Mexican Journal of Phytopathology
ISSN: 2007-8080

Crossref
Open access

Scientific Article

Establishment of an efficient protocol for *in vitro* disinfection of seeds of seven *Agave* spp. species

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Section:
Periodical Issue

Received:
02 October, 2023
Accepted:
29 November, 2023
Published:
20 December, 2023

Citation:
Aguilar-Rito MG, Arzate-Fernández AM, García-Núñez HG and Norman-Mondragón TH. 2024. Establishment of an efficient protocol for *in vitro* disinfection of seeds of seven *Agave* spp. species. Mexican Journal of Phytopathology 42(1): 5. https://doi.org/10.18781/R.MEX.FIT.2310-1

ABSTRACT

Background. Disinfection of *Agave* seeds is a crucial step in *in vitro* culture to prevent contamination, which can be caused by microorganisms such as bacteria, fungi and viruses that can affect seedling growth and reduce seed germination rate. Therefore, proper seed disinfection is essential to ensure vigorous and healthy plant growth.

Objective. Generate an efficient seed disinfection protocol in seven species of *Agave*; *Agave marmorata*, *A. karwinskii*, *A. potatorum*, *A. angustifolia*, *A. cupreata*, *A. horrida* and *A. salmiana* to reduce pollution levels.

Materials and methods. A total of 12 disinfection treatments with disinfectants and different combinations were evaluated. The disinfectants used were; 3 % Hydrogen Peroxide for 24 h, Commercial Sodium Hypochlorite 5 % (v/v) for 5 min, Calcium Hypochlorite 8 % (w/v) for 15 min, Copper Sulfate 30 % (v/v) for 10 min, Mercury Chloride II 0.1 % (w/v) for 10 min. Before each treatment was tested, the seeds were pre-washed with liquid soap and subjected to the treatments, Subsequently, they were sown in DM medium and the percentage of germination and contamination for each treatment was evaluated weekly for a period of 30 days. Additionally, the contaminating microorganisms found were identified.

Results. The best treatment for seed disinfection was 30 % copper sulfate (v/v) for 10 min, 0.1 % mercuric chloride II for 10 min and 3 % hydrogen peroxide for 24 h, obtaining 100 % disinfection.

Conclusion. Four genera of fungi were identified: *Monilinia* sp., *Aspergillus* sp., *Penicillium* sp., and *Alternaria alternata*, a bacterium; *Bacillus* sp., and a yeast, *Schizosaccharomyces* sp.

Keywords: Seeds, agave, disinfection methods, contamination, germination.

Introduction

The *Agave* genus is one of Mexico's most representative, with around 211 species, out of which 159 are found in Mexico, that is, 75.3 % of the total (García *et al*, 2019). Several products are obtained from these, such as foods, beverages, fibers and others (García, 2007). Their uses in Mexico are linked to the economic, social, cultural and environmental development of numerous populations. In recent decades, *Agave* has had an increasing social and economic rise due to its use in as raw material for the production of fermented and distilled alcoholic beverages such as pulque, mezcal and tequila. In Mexico, several species of wild agave are gathered to distil, with *A. marmorata, A. karwinskii, A. potatorum, A. angustifolia, A. cupreata, A. horrida* being the most noteworthy, and for the production of pulque, only one species is gathered: *A. salmiana.* According to the International Union for Conservation of Nature's (IUCN's) Red List and Mexico's Secretariat for the Environment and Natural Resources (Secretaría de Medio Ambiente y Recursos Naturales - SEMARNAT) *Agave* has become the target of over-gathering via illegal plundering. The lack of sexual propagation has reduced wild populations and threatened the genetic variability and structure in the cultivars, along with inflorescence usually being removed to ensure the accumulation of sugars in the plant hearts, eventually preventing the formation of seeds (Peña *et al.* 2006), which confirms the need to preserve the germplasm of these species in danger of extinction and threatened from seeds, using strategies based on the development and optimization of protocols for their disinfection and propagation *in vitro.*

The disinfection of agave seeds is a crucial step for the *in vitro* planting, since the success of the propagation systems depends largely on the control and prevention of contamination. Phytopathogens cause substantial losses of plant material in the production or research processes.

The purpose of disinfecting the seed is to remove contaminating microorganisms found in the seed coat and embryo, thus freeing the plants produced from bacteria and fungi related to the seeds. In addition to preventing contamination and diseases, the disinfection of *Agave* seeds can also improve germination rates, which is essential for the success of the *in vitro* planting of *Agave*, since a low germination rate can affect the efficiency and productivity of the *in vitro* planting process (Zurita *et al*.,

2014; Flores *et al.,* 2019) It is therefore important to follow adequate disinfection procedures to guarantee the success of the crop, improving the quality, safety, yield and conservation of the germplasm.|

Diverse seed disinfection protocols have recently been reported in species such as *A. victoria reginae* (Domínguez *et al*., 2008; Ramírez *et al*., 2008), *A. fourcroydes* (Monja, 2013), *A. angustifolia* (Arzate *et al*., 2016), *A. marmorata* (Álvarez *et al*., 2020) and *A. tequilana* (Delgado *et al*., 2021), in which most authors coincide in the use of ethanol and sodium hypochlorite and calcium. Nevertheless, there have been no reports on the use of mercury chloride ($HgCl_2$), Hydrogen peroxide (H_2O_2) and fungicides in *Agave*, as well as the rate of contamination and the contaminating microorganisms found, which, in large percentages, raise the production costs, therefore an efficient disinfection protocol may help reduce these costs.

The aim of this study was to evaluate 12 treatments (Table 2) to generate an efficient *in vitro* disinfection protocol in six species of mezcal *Agave* (*A. marmorata, A. karwinskii*, *A. potatorum*, *A. angustifolia, A. cupreata*, *A. horrida* and a pulque *A. salmiana*), using five disinfectants that help widely disinfect: hydrogen peroxide (H_2O_2), commercial sodium hypochlorite (NaClO), calcium hypochlorite $Ca(ClO)_2$, copper sulfate ($CuSO_{4)}$ commercial Product PENTAMAX®, mercury (II) chloride ($HgCl_2$) and ethanol (C_2H_6O).

Materials and Methods

This study was carried out in the Plant Molecular Biology Laboratory, found in the Center for the Plant Breeding Research and Advanced Studies in the School of Agricultural Science, of the Autonomous University of the State of Mexico, "El Cerrillo" Campus, Toluca, State of Mexico.

Biological material. Mature seeds taken from seven agave seeds were used, gathered in different Mexican states in periods between 1 and 7 years, stored in a germplasm bank during their storage (Table 1).

Disinfection of the biological material. Twelve treatments with disinfectant solutions and different combinations (Table 2) plus a control (T0) were evaluated, selected based on reports obtained in the disinfection of seeds of other genera, which displayed acceptable results for disinfection (Flores *et al*., 2008; Billard *et al.,* 2014; Tamayo *et al.,* 2017; Campos *et al.,* 2020; Zúñiga and Beauregard, 2020). The disinfectants used were hydrogen peroxide (H_2O_2) at 3% for 24 h, commercial sodium hypochlorite (Cloralex® at 4 %) (NaClO) with a final concentration of 5 % v/v) for 5 min, calcium hypochlorite $Ca(ClO)_2$ at 8 % (p/v) for 15 min,

Table 1. *Agave* species and seed collection data.

Specie	Lugar of collect	Date
Agave horrida	Ocuilan, México	2015
Agave salmiana	Acambay, México	2015
Agave karwinskii	Oaxaca, México	2017
Agave potatorum	Oaxaca, México	2017
Agave marmorata	Oaxaca, México	2017
Agave angustifolia	Zacualpan, México	2019
Agave cupreata	Puebla, México	2021

Table 2. Disinfection treatments evaluated in seven *Agave* species.

Treatments	Disinfectants				
	$CuSO_4$ 30 % v/v	$HgCl_2$ al 0.1 % p/v	$Ca(ClO)_2$ al 8 % p/v	NaClO al 5 % v/v	H_2O_2 al 3 %
0	*Control treatment				
1			15 min		
2				5 min	
3			15 min	5 min	
4					24 h
5	10 min		15 min		
6	10 min			5 min	
7	10 min		15 min	5 min	
8	10 min				24 h
9	10 min	10 min	15 min		
10	10 min	10 min		5 min	
11	10 min	10 min	15 min	5 min	
12	10 min	10 min			24 h

$CuSO_4$= Copper sulfate**; $HgCl_2$ =** Mercury (II) chloride**; Ca $(ClO)_2$ =** Calcium hypochlorite**; NaClO =** Sodium hypochlorite**; H_2O_2 =** Hydrogen peroxide**.** *Control (Without disinfectants).

PENTAMAX® brand copper sulfate ($CuSO_4$) at 30 % (v/v) for 10 min, mercury (II) chloride ($HgCl_2$) at 0.1 % (p/v) for 10 min. The 12 treatments were evaluated with 10 seeds per treatment (every seed was considered as one experimental unit), and for each treatment, three repetitions were considered, with a total of 390 seeds being evaluated for every species (considering a control). The experiment was monitored every week for a 30-day period.

Initially, the seeds were placed in sterile, 50 mL Falcon tubes, and they were washed with 15 mL sterile osmosis water plus 1 mL of commercial detergent

(Axión®) and two drops of Tween 20® for 15 minutes, and they were constantly shaken; after this time, they were rinsed three times with sterile distilled water. Next, they were submerged in 15 mL of ethanol at 70 % (v/v) for one minute and rinsed twice with sterile distilled water. Later, the disinfection treatments were applied, the seeds were shaken constantly in all treatments and they were rinsed three times with sterile distilled water after every disinfectant was applied to eliminate the residues of disinfectants, except for the treatments with H_2O_2. Everything was carried out under aseptic conditions, inside a laminar flow hood.

Seed germination. After being disinfected, the seeds were planted in 100 mL culture jars, placing 10 seeds in every jar. In each jar, 20 mL of culture medium were added, with MS salts (Murashige and Skoog, 1962), supplemented with 30 gL^{-1} of sucrose, 0.5 gL^{-1} of activated charcoal, without plant growth regulators and gelled with 8 g L^{-1} of agar. The pH of the medium was adjusted at 5.7 and it was sterilized in an autoclave at 1.5 kg/cm^2 of pressure and a temperature of 121.5 °C for 15 minutes. The cultures were incubated for 30 days in an incubation room with a photoperiod of 16 h of light and eight hours of darkness, with a light intensity of 18.83 µM m^{-2} s^{-1} at a temperature of 25 ± 2 °C; a seed was considered germinated when the radicle was visible.

Identification of contaminating microorganisms found in the agave seeds. The contaminating microorganisms (fungi, bacteria and yeasts) found in the treatments that presented any contamination were isolated, they were planted in Petri dishes with a PDA (Potato Dextrose Agar) medium for their isolation and purification and later sent to the Microbiology Laboratory of the Center for Research and Advanced Studies in Animal Health (Centro de Investigación y Estudios Avanzados en Salud Animal - CIESA) of the Autonomous University of the State of Mexico for their taxonomic identification, where the samples were replanted in a PDA medium and sabouraud agar at 25 ± 2 °C for 26 days, constantly monitored for their morphological identification, using lactophenol stains. For the identification of bacteria, the samples were planted in blood agar and TSA (Tryptone-Soy Agar) incubated at 36 °C and constantly monitored for 5 days.

Evaluated variables. In the 30 days of the experiment, the following variables were evaluated on a weekly basis:

Percentage of contamination of the seeds: ([Number of contaminated seeds * 100] / total number of seeds planted)
Percentage of germination of the seeds: ([Number of seeds that germinated * 100] / total number of seeds planted)

Days to germination: The average number of days to emergence of the radicle per seed in each species was registered.

Statistical analysis. The experimental design for this study had a total random distribution in a bifactorial arrangement with three repetitions. The data registered in the variables of seed contamination and germination underwent a simple classification Analysis of Variance (ANOVA) and Tukey's mean comparison test ($p<0.05$) was performed using the Sofware InfoStat 2017 Sofware.

Results

The analysis of variance for contamination and germination revealed a determination coefficient of 0.78 and 0.97, respectively, indicating that the model used was adequate to explain the variability of these variables. Likewise, the coefficient of variation was 34 % for contamination and 103 % for germination, explaining the latter with extrinsic (treatments) and intrinsic factors (genetic-physiologic) of each seed and species and confirmed statistically with the registration of highly significant differences ($p<0.01$) in the species x treatments interaction, indicating that at least one treatment was enough for both variables (contamination and germination) in at least one species (Table 3).

Germination. When processing the germination data, the means tests displayed significant statistical differences ($p<0.05$) in the different treatments. The germination response was highly variable in the different treatments and independent for all

Table 3. Analysis of variance for the variables contamination and germination, evaluating 12 disinfection treatments and seven agave species.

F.V	Contamination					Germination				
	SC	gl	CM	F	p-valor	SC	gl	CM	F	p-valor
Species	507.03	6	84.51	67.5	<0.0001	187.11	6	31.18	11.2	<0.0001
Treatment	1221.05	12	101.75	81.2	<0.0001	235.34	12	19.61	7.01	<0.0001
Species x Treatment	1999.25	72	27.77	22.2	<0.0001	481.89	72	6.69	2.39	<0.0001
Error	114	91	1.25			254.50	91	2.80		
Total	3841.34	181				1158.84	181			
		R^2= 0.97		CV= 34.29%			R^2= 0.78		CV= 103.17%	

SC: Sum of squares, gl: Degrees of freedom, CM: Mean squares, F: F calculated, CV: Coefficient of variation and R^2: Coefficient of determination.

species, since each species responded in different ways and their germination times were also variable; for the most part, this variable was low (0 – 20 %) (Table 4).

Table 4. Means comparison for the variables contamination and germination in seven *agave* species, according to Tukey's test ($p<0.05$).

Treatments	*A. salmiana* DG (21d)		*A. horrida* (14d)		*A. marmorata* (8d)		*A. potatorum* (27d)		*A. karwiskii* (20d)		*A. angustifolia* (20d)		*A. cupreata* (14d)	
	C	G	C	G	C	G	C	G	C	G	C	G	C	G
0 Sin ningún desinfectante	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}
1 NaOCl	100 ^{D}	0 ^{C}	10 ^{AB}	10 ^{BC}	0 ^{A}	80 ^{AB}	0 ^{A}	10 ^{BC}	100 ^{D}	0 ^{C}	0 ^{A}	80 ^{AB}	0 ^{A}	10 ^{AB}
2 $Ca(ClO)_2$	100 ^{D}	0 ^{C}	0 ^{A}	85 ^{A}	100 ^{D}	40 ^{ABC}	100 ^{D}	0 ^{C}	0 ^{A}	0 ^{C}	0 ^{A}	0 ^{C}	0 ^{A}	20 ^{ABC}
3 $Ca(ClO)_2$/NaOCl	0 ^{A}	60 ^{ABC}	0 ^{A}	85 ^{A}	100 ^{D}	85 ^{A}	0 ^{A}	0 ^{C}	100 ^{AB}	0 ^{C}	100 ^{D}	0 ^{C}	0 ^{A}	20 ^{ABC}
4 H_2O_2	0 ^{A}	10 ^{BC}	0 ^{A}	0 ^{C}	0 ^{A}	70 ^{ABC}	0 ^{A}	85 ^{A}	0 ^{A}	0 ^{C}	40 ^{ABC}	20 ^{ABC}	0 ^{A}	10 ^{BC}
5 $CuSO_4$/ $Ca(ClO)_2$	100 ^{D}	0 ^{C}	0 ^{A}	10 ^{BC}	0 ^{A}	50 ^{ABC}	80 ^{CD}	0 ^{C}	10.0 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	0 ^{A}	50 ^{ABC}
6 $CuSO_4$/NaOCl	0 ^{A}	0 ^{C}	0 ^{A}	0 ^{C}	0 ^{A}	80 ^{AB}	100 ^{D}	0 ^{C}	0 ^{A}	0 ^{C}	50 ^{BC}	0 ^{C}	0 ^{A}	85 ^{A}
7 $CuSO_4$/ $Ca(ClO)_2$/NaOCl	10.0 ^{D}	0 ^{C}	0 ^{A}	20 ^{ABC}	0 ^{A}	10 ^{BC}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	100 ^{D}	0 ^{C}	0 ^{A}	80 ^{AB}
8 $CuSO_4$/H_2O_2	0 ^{A}	20 ^{ABC}	100 ^{D}	10 ^{BC}	0 ^{A}	10 ^{BC}	0 ^{A}	0 ^{C}	0 ^{A}	10 ^{BC}	0 ^{A}	10 ^{ABC}	0 ^{A}	0 ^{C}
9 $CuSO_4$/$HgCl_2$/ $Ca(ClO)_2$	0 ^{A}	0 ^{C}	10 ^{AB}	10 ^{BC}	0 ^{A}	40 ^{ABC}	0 ^{A}	0 ^{C}	0 ^{A}	20 ^{ABC}	30 ^{AB}	0 ^{C}	0 ^{A}	80 ^{AB}
10 $CuSO_4$/$HgCl_2$/NaOCl	100 ^{D}	0 ^{C}	0 ^{A}	30 ^{ABC}	0 ^{A}	10 ^{BC}	0 ^{A}	0 ^{C}	0 ^{A}	20 ^{ABC}	100 ^{D}	0 ^{C}	0 ^{A}	80 ^{AB}
11 $CuSO_4$/HgCl/$Ca(ClO)_2$/NaOCl	100 ^{D}	20 ^{ABC}	0 ^{A}	30 ^{ABC}	0 ^{A}	20 ^{ABC}	0 ^{A}	10 ^{BC}	40 ^{ABC}	10 ^{BC}	100 ^{D}	0 ^{C}	0 ^{A}	80 ^{AB}
12 $CuSO_4$/$HgCl_2$/H_2O_2	0 ^{A}	50 ^{ABC}	0 ^{A}	30 ^{ABC}	0 ^{A}	90 ^{A}	0 ^{A}	30 ^{ABC}	0 ^{A}	20 ^{ABC}	00 ^{A}	85 ^{A}	0 ^{A}	80 ^{AB}

T: Treatment, 0: Control; DG = Days to germination; from in vitro planting; C =Contamination; G=Gemination. Same letters indicate no significant differences ($p<0.05$) between treatments.

However, the results coincide with those for the contamination variable. The only treatment that stimulated germination for the seven *Agave* species was treatment 12, where the percentage of germination for most of the species was above the other treatments, in *A. marmorata* (90 %), *A. angustifolia* (85 %), *A. salmiana* (50 %), *A. horrida* (30 %), *A. potatorum* (30 %), *A. cupreata* (30 %), and *A. karwinskii* (20 %). On the other hand, *A. marmorata* gave a better response, reaching an average of 90 % of germination, as well as being the earliest species, germinating 8 days after planting, thus turning out to be the species that statistically stood out from the rest (Table 4).

Nevertheless, despite the low percentage of germination for the other treatments (0 – 20 %) none of the seedlings obtained displayed abnormalities in their germination and development, regardless of the treatment used and of the species (Figure 1).

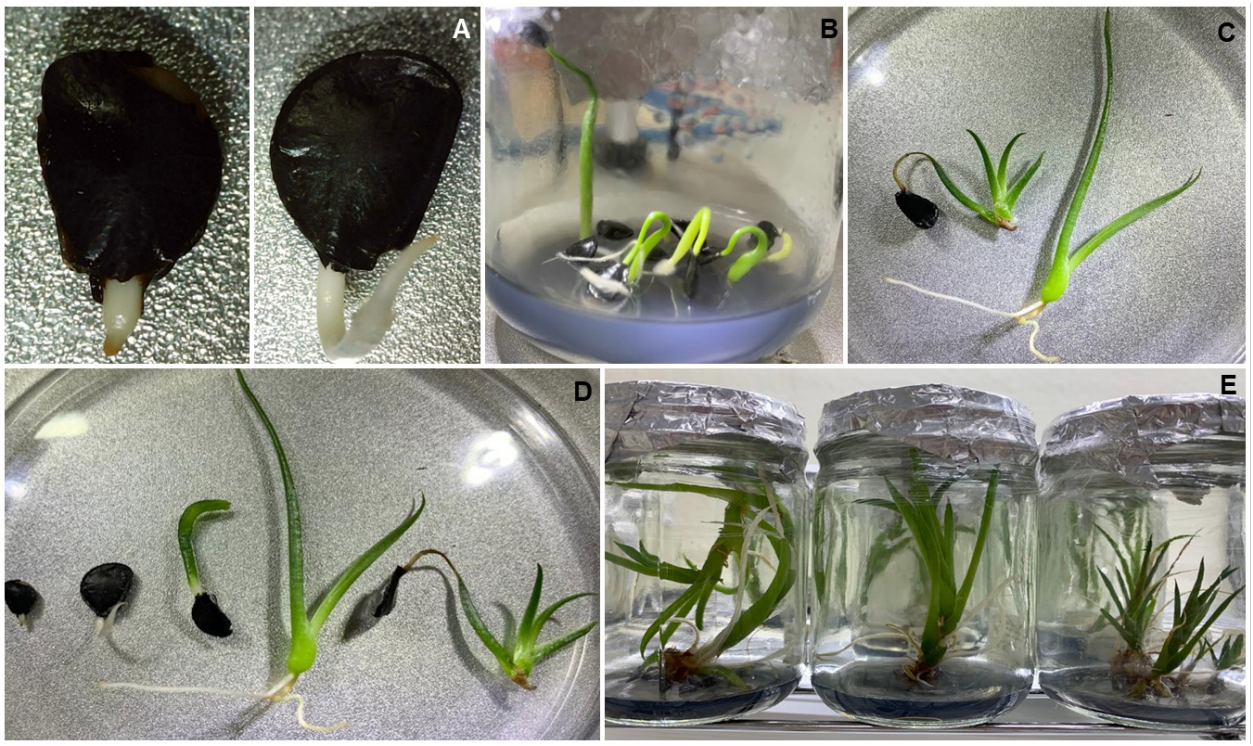

Figure 1. *A. salmiana* seed germination. A) root emergence and elongation. (14 dap) B) Elongation of the plumule. (21 dap) C) Diferenciation of the root cotyledons and development. (30 dap) D) Full germination and development. (14-30 dap) E) Normal, completely developed *A. salmiana* seedlings. (50 dap)**;** dap days after planting.

Identification of pathogens

Contamination. The Tukey's test ($p<0.05$) determined that there are highly significant differences between all the treatments applied, since most treatments differentially reduced contamination in seeds. However, statistically, only one treatment was better than the rest (Treatment 12), in which contamination was reduced completely (100 %) for all the species studied, and which consisted of applying hydrogen peroxide (H_2O_2) at 3 % for 24 h, PENTAMAX (PM) at 30 % (v/v) for 10 min and Mercury (II) chloride ($HgCl_2$) al 0.1 % (p/v) for 10 min. In contrast with the above, the highest contamination levels were observed in the calcium hypochlorite and sodium hypochlorite treatments. It is worth pointing out that the contamination of *Agave* spp. seeds in the different treatments was mainly due to fungi and, less frequently, to bacteria (Table 4).

Identification of contaminating microorganisms. In general terms and for the first time, six genera of microorganisms were found which were related to the

Agave seeds. The results of the taxonomic study for the six samples analyzed reveal four fungi genera (*Monilinia* sp., *Aspergillus* sp., *Penicillium* sp. and *Alternaria alternata*), one bacterium (*Bacillus* sp.) and one yeast (*Schizosaccharomyces* sp.) (Figure 2). It is noteworthy that in the isolation of the contaminating species *Aspergillus* and *Penicillium* all species were displayed, whereas others were exclusive to some species. *Schizosaccharomyces* sp., was found in *A. marmorata, Alternaria alternata* in *A. angustifolia* and *Monilinia* sp., in *A. horrida.*

Evaluating the efficiency of treatments on the control of the pathogens revealed that most treatments had an effective control over bacteria, yet it was deficient for

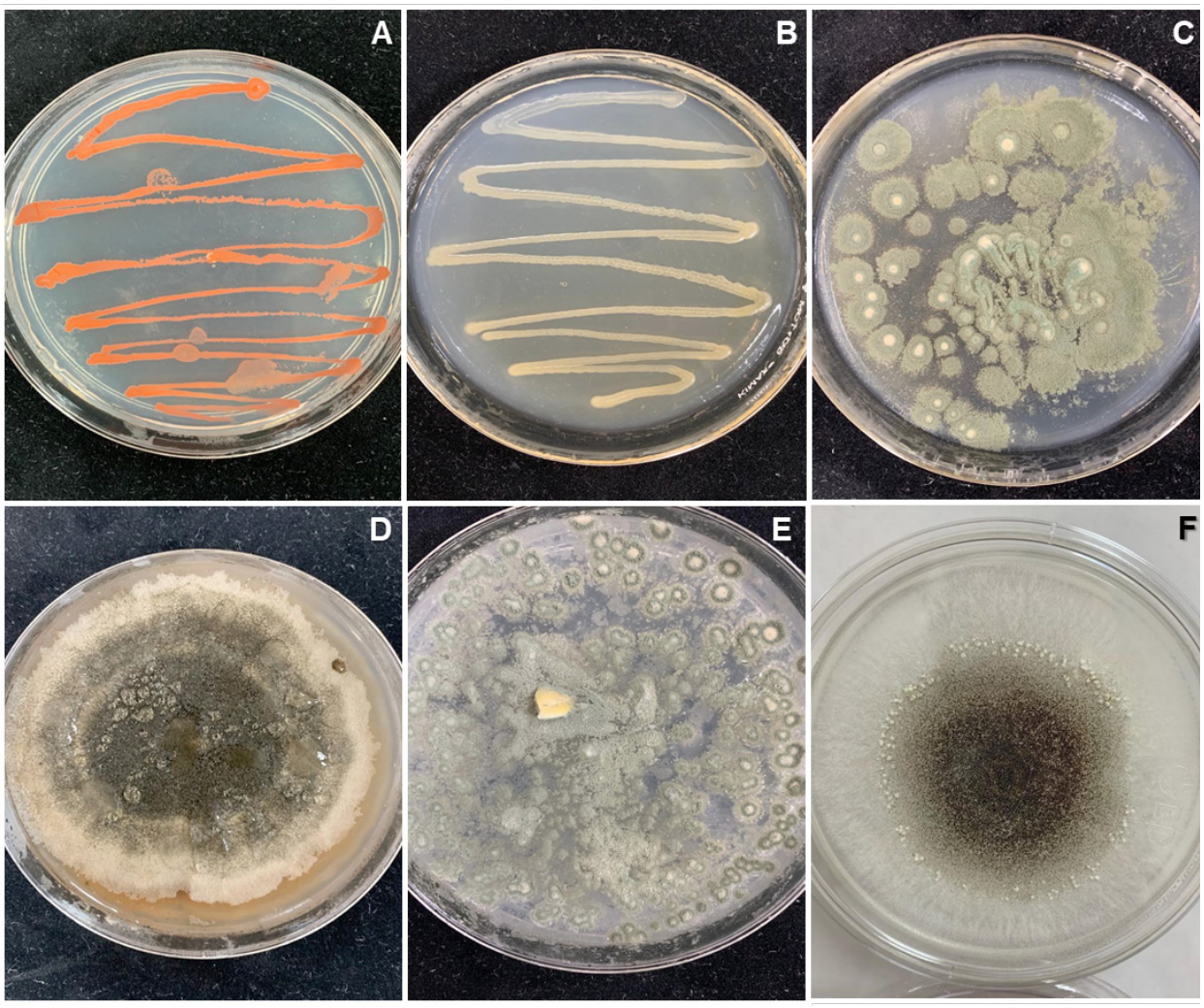

Figura 2. Bacterial and fungal strains found in the seeds of *Agave* spp. A) *Schizosaccharomyces* sp. B) *Bacillus* sp. C) *Penicilllium* sp. D) *Alternaria alternata.* E) *Aspergillus* sp. F) *Monilinia* sp.

fungi in comparison with the control treatment. However, H_2O_2 presented favorable results greater than the other treatments, even in one which used the disinfectant only (Table 5).

Table 5. Contaminant microorganisms found in the *Agave* seeds after applying the treamtnets.

Treatments	*A. salmiana*			*A. angustifolia*			*A. horrida*			*A. marmorata*			*A. potatorum*			*A. karwiski*			*A. cupreata*		
	H	B	L	H	B	L	H	B	L	H	B	L	H	B	L	H	B	L	H	B	L
1 $Ca(ClO_2)$	x						x	x								x	x	x			
2 NaOCl	x									x	x			x							
3 $Ca(ClO_2)$/NaOCl				x						x						x	x	x			
4 H_2O_2				x																	
5 CuSO4/$Ca(ClO_2)$	x			x										x		x					
6 CuSO4/NaOCl				x										x							
7 CuSO4/$Ca(ClO_2)$/NaOCl	x			x										x		x					
8 CuSO4/H_2O_2							x														
9 CuSO4/$HgCl_2$/$Ca(ClO_2)$				x			x														
10 CuSO4/$HgCl_2$/NaOCl	x			x																	
11 CuSO4/$HgCl_2$/$Ca(ClO_2)$/NaOCl	x			x												x					
12 CuSO4/$HgCl_2$/H_2O_2																					

H: Fungi; B: Bacteria; L: Yeasts. X: Microorganisms found.

Discussion

Germination. The total germination percentages for most of the species were higher than those reported by Ramírez *et al*. (2016) for *A. mapisaga* (70 %) and *A. angustifolia* with 28 % germination. Additionally, the seeds of the seven *Agave* species displayed percentages of germination above 50 %, even in species with the most years of gathering, such as *A. horrida* and *A. salmiana,* coinciding with Castillo *et al.* (2022), who reported that *A. victoriae reginae* seeds were viable after one year of having been gathered and suggest that it is possible to store this species' seeds for at least one year and have acceptable germination percentages. According to the results from this study, the seeds displayed a viability of over 50 % in storage in a germplasm bank for longer periods. However, for future studies, we suggest starting with the viability test with tetrazolium chloride, which will provide clarity in the viability obtained.

In addition, by analyzing the days of germination, it is possible to claim that the seeds displayed no latency, a typical characteristic of other *Agave* species (Freeman *et al*., 1977; Freeman, 1975). This lack of latency in evaluated seeds is similar to those reported by Peña *et al.* (2006) for *A. salmiana* seeds stored for two years and which had a lower latency than those planted in the same year of gathering.

Finally, in the treatments with no germination for any species, most notably the control treatment (T0), in which no species germinated, this may be attributed to the presence of fungi and bacteria (contamination), since the seeds affected by pathogenic fungi may display sclerotization, stromatization, decoloring, necrosis, rotting of the root, size reduction, abortion and inability to germinate (Ellis and Gálvez, 1980; Agarwal and Sinclair, 1987).

Contamination. In *Agave*, no specific data have been reported for the topic of disinfection. In this study, the lowest average number of contaminated seeds (0-10 %) was displayed in treatments in which H_2O_2 was applied. However, when applying H_2O_2 at 3 % for 24 h in combination with copper sulfate **(**PENTAMAX®) at 30 % for 10 min and mercury (II) chloride at 0.1 % for 10 min, the seeds were totally disinfected. In this genus, no similar results have been reported with these disinfectants. Nevertheless, Flores *et al.* (2019) obtained high levels of disinfection by applying H_2O_2 (3 % v/v) for 24 horas, shaking constantly. This may be due to the hydrogen peroxide exerting its oxidative activity with the production of free radicals, which produce oxidative damage on proteins and lipids in the cell membranes of the pathogens, confirming is bactericidal, viricidal and fungicidal abilities. When the H_2O_2 is shaken constantly, the oxygen demand is reduced, therefore H_2O_2 has a higher oxidation ability than chlorine or chlorine dioxide. In addition, its molecules leave no toxic residues when they release oxygen (CNIB, 2023; Rodríguez *et al*., 2009).

On the other hand, Bedoya *et al*. (2016) reported that the immersion of *Aloysia triphylla* leaves in $HgCl_2$ at 0.2 % p/v for 5 minutes helped obtain over 80 % of viable, contamination-free explants. Regarding the disinfection with, Barney (2003) reduced the amount of endophytic fungi in *Lepanthes rupestris* or *Lolium perenne* seedlings by applying Propiconazol or Procloraz. In sum, all the disinfectants mentioned are efficient, although for agave, H_2O_2 combined with $HgCl_2$ and copper sulfate is highly efficient to reduce seed contamination, helping obtain disinfections of 100% in the seven species evaluated.

An intermediate contamination level was achieved by applying $Ca(ClO)_2$ and NaOCl, contrasting with results from other species. Martínez *et al*. (2003) obtained pathogen-free *Agave victoriae reginae* seeds using NaOCl as a disinfectant, which has been effectively proven aganst bacteria, viruses and fungi, since its dilution in water produces hypochlorous acid (HClO), which easily penetrates the cells of microorganisms and acts on its proteins and nucleic acids (Auccasi, 2016). Nevertheless, according to Lenntech (2008), in order to achieve a successful disinfection, a concentration must be applied that is adequate to the degree of infection of the pathogenic organisms. This coincides with Álvarez *et al.,* (2008),

who mention the adequate effectiveness of chlorine and ethanol as disinfectants in pathogenic bacteria, yet deficient against fungi and viruses.

Although the highest contamination levels were observed in seeds free of chemical solutions (control treatment), which displayed a complete contamination in the seeds, which may lead to deduce that washing the seed with running water and soap is not enough, chemical disinfectants must be applied to reduce the presence of pathogenic agents. Finally, comparing different species and solutions used, the results show the effectiveness of each one for *Agave* spp.

Identification of microorganisms. According to the National Agricultural Technology Insistute (Instituto Nacional de Tecnología Agropecuaria - INTA, 2022), the three fungal genera can be classified into three functional group: field fungi (*Fusarium* and *Trichoderma*), storage fungi (*Aspergillus* and *Penicillium*) and generic contaminating fungi (*Monilia* and *Rhizopus*), each one with a particular origin and habitat.

Aspergillus and *Penicillium* are typical genera under storage conditions, with the ability to invade seeds and grains with a low moisture content, to grow in a wide range of temperatures and, with few exceptions, to infect before the harvest. They actively colonize the seeds, where they cause deterioration and reduce germination, via enzymatic principles and toxins (Howlett, 2006), thus confirming that the seeds which displayed an infestation with these genera did not germinate, being the main reason. In moringa seeds, the genus *Aspergillus* turned out to be the most versatile, since it was displayed the most frequently, being present in the moringa seed both internally and externally (Sabu *et al*., 2022). Information regarding *Monilinia* is scarce, since it has not been reported in *Agave* or other seeds. It lives mostly in humid areas and mainly attacks fruit trees, being the causal agent of brown rot (Malvárez *et al*., 2001). It behaves like a wound pathogen, since it infects fruits from lesions caused by insects and/or mechanical grazes (Zúñiga *et al*., 2011).

Alternaria alternata was found to have the highest incidence and exclusively in *A. angustifolia*. This pathogen is a saprophytic fungus and it is spread via spores in wind and the mobilization of infected seeds (DGSV, 2023). *A. alternata* causes diseases in several economically important plants such as broccoli, tomato, chili pepper, potato, citrus fruits, apple, etc. (Meena and Samal, 2019). Kurowski and Wysocka (2009) point out that *A. alternata* is commonly found as a contaminant in cereal grains and is the main fungal disease isolated in amaranth and oat seeds (Noelting *et al*., 2016; Leyva *et al.,* 2014). However, there are no reports for *A. angustifolia.*

Finally, there are reports of the genera *Bacillus* sp. and *Schizosaccharomyces* sp. as beneficial agents, particularly *Bacillus,* which has been reported as one

of the main microbial antagonism genera and as a biological control agent for agriculture. In addition, there have been reports of *Agave* as an endophytic agent that promotes plant growth and development (Beltran *et al.,* 2014). However, in this investigation, both genera were found as contaminant saprophytic agents in *Agave* seeds, of which there are no reports in other seeds, which could open doors for future investigations.

Conclusions

The 12 treatments proposed were evaluated and the results show that treatment 12, consisting of hydrogen peroxide at 3 % for 24 h in combination with copper sulfate (PENTAMAX®) at 30 % (v/v) for 10 min and mercury (II) chloride at 0.1 % (p/v) for 10 min, was the best in the control of contaminating microorganisms found in the *Agave* spp. seeds, achieving a 100% disinfection rate and a germination of 20 to 90 %, depending on the species, therefore it is recommended for the disinfection of *Agave* spp. seeds.

For the first time in *Agave* seeds, the taxonomic identification of the contaminating microorganisms found was carried out, reporting four fungi (*Penicilllium* sp., *Alternaria alternata, Aspergillus* sp*., Monilinia* sp.), one bacterium (*Bacillus* sp.) and one yeast (*Schizosaccharomyces* sp.), out of which *Alternaria alternata., Monilinia* sp. and *Schizosaccharomyces* sp. were specific to *para A. angustifolia, A. horrida* and *A. karwiski*, respectively. In addition, the disinfecting agents that control them were also reported for the first time.

Acknowledgments

I thank the research team of the Laboratory of Laboratorio de Biología Molecular Vegetal of Centro de Investigación y Estudios Avanzados en Fitomejoramiento, Facultad de Ciencias Agrícolas, of the Universidad Autónoma del Estado del Estado de México for the support and guidance in the elaboration of this research work.

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