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ARTICLE

Molecular Fingerprinting of Three *Ex-Situ* Cultivated Populations of *Acalypha gaumeri* Pax & K. Hoffm (Female and Male) and Evaluation of Their Antifungal Activity Against Phytopathogens

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ABSTRACT: *Acalypha gaumeri* (Euphorbiaceae) is the only endemic species of the genus in the Yucatan Peninsula. It is dioecious and has antifungal properties against various phytopathogens. In the present study, molecular identification of *A. gaumeri* was performed using the *rbcL* region, confirming its belonging to the *Acalypha genus*. Its genetic diversity was evaluated using 10 SPAR markers (ISSR and DAMD) from 60 individuals collected from female and male plants of the Kiuic, Tinum and Yaxcaba *ex-situ* populations. The results showed a high level of genetic polymorphism (PIC = 0.980) and significant differences among the populations. Ethanol and aqueous extracts from leaves, stems, and roots of both genders and three populations were evaluated against three phytopathogenic fungi. Only the ethanol extracts of the roots showed inhibitory antifungal activity, whereas Kiuic and Tinum, both male and female individuals, showed inhibitory effects at 1000 μg/mL against the three pathogens. The ethanol extract of the female flowering plant of Kiuic showed activity at minimum inhibitory concentrations of 250 μg/mL against *Alternaria chrysanthemi* CICY004 and 500 μg/mL against *Colletotrichum gloeosporioides* CICY002 and *Penicillium oxalicum* ITC25. Chromatographic profile of the ethanol extracts confirmed the presence of alkaloidal components in the *ex-situ* cultivated plants, which were analyzed by HPLC. The results revealed that the peaks at T_R of 7.60, 7.88, and 8.49 min were the most abundant components (9.95%–21.93%), with differences between female and male plants of the three populations. This research confirms that *A. gaumeri* cultivated and genetically characterized is a potential raw material to develop an eco-friendly product for the control of fungal diseases in crops.

KEYWORDS: *Acalypha gaumeri*; antifungal activity; alkaloidal compounds; eco-friendly crop protection; genetic diversity; phytopathogenic fungi

1 Introduction

Plant-pathogenic fungi cause significant economic losses [1]. Species of the *Colletotrichum* genus are considered to be among the "Top 10" in terms of their economic and scientific importance [2]. *Alternaria chrysanthemi*, for example, is responsible for losses in chrysanthemum species, which are the second most productive cut flower in Mexico [3,4], while *Penicillium oxalicum* causes stem rot in tomato plants and is a secondary pathogen of postharvest fruit of *Capsicum chinensis* [5]. Synthetic fungicides, such as mancozeb, chlorothalonil, and difenoconazole, are generally used to control phytopathogens [6]. These fungicides are effective to a certain extent, but they have adverse effects on human health and the environment. They also generate resistance to the pathogens [7]. Among viable alternatives to the safe replacement of synthetic fungicides are aqueous plant extracts. In a search for antifungal plant extracts to control phytopathogens, a botanical screening effort revealed that *Acalypha gaumeri* exhibits broad-spectrum antifungal activity.

In the Yucatan Peninsula, 14 species of *Acalypha* (Euphorbiaceae) have been documented. Among these, *A. gaumeri* Pax & K. Hoffm. is the sole species classified as an endemic, small and dioecious shrub [8,9]. Extracts of *A. gaumeri* have demonstrated insecticidal [10], nematicidal [11], and antifungal [12,13] activity on phytopathogens and parasites. Ethanol extracts (EEs) of *A. gaumeri* root inhibited mycelial growth (IMG) of *Alternaria tagetica* (ATTC 53771), *A. chrysanthemi* (CICY004), *Corynespora cassiicola* (ITC03), *Alternaria* sp. (ITC02), *Colletotrichum capsici* (CC4), *Colletotrichum gloeosporioides* (CG4), *Curvularia* sp. (ITC10) and *Helmintosporium* sp. (ITC04) [12,13]. Furthermore, the aqueous extracts of the root of *A. gaumeri* are effective in controlling leaf blight in chrysanthemum, which is caused by *A. chrysanthemi* [14]. Samples from three different populations of *A. gaumeri* collected in Oxkutzcab, Tinum and Yaxcaba (Yucatan state, Mexico) showed antifungal activity against *A. chrysanthemi* (CICY004), *C. gloeosporioides* (CG4) and *Pseudocercospora fijiensis* [15]. The three populations of plants with female flowers and male flowers are maintained in *ex-situ* propagation in the experimental nursery of Centro de Investigación Científica de Yucatán A. C. (CICY). Therefore, these *ex-situ* cultivars should be evaluated for biological efficacy and their spectrum of activity against other phytopathogenic fungi expanded.

The species *A. gaumeri* (Fig. 1) has been identified morphologically by botanical characteristics (Pax, F. and K. Hoffmann) and molecularly by amplification of the *ITS*, *ndhF*, and *trnL-F* regions [16]. Molecular studies are relevant for species identification and have been used to complement accurate and reliable characterization [17–19]. This is the first step in ensuring the origin of an extract's efficacy, quality and safety [20]. In recent years, single-primer amplification reaction (SPAR) methods have been used to detect genetic variation in plants, particularly in poorly studied species. This method includes targeted amplification of Deoxyribonucleic acid (DNA), Random amplification of polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and Directed amplification of minisatellite DNA (DAMD) [21]. Genetic diversity studies have been conducted on various members of the family Euphorbiaceae. For example, the genetic diversity of 35 sun poinsettia (*Euphorbia* spp.) genotypes, collected in Morelos, México, were examined using RAPD molecular markers [22]. *Sapium macrocarpum*, collected in three distinct locations within the medium-subtropical evergreen forest of the Selva, El Ocote Biosphere Reserve (REBISO) in Chiapas, Mexico, exhibited significant genetic diversity. However, minimal differentiation was observed among the localities [23].

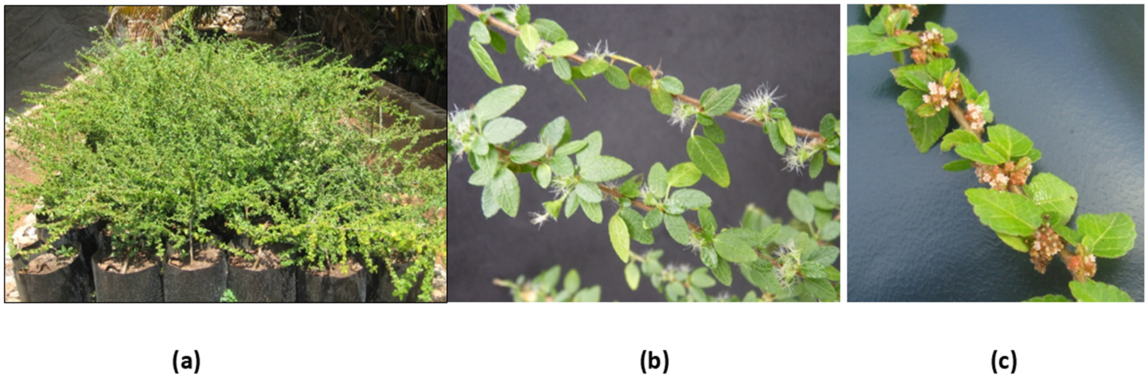

Figure 1: *Acalypha gaumeri* (**a**) Plants *ex-situ* cultivated, (**b**) female flowers and (**c**) male inflorescence.

This study contributes to ongoing research on *A. gaumeri*. The aims were the molecular identification of *A. gaumeri* collected in the state of Yucatan, the analysis of the genetic fingerprint of three Kiuic, Tinum and Yaxcaba populations (females and males) grown *ex-situ* using SPAR-type molecular markers and the evaluation of the antifungal activity of aqueous and ethanol extracts of the roots, stems and leaves against *A. chrysanthemi*, *Colletotrichum gloeosporioides* and *P. oxalicum*, and also the chromatographic profile of the ethanol extracts of these *ex-situ* plants cultivated.

2 Methodology

2.1 Plant Material

This study utilised *A. gaumeri* plants (both male and female) from the Kiuic (Municipality of Oxkutzcab), Tinum, and Yaxcaba populations. These were grown in pots from cuttings at the experimental nursery, Centro de Investigación Científica de Yucatán A. C.

Sixty samples of young, symptomless leaves, preferably from the basal part, were collected from 10 male and 10 female plants from each population in November 2022. These fresh samples (3 g) were washed and frozen at −80°C for molecular identification.

2.2 DNA Extraction

Genomic DNA was extracted from the samples that had been individually deep-frozen using the method established by Tapia-Tussell et al. [24]. DNA concentration (ng/μL) and purity (A260/A280) were measured using a spectrophotometer (Nanodrop 2000, Madrid, Spain). DNA integrity was determined by 0.8% agarose gel electrophoresis. Samples were diluted to 20 and 5 ng/μL concentration with sterile ultrapure water and stored at −20°C for subsequent use.

2.3 Molecular Identification of Acalypha gaumeri

Genomic DNA obtained from leaves of a female Yaxcaba (YF) plant with the primers *rbcL*-R and *rbcL*-F was amplified [25]. The amplicons were sent to Macrogen in South Korea for sequencing. The sequences obtained were edited using BioEdit software version 7.0.5.3 [26] and compared with the National Centre for Biotechnology Information GenBank database (http://blast.ncbi.nlm.gov) using the BLAST program.

A phylogenetic tree was constructed using the Neighbour-Joining method [27] with MEGA software version 11 to identify samples at the species level. An *in silico* analysis was conducted to compare the restriction profiles of the *A. gaumeri* sample under study with those of a sequence of *A. chamaedrifolia*. This analysis utilized a total of 60 restriction enzymes and was performed using the pDRAW32 program edition 1.1.147 [28].

2.4 Analysis with ISSR and DAMD Markers

The genetic diversity of the three *A. gaumeri* populations was determined using 10 SPAR markers. The markers comprised 5 ISSR and 5 DAMD loci [27,29]. The ISSR markers were IS01, IS21, IS14, IS17 and IS19. The DAMD markers were HBV, M13, HVA, 33.6 and HVR (Table 1).

The polymerase chain reaction (PCR) reactions for the ISSR primers included a final volume of 25 μL for each reaction, containing 10 ng of genomic DNA and 1× PCR buffer (Invitrogen, Carslbad, CA, USA), 2.5 mM of $MgCl_2$ (Invitrogen, Carslbad, CA, USA), 0.25 mM of dNTPs (Invitrogen, Carslbad, CA, USA), 1 μM primer and 1U of Taq DNA polymerase (Invitrogen, Carslbad, CA, USA). The amplification reactions were performed on a C1000 Touch (Bio-Rad, Hercules, California, USA). Amplification conditions included an initial denaturation at 94°C for 2 min, followed by 36 cycles of amplification, each at 94°C for 40 s, 54°C for 45 s, and 72°C for 1.5 min, and a final extension step at 72°C for seven min.

Table 1: List of SPAR markers used to obtain the genetic fingerprint of *Acalypha gaumeri* populations.

Type of Primer	Primer	Sequence
ISSR	IS01	5′-GAC AGA CAG ACA GAC A-3′
	IS21	5′-GTC GTC GTC GTC GTC GTC-3′
	IS14	5′-AGA GAG AGA GAG AGA GTA-3′
	IS17	5′-TGT GTG TGT GTG TGT GGT-3′
	IS19	5′-AGA GAG AGA GAG AGA GTC-3′
DAMD	HBV	5′-GGT GTA GAG AGG GGT-3′
	M13	5′-GAG GGT GGC GGT TCT-3′
	HVA	5′-AGG ATG GAA AGG AGG C-3′
	33.6	5′-AGG GCT GGA GG-3′
	HVR	5′-CCT CCT CCC TCC T-3′

The PCR reactions for the DAMD primers were performed using the methodology outlined by Verma and Rana (2013). The final volume of each reaction was 25 μL containing 60 ng of genomic DNA, 1× PCR buffer (Invitrogen, Carslbad, CA, USA), 2 mM of $MgCl_2$ (Invitrogen, Carslbad, CA, USA), 0.25 mM of DNTP's (Invitrogen, Carslbad, CA, USA), 0.2 μm primer and 1U de Taq DNA polymerase (Invitrogen, Carslbad, CA, USA). Amplification included an initial denaturation for 5 min at 92°C, followed by 40 cycles of amplification, each at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and a final extension step for seven min at 72°C.

The fragments obtained with ISSR and DAMD markers were separated at 6% on polyacrylamide gels in a 29:1 ratio (acrylamide: bisacrylamide) (Millipore-Sigma, St. Louis, MO, USA). The gels were run at 200 V for 2.5 h in 1× Tris-glycine (TG) buffer (Millipore-Sigma, St. Louis, MO, USA). The DNA band sizes were subsequently determined relative to the 1 Kb molecular marker (Invitrogen, Carlsbad, CA, USA). Gel staining was performed using the silver staining technique [30].

3 Preparation of Extracts

Roots of adult plants of *A. gaumeri* were collected in September at the CICY nursery after two years of culture. Each population (3) was separated by the sex of the flowers (female or male) and the type of plant tissue (leaf, stem, and root). Roots were washed with plenty of water, dried for 4 weeks in natural light at 40°C, and then crushed using a Pagani mill (Pagani-Dycomet, SA de CV, Mexico DF, Mexico) with a mesh number of 5. Finally, the samples obtained were extracted with water and ethanol (3 × 2 × 3× 2), resulting in 36 extracts.

3.1 Ethanol Extracts

The dried and ground plant material of each *A. gaumeri* sample (30 g) was macerated with ethanol (300 mL) at 24°C for 2 days (3 times). The solvent with the sample was filtered on paper (Whatman no. 1) and concentrated under reduced pressure in a rotary evaporator (IKA RV-10, Staufen, Germany) until the dry ethanol extract was obtained. The samples were stored under refrigerated conditions (4°C) until they were used.

3.2 Aqueous Extracts

The dried and ground plant material (1.5 g) was extracted with distilled water (20 mL), heated to boiling for 3 min, and then cooled to room temperature. The mixture was then filtered using cotton gauze and

Whatman no. 1 filter paper. The filtered volume was adjusted to 25 mL to obtain the aqueous extract at an initial concentration of 6% w/v. The aqueous extracts (AEs) were sterilized using a 0.22 μm Millipore filter (Merck-Millipore, Burlington, MA, USA), lyophilized, and stored frozen for evaluation.

4 Phytopathogenic Fungi

The phytopathogenic fungi (Table 2) used to evaluate antifungal activity were obtained from the Biotechnology Unit's stock at the CICY and correspond to fungal isolates collected from plants in the region. They were grown on potato dextrose agar (PDA) for 7 days. *A. chrysanthemi* (CICY004) was maintained at 18 ± 2°C in the dark, while *C. gloeosporioides* (CICY002) and *P. oxalicum* (ITC25) were maintained at 23 ± 2°C for seven days [13].

Table 2: Phytopathogenic strains tested for antifungal activity in *Acalypha gaumeri* extracts.

Strain	Key	GenBank Number	Host
Alternaria chrysanthemi	CICY004	MH846127.1	*Dendranthema grandiflorum*
Colletotrichum gloeosporioides	CICY002	HM562713.1	*Carica papaya*
Penicillium oxalicum	ITC25	KP868627	*Capsicum chinense*

4.1 Antifungal Assay by Microdilution

The spore suspension of *A. chrysanthemi* was prepared at 2.5 × 10^4 spores/mL, while those of *C. gloeosporioides* and *P. oxalicum* were prepared at 1 × 10^5 spores/mL from PDA medium.

The ethanol extracts were resuspended in a mixture of dimethyl sulfoxide (DMSO) and 0.5% Tween 20 at a concentration of 80 μg/μL (stock solution). The AEs were suspended with sterile distilled water at a concentration of 6% w/v (stock solution). A serial dilution was performed using the stock solution in Roswell Park Memorial Institute (RPMI) 1640 medium (1:1 v/v) to a final volume of 100 μL/per well. Subsequently, 100 μL of the spore suspension was added to each well, bringing the final volume to 200 μL. The final concentrations of the ethanol extracts were 2000, 1000, 500, 250, 125 and 62.5 μg/mL with 0.62% of 0.5%-DMSO-tween 20 solution. Concerning the aqueous extracts, each one (100 μL) was deposited in a microwell, after which the spore suspension (100 μL) was added at a final concentration of 3% w/v. The negative growth control was the blank, RPMI 1640 medium and spore suspension with 0.5% DMSO-tween 20 solution; and the positive control was the commercial fungicide prochloraz (CE45) at 0.11% (w/v) and 40% DMSO solution. All samples were replicated four times. As previously indicated, the microplates were incubated at different temperatures and photoperiod conditions. The assay was terminated at 96 h, and plate reading were performed at 24, 48, 72, and 96 h using the National Committee for Clinical Laboratory Standards [13,31] numerical scale. The percentages of mycelial growth inhibition (MGI) were determined using the Abbott formula [(%MGI in the negative control—%MGI in the treatment)/%MGI in the negative control] × 100 [32].

4.2 Fungicidal or Fungistatic Effect

Samples (10 μL) without fungal growth in the microwells were transferred to Petri dishes containing PDA medium. These samples were incubated under the same conditions previously outlined for each pathogen, and their progress was observed for 3 days. The effect of the extract was classified as fungicidal, indicated by the absence of mycelial growth, and fungistatic as evidenced by hyphal growth after several days to weeks of observation.

4.3 Data Analysis

The presence or absence of each PCR amplicon was determined by assigning a value of "1" for the presence of the fragment and a value of "0" for its absence. Binary data obtained from ISSR and DAMD profiles were used to construct a similarity matrix using Jaccard coefficients [33] and the Neighbour-Joining method to generate a dendrogram with the Sequential Agglomerative Hierarchical Nested Agglomerative Clustering (SAHN) module of the NTSYS pc software, version 2.21q [34].

Principal component analysis (PCoA) was performed to differentiate the *A. gaumeri* accessions. Polymorphic information content (PIC) values were also calculated using the equation developed by Anderson et al. [35].

One-way analysis of variance was performed on the percentage inhibition of mycelial growth data, with the original data first being transformed using the formula $y = \arcsin[\sqrt{(y/100)}]$. Treatment means were compared using the Tukey multiple range test ($p = 0.05$). Analyses of variance were performed using SAS version 9.4 for Windows.

4.4 Dragendorff Test

The presence of alkaloids in the ethanol extracts was detected using Dragendorff reagent with a butanol:acetic acid:water (7:1:2, v/v/v) eluent system, as previously reported by Vargas-Díaz et al. [15]. The orange spots detected on the plate were recorded as a frontal reference (F_R).

4.5 Liquid Chromatography Analysis

The liquid chromatograms of the ethanol extracts were obtained using an Agilent Technologies 1260 chromatograph coupled with a multichannel UV/Visible detector. The six ethanol extracts of the roots of *A. gaumeri ex-situ* cultivated and a sample collected in the field were analyzed. The samples were diluted to 1 mg/mL in HPLC-grade methanol and then filtered through a 0.22 μm Millipore filter. A 20 μL aliquot of each sample was injected into a Grace C18 column with an internal diameter of 4.6 mm, a length of 250 mm and particles measuring 5 μm in thickness. The mobile phase consisted of a mixture of solvents A: 30 mM of sodium acetate buffer (80 mL), acetonitrile (20 mL) and acetic acid (1 mL); and B: acetonitrile (45 mL), methanol (45 mL), water (10 mL), and acetic acid (0.25 mL). The method starts with A:B 40:60 (6 min), 0:100 (2 min), 40:60 (2 min), 0:100 (1 min). The retention times (R_t) of the peaks (min) were recorded at a wavelength of 280 nm, and the percentage of absorption area was calculated.

5 Results

5.1 DNA from Acalypha gaumeri

Leaf samples of *A. gaumeri* (60) were obtained from female and male plants of the Kiuic (Oxkutzcab), Tinum and Yaxcaba populations *ex-situ* cultivated at CICY. The genomic DNA concentration of each sample ranged from 142 to 1500 ng/μL, with a mean of 821 ng/μL. The DNA purity value (A260/A280 ratio) ranged from 1.74 to 2.03. Furthermore, a 330 base pairs (bp) PCR product was obtained for all samples when amplified from the 16S ribosomal region of the chloroplast, indicating that there were no PCR reaction inhibitors in these DNA samples.

5.2 Sequencing

The sequence obtained with the *rbcL* primers was compared with sequences deposited in NCBI, showing identities values of 98–100% with different species of the genus *Acalypha* (Table 3). In order to corroborate the identification of the sample under study, a phylogenetic tree was constructed using the neighbour-joining method (Fig. 2). The phylogenetic analysis unequivocally demonstrated that the *A. gaumeri* specimen is distinctly categorized within the clade corresponding to the genus *Acalypha*. However, the sequence obtained was not directly grouped with any of the other species analyzed; instead, it was positioned on an independent branch, which demonstrates its molecular differentiation from the other species of the genus. The final outcome of the sequence obtained from *A. gaumeri* was deposited in GenBank with the accession number PP819641.1.

Table 3: The accession number and maximum identity of the *Acalypha* species.

Specie	Accession	Percentage of Identity	Query Cover
A. gaumeri	PP819641.1	100	100
A. chamaedrifolia	MH549716.1	100	99

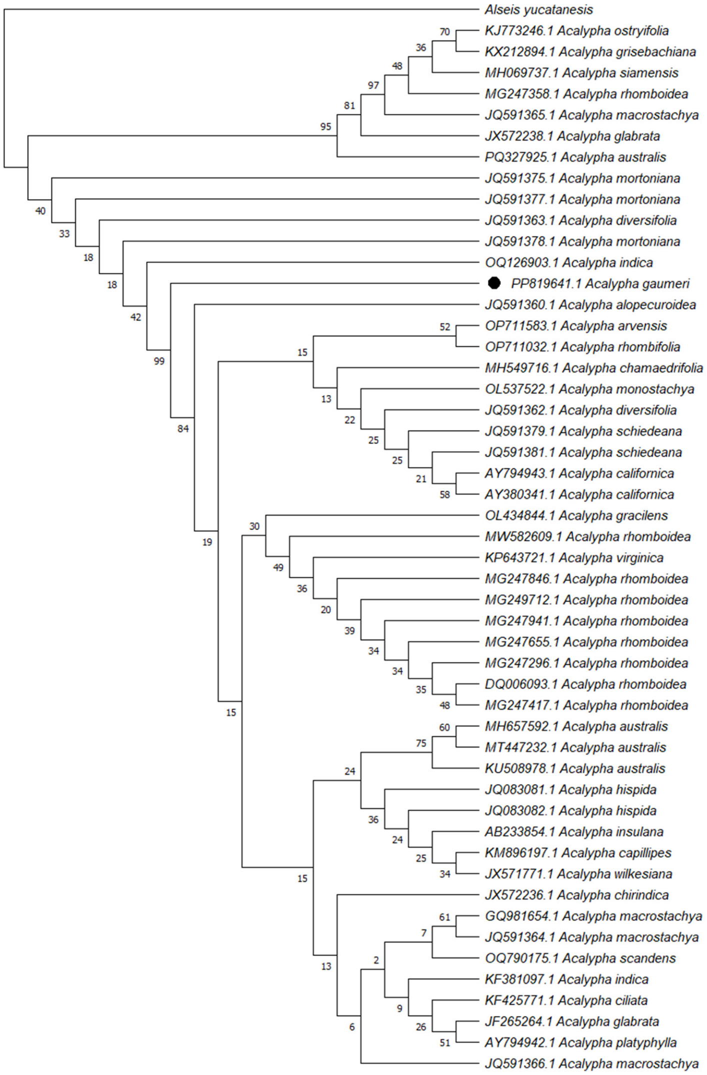

Figure 2: Phylogenetic analysis of *Acalypha gaumeri* was inferred using the neighbour-joining method with 1000 bootstrap replicates, performed in MEGA 11 with the external sequence of *Alseis yucatanensis*.

5.3 ISSR and DAMD Markers

Among the 10 microsatellite PCRs, several samples did not amplify with the ISSR 14 and ISSR 21 markers and were therefore discarded from the analysis. With the remaining three ISSRs and the five DAMDs, a PIC value of 0.980 was obtained, indicating a highly significant correlation. Tests with DAMD primers demonstrated an average polymorphism value of 0.984, higher than that of ISSR primers (0.975). The HVR (0.999) and 33.6 (0.995) markers provided the most polymorphic information in this study.

A dendrogram of the 60 samples under study was generated (Fig. 3) with data from the absence-presence matrices of the 10 primers processed (5 ISSR + 5 DAMD). The dendrogram analysis revealed two large clades (Fig. 3, clade 2 and 3) and one small clade (clade 1). The 60 samples were almost equally divided as follows: 4 samples were allocated to clade 1; 33 samples to clade 2; and 21 to clade 3. A substantial genetic discrepancy was identified between the samples. The most genetically similar accessions, as indicated by a similarity coefficient of 0.44, were TF5 and TF6 from the propagated plants of the Tinum population in clade 2c. Accessions YF3 and YF4, which exhibited a similarity coefficient of 0.34, are derived from the Yaxcaba population and are classified within subclade 3a (Fig. 3).

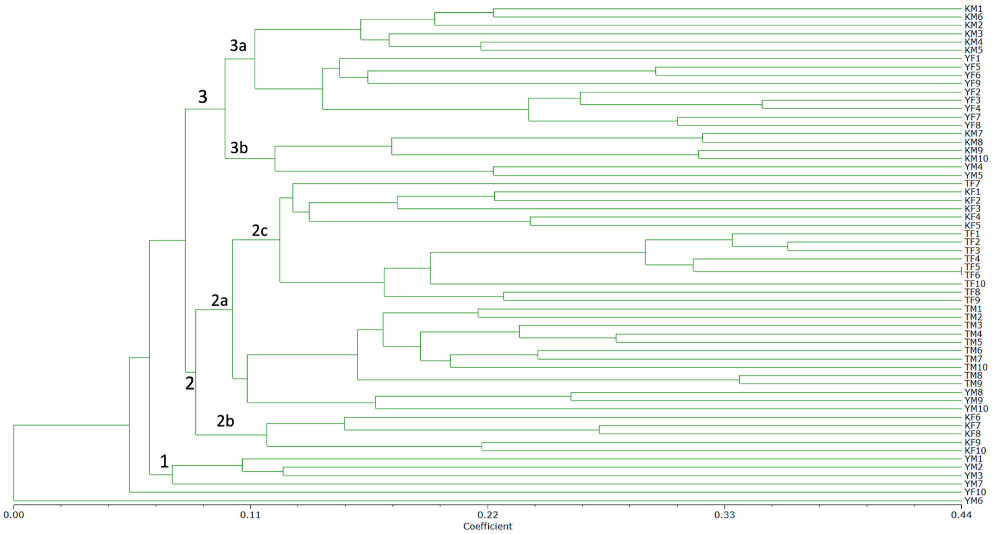

Figure 3: ISSR and DAMD consensus of the unweighted pair group arithmetic mean (UPGMA) dendrogram based on Jaccard's coefficient, showing relationships among 60 *Acalypha gaumeri* samples determined using cumulative data. The numbers 1, 2, and 3 are indicative of the primary clades, while each number accompanied by a letter (a, b, or c) signifies the subclades that are formed according to their respective groupings.

The second clade (subclades 2a and 2b) comprises ten samples of female Kiuic, ten samples of female and male Tinum, and three samples of male Yaxcaba. Clade 3 comprises ten samples from the male Kiuic population, eight samples from the female Yaxcaba population, and two samples from the male Yaxcaba population. Conversely, clade 1 comprises four samples from the male Yaxcaba population and a single sample from the female Yaxcaba population (Fig. 3). The majority of the samples classified within clade 2 exhibited geographical proximity to specimens from the Tinum population. In contrast, individuals from clade 3 exhibited increased dispersion, as demonstrated by specimens from the Kiuic and Yaxcaba populations.

In the two-dimensional representation of the data (Fig. 4A), all clusters demonstrate a clear separation between clades. However, the three-dimensional graph provides a more detailed illustration of this separation between clades (Fig. 4B).

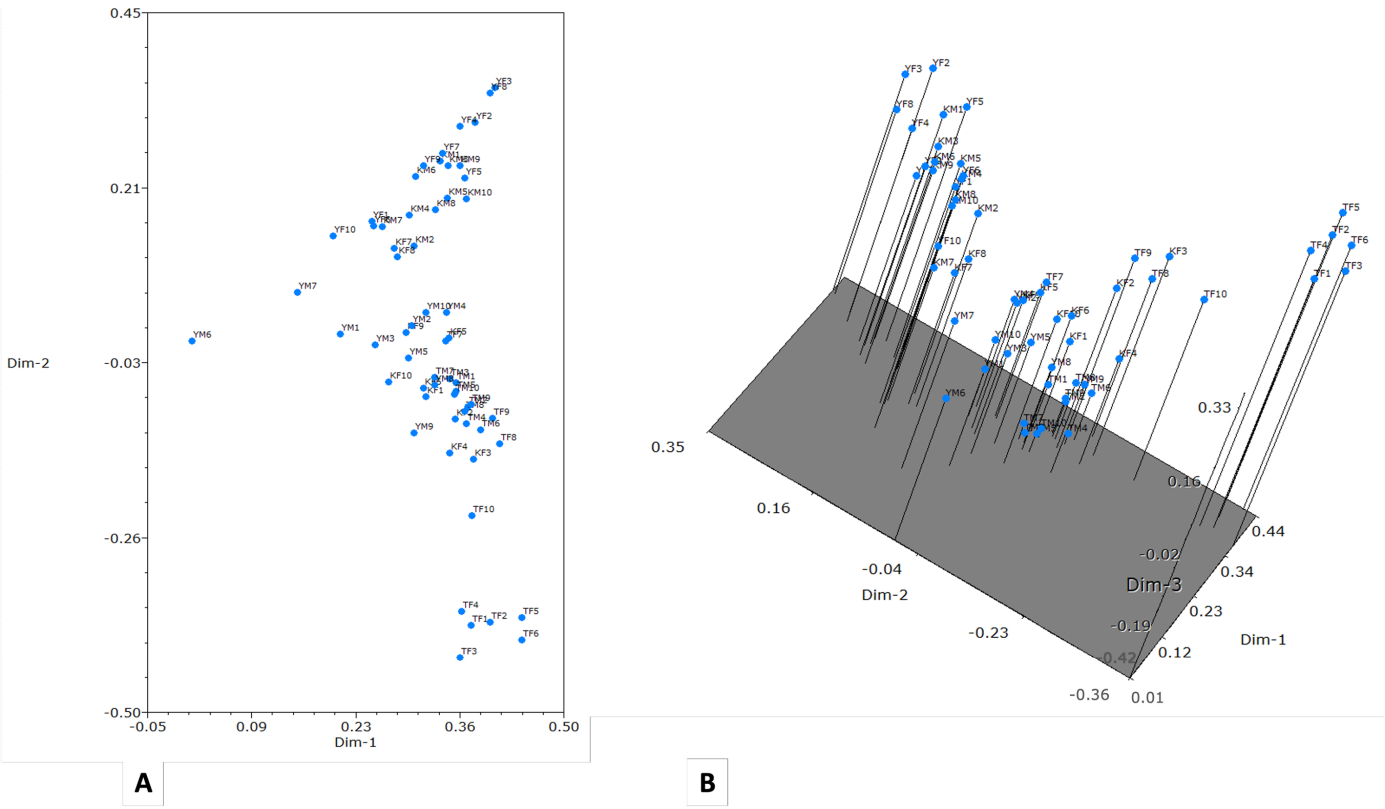

Figure 4: Principal component analysis obtained with NTSYS software, version 2.21q. (**A**) Two-dimensional plot; (**B**) Three-dimensional plot.

5.4 Extraction Yield of Dried and Ground Roots

The yield of the extracts is shown in Table 4. In general, the leaves yielded the highest ethanol extract, ranging from 9.27% to 16.61%. The stems yielded between 1.79% and 3.74%, while the roots yielded between 1.88% and 2.49%. The highest yield was obtained from the leaves of the Kiuic female population, at 16.61%.

Conversely, the aqueous extracts from the leaves of *A. gaumeri* across the three populations had higher solids yields after freeze-drying, ranging from 15.83% to 25.8%, compared to the stem (0.13% to 3.23%) or roots (0.56% to 11.26%). The highest yield was found in the female Kiuic leaves (25.53%), while the lowest yield was found in the male leaves of the same population (12.4% solids) (Table 4).

Table 4: Percentage yield of the ethanol and aqueous extracts from three populations of *Acalypha gaumeri.*

Population	Plant Tissue	Flower Sex	Key	Yield EE (%)	Yield Lyophilized AE (%)
Kiuic	L	F	KLF	16.61	25.53
		M	KLM	11.79	12.4
	S	F	KSF	3.12	0.13
		M	KSM	3.74	3.2
	R	F	KRF	2.01	0.56
		M	KRM	2.31	2.36
Tinum	L	F	TLF	11.52	25.8
		M	TLM	10.66	18.03
	S	F	TSF	1.79	2.43
		M	TSM	3.00	3.23
	R	F	TRF	2.35	2.96
		M	TRM	2.13	0.83
Yaxcaba	L	F	YLF	9.73	15.83
		M	YLM	9.27	19.56
	S	F	YSF	2.81	2.4
		M	YSM	2.81	1.83
	R	F	YRF	2.49	3.23
		M	YRM	1.88	11.26

L: Leaves, S: Stem, R: root; F: Female flower, M: Male inflorescence; EE: Ethanol extract; AE: Aqueous extract.

5.5 Antifungal Activity of Acalypha gaumeri Extracts

The antifungal activity of the 36 extracts (ethanol and aqueous) of leaves, stems, and roots of *A. gaumeri*, male and female specimens from three populations cultivated in *ex-situ* conditions, against *A. chrysanthemi, C. gloeosporioides* and *P. oxalicum* showed few differences between flower sex, but high differences between populations were found (Table 5). All ethanol extracts obtained from the roots of the three populations of *A. gaumeri* exhibited inhibitory effects against the three phytopathogens at 2000 μg/mL. However, only the ethanol extracts of female plant stems from Tinum (TSF; MGI = 50%), Yaxcaba (YSF), and plant leaves (YLF) exhibited a slight (MGI = 25%–50%) antifungal effect on at least one of the phytopathogens tested.

There are no aqueous extracts from *A. gaumeri* with activity against the three fungal pathogens evaluated at 3% w/v (Table 5). Therefore, the minimum inhibitory concentration (MIC) was determined using serial dilutions of the six ethanol extracts that were active against each evaluated fungus (Tables 6–8).

Table 5: Percentage of mycelial growth inhibition (MGI) of ethanol (2000 μg/mL) and aqueous (3% w/v) extracts from *Acalypha gaumeri* (leaves, stem, and roots) of three populations *ex-situ* cultivated against three fungal phytopathogens.

Population/Controls	**Plant Tissue**	**Flower Sex**	**Key/Control**	**Mycelial Growth Inhibition (%)**					
				Alternaria chrysanthemi		***Colletotrichum gloeosporioides***		***Penicillium oxalicum***	
				EE	**AE**	**EE**	**AE**	**EE**	**AE**
Kiuic	L	F	KLF	0	0	0	0	0	0
		M	KLM	0	0	0	0	0	0
	S	F	KSF	0	0	0	0	0	0
		M	KSM	0	0	0	0	0	0
	R	F	KRF	100	0	100	0	100	0
		M	KRM	100	0	100	0	100	0
Tinum	L	F	TLF	0	0	0	0	0	0
		M	TLM	0	0	0	0	0	0
	S	F	TSF	0	0	50	0	0	0
		M	TSM	0	0	0	0	0	0
	R	F	TRF	100	0	100	0	100	0
		M	TRM	100	0	100	0	100	0
Yaxcaba	L	F	YLF	25	0	0	0	0	0
		M	YLM	0	0	0	0	0	0
	S	F	YSF	25	0	0	0	0	0
		M	YSM	0	0	0	0	0	0
	R	F	YRF	100	0	100	0	100	0
		M	YRM	100	0	100	0	100	0
			NC	0		0	0	0	0
			SS	0		0	0	0	0
			Blank	0		0	0	0	0
			PC	100		100	100	100	100

L: Leaves, S: Stem, R: root; F: Female flower, M: Male inflorescence; EE: Ethanol extract; AE: Aqueous extract, NC: RPMI 1640: Roswell Park Memorial Institute Medium; SS: Spore suspension, Blank: Medium + SS; PC: prochloraz 0.11% (w/v).

5.5.1 Alternaria chrysanthemi

The antifungal effects of the six ethanol extracts were inhibitory at 2000 and 1000 μg/mL (Table 6). These same extracts showed a statistically significant difference in the percentage of MGI at 500, 250, and 125 μg/mL against *A. chrysanthemi*, with MGI values ranging from 0% to 100%. The lowest MIC, at 250 μg/mL, was observed with the KRF and TRM ethanol extracts, indicating a fungicidal effect. Followed by KRM and TRF with MIC values of 500 μg/mL and fungistatic effect (Table 6).

Table 6: Minimum inhibitory concentration (MIC) of ethanol extracts from the roots of female and male *Acalypha gaumeri* plants from three populations *ex-situ* cultivated against *Alternaria chrysanthemi*.

Population	**Sample**	***Alternaria chrysanthemi* (% MGI) Ethanol Extract Concentration (μg/mL)**						**MIC (μg/mL)**
		2000	**1000**	**500**	**250**	**125**	**62.5**	
Kiuic	KRF	100a	100a	100a	100a	50b	0b	250++
	KRM	100a	100a	100a	50c	0d	0b	500+
Tinum	TRF	100a	100a	100a	75b	25c	0b	500+
	TRM	100a	100a	100a	100a	50b	0b	250++
Yaxcaba	YRF	100a	100a	75b	50c	0d	0b	1000+
	YRM	100a	100a	75b	25d	0d	0b	1000+
	NC	0b	0b	0c	0e	0d	0b	
	SS	0b	0b	0c	0e	0d	0b	
	Blank	0b	0b	0c	0e	0d	0b	
	PC	100a	100a	100a	100a	100a	100a	

MGI: Mycelium growth inhibition; F: Female M: Male inflorescence; NC: RPMI 1640: Roswell Park Memorial Institute Medium; SS: Spore suspension, Blank: Medium + SS; PC: prochloraz 0.11% (w/v); (++): fungicidal; (+): fungistatic. a, b, c, d, e: Means with different letters within columns differ significantly (Tukey's test, $p < 0.05$).

5.5.2 Colletotrichum gloeosporioides

The six ethanol extracts were inhibitory at concentrations of 2000, 1000, and 500 μg/mL, except for KRM, which had MGI values of 75% on *C. gloeosporioides* (Table 7). At 250 μg/mL, the six ethanol extracts showed statistically significant differences in their MGI percentages, ranging from 0% to 100%. Therefore, KRF, TRF, and TRM ethanol extracts showed the lowest MICs of 500 μg/mL against *C. gloeosporioides*, exhibiting a fungicidal effect (Table 7).

Table 7: Minimum inhibitory concentration (MIC) of ethanol extracts from the roots of female and male *Acalypha gaumeri* plants from three populations *ex-situ* cultivated against *Colletotrichum gloeosporioides*.

Population	**Sample**	***Colletotrichum gloeosporioides* (% MGI) Ethanol Extract Concentration (μg/mL)**						**MIC (μg/mL)**
		2000	**1000**	**500**	**250**	**125**	**62.5**	
Kiuic	KRF	100a	100a	100a	50c	0b	0b	500++
	KRM	100a	100a	75b	0e	0b	0b	1000+
Tinum	TRF	100a	100a	100a	75b	0b	0b	500++
	TRM	100a	100a	100a	0e	0b	0b	500++
Yaxcaba	YRF	100a	100a	0c	0e	0b	0b	1000+
	YRM	100a	100a	0c	25d	0b	0b	1000+
	NC	0b	0b	0c	0e	0b	0b	
	SS	0b	0b	0c	0e	0b	0b	
	Blank	0b	0b	0c	0e	0b	0b	
	PC	100a	100a	100a	100a	100a	100a	

MGI: Mycelium growth inhibition; F: Female M: Male inflorescence; NC: RPMI 1640: Roswell Park Memorial Institute Medium; SS: Spore suspension, Blank: Medium + SS; PC: prochloraz 0.11% (w/v); (++): fungicidal; (+): fungistatic. a, b, c, d, e: Means with different letters within columns differ significantly (Tukey's test, $p < 0.05$).

5.5.3 Penicillium oxalicum

The six ethanol extracts of *A. gaumeri* from the three populations exhibited inhibitory effects on *P. oxalicum* at 2000 and 1000 μg/mL. The serial dilution at 500–62.5 μg/mL indicated statistically significant differences in the MGI for the pathogenic fungi. The KRF extract was the only sample to exhibit the lowest MIC (500 μg/mL) against *P. oxalicum* and a fungicidal effect. In addition, only RKF induced MGI of 75% at 250 μg/mL (Table 8).

The ethanol extract of KRF demonstrated the capacity to inhibit the growth of the three test fungal pathogens, with MIC values ranging from 250 to 500 μg/mL. This extract exhibits a fungicidal effect against the three phytopathogens (Table 8).

Table 8: Minimum inhibitory concentration (MIC) of the ethanol extracts (EE) from the roots of female and male *Acalypha gaumeri* plants from three populations *ex-situ* cultivated against *Penicillium oxalicum*.

Population	Sample	***Penicillium oxalicum*** **(% MGI)** **Ethanol Extract Concentration (μg/mL)**						MIC (μg/mL)
		2000	**1000**	**500**	**250**	**125**	**62.5**	
Kiuic	KRF	100a	100a	100a	75b	50b	25b	500++
	KRM	100a	100a	75b	50c	25c	0c	1000+
Tinum	TRF	100a	100a	75b	50c	25c	0c	1000+
	TRM	100a	100a	50c	25d	0d	0c	1000+
Yaxcaba	YRF	100a	100a	75b	50c	25c	0c	1000+
	YRM	100a	100a	75b	50c	25c	0c	1000+
	NC	0b	0b	0d	0e	0d	0c	
	SS	0b	0b	0d	0e	0d	0c	
	Blank	0b	0b	0d	0e	0d	0c	
	PC	100a	100a	100a	100a	100a	100a	

MGI: Mycelium growth inhibition; F: Female M: Male inflorescence; NC: RPMI 1640: Roswell Park Memorial Institute Medium; SS: Spore suspension, Blank: Medium + SS; PC: prochloraz 0.11% (w/v); (++): fungicidal; (+): fungistatic. a, b, c, d, e: Means with different letters within columns differ significantly (Tukey's test, $p < 0.05$).

5.6 Chromatographic Profile

All ethanol extracts tested positive in the Dragendorff test, producing several orange spots at different F_R values of 0.27, 0.39, 0.47, 0.54 and 0.59.

The ethanol extracts from the roots of *A. gaumeri ex-situ* cultivated showed two to four peaks each with percentages of at least 8% (Table 9). The plants from Kiuic and Tinum exhibited a similar chromatographic profile, displaying four peaks (R_t of 4.5, 4.84, 6.5, and 7.88 min) in female plants and three (R_t of 4.5, 4.84, and 8.49 min) in male plants. The population of Yaxcaba exhibited a different chromatographic profile: with two peaks (R_t of 4.5 and 8.49 min) in female plants and three peaks (R_t of 4.3, 4.5, and 7.6 min) in male plants. The most abundant peak corresponded to the least polar compound with a T_R of 8.49 in KRM (21.93%) and YRF (18.40%) min. The field sample from Yaxcaba revealed three prominent peaks (R_t of 4.8, 5.91 and 6.75 min) with 11.09–15.60% of area.

Table 9: Percentage of component area of the components in the liquid chromatograms of the ethanol extracts obtained from the *ex-situ* cultivated roots of *Acalypha gaumeri* from the Kiuic, Tinum and Yaxcaba populations.

Population	**Sample**	**Retention Time (min)**								
		4.30	**4.50**	**4.84**	**5.81**	**6.50**	**6.72**	**7.60**	**7.88**	**8.49**
Kiuic	KRF		14.00			16.81			16.49	
	KRM		7.12	7.76						21.93

Table 9: *Cont.*

Population	Sample	Retention Time (min)								
		4.30	4.50	4.84	5.81	6.50	6.72	7.60	7.88	8.49
Tinum	TRF		15.63			10.29			15.16	
	TRM		7.57	10.05						9.95
Yaxcaba	YRF		11.01							18.40
	YRM	6.99	7.00					17.24		
Yaxcaba field				11.09	15.60		12.92			

F: Female, M: Male.

6 Discussion

This research contributes to the comprehensive knowledge of *A. gaumeri.* Therefore, the first part of the work focused on molecular identification and characterization of the populations from Kiuic, Tinum, and Yaxcaba, which were maintained in our nurseries.

In Mexico, the *Acalypha* genus includes 108 species, of which 61% are catalogued as endemic to the country. Among these, *A. gaumeri* is currently the only species endemic to the Yucatan Peninsula and is classified as "near threatened" [8,9]. This research employed a dual approach to identifying *A. gaumeri*, integrating morphological characteristics with molecular identification from genomic material. The specimens used in this study were collected and propagated in the Yucatan Peninsula. Previous deposits of *A. gaumeri* in GenBank include sequences obtained with *ITS* (OM140897), *ndh-F* (OM066409) and *trnL-F* (OM066565) primers, using herbarium specimens [16]. In our study, *ITS* and *matK* primers were used with fresh leaf material from *A. gaumeri* without success. Therefore, this is the first report of the *A. gaumeri* sequence deposited in GenBank using the *rbcL* primer. The *rbcL* gene has been the most thoroughly characterized and is considered to be one of the most reliable and useful for molecular identification studies [36]. The results of the phylogenetic analysis demonstrate the separation of *A. gaumeri* from the other sequences analyzed, with a high bootstrap support value (99%), indicating a genetic difference between the species. In this study, the use of the *rbcL* primer is demonstrated to be an effective method for molecular species identification. In this context, it has been confirmed that the *rbcL* primer is suitable for molecular identification of *A. gaumeri*, as other alternative markers have limitations in terms of amplification. Consequently, it is recommended that subsequent studies evaluate additional markers, such as *trnL-F* and *ndh-F*, to consolidate molecular identification. This finding is consistent with previous reports indicating that one of the main regions of the plant DNA barcode is *rbcL*. When used in conjunction with complementary regions, it facilitates accurate identification in taxonomic studies [37].

As part of this study, we present the first report on genetic diversity in the *ex-situ* cultivated Kiuic, Tinum, and Yaxcaba populations of *A. gaumeri.* This documentation used ISSR and DAMD markers and revealed a high degree of polymorphism. Previously, a high degree of polymorphism was reported in genetic diversity studies of 54 varieties of *Ricinus communis* (Euphorbiaceae), with an average of 74.59% using ISSR markers [38]. On the other hand, DAMD markers showed greater polymorphism than ISSR markers. These data are consistent with the report on *Jatropha curcas*, in which DAMD and ISSR markers displayed PIC values of 0.872 and 0.86, respectively [39]. Furthermore, this report indicates that markers 33.6 and HVR had the highest PIC values (0.914 and 0.898, respectively) [39], consistent with those obtained for *A. gaumeri.* The findings of this molecular marker study showed that individuals within populations possess a high degree of genetic diversity. This behaviour is generally associated with geographic distribution, reproductive mode, fecundity, and seed dispersal [39]. Another critical factor influencing genetic diversity within *ex-situ* cultivated populations is the genetic variation present in the parental populations [40].

However, this study did not directly compare populations with their parental populations. Given that the individuals analyzed in each population descended from wild parents, it can be postulated that the genetic variability present *in situ* was maintained *ex situ.* This hypothesis is supported by the dendrogram, which shows genetic differentiation between populations from different locations and within populations, as evidenced by differences between male and female individuals. These results on genetic variability indicate that, despite *ex-situ* propagation, these populations maintain their genetic differences. Growers should take this information when selecting individuals for reproduction to produce antifungal extracts.

Among the 36 ethanol and aqueous extracts, 22% showed antifungal activity against the tested plant pathogens. The active extracts included ethanol extracts from roots (KRF, KRM, TRF, TRM, YRM, YRF) against the three phytopathogens, which were inhibitory at 1000 µg/mL. The most active ethanol extracts, as indicated by the lowest MIC, were from *A. gaumeri* roots collected at Kiuic from female plants (KRF) against *A. chrysanthemi*, *C. gloeosporioides* and *P. oxalicum* at 250 µg/mL, 500 µg/mL and 500 µg/mL, respectively. This is the first study of the antifungal activity of *A. gaumeri* cultivated outside its natural environment, confirming that the roots of cultivated plants continue to inhibit the mycelial growth of *A. chrystanthemi* and *C. gloeosporioides* when evaluated using the microdilution bioassay. In addition, separate evaluations of male and female specimens are provided. The ethanol extracts were found to be more efficacious than those reported from field-collected plant extracts, with MIC values of 500 µg/mL and 1000 µg/mL against *A. chrysanthemi* CICY004 and *C. gloeosporioides* CG4, respectively [15].

According to previous reports, ethanol root extract of *A. gaumeri* at 1 mg/mL induced 68%–97% IGM on *A. chrysanthemi* (CICY004), *Alternaria tagetica* (ATTC 53771), *Alternaria* sp. (ITC02), *Colletotrichum capsici* (CC2), *C. gloeosporioides* (GC4), *C. cassiicola* (ITC03), *Curvularia* sp. (ITC10) and *Helminthosporium* sp. (ITC04) using the agar dilution assay [12,13]. Also tested on *C. gloeosporioides* (CICY002) and *Fusarium oxysporum* (CICY003) without activity [12]. The spectrum of antifungal activity of *A. gaumeri* was enriched by the effect of root ethanol extracts against *P. oxalicum* ITC25 isolated from habanero pepper (*Capsicum chinenese*).

The aqueous extracts from *A. gaumeri* did not inhibit *A. chrysanthemi* CICY004, *C. gloeosporioides* CICY002 and *P. oxalicum* ITC25 at 3% w/v. Other studies conducted with aqueous extracts from *A. gaumeri* roots showed no effect on the MGI against *A. alternata* ITC24, *Corynespora cassiicola* ITC23, *Curvularia lunata* ITC22, and *F. equiseti* ITC32. However, these completely inhibited the sporulation of *C. lunata* ITC22 and the conidial germination of both *C. lunata* ITC22 and *F. equiseti* ITC32. In addition, aqueous extracts from *A. gaumeri* roots exhibited 69% growth inhibition on *A. chrysanthemi* CICY004 using 3% w/v microdilution assay [13]. Plant material was collected in the field (Yaxcaba), and the agar dilution assay was used [41]. In addition, weekly application of aqueous extracts (3%) to chrysanthemum plants for three weeks under field conditions reduced the severity of leaf blight disease, as did the fungicide Captan® [13].

The chromatographic profile of ethanol extracts from *A. gaumeri* reveals differences among populations and genera, as well as between field-collected and *ex-situ* cultivated plants. Field and nursery extracts showed the presence of two major alkaloidal components, as determined by the Dragendorff test in thin-layer chromatography [15] and by HPLC at 280 nm. But the difference in their retention time is due to structural differences. Only one peak was found in all samples at R_t of 4.5 min (7–15.63%), while peaks at R_t of 5.91 min (15.60%) and 6.75 min (12.92%) were only detected in the field sample. Among the female populations of Kiuic and Tinum, KRF was the most active extract, showing higher peak areas at R_t of 6.5 (16.81%) and 7.88 (16.49%) min than other extracts. However, among the male populations, the most active was TRM exhibited higher peak abundances at R_t 4.5 min (7.57%) and 4.84 min (10.05%). This is consistent with the activity observed in the roots of *A. gaumeri* from the Tinum population, which were collected in the field and showed MIC values of 500 and 1000 μg/mL against *A. chrysanthemi* and *C. gloeosporioides* [15].

It was also found that female individuals from Kiuic, belonging to clade 2, as well as male and female individuals from Tinum, were more active and had the lowest MIC. According to Wang et al. [42], these results may correlate with antifungal activity against the tested phytopathogenic fungi. Genetic variability within a species results in differences in the kinds and amounts of secondary metabolites, as evidenced by chromatographic profile and biological effects.

The demonstrated antifungal activity of *A. gaumeri ex-situ* propagated, and its complementary molecular identification are promising biorational product options. This finding highlights the need for ongoing evaluations against other phytopathogens. The chemical identification of alkaloid compounds in field-collected plants is being carried out to verify their activity and toxicity. Additionally, the purification and identification of the compounds detected in *ex-situ* cultivated plants must be carried out. Seasonal monitoring of activity and chemical profile is also required.

7 Conclusions

The molecular identification of *A. gaumeri* from Yucatan using the *rbcL* primer complements its taxonomy. The *A. gaumeri* plants sampled from the Kiuic (Oxkutzcab), Tinum, and Yaxcaba, populations in Yucatan showed a higher genetic diversity than expected. The genetic fingerprints obtained for female individuals in the Kiuic population showed slight differences compared with those from the Tinum and Yaxcaba populations. The molecular markers ISSR and DAMD were useful for detecting polymorphisms within and between individuals and populations. Antifungal activity is maintained in specimens propagated and cultured outside their environment. The activity of *A. gaumeri* varies depending on the sex, organ, and population. The root is the main plant tissue of *A. gaumeri* that inhibits the growth of phytopathogenic fungi. The spectrum of antifungal activity of *A. gaumeri* is broadened by the sensitivity of *P. oxalicum*. The differences in the genetic fingerprint of the female population in Kiuic could be related to its significant biological activity and chemical profile. This information will enable important decisions on the selection of materials for further study, ensuring the sustainability of the raw materials used to produce botanical extracts.

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