Disinfestation of *Agave angustifolia* Haw. collected in the field prior to *in vitro* culture

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ABSTRACT

Objective: to determine a disinfestation procedure for *in vitro* establishing of *Agave angustifolia* Haw. explants collected in the field.

Design/Methodology/Approach: seven agave plants per treatment were placed in perlite to contrast six treatments; two agrochemicals (Benomyl 2 g L^{-1} and Actara 0.5 g L^{-1}) and three contact times (10, 20 and 30 days). The stems of the plants were sectioned in 4-6 segments, after treatment with alcohol 80%, chlorine 30% and hydrogen peroxide 20%, to later place them *in vitro*. Usually there is contamination of explants mainly by bacteria, so Pursue® was used at 25 and 50%, placing 20 explants in each concentration for 1 minute. The variables evaluated were percentage of visibly healthy explants, type of contamination. Due to the difference in the number of explants, no statistical tests were performed.

Results: the plants treated with Benomyl presented less contamination by fungi without considering the contact time. Bacteria were present in the different treatments used, however, in the explants with pretreated with Benomyl, 85% was obtained visibly healthy when Pursue® at 50% was used.

Study limitations/Implications: other agrochemicals, such as specific bactericides are desirable to determine through their use, whether they mitigate *in vitro* contamination by pathogenic bacteria.

Findings/Conclusions: pretreatments with agrochemicals for the *in vitro* establishing of plants collected from the field are necessary to increase the number of visually healthy explants. With the Pursue® product at 50%, up to 85% of explants can be obtained without the presence of fungi or bacteria.

Keywords: bacteria, contamination, explant, fungicide, pretreatments.

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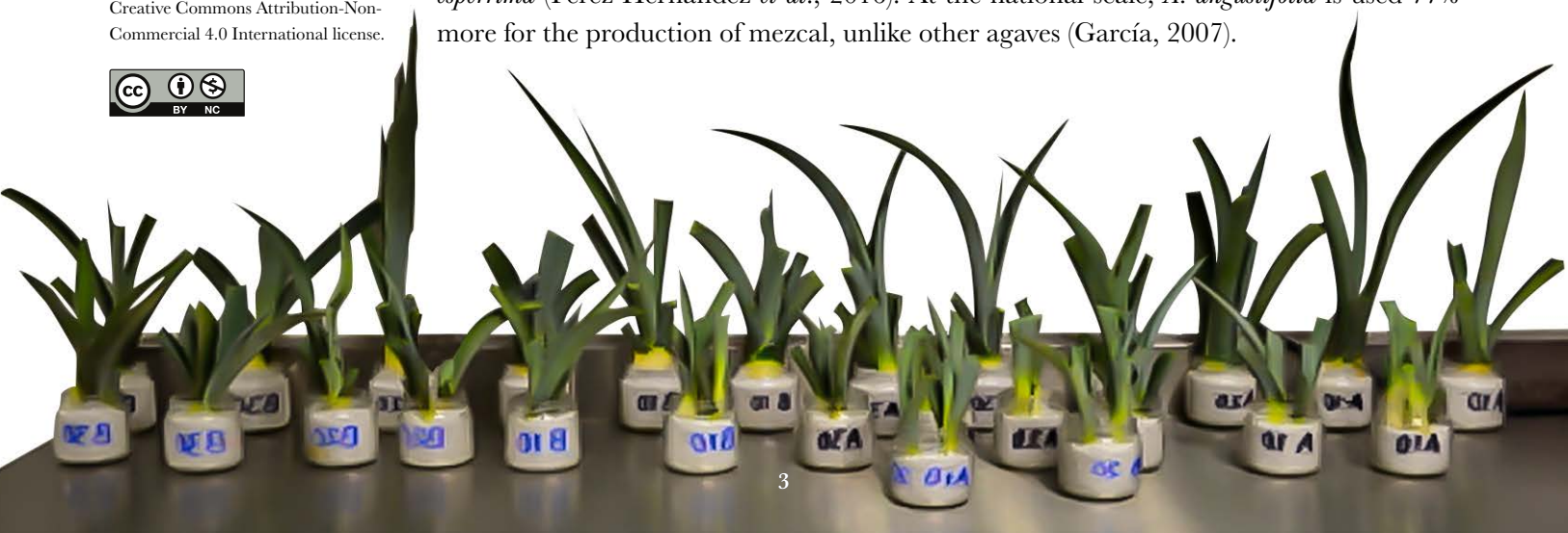
INTRODUCTION

The genus *Agave* is endemic to America; within the 200 existing species, 150 are native to Mexico (Lara-Ávila and Alpuche-Solís, 2016). The uses of these plants are variate: food, beverages, medicinal, fibers (García, 2007). Several species of agave are used for mezcal production, among them are *Agave angustifolia*, *A. cupreata*, *A. potatorum*, *A. salmiana* and *A. esperrima* (Pérez-Hernández *et al.*, 2016). At the national scale, *A. angustifolia* is used 77% more for the production of mezcal, unlike other agaves (García, 2007).



Currently in the municipality of Chilapa, Guerrero, plantations have been made with this species, to obtain mezcal (Barrientos-Rivera *et al.*, 2019). However, it is mentioned that there have been problems for the sexual and asexual multiplication of the species, so in vitro culture is an alternative for obtaining plants (Domínguez *et al.*, 2008). For the genus *Agave*, protocols have been established from the *in vitro* multiplication stage (Aguilar and Rodríguez, 2018). However, these start from plants already established in culture medium. On the other hand, when plants are collected in the field, for subsequent *in vitro* placement, contamination of explants is a difficult problem to solve. Among the most used products for disinfestation are: sodium hypochlorite, ethanol, mercury chloride, tween 20 in different concentrations (Hernández and González, 2010). Even including antibiotics such as sodium ampicillin and chloramphenicol (Alves-Pereira *et al.*, 2014) which have been insufficient options for some species from the field.

Disinfestation protocols are necessary to initiate *in vitro* culture (Bedoya-Pérez *et al.*, 2016), since the rest of the stages will depend on this: multiplication, rooting, and acclimatization, before being stablished in the field. Due to the morphology of agave in addition to the development in the field, the accumulation of dust, presence of fungi and bacteria is greater. Also, by placing fractions of stems in visually aseptic *in vitro* culture medium, the probability of contamination increases.

Therefore, the objective of this study was to determine a disinfestation protocol for the *in vitro* establishing of *Agave angustifolia* explants, from plants collected in the field.

MATERIALS AND METHODS

Plant material

To obtain agave plants *in vitro*, several experiments were conducted without satisfactory results. It was then decided to define an efficient disinfestation protocol for *in vitro* establishing of stem sections.

In April 2017, outstanding (elite) plants of *A. angustifolia* 15-30 cm in length and 3-5 cm in diameter were collected in the field, transported to the *in vitro* culture laboratory of the Genetics building, College of Postgraduates, Campus Montecillo. In the laboratory, the roots were pruned to leave them in approximately 2 cm; then the plants were washed with soap and water, to remove the soil, and they were placed in a cotton cloth to remove the excess of water.

Pretreatments

The pretreatments for disinfestation were six, product of solutions of benomyl 2 g L^{-1} (1-(butylcarbamoyl) benzimidazole-2-ylcarbamate methyl), and actara 0.5 g L^{-1} (3-(2-Chloro-1,3-thiazol-5-ylmethyl)-1,3,5-oxadiaznan-4-ylidene (nitro) amino), plus the contact time of the solutions with the plants of 10, 20 and 30 days. Seven plants of *A. angustifolia* were used per treatment, placed in 100 mL Gerber® bottles with agrolite (Figure 1), 15 mL of the solutions on the substrate and 2 mL on the leaves (depending on the treatment) were added in the first application.

The products added to the substrate were prepared on the same day of application. Solution applications were made on days 1, 10 and 20; with one, two and three applications

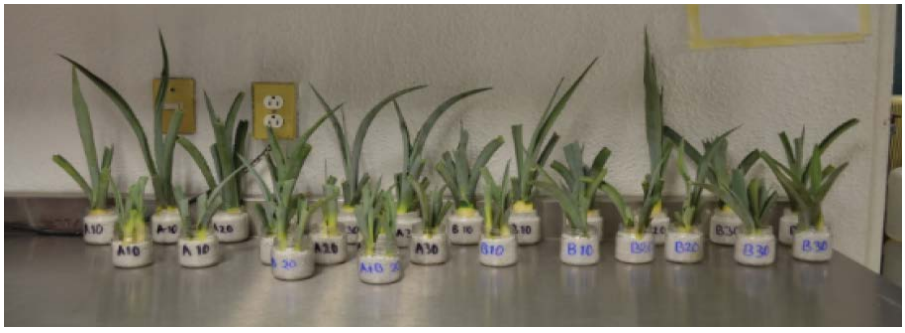


Figure 1. Pretreatments of disinfestation of *A. angustifolia* from the field. A: actara; B, benomyl; 10, 20, 30: days of contact of plants with chemicals.

for days 10, 20 and 30 contact days, respectively. *In vitro* seeding was carried out one day after the aforementioned contact time.

In vitro disinfestation

Plants were extracted from agrolite. The portion corresponding to the stem was washed with soap and water, the roots and leaves were removed.

In a laminar flow hood, ethanol 80%, sodium hypochlorite (commercial) 30%, and hydrogen peroxide (commercial) 20% were used for 3, 2 and 2 minutes respectively. After the disinfestation process, the stems of each pretreatment were cut into a cube shape (Figure 2). Finally, three washes with sterile de-ionized water were applied for 3, 2 and 1 minutes. This process was performed for all nine treatments.

Establishing the *in vitro* culture

After the process of disinfestation, the stems were divided into 4-6 sections. The tissue damaged by the products used was removed and sections of approximately 0.5-0.8 cm were obtained to be placed in test tubes (Figure 2) with 10 mL of culture establishing medium. Which is composed of commercial sugar 20 g L^{-1} and Sigma® agar 6 g L^{-1}, pH 5.7 and sterilized at 20 pounds of pressure in autoclave.

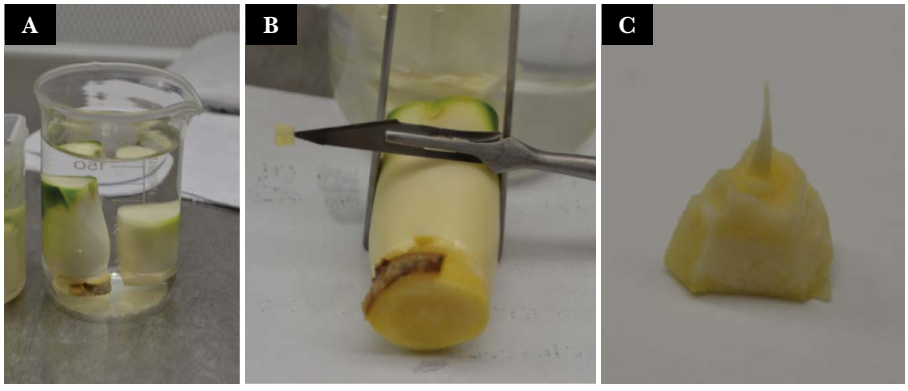


Figure 2. Conditioning of agave stem cuts to be placed in *in vitro* conditions. A: Agave rinse in sterile deionized water; B: Agave stem cutting; C: Stem section for *in vitro* placement.

If the explants were not contaminated, they were sub-cultured two weeks later in test tubes with 10 mL of culture medium with salts and developmental regulators (Table 1), where they remained for four weeks. The subsequent subcultures were made in 200 mL Gerber® bottles with 30 mL of culture medium (Table 1), for the multiplication of agaves.

The number of segments placed per treatment was variable, as those were in function of the stem size of the agave plants. The percentage and type of contamination in the tissues during the first two weeks of the established culture were evaluated to contrast the effectiveness of the disinfestation processes.

Due to the incidence of bacteria in the explants *in vitro*, in November 2017 the product Pursue® (quaternary ammonium salts) was used in concentrations of 25 and 50%; 20 explants were placed in the solutions for 1 minute, then three rinses were made with sterile deionized water of 3, 3 and 1 minutes. These were placed in test tubes with 10 mL of culture medium (Table 1). Only the percentage of contamination was evaluated in these explants.

Experimental design and variables evaluated

The experimental design used was completely randomized with factorial arrangement for treatments; two agrochemical solutions, and three resting times of the explants (Table 2).

Table 1. *In vitro* culture medium used for acclimatization and multiplication of *Agave angustifolia* explants.

Reagent	Concentration
$NH_4\ NO_3$	8 mM
KNO_3	10 mM
$Ca(NO_3)\ 4H_2O$	2.0 mM
$MgSO_4\ 7H_2O$	1.4 mM
$KH_2\ PO_4$	1.3 mM
Chelates$^{\times}$	0.1 mM
Micronutrients†	0.1 mM
Thiamine†††	0.1 mM
Mioinositol††	0.1 mM
BA††	4.43 μM
IBA††	0.42 μM
Sucrose	20 g
Agar	6 g

Chelates$^{\times}$: $FeSO_4\ 7H_2O$ (695 mg) and Na EDTA (931 mg), prepared in 250 mL of deionized water. † Micronutrients: H_3BO_3: 155 mg, $MnSO_4\ H_2O$: 422.5 mg, $ZnSO_4\ 7H_2O$: 215 mg, $Na_2\ MoO\ 2\ H_2O$: 6.25 mg, $CuSO_4\ 5H_2O$: 0.625 mg, prepared in 250 mL of deionized water. †† 10 mg of reactive prepared in 100 mL of deionized water. ††† Thiamine:1 g of reactive in 100 mL of deionized water. All the active chemicals by Sigma®.

Table 2. Treatments used in the process of disinfestation of *A. angustifolia* in *in vitro* culture.

Agrochemical	Exposure days
Benomilo®	10
	20
	30
Actara®	10
	20
	30

Benomyl: 2 g L^{-1}; Actara: 0.5 g L^{-1}; 10, 20 and 30: days of rest in perlite with agrochemicals.

RESULTS AND DISCUSSION

Preliminary results

The first attempts to establish *in vitro* plants of *A. angustifolia* were in November 2016, using seeds and applying alcohol (80%), chloralex (20%) and Timsen® (1 g L^{-1}) for 5, 10 and 15 minutes, respectively. There was 95% of germination, and 66% of the seeds presented contamination, the presence of fungi and bacteria predominated. It is worth mentioning that the explants with fungi were discarded, and treatments were applied to those with bacteria, using different products to try to eliminate pathogens while the plants remained at in vitro conditions.

Puente-Garza *et al.* (2022) performed *in vitro* germination of *A. salmiana* with 93% germination, but did no mention the presence or absence of contamination. Whereas Hernández-Castro *et al.* (2021) obtained 95% germination with seeds of *A. angustifolia* placed in substrate. Therefore, although *in vitro* culture is a novel technique, for *A. angustifolia* its use in seed germination is not justified. It is a more expensive technique, and moreover, germination in substrate is equal to that reported *in vitro*.

Regarding the products used for seed disinfestation, the most used are chlorine and alcohol, or even antibiotics for human use (Hernández and González, 2010). In recent years, protocols have been designed for the *in vitro* disinfestation of explants using hydrogen peroxide, chlorine dioxide and silver nanoparticles (Ramírez *et al.*, 2014; Pastelín-Solano *et al.*, 2020) which are not harmful to people who use them.

Pretreatments with agrochemicals

Although the number of plants placed in agrolite were seven per treatment, the number of explants was different due to the initial size of the plant, which is why no statistical analysis was made.

From the agrochemicals used with Benomilo, 22, 13 and 9 visually healthy explants were obtained with 10, 20 and 30 days of exposure to the products. There was also less contamination by fungi and bacteria; contrary to what occurred when actara was used (Table 3).

The implementation of pretreatments for *in vitro* establishing is little used. This usually happens because many of the agave multiplication protocols are reported on

Table 3. Quantity and condition of explants from *A. angustifolia* placed *in vitro*.

Agrochemical	Exposure days	Explants contidions (%)		
		Fungus	Bacteria	Visually healthy
Actara	10	4	10	9
Benomilo		0	6	22
Actara	20	18	17	7
Benomilo		3	6	13
Actara	30	7	8	4
Benomilo		7	13	9
Total explants		39	60	64

plants already established *in vitro* (Ríos-Ramírez *et al*., 2017) and the disinfestation stage is omitted. Despite the acclimatization, multiplication and rooting *in vitro* are facilitated. This suggests that the methodology is easy for many species in which it is implemented, but there is no mention of methodologies for establishing *in vitro*, those plants collected from the field.

Bedoya-Pérez *et al.* (2016) mentioned a disinfestation protocol similar to the one proposed here, with *Aloysia tryphilla* plants, applying the fungicides Fosetal and Benomil. However, due to the application of other *in vitro* disinfestation treatments, those authors did not report the results corresponding to fungicides.

Of the treatments applied, a lower percentage of fungal contamination was obtained when Benomyl was used, even with 10 days of exposure there was no presence of those pathogens (Figure 3). In a similar way, it can be ratified that it is not necessary for the plants to be maintained for 30 days with the chemicals. In fact, with that time of exposure there were fewer visually healthy explants, 21 and 31 with actara and benomyl, respectively.

With the pretreatments used, a total of 39, 37 and 24% visually healthy explants were obtained, without bacteria or fungi, respectively. But before using the pretreatments, the contamination was 80-100% predominating fungi, even in the explants previously contaminated with bacteria.

Despite the use of agrochemicals, the presence of bacteria in the environment was evident, this could be due to two reasons; that they were endogenous pathogens, or else, the proliferation of those occurred because a specific bactericide was not used in the treatments. Martínez-Rodríguez *et al.* (2014) mentioned that there are endophytic bacteria associated with species of the genus *Agave*, which promote plant growth. The bacteria that proliferated under *in vitro* conditions did not cause damage to the explants, which suggests that they were in symbiosis with the plants.

Seven months after applying the pretreatments, most of the explants had bacteria even when products such as alcohol, chlorine, Timsen®, hydrogen peroxide had been used, so it was decided to use Pursue®. With this procedure applied, an average of 65% of visually healthy explants were obtained (Figure 4). With those we started the process of acclimatization and *in vitro* multiplication of agave.

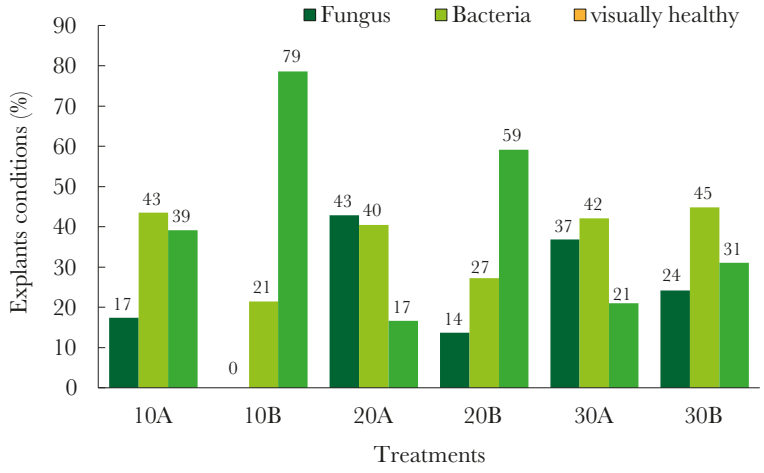


Figure 3. Percentage of condition of *in vitro* explants of *A. angustifolia*, treated with agrochemicals under *ex vitro* conditions. 10, 20, 30 days of exposure to chemicals; A: Actara; B: Benomyl.

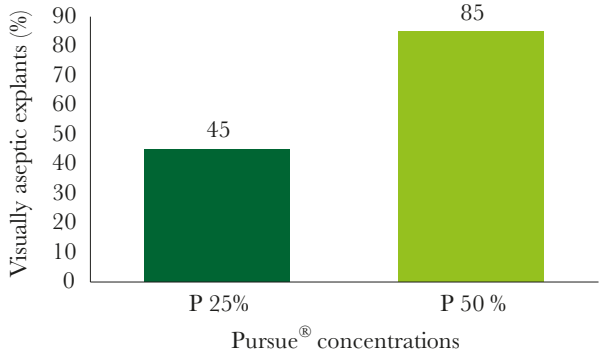


Figure 4. Percentage of visually aseptic explants of *A. angustifolia*, treated with Pursue®. Q: Pursue; n=20.

Alves-Pereira *et al.* (2014) mentioned that contamination by endogenous bacteria is common in *in vitro* culture; moreover, that the application of chlorine or alcohol is not sufficient. This makes it necessary to use antibiotics in the culture medium.

It is worth emphasizing that most *in vitro* studies do not mention how they addressed or solved the problems of contamination of the explants. In this study it was important to obtain visually healthy explants. That is why various products were used, until determining that with Pursue® at 50%, better results are obtained, in regard to the number of explants without visible contamination.

CONCLUSIONS

The use of fungicide and insecticide in agave plants, collected from the field prior to in vitro culture, is an alternative to reduce contamination of explants established in laboratory conditions. The concentration of Pursue® at 50% to disinfect explants with bacteria is satisfactory up to 85% of the treated explants.

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