: https://doi.org/10.30574/gscbps.2023.24.3.0390

Abstract

Citrus fruits are among the priority crops for Uganda. Government has invested significant resources to boost citrus production due to their potential to increase household income, nutritional security and national exports. However, soil-borne pathogens are among several biotic-factors significantly constraining production. Whereas inorganic chemicals have been applied in the past, they have potential detrimental effects. Therefore, an experiment was conducted in the greenhouse of National Forestry Resources Research Institute (NaFORRI) at Kifu - Mukono in 2020-2021 to determine dominant soil and Citrus pathogens and test efficacy of different non-inorganic citrus pathogen control practices. A randomized complete block design with five treatments replicated thrice was used. The treatments included; hot water, heated wet-soil, fumigated soil, solarized wet-soil, and untreated control. A total of 30 Citrus seeds per variety were treated with sodium-hypochlorite-solution (1:7) before being sown. Soils from above treatments were cultured on Potato and Sabouraud-Dextrose Agar (PDA and SDA) and de Man, Rogosa and Sharp (MRS) agar. Results indicated that *Aspergillus niger* was the dominant soil-borne pathogen identified in pure cultures. Others included *A. flavus*, *A. fumigatus*, *A. viridinutans, Penicillium chrysogenum* and *Fusarium oxysporum.* Ranking based on scores in: cost-effectiveness, leaf-health, chlorophyll-intensity and general-health of seedlings revealed solarization (35) as most effective technique for managing soil-borne pathogens, followed by boiling (33), heating (31), untreated control (27) and lastly fumigation (26). Identified pathogens induce aflatoxins in Citrus and lead to great losses during post-harvest handling; they also cause root and fruit-rots. Solarization creates solar-radiation in a miniature greenhouse that kills pathogens. There is a need to study non-inorganic control practices of citrus pathogens under field conditions for improved yields in Uganda.

Keywords: Citrus-production; Soil-borne-pathogens; Solarization; Non-inorganic-control-practices; Greenhouse

1. Introduction

Citrus contains important nutrients to the body. Its consumption provides vitamin C which is an important nutrient in boosting immune system and other important nutrients including folate and potassium, in fresh form, it's a good source of dietary fiber (Verheye, 2010). In Uganda, its production contributes to Growth Domestic Product (GDP) with export of this fruit standing at 13,582 tonnes in 2017, according to UN comtrade (Dijkxhoorn *et al*., 2019). However, production of Citrus in Uganda is severely endangered by several biotic constraints such as pests and diseases (Obonyom and Kumakech, 2018). In citrus growing parts of Uganda like Eastern region, farmers are suffering from heavy losses in their Citrus orchards due to pests and diseases. These originate from nurseries and/ or in field and have caused many to get discouraged and abandon their orchards (field observation in Soroti District). Among these, soil-borne pathogens are of significant importance. Soil-borne diseases are considered a major limitation to crop production. Soil-borne plant pathogens such as *Rhizoctonia* spp., *Fusarium* spp., *Verticillium* spp., *Sclerotinia* spp., *Pythium* spp., and *Phytophthora*

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spp can cause 50%–75% yield loss for many crops such as wheat, cotton, maize, vegetables, fruit and ornamentals (Mihajlovic´ *et al*., 2017; Baysal-Gurel *et al*., 2018). There is a growing need to produce large quantities of citrus fruits to supply processing factories. However, the apparent low yield and quality of orchards limits expansion of this investment. Farmers have resorted to using pesticides, fungicides and fertilizers to overcome above challenges and improve yields. However, these methods are associated with detrimental effects especially to environment like pollution, carcinogenic effects (Lu, 1995), pest resurgences and disease resistances (Sofo *et al*., 2014). Also, it should be noted that there are evident issues with use of synthetic chemicals which include ecological disturbance, human health hazards, damage to aquatic ecosystems, reduction of beneficial soil microorganisms and even ozone layer depletion (Bell, 1996). In many parts of the world, methyl bromide, a soil fumigant was extensively used to control those pathogens before implementation of Montreal Protocol in 1986, an international treaty to protect ozone layer (Bell, 1996) which also agreed to phase down production and use of hydrofluorocarbons in 2016 with the Kigali amendment. Farmers make heavy losses from purchase of inorganic chemicals to control and manage these pathogens. Nevertheless, over reliance on synthetic chemicals is detrimental to environment, animal and human health (Sarkar *et al*., 2021; Pathak *et al*., 2022). These constraints have necessitated research into more eco-friendly, effective and relatively cheap non-inorganic technologies to control soil-borne Citrus pathogens to mitigate these economic and ecological losses. Elsewhere, there are several practices which have minimized use of fungicides in Citrus Nursery (Gade and Giri, 2005).

This study compared efficacy of using soil solarization, soil fumigation, steam sterilization (heating moistened soil), hot water treatment and untreated control, all integrated with cultural practices in control of soil-borne diseases in citrus seedlings under greenhouse conditions. *In vitro* laboratory analysis was done to investigate colony forming units of soil-borne pathogens for each of management practices above to assess their effectiveness and *in vivo* analysis of Citrus seedlings and economics of the treatments in greenhouse by comparing their respective scores in different attributes viz., cost-effectiveness of treatments, and leaf-health/ disease incidences/ percentage control levels of pathogens, chlorophyll-intensity and general-health of citrus seedlings under different treatments. The study specifically aimed at determining dominant soil and Citrus pathogens and testing the efficacy of different non-inorganic soil-borne citrus pathogen control practices. The choice of Citrus as a test plant specimen in the experimental analysis was based on our observations that it was more susceptible to soil-borne pathogens during production in the greenhouse in the years 2018, 2019. Hence, this would validate our findings compared to when other fruits like avocado, mango, guavas, and soursop were used. The experiment aimed at rectifying the biotic production constraints of citrus in greenhouse reflected in Fig. 13 and consequently extenuating the farmers' outcries after adoption of the non-inorganic soil-borne citrus pathogen control technique(s) recommended in our study.

2. Material and methods

2.1. Study area

The experiment was set-up inside greenhouse located on coordinates (Longitude: E 032^0 45.592, Latitude: N 00^0 27.224 and elevation: 1188 m asl) at National Forestry Resources Research Institute (NaFORRI), Kifu. Laboratory analysis was conducted in soil laboratory of NaFORRI.

2.2. Determination of dominant soil and Citrus pathogens

2.2.1. Culturing and quantification of soil-borne citrus pathogens from different treatments using serial dilution and microscopic techniques

This involved use of culture media to cultivate bacteria and fungi. Citrus samples that expressed clear symptoms like rots, spots, scab, greening, soot and unsterilized soils (Fig. 1A) were used as sources of inocula. From rotten and symptomatic citrus leaves, fungal pathogens were isolated by washing them thoroughly in sterile water, followed by surface sterilization using a solution of sodium hypochlorite for 60 seconds. Using flame-sterilized scalpel, 3 leaf discs were cut and their outer layers were removed rapidly. Then, small pieces from central core of tissues in the advancing margin of infection were cut and dipped in 90% alcohol and rinsed in sterile water. These were cultured on SDA (Fig. 3) and PDA (Fig. 4) supplemented with streptomycin sulphate (to stop growth of bacteria) for fungi and incubated at 30 °C for 3 and 5 days and MRS agar supplemented by fluconazole (to stop growth of fungi), at a rate of five pieces of tissues per petri dish and incubated upside down at 30 °C for 3, 5 and 10 days (for bacteria). To determine the microorganisms in untreated control and differently treated soil, minute samples were inserted in sterile vials and one (1) gram of soil was inserted in 9 mL of sterile water and these were serial diluted 10 times. One milliliter of the 5^{th} and 8^{th} dilutions were cultured on PDA (for fungi) and MRS agar (for bacteria) respectively and incubated at 30 °C to obtain colony forming units. These were counted under dissecting microscope.

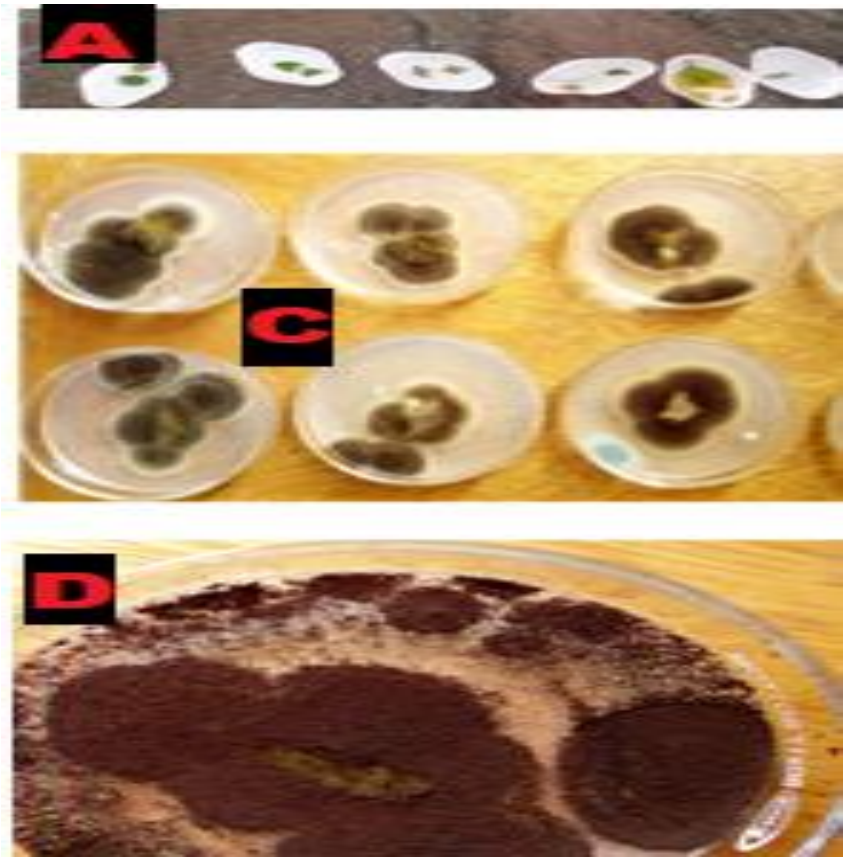

Figure 1 A- Rotten Citrus leaf samples obtained from seedlings grown on untreated control, surface sterilized with sodium hypochlorite and rinsed in double distilled water before culturing; B-Individual spores of *Aspergillus niger* observed under a microscope on a slide, C - culture of rotten Citrus leaf samples from untreated control for fungi on Sabouraud Dextrose Agar revealed *A. niger*, as the dominant soil-borne pathogen; D- Old colonies of *A. niger*; E- mycelia of fungi observed under a microscope for identification (Photo credit: Ronald Kisekka)

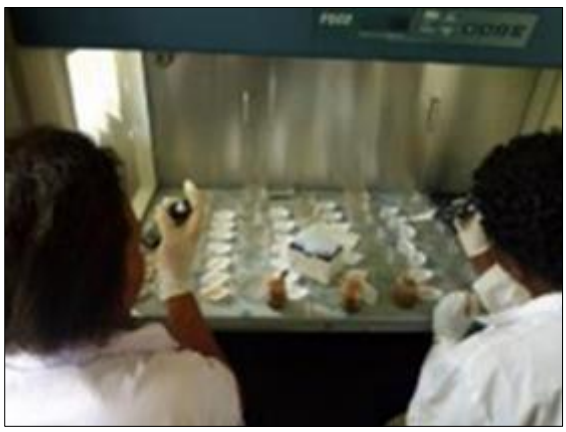

Figure 2 Research team performing serial dilutions of soil under laminar flow prior to culturing on PDA and SDA (fungi) and MRS agar (bacteria) (Photo credit: Ronald Kisekka)

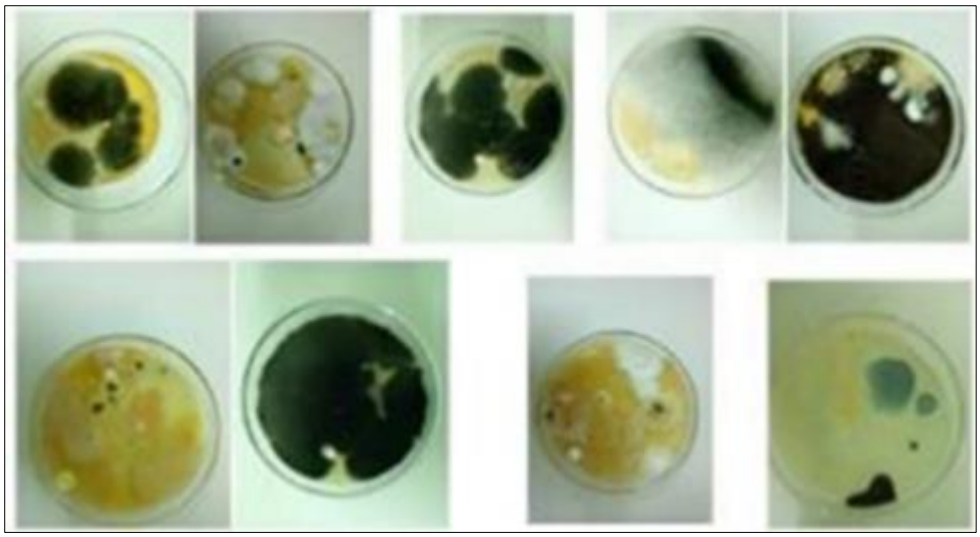

Figure 3 Colony forming units of fungi cultured on SDA (Photo credit: Ronald Kisekka)

Figure 4 Colony forming units of fungi cultured on PDA (Photo credit: Ronald Kisekka)

2.2.2. Detection and identification of dominant soil-borne citrus pathogens by using pure culture and mycology taxonomic keys

To obtain pure cultures, individual spores from colonies were aseptically transferred and inoculated on slants of PDA and MRS Agar for further selection and identification (Fig. 5). We used single hyphal tip method where by a small segment of fungal growth in agar medium was transferred to center of slanted agar media in test tubes containing nutrient medium, using a flame-sterilized inoculation needle and incubated at 30 ℃ for 10 days. The type of bacteria and fungi obtained in respective diseased citrus samples and soil was identified using colony morphology. For fungi, there was observation of type of hyphae, colour of conidia (spores), and other microscopic properties of fungal pathogens by mounting slides and Petri dishes with individual spores and cultures respectively on a dissecting microscope for identification purposes (Fig. 1B-E). On the other hand, colony-forming units of bacteria were mounted on slides for observation under light microscope and only shape of bacterial cells was used for identification.

Figure 5 Left; test tube slants of Sabouraud Dextrose Agar in the oven used to isolate pure cultures prior to identification; Middle and right- Pure cultures of Fungi on slanted SDA in test tubes incubated at 30 ℃ (Photo Credit: Ronald Kisekka)

2.3. Efficacy of different non-inorganic citrus pathogen control practices

2.3.1. Description of pathogen control practices used in the experiment

Soil solarization

This involved laying a white transparent polythene bag (180 microns) on top of moistened soil for a period of 8 weeks before sowing (Fig. 6). Soil was cleaned by removing weeds, foreign objects and rocks. The soil was also well sorted to remove unwanted residues before potting. After potting, the soil was watered and immediately the pots were covered with a clear plastic sheet (180 microns) for eight weeks during the dry season of December 2019 to January 2020. The plastic sheet was laid very close to the pots to maximize solar radiation. To ensure that the soil kept moisture, water was applied once and borders of the transparent polythene bag sealed with potted soil to maintain air tight condition throughout the experimental period. Wet soil conducts heat better than dry soil and makes soil organisms more vulnerable to heat (Rajwade & Garg 2020).

Figure 6 Left-Soil solarization treatment; Right-Healthy citrus seedlings raised on solarized soil (Photo credit: Ronald Kisekka)

Soil fumigation

This involved use of a soil fumigant, particularly Dazomet following the manufacturer's guide. The reason for using Dazomet is because it is accessible compared to methyl bromide which was banned. Five hundred milliliters (500 mL) of Dazomet were mixed with 1 litre of water in 1:2 ratio and poured the mixture on potted soil and allowed a 30- minute exposure. Then high quantities of sterile water were subsequently poured on potted soil before sowing Citrus seeds (Fig. 7). The aim of pouring excess water was to wash-off Dazomet so that seedlings were not scorched and the more water poured the better.

Figure 7 Left-Fumigated potted soil; Right-Citrus growing on fumigated potted soil showing mild symptoms of scorching due to abiotic cause (corrosive fumigant) (Photo credit: Ronald Kisekka)

Steam sterilization

This involved heating moistened soil in a metallic drum to generate steam that sterilized the soil (Fig. 8). The soil was allowed 24 hours of cooling before potting and sowing Citrus seeds. Incidence of soil-borne pathogens (colony forming units, c.f.u) after steam sterilization was assessed by culture techniques (SDA and PDA for fungi).

Figure 8 Left-steaming of soil in a metallic drum; Right-Health citrus seedlings growing on steamed soil (Photo credit: Ronald Kisekka)

Hot water treatment

This involved boiling water in a kettle. Hot water at 100 ℃ under normal atmospheric pressure was poured on potted soil to kill fungi, bacteria and some viruses. This was allowed to cool before sowing Citrus seeds (Fig. 9). Number of c.f.u after hot water treatment was assessed by culture techniques.

Figure 9 Left-young citrus on treated soil using hot water at 100 ℃; Right-older health citrus seedlings on soil treated with hot water (Photo credit: Ronald Kisekka)

Untreated control

This consisted of soil in a form in which it was obtained without any treatment (Fig. 10). The purpose of this was to act as a control but it was equally integrated with cultural practices. Colony forming units were also obtained on SDA and MRS agar.

Figure 10 Untreated soil (control): Left-soil in the form it was obtained; Middle- young citrus on untreated soil; Right-older citrus grown on untreated soil. Note that some leaves from control were rotting as shown in Figure 1A (Photo credit: Ronald Kisekka)

Cultural practices integrated in all above treatments

The plants were watered uniformly once in the morning, hand weeded, rogued infected Citrus parts, and 500 mL of Poly-Feed fertilizer (solution mixed at a dose of 8g/ 20 liters of water) that contained Nitrogen (N), phosphorous (P) Potassium (K) and other micro nutrients were applied (Fig.11).

Figure 11 Cultural practices integrated in the above treatments: Left and middle – hand weeding; Right- application of 500 mL of Poly-Feed foliar fertilizer at 8g/ 20 liters of water (Photo credit: Ronald Kisekka)

2.3.2. Greenhouse experiments

A randomized complete block design was set-up in greenhouse (Table 1) containing all the described pathogen control practices above as treatments. Citrus seeds of three different species were sterilized in 1:7 solution of sodium hypochlorite to eliminate seed-borne pathogens and seed coat contaminants, then sown in the 5 treatments and monitored for germination and disease responses (Fig. 12). *In vitro* studies for number of colony forming units of fungi and bacteria in the untreated control and treated soils were done. Scores for growth parameters of citrus using an arbitrary scale of 5-10 were carried out. Ranking of control practices was finally conducted.

Figure 12 Pictorial experimental design (Photo credit: Ronald Kisekka)

Table 1 Experimental design

R	**Block 1 = Replicate 1**			**R**	**Block 2 = Replicate 2**			**R**	**Block 3 = Replicate 3**		
V	**A**	**B**	**C**	**V**	**A**	**B**	**C**	**V**	**A**	**B**	**C**
T_1	N=30	N=30	N=30	T_1	N=30	N=30	N=30	T_1	N=30	N=30	N=30
T_2	N=30	N=30	N=30	T_2	N=30	N=30	N=30	T_2	N=30	N=30	N=30
T_3	N=30	N=30	N=30	T_3	N=30	N=30	N=30	T_3	N=30	N=30	N=30
T_4	N=30	N=30	N=30	T_4	N=30	N=30	N=30	T_4	N=30	N=30	N=30
T_5	N=30	N=30	N=30	T_5	N=30	N=30	N=30	T_5	N=30	N=30	N=30

Key: T for treatments: T_1 = Control/ untreated soil with Cultural practices; T_2 = Hot water at 100 ℃ with Cultural practices; T_3 = Moistened and steamed/ heated soil in a metallic drum at 180 ℃ with Cultural practices; T_4 = Fumigated soil using Dazomet with Cultural practices; T_5 = Solarized soil for eight weeks with Cultural practices, R = Replicate, V = Variety where A = *Citrus sinensis* ("*Emicungwa"*), B = *Citrus limon* ("*Enniimu"*) and C = *Citrus reticulata* ("*Mangadda"*)

Figure 13 Biotic constraints of Citrus production in greenhouse and nursery: Top Left: Severely infected citrus inside green-house; Top Right: Severely infected seedlings in the citrus nursery; Bottom Left: Anxiety of one of the scientists about the citrus infection levels in green-house; Bottom Right: Grafted Citrus expressing severe symptoms of infection (Photo credit: Ronald Kisekka)

Efficacy of a treatment was calculated from formula,

$$E = (C-T)/C*100$$ where, E = Efficacy, C= cfu of control and T = cfu of treatment.

Percentage saving per treatment was calculated as,

$$\% \text{ saving} = (\text{cost of control} - \text{cost of treatment})/\text{cost of control}*100.$$

3. Results

3.1. Dominant soil-borne pathogens affecting Citrus production in greenhouse

Figure 14 Cultures of some of the identified soil-borne pathogens: Left - *Aspergillus niger;* Centre-*Penicillium chrysogenum;* Right - *Aspergillus flavus* (Photo credit: Ronald Kisekka)

The three detected pathogenic genera were; *Aspergillus*, *Penicillium* and *Fusarium* (Table 2a). *Aspergillus niger* was the dominant soil-borne pathogen identified based on morphological characteristics in pure cultures (Fig. 15). This was the sole pathogen isolated from rotten citrus leaves sampled from seedlings raised on untreated control. Others isolated from soil included: *A. flavus*, *A. fumigatus*, *A. viridinutans*, *Fusarium oxysporum* and *Penicillium chrysogenum* (Table 2b; Fig. 14). In addition to these, beneficial bacteria of *Bacillus* spp. were isolated and these are not pathogenic but mostly used as microbial control agents or plant growth/ health promoting bacteria (Table 3).

Table 2a Number of fungal species isolated from soil samples. In parenthes are percentages

Isolated soil-borne fungal pathogen	Number and percentage (%)
Aspergillus spp.	4 (66.6)
Penicillium spp.	1 (16.7)
Fusarium spp.	1 (16.7)
Total	6 (100)

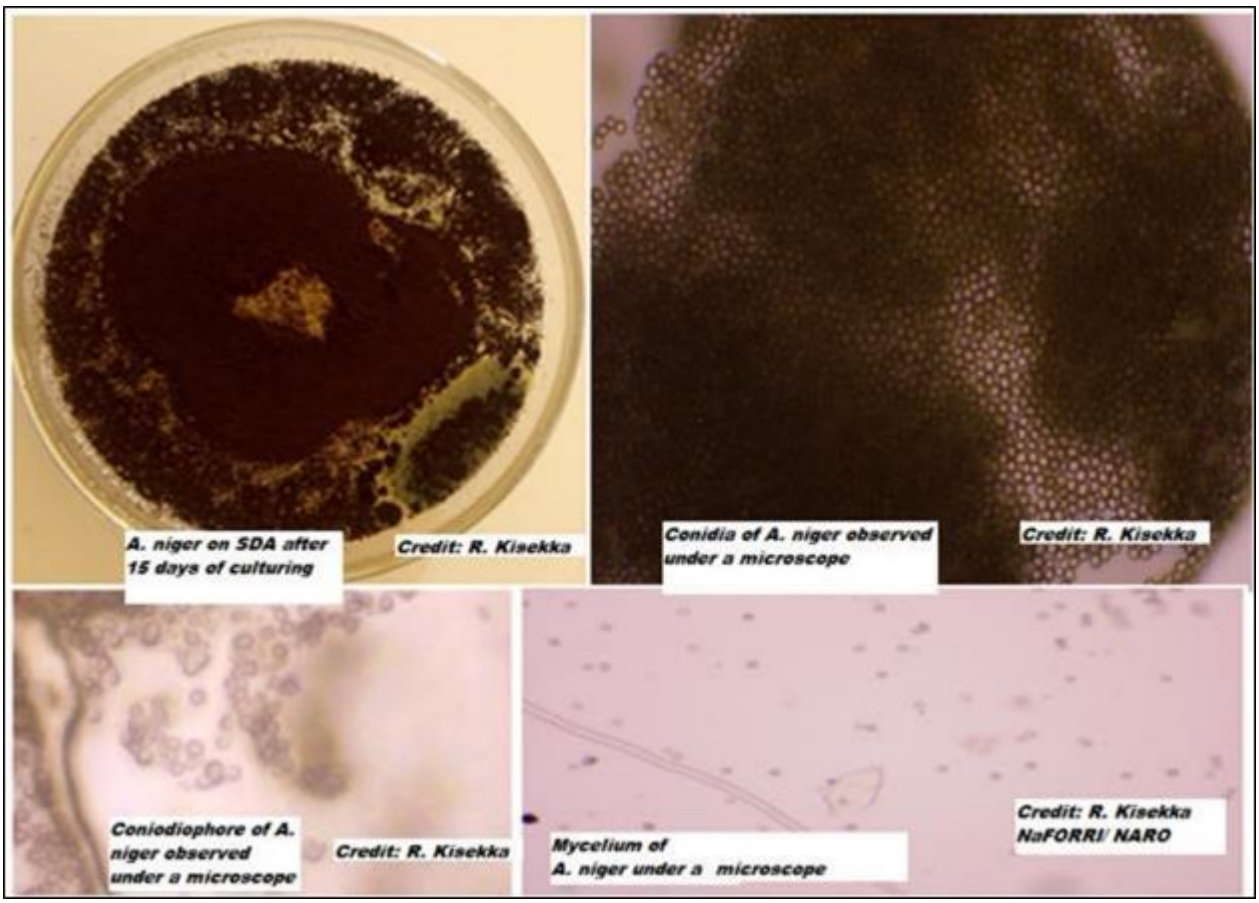

Figure 15 Microscopic and macroscopic morphological features of *Aspergillus niger*, one of the isolated predominant soil-borne citrus pathogens in our study

Table 2b Colony characteristics based on our observations, confirmatory fungi and their pathogen status in citrus

Description of colony characteristics	Identified fungi; Pathogen status; References
Colony with white and typical black spores on the surface. The lower surface is yellow and heavily furrowed. Sporulation is heavy.	*Aspergillus niger;* causes fruit rot, collar rot (Aspergillus rot); Dey *et al.* (2004)
On SDA, colonies showed typical blue-green pigmentation with suede-like surface consisting of a dense felt of conidiophores	*A. fumigatus;* induces aflatoxins; Dennis, (1983).
Cottony white colour on surface with reverse, yellow; texture: rough	*A. viridinutans;* induces aflatoxins; Highley *et al*., 1994;
Surface: Magenta pink; Reverse: Meganta red turning violet with floccose texture. Sporulation is poor	*Fusarium oxysporum; Fusarium* wilt, and dry root rot, Citrus blight, mycotoxins; Nelson *et al*., 1993; Yaseen & D'Onghia,, 2012; Sakovich, 2003
Cottony look with dull green centre	*Penicillium chrysogenum;* induces aflatoxins; Moline, 1984

3.2. Efficacy of treatments for control of soil-borne pathogens in Citrus production under greenhouse conditions

Based on results from cultures of fungi (Table 4; Fig. 16) and bacteria (Table 5; Fig. 17) of soil on SDA and MRS agar (Tables 2b and 3 respectively), and based on the overall ranking of the treatments using scored parameters (Table 7), soil solarisation was the most cost-effective technique saving 77.52% (Table 6), eco-friendly (with relatively high survived population of soil beneficial *Bacillus* bacteria that is to say 19.33 x 10^8 (49.99%), 12.67 x 10^8 (43.20%) and 13.00 x 10^8 c.f.u (162.5%) after 3, 5 and 10 days of incubation respectively at 30 ℃; see Table 5) and effectual non-inorganic soil-borne control technique during Citrus seedling production (66.8%), followed by hot water treatment (63.2%) whereas the least were use of soil fumigant/ dazomet (34.6%) and the control (0.0%) where no treatment was done other than cultural practices (Table 4). All the others were also integrated with cultural practices.

Table 3 Colony characteristics based on our observations, confirmatory bacteria and their pathogen status in citrus

Description of colony characteristics	Identified bacteria; Pathogen status; References
All bacteria were observed to be rod-shaped	*Bacillus* spp. Not pathogenic but soil beneficial
	microorganisms; Ajilogba *et al*., 2013; Pertot *et al*., 2015; Shafi *et al*., 2017

Table 4 Colony forming units of fungi after three and five days of culturing on SDA

Treatments	Three days						Five days					
	Plate number			Aver colonies/ plate	*CFUs/cm^3	Efficacy (%)	Plate number			Aver colonies/ plate	*CFUs/cm^3	Efficacy (%)
	1	2	3				1	2	3			
Steaming	22.0	14.0	17.0	17.7	17.7 x $10^{5\,a}$	36.8^a	11	13	26	16.7	16.7 x $10^{5\,b}$	71.2^a
Solarization	**5.0**	**16.0**	**7.0**	**9.3**	**9.3 x $10^{5\,bc}$**	**66.8^b**	**8**	**24**	**11**	**14.3**	**14.3 x 10^{5} bc**	**75.3^a**
Fumigation	7.0	18.0	30.0	18.3	18.3 x$10^{5\,a}$	34.6^a	15	16	5	12.0	12.0 x$10^{5\,ab}$	79.3^a
Control	31.0	33.0	20.0	28.0	28.0 x$10^{5\,c}$	0.0^c	24	30	120	58.0	58.0 x $10^{5\,c}$	0.0^b
Hot water	11.0	8.0	12.0	10.3	10.3 x$10^{5\,bc}$	63.2^b	7	7	14	9.3	9.3 x $10^{5\,a}$	83.9^a

*CFU per cm^3 of soil = average number of colony forming units x dilution factor; values within columns followed by the same letters are not statistically different at P<0.05 level of significance

Table 5 Colony forming units of bacterial culture on MRS agar from different treatments as a measure of eco-friendliness

Culture duration	Treatment	R 1	R 2	R 3	Aver colonies/ plate	*CFUs/cm3	Efficacy (%)
After 3 days of incubation at 30 ^{O}C	Steaming	4	13	1	6.00	6.00 x $10^{8\,a}$	84.45^{a}
	Solarization	10	14	34	19.33	19.33 x $10^{8\,b}$	50.01^{b}
	Fumigation using Dazomate	7	7	12	8.67	8.67 x $10^{8\,c}$	77.58^{a}
	Control	20	40	56	38.67	38.67 x $10^{8\,d}$	0.00^{c}
	Hot water at 100 ^{O}C	36	16	65	39.00	39.00 x $10^{8\,e}$	-0.85^{d}
After 5 days of incubation at 30 ^{O}C	Steaming	5	12	1	6.00	6.00 x $10^{8\,a}$	79.54^{a}
	Solarization	14	8	16	12.67	12.67 x $10^{8\,f}$	56.80^{b}
	Fumigation using Dazomate	8	6	15	9.67	9.67 x $10^{8\,c}$	67.03^{a}
	Control	33	22	33	29.33	29.33 x $10^{8\,g}$	0.00^{c}
	Hot water at 100 ^{O}C	47	45	43	45.00	45.00 x $10^{8\,d}$	-53.43^{d}
After 10 days of incubation at 30 ^{O}C	Steaming	8	2	1	3.67	3.67 x $10^{8\,h}$	54.13^{b}
	Solarization	10	14	15	13.00	13.00 x $10^{8\,f}$	-62.50^{d}
	Fumigation using Dazomate	7	1	10	6.00	6.00 x $10^{8\,a}$	25.00^{e}
	Control	3	9	12	8.00	8.00 x $10^{8\,c}$	0.00^{c}
	Hot water at 100 ^{O}C	7	9	8	8.00	8.00 x $10^{8\,c}$	0.00^{c}

*CFU per cm^3 of soil = average number of colony of soil = average number of colony; R = Replicate; CFU/cm^3 and efficacy within columns followed by the same letters are not statistically different at P<0.05 level of significance

Table 6 Cost-effectiveness of treatments used in the experimental study

Treatment	Necessary items	Total cost ('000 UGX)	% saving	Rank of cost effectiveness
Solarization	10 m^2 clear polythene sheet 180 microns, potting bags, labour	47	77.52	1
Fumigation	Dazomate, potting bags, labour	92	52.08	2
Steaming	Metalic drum, firewood, potting bags, labour	122	36.46	4
Hot water	Firewood, 5L - kettle, potting bags, labour	102	46.88	3
Untreated control	Potting bags, labour, cost of anticipated inorganic chemicals	192	0.00	5

Table 7 Summary scores under various parameters and overall ranking of different techniques tested in the experimental study

Treatment	**Scored parameters based on observation (Arbitrary scale 5-10)**					
	Cost-effectiveness (Cheapness)	**Leaf health (good)**	**Chlorophyll intensity (high)**	**General appearance of seedlings (Health)**	**Total scores**	**Over all Treatment ranking**
Solarisation	**9^{a}**	**8^{a}**	**8^{ab}**	**10^{ac}**	**35^{a}**	**1**
Fumigation	8^{b}	5^{b}	9^{a}	6^{b}	28^{b}	5
Steaming	7^{bc}	7^{bc}	9^{a}	8^{c}	31^{c}	3
Hot water	6^{bc}	9^{a}	8^{ab}	10^{ac}	33^{d}	2
Control	10^{a}	6^{bc}	4^{c}	7^{b}	27^{b}	4

Key considerations: Scores: In the column for cost-effectiveness, the highest score shows the cheapest treatment whereas the lowest score stands for the most expensive treatment and this parameter gives a measure of affordability of the technology, ecological friendliness results from colony forming units (cfu) of bacteria (conserved Bacilli) and this consequently led to high scores in general appearance of seedlings . Within columns, scores followed by the same letters are not statistically different at $P<0.05$ level of significance. In the columns for total scores and overall ranking, treatment with highest score was ranked best and lowest last.

Figure 16 Colony forming units of fungi after 3 days of culturing on SDA (Photo credit: Ronald Kisekka)

Figure 17 Bacterial cultures of soil samples from different treatments on MRS agar used for establishing colony forming units. The bacteria were all observed to be rod-shaped and identified as *Bacillus* spp. and beneficial because they didn't induce any observable symptoms of infection on the Citrus (Photo credit: Ronald Kisekka)

4. Discussion

Identified predominant soil-borne pathogens, *Apergillus* spp., led by *A. niger* in our study can induce aflatoxins in Citrus fruits during production hence making postharvest handling cumbersome and consumption of Citrus unsafe to human and animal life if no management measure is applied. Our study agrees with previous studies for example Sharma, 2012 found out that *A. niger* is a fungus and one of the most common species of the genus *Aspergillus*. Raghukumar, 2017 revealed that *A. niger* is widely spread due to its spore-forming ability. Dijksterhuis *et al*., 2013 reported that it is a versatile fungus that is cosmopolitan in distribution and has been observed in a wide range of habitats like soil, air, water, and on decaying plant materials. It is normally found as a saprophyte growing on dead fruits, leaves, compost piles, stored grains, and other decaying vegetation according to Gautam *et al*., 2011. *Aspergillus niger* can cause various plant diseases which lead to great economic losses. Bukar *et al*., 2009 revealed that 90% orange samples were infected with one or more than one fungal species dominated by *Aspergillus* (32.5%), *Mucor* (25%), *Penicillium* (15%), *Rhizopus* (15%), *Fusarium* (7.5%), and *Alternaria* (5%) in that order. Our study agrees with their findings since it also identified 4 species of *Aspergillus* (66.6%), one species of *Penicillium* (16.7%) and another one species of *Fusarium* (16.7%) (Table 2a), all implicated in hampering Citrus production. Research shows that fungi in these three genera: *Aspergillus*, *Penicillium* and *Fusarium* secrete mycotoxins that induce diseases known as mycotoxoces in humans and animals (Awuchi *et al*., 2021). Some may lead to severe illness and death. Similarly, Fatima *et al*. (2009) also stated that *Aspergillus sp.* and various other fungi were major causes of post-harvest diseases. Several studies revealed the dangers of *Aspergillus* spp. on plant, human and animal health for example: *Aspergillus niger* has been reported to cause root and fruit-rots (Dey *et al.*, 2004) and this is also evidenced by the fact that during the duration of our experimental study, some of the Citrus from untreated control were rotting and their leaves were collected, cultured and found to contain the same detected rot-causing pathogen (*A. niger*). It is imperative to know that this fungus could have also caused rotting in other Citrus seedlings if there were not any other treatments included in our study design other than untreated control. The effect of rotting in these seedlings would have been equally high as observed in the control where survival rate was very low. Similar studies elsewhere revealed that *A. niger* is associated with dangers to human and animal health for example: *A. niger* produces aflatoxins, ochratoxin A (OTA) in stored products (Soares *et al*., 2013). Production of Fumonisin B2 and OTA from *A. niger* was also reported by Frisvad *et al*., 2007, Krueger *et al*., 2009 and Logrieco *et al*., 2011. Fumonisin B2 can cause diseases both in man and animals, and is a known carcinogen (Frisvad *et al*., 2007; Logrieco *et al*., 2011). *Aspergillus* spp. - infected plants induce human fungal infections predominantly in immuno-compromised people (Diba *et al*., 2007). *Aspergillus fumigatus* was reported to result in a high level of abortion in cattle feeding on contaminated food and may also infect human lungs (Pandey & Prasad, 1993). *Aspergillus flavus* was reported to produce aflatoxin in many kinds of plants and plant products although groundnut, maize and cotton seeds

were the major agricultural crops with severe aflatoxins (Diener et *al*., 1987). Its aflatoxins have been confirmed to be greatly toxic to man and farm animals. It is a liver toxin that can stimulate cancer in susceptible animals (Highley *et al*., 1994). Other studies also associated several *Aspergillus* species and other fungi in different genera with stored products of which some may produce other important mycotoxins (Jacobsen *et al*., 1995). Therefore, caution should be taken in managing soil-borne pathogens during Citrus production because fungi like *Aspergillus* spp. have potential to induce aspergillosis especially in immuno-compromised individuals (Diener *et al*., 1987). Research shows that *Penicillium chrysogenum* is among *Penicillium* spp. that are known to cause blue and green mold rots, also regarded as *Penicillium* rots. They are the most common and habitually the most destructive of all postharvest diseases, affecting most kinds of fruits and vegetables (Dennis, 1983). On fruits like citrus, some infections may take place in the field, but blue molds or green molds are essentially postharvest diseases and often account for up to 90% of decay in transit, in storage, and in the market. *Penicillium* enters tissues through wounds. However, it can spread from infected fruit in contact with healthy ones through the uninjured skin (Moline, 1984). Research further reveals that the losses caused by the rotting of fruits and vegetables by *Penicillium* spp., include the fungus producing numerous mycotoxins, such as patulin, in the affected products, which contaminate juices and sauces made from healthy and partly rotten fruits (Dennis, 1983). *Fusarium* spp., including *F. oxysporum* is known to be associated with three citrus diseases namely; citrus fusarium wilt common to Citrus seedlings, citrus blight and citrus dry root rot. Fusarium dry root rot can be symptomless for years, but once enough root tissue has been damaged, sudden collapse can occur under dry hot conditions (Sakovich, 2003).

Therefore, citrus farmers should acquire Citrus seedlings from certified nurseries that ensure obligatory precautions during production since the soil-borne pathogens discussed above may remain latent and express symptoms later in the field or lead to post harvest losses. This eventually increases their cost of production via high purchase of farm inputs like agricultural chemicals to mitigate the soil-borne pathogens in their orchards. Similarly, cultural practices like hand weeding and foliar application of fertilizer like Poly-Feed integrated with solarization, the best technique assessed in our study are very pivotal. Studies elsewhere also revealed solarization as an effective technique for managing soil-borne pathogens for instance Gade and Giri 2005 established a significant decrease in mortality caused by *Phytophthora* spp*.* in seedlings of *Citrus jambhiri* caused by solarization followed by drenching and spraying of Metalaxyl @ 0.2 percent. Katan, 1976 found that mulching for four to five weeks resulted in a significant reduction in both the incidence and severity of *Verticillium* wilt in eggplant. He also observed almost complete control of several weed species in the mulched plots. His findings agree with our results that solarization integrated with cultural practices regulated soil-borne pathogens during Citrus production as reflected in Tables 4, 5 and 6. Control of weeds was not the primary objective in our study, however, observations revealed that most of the annual weeds were also equally controlled by soil solarization viz., *Setaria viridis*, *Chenopodium album*, *Euphorbia hirta*, *Cyperus rotundus*, *Cynodon dactylon*, *Amaranthus* spp., *Abelmoschus esculentus*, *Commelina benghalensis*, *Aerva lanata*, *Epimedium* spp., *Bidens pilosa*, *Sida cordifolia*, *Ageratum houstonianum*, *Chromolaena odorata*, *Senna occidentalis*, *Galinsoga parviflora*, *Calliandra* spp., *Solanum nigrum*, and *Ageratum conyzoides*. This agrees with Gade and Giri 2005 who noticed that annual weeds like *Cyperus rotundus*, *Cynodon dactylon*, *Portulaca oleracea*, *Euphorbia* sp. and *Trideix procumbence* were controlled by solarization. Other researchers reported that through direct thermal destruction, soil solarization changes microbial population of the soil and can eliminate most of the pests and that during the hot summer months, it can increase the soil temperature to a level which kills many important soil-borne pathogens like *Verticillium dahliae*, certain *Fusarium* spp., *Sclerotinia* spp., *Agrobacterium tumefaciens*, *Streptomyces scabies*, and nematodes, in addition to controlling many weeds (Baysal-Gurel *et al*., 2018). Nevertheless, soil solarisation works well during dry season when there is long hours of availability of intense solar energy (Krueger & Mcsorley, 2012) or inside green house in moderately hot days. Mihajlovic´ and other researchers also stated that soil solarization is a climate-dependent measure and is adapted to those regions and seasons (Mihajlovic´ *et al*., 2017) that tolerate abundant sunshine and high temperature (Baysal-Gurel *et al*., 2012). Related research revealed that the technique is effective in tropical and sub-tropical regions where summer temperatures go up to 45 °C (Nakamura *et al*., 2011). Hence, Uganda being a tropical country, the technique is a suitable and reliable approach which when embraced will save the country in various aspects like reduction of over reliance on synthetic chemicals, climate change mitigation and other human health and environmental benefits. One drawback is that the approach is not applied instantly since it requires at least six weeks of exposure for better results. A study by Panth and colleagues also reported that although soil solarization is an economical method, it requires a comparatively long period of time to work and is only dependent upon climatic conditions, hence its application in crop production is limited (Panth *et al*., 2020).Therefore, proper planning when to use the technique before sowing citrus seeds needs to be done. Phenotypic expression of Citrus seedlings in all the treatments in the current study showed that they were health-looking. This implies that majority of soil treatment technologies integrated with good cultural practices, namely: pot weeding, use of foliar fertilizers improve production of Citrus under greenhouse conditions albeit laboratory analysis (colony forming units) revealed some differences at microbiological levels among the different treatments. Hot water treatment was the second-best technique in cost-effectiveness and efficacy levels although steam sterilization and other tested techniques did not highly deviate from it as well unlike untreated control (Table 6; Table 7). Related research elsewhere supports our findings that integration

of more than one technique can effectively manage soil-borne pathogens (Ramirez-Villapudua and Munnecke, 1987; Chellemi *et al*., 1997). However, some tested techniques in our study for instance hot-water, steam sterilization/ heated-soil required ready sources of energy like fuel wood or charcoal which made them comparatively expensive and saved only 46.88% and 36.46% respectively (Table 6). Moreover, these become very costly due to high energy demand which is not sustainable to resource poor farmers and not eco-friendly due to hydrofluorocarbon emissions in the ozone layer and their adverse effects to soil microbiota for instance, in our study, steam sterilization saved only 15.55% of soil beneficial *Bacillus* spp. Over reliance on these techniques may lead to depletion of forestry sector in the country and worsen impacts of climate change. A related study revealed that fuel, labour and time consumed to apply steam/ heating technique in the field makes its adoption unpleasant (Luvisi *et al*., 2015). Also, Samtani and his colleagues reported a reduction of net return to the growers due to high fuel and equipment costs of the same method (Samtani *et al*., 2012; Samtani *et al*., 2011). Therefore, soil-solarization which derives its energy needs naturally from the sun remains real and self-sustainable and will save nursery operators' and horticulturalists' income and boost sustainability of the forestry sector hence mitigating impacts of climate change. This is in agreement with Panth and other researchers who reported that soil solarization can be a cost-effective technique for management of soil-borne diseases, especially for organic growers as it does not require any extra ordinary skill and technology (Panth *et al*., 2020). Similar studies revealed that soil solarization utilizes solar radiation from the sun and this kills soil-borne pests and diseases (Krueger & Mcsorley, 2012). This technology does not destabilize the soil-microbial community compared to other techniques (like soil fumigation and steaming) and improves the absorption of soil inorganic macro and micro nutrients by the plants (Persello-Cartieaux *et al*., 2003). Related studies like Ramirez-Villapudua and Munnecke, 1987 portrayed that both solar heating alone and cabbage amendments reduced soil-borne populations of *Fusarium oxysporum f. sp. conglutinans*, but these treatments were not as effective as the combination of the two treatments. Greenberger *et al*., 1987 had suggested that suppressiveness in solarized soils may result from a shift in microbial populations in favour of heat-resistant antagonists. The citrus seedlings from untreated control showed good health due to integration of other cultural practices like roguing, hand weeding and Poly-Feed fertilizer application. However, they latently contained the *in vitro*-detected pathogens (*A. niger*, *A. flavus*, *A. fumigatus*, *A. viridinutans*, *F. oxysporum* and *P. chrysogenum*) that are risk factors of post-harvest losses (rots, mycotoxins and others) during citrus production among farmers hence likely to increase their cost of production through purchase of chemicals for on-farm/ orchard and post-harvest disease management. The findings strongly emphasize the fact that good cultural practices are a precursor to production of clean and health nursery seedlings. Related to our study, Gade and Giri 2005 revealed that solarization in combination with Metalaxyl reduced the schedule of the fungicides by 50 per cent and thus ultimately reduced the cost of production in grafts of *C. jambhiri* with extra benefit of weed control and vigorous growth of seedlings. Previous studies also ascertained effect of solarization on yield and their benefits to general crop performance. These benefits in moderate temperature conditions provide hope for the future. Due to reductions of pesticides, threats of ground water pollution, and the everlasting need to control a wide range of soil-borne pathogens and pests, solarization provides many possibilities for improved yield (Krueger & Mcsorley, 2012). Perhaps more importantly, solarization may also improve quality with increases of yield (Yücel *et al*., 2007). In certain instances, these yield benefits were infinitive since crops in un-solarized soil were totally destroyed by pathogens (Jacobsohn *et al*., 1980). These studies also ascertained that increases in yield by solarization may depend upon a variety of factors viz., damage resulting from the disease being controlled, inoculum potential, and efficacy of control, compensation by neighboring plants, and the phenomenon of increased growth response (Katan, 1976). Notably, the survival of beneficial soil microorganisms particularly *Bacillus* spp., also supports the improvement in quality and health of produced Citrus under the different methods tested in our study. Research elsewhere revealed that *Bacillus species* from the rhizosphere were effective against a variety of soil borne pathogens (Jacobsen *et al*., 2004). Various species of *Bacillus* were reported to promote the health of crops and control diseases via diverse mechanisms viz., producing antibiotic metabolites, suppressing plant pathogens, others antagonize plant pathogens by competing for nutrients like iron and phosphate, others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake (Gardener 2004). This calls for the use of climate-smart techniques that conserve the beneficial *Bacillus* spp. Research elsewhere showed that where *Bacillus* spp. or/ and their by-products are available to plants, the outcome is disease control (Gardener 2004). Singh *et al*., 2008 found out that chir-pine seeds subjected to *B. subtilis* BN1 showed early seed emergence, viability and increased biomass. *Bacillus sphaericus* and *B. brevis*-2 increased plant length significantly while *B. megaterium*, *B. polymyxa*, *B. sphaericus*, *B. brevis* -1 and *B. thuringiensis* increased significantly by 30-54% the number of pods. Similarly, pod weight was increased by 25% while seed yield by 35% in plants treated with *B. thuringiensis* (de Freitas *et al*., 1997). Ajilogba *et al*., 2013 also revealed that subtilis FZB24 and FZB37 inhibited mycelial growth of *F. oxysporum*, *R. solani* and *Sclerotinias sclerotiosum* in vitro. Incidence of *F. oxysporum* disease was significantly reduced by up to 50% whereas plant height, root and shoot fresh weight increased significantly compared to the control (Ajilogba *et al*., 2013). Importantly, result of greenhouse differed from result *in vitro* which means that antifungal activities *in vitro* did not always correlate with disease reduction *in vivo* (Schmledeknecht *et al*., 2001). Their findings concur with other studies where bacteria that antagonize soil-borne pathogen *in vitro* are not necessarily the most effective *in vivo* and vice versa (Chérif *et al*., 2002) and with the current study, hence a need to study these in the field.

5. Conclusion

Aspergillus niger was the dominant soil-borne fungal pathogen affecting Citrus production. Other minor fungal pathogens included: *A. flavus*, *A. fumigatus*, *A. viridinutans*, *Fusarium oxysporum* and *Penicillium chrysogenum. Bacillus* spp. was the beneficial soil microorganism (bacteria) isolated during Citrus production. Soil solarization integrated with cultural practices was the most effective non-inorganic technique for managing soil-borne pathogens during production of Citrus seedlings under greenhouse conditions. Therefore, farmers/ nursery operators should use the solarization technique integrated with cultural practices as an effective ecological tool for better results in the production of Citrus in Uganda. Through use of the recommended technique(s), disease-free Citrus seedlings from certified nurseries will be easily accessed by farmers for improved productivity. This will reduce the inoculum density of soil-borne pathogens in their established citrus orchards and directly reduce their expenses on purchase of chemicals used to treat them hence lessen dependency on synthetic chemicals.

Recommendations

The recommended soil-borne citrus pathogen management technique(s) by our study need to be disseminated using several technology uptake pathways in order to be widely adopted by nursery operators and citrus growers in Uganda. It is worth noting that soil solarization can be also up scaled and applied in the citrus orchards for large scale management of soil-borne pathogens hence, studies to investigate use of this technology in the management of soil-borne pathogens at orchard level and its effect on yield should be conducted in Uganda. Use of climate-smart non-inorganic techniques of soil-sterilization integrated with cultural practices to control soil-borne pathogens is always pivotal. These conserve the beneficial soil microorganisms which are not conserved by other inorganic methods like use of synthetic chemicals. To minimize post-harvest losses, citrus fruits must be stored at temperatures below 15 °C since *A. niger* is very problematic when fruits are stored at temperatures greater than 15 °C. Also, damage of fruits should be minimized during transit to regulate infection. Good cultural practices in combination with biological control formulations containing antagonistic yeasts and bacteria such as *Pseudomonas syringae*, *Bacillus* spp., including *B. subtilis*, *Trichoderma* spp., and *Agrobacterium radiobacter* are recommended to minimize postharvest losses. These techniques combined with the solarization technology, a focus of our study form an integrated management system that will sustainably boost the citrus industry in Uganda.

Compliance with ethical standards

Acknowledgments

The authors would wish to thank National Agricultural Research Organization for funding the study via Competitive Grant Scheme (CGS) project 'Refurbishment and establishment of National Forestry Research laboratories at Kifu: Functionalizing acquired Lab equipment" (Grant Number: CCGS No. 5/41/18).

Disclosure of conflict of interest

The authors declare no conflict of interest.

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