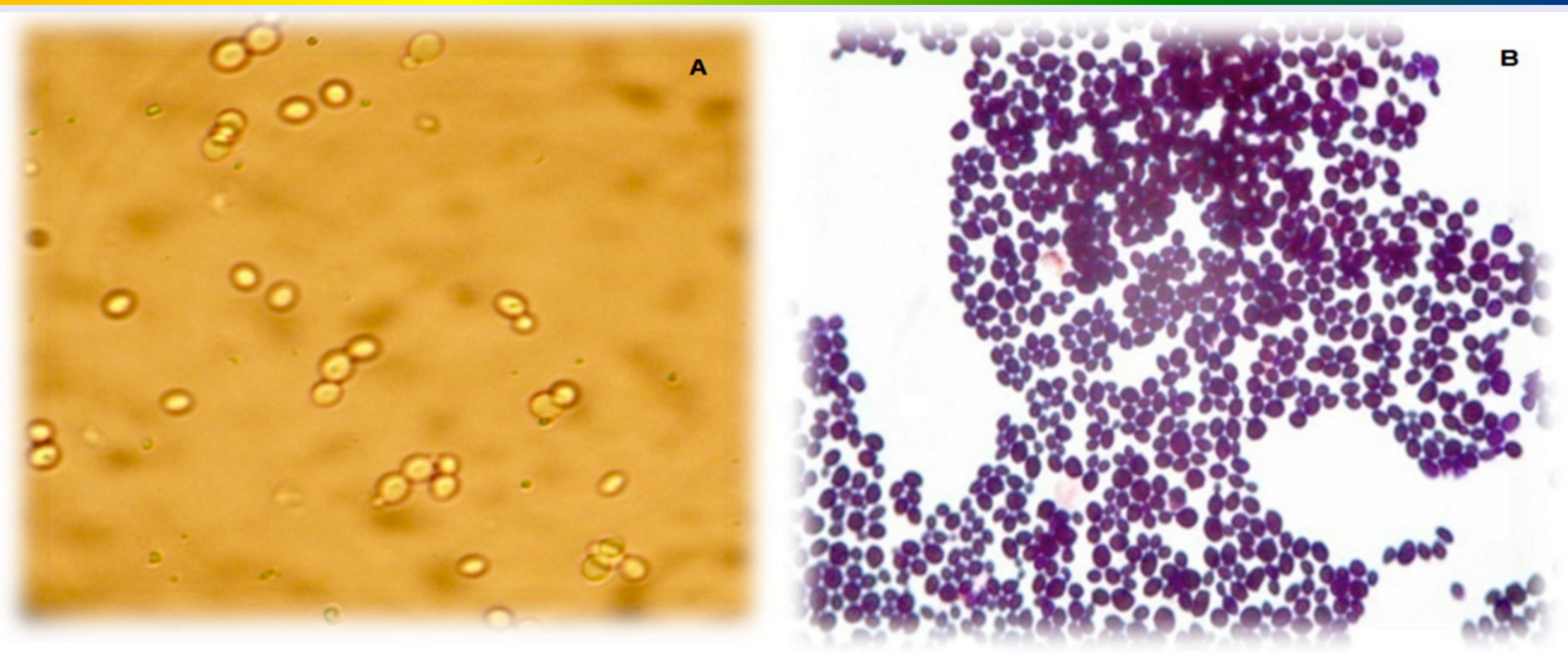
Number 2 Issue 2 June 2012

International Biotechnology Color Journal

A Scientific Peer Reviewed Journal with Focus on BIOTECHNOLOGY
and Covering Its Many Hues, Tints, Tones & Shades



Regular issue:
Prevalence of *Candida* spp. in women at Chiapas
2012.

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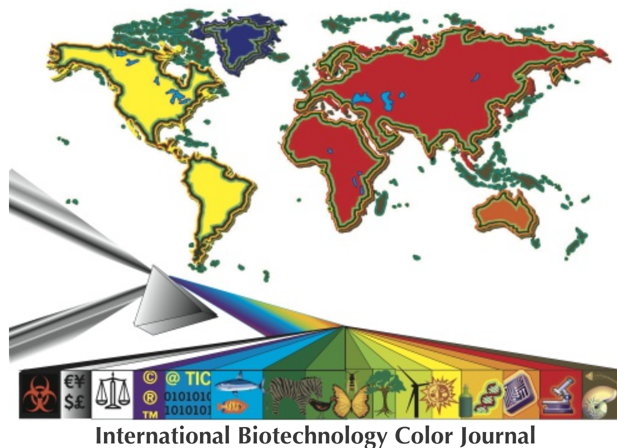

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Editorial section

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If you are interested in participating as reviewer, please send us a letter, and a CV stressing your experience in the filed. Send this information by e-mail to "Dr. Jose Juan Zúñiga Aguilar" <zuniga@cicy.mx>

Editorial comments to the contents of this issue

By José Juan Zúñiga-Aguilar, Chief editor.

In the first report, Monjaraz-Gutiérrez *et al*. developed an integral study, which included clinical and molecular tests to identify the etiological agent of vaginitis, a disease that constitutes a public health problem with stronger impact into the low-income stratus of the southern Mexico. In contrast to the traditional reports based on clinical inspection, this study identified *Candidus glabrata* instead of *C. albicans* as the predominant pathogen found in the affected population; and found C. glabrata to be prevalent in asymptomatic women. These are fundamental elements to help the government in the design of better drug treatments and to establish suitable programs for the eradication of this endemic disease.

In the second contribution, Hernández-Domínguez and Rodríguez-Sotres developed an interesting studio, which reports the first description of the inorganic pyrophosphatases in *Taxodium mucronatum*, a tree that is particularly important in Mexico. Even though, inorganic pyrophosphatases are ubiquitous key elements in the coordination of the anabolic activity with the developmental growth in all organisms, they are poorly characterized in trees. In their contribution, authors reported the characterization of two PPases activities from *Taxodium mucronatum*, under two different physiological conditions where the production of ATP might be either highly demanded or compromised. Their results support the proposal of an important role of these enzymes in the plant development and the adaptation to stress in conifers. The purification of one of these PPases, which they have initiated, should provide an important tool to understand the biochemical and physiological roles of these enzymes in plants.

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A molecular epidemiological study of prevalence of *Candida* spp. in women in the City of Tuxtla Gutierrez, Chiapas.

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ABSTRACT

Vaginitis caused by *Candida spp*., is a frequent fungal infection of epidemiological significance that affects particularly to women of childbearing age. Historically vaginits has been associated with C. albicans; nevertheless an important increase in incidence of other species such as *C. glabrata*, *C. parapsilosis* and *C. krusei* has been recently reported. Our main objective was to characterize diferentially through biochemical, microbiological and molecular assays four species of Candida in order to determine its prevalence in Tuxtla Gutiérrez region. Samples were obtained from vaginal exudates of 167 women of 15 to 45 years residing in Tuxtla Gutierrez from August 2007 to October 2008. The species identification was performed through chromogenic medium and induction of fungal germ tube formation. The genus identification was done by PCR analysis using oligonucleotide primers (5'– AAGTATTTGGGAGAAGGGAAAGGG–3' and 5'– AAAATGGGCATTAAGGAAAAGAGC –3'), which were designed based on an intron of a ribosomal gene of *Candida albicans*. The prevalence of vaginal candidiasis in the women population studied was 21%. The isolated species were: *C. glabrata* (13 %, n = 21), *C. albicans* (6 %, n = 10), *C. krusei* (1 %, n = 2) and *C. parapsilosis* (1 %, n = 2). The symptoms associated with vaginal candidiasis are caused by four different species; thus, the identification of species in the diagnosis of candidiasis with antifungal susceptibility testing is crucial for effective treatment. The PCR amplification produced bands of 100–350 bp in different species of *Candida*. Changes in vaginal ecology caused by candidiasis were observed in 43 % of clinical isolates. A written test was applied to the population under study to find out the relation between clinical, demographic and socioeconomic factors. According to the results, most of the candidiasis cases were found in asymptomatic women. There is a higher prevalence of *C. glabrata* (12.6 %) than other species, and the disease incidence is associated to low–income women with active sexual life.

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Abbreviations: VC, Vaginal candidasis; ENCB-IPN, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional; YPD, yeast extract-peptone-dextrose; SPSS: Statistical Package for the Social Sciences; OR: odds ratio; UW: Unpaid works; OUW: other unpaid works; SAH: Systemic Arterial Hyepertension; DM Diabetes mellitus; ID, immune disease; CVE, cervicovaginal erythema; AWP, Adherent withish plaques; STI, sexually-transmitted infections; S, sensibility; E, specificity; PV, predictive value; INT1, primer 5' -AAGTATTTGGGAGAAGGGAAAGGG-3'; INT2, primer 5' -AAAATGGGCATTAAGGAAAAGAGC-3'; CaTX1 to CaTX35, vaginal exudates of 1 67 women who visited the cytology vaginal module at "Tuxtla" Health Center (Ministry of Health) in Tuxtla Gutiérrez, Chiapas, México..

Keywords: *Candida spp*., *C. glabrata*, PCR, sexually-trasmitted diseases, Vaginal candidiasis.

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INTRODUCTION

Vulvovaginitis is one of the major causes for gynecologic consultation in the world. Among them, vaginal candidiasis (VC) is considered as the leading cause of vulvovaginitis in Mexico and Central America (1). It is estimated that 75% of the women in their reproductive age have presented this infection, at least once in their lifetime, and 5% presented recurrence (2,3). In Mexico, in 2007, the Ministry of Health reported prevalence below 1% in women of reproductive age, but other authors have found increased presence of the disease (1,4). It is well known that *Candida albicans* is the main agent of vaginal fungal infections (5) and other gynecological disorders (6). Furthermore, less frequently there have been involved another Candida species known as "non-albicans", like *C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei,* among others (7). Nevertheless, in recent decades non-albicans species had increased their frequency and gained greater clinical significance (5,7).

Since not all species of the genus *Candida* share the same antifungal susceptibility pattern, it is necessary to identify the genus and species and their associated clinical characteristics for epidemiological studies in order to provide reliable data to carry out preventive programs for disease control. (7). The objective of this study was to characterize differentially through biochemical, microbiological and molecular assays four species of Candida in order to determine its prevalence in Tuxtla Gutiérrez region. A pair of oligonucleotide primers (INT1 and INT2) were used in this study, because the sequence from which they were designed has a high homology among *Candida* species(8).

MATERIAL AND METHODS

Patients and methods

Women who participated in this study were selected according to the following criteria: (1) age between 15 to 45 years old; (2) sexually active women (3) residence in Tuxtla Gutiérrez, Chiapas (4) a 72h period of sexual abstinence (5) informed written acceptance. Women were excluded when using antimicrobials and antifungals in past three weeks prior to the date of sample collection, or showing menstrual and/or abnormal uterine bleeding. All women were informed about the study, agreed to answered a questionnaire and gave the consent to all clinical procedures. The questionnaire collected data on demography (age, place of residence, occupation, level of education, family income), sexual risk behavior (pregnancy, number of sexual partners ever, sexual activity), potential risk factors associated to candidiasis (drug use, history of sexual transmitted infections, associated diseases) and the clinical details relating to infection recorded by the attending physicians (signs and symptoms of candidasis, characteristic of vaginal exudates, vaginal abnormalities, speculoscopy observations).

Clinical isolates (CaTX1 to CaTX35) were obtained from vaginal exudates of 167 women who visited the Cytology Vaginal Module at “Tuxtla” Health Center (Ministry of Health) in Tuxtla Gutiérrez, Chiapas, México, from August 2007 to October 2008. *C. albicans* (CAL1), *C. parasilopsis* (CPA4), *C. tropicalis* (CTR34) strains were kindly provided by Dr. César Hernandez from ENCB-IPN, México.

Sample collection

Vaginal exudates were collected from secretions of the bottom of the sac and vaginal walls with sterile swabs and inoculated on YPD medium with 0.05% chloramphenicol. Vaginal pH and Gram staining were also registered.

Biochemical and microbiological assays

Biochemical identification of all isolates was performance with Whiff (10% KOH) and urease test (9). Isolates were considered presumptive for *Candida* spp according to Amsbel criteria (14). In addition, isolates were subcultured in rich and chromogenic medium for further identification according to their morphology on solid medium. *Candida* species were identified through colonial growth on YPD medium and CHROMagar Candida ® (11,12) Isolates of *Candida* spp were identified as positive of *Candida* when colonies grown on solid YPD medium presented yeast-like morphology and Gram positive stain. *Candida* species were defined as *albicans*, *krusei, glabrata* or *parasilopsis* using chromogenic media CHROMagar Candida® (CHROMagar Microbiology, France) and CandiSelect® (Bio-Rad) according to morphology, as described in the manufacturer's instructions. Germ tube formation of isolates cultured on 0.5ML of human serum, and incubated at 37 °C for 2-3 h, was also determined.

DNA extraction and amplification

Total DNA was extracted with Aqua Pure® extraction kit (Bio-Rad). Polimerase Chain Reaction was done according to Sambrook *et al.* (13). INT1 and INT2 primers were designed by Baquero *et al.* (8) from a intron secuence of a ribosomal gene of *Candida albicans* (CaYST1). The reaction mixture was as follows: 500 ng of DNA, 1U Taq Polimerase (TaqKara Bio USA ®), 0.3 mM of INT1 primer (5’-AAGTATTTGGGAGAAGGGAAAGGG-3’), 0.3 mM of INT2 primer (5’-AAAATGGGCATTAAGGAAAAGAGC-3’), 0.2 mM

dNTP´s Mix (Promega®), 2 µL of 10X EX buffer extraction. DNA was amplified in a PCR thermal cyler (Cicler Bio-Rad®), by using 1 cycle at 95 °C 3 min, and then 40 cycles as follows: 60 s of denaturation at 94 °C, 30s of annealing at 55 °C, and 45 s of primer extension at 62 °C. At the final cycle, an additional 5 min of incubation at 72 °C was carried out ensure complete polymerization of any remaining PCR products.

Data processing, analysis and results

Data were analyzed using SPSS version 15. Frequency distribution of demographic data, characteristics of the population, sexual history and clinical manifestations were analyzed. The relationship between selected risk factors and the prevalence of candidiasis was determined by using unadjusted Odds Ratios (OR) and Chi square tests. In this study, a transversal, analytical and experimental design with a confidence level of 95% and 80% power was used. To determine the disease prevalence, we used the operational definition of positivity for *Candida spp*.

Ethical considerations

Ethical approval for the study was obtained from the Ethics Committee, Faculty of Medicine, Autonomy University of Chiapas. Informed verbal consent was obtained from all participants, after explaining them the purpose of the study. Patient identity was kept confidential.

RESULTS

Epidemiological analysis

Women´s profiles were consistent in this study. According to our results, one half were mature young women among 35 and 45 years old (48%; 80 cases). As shown in Table I, they lived in urban areas (56%; 94 cases) without any remuneration to their job (76%; 126 cases); thus, almost all have a low socioeconomic status (87%; 145 cases). These women were sexually active (99%; 166 cases) within a low risk profile, because most of them had one sexual partner during their lifetime (69%; 115 cases). Among them, only 14% (23 cases) were pregnant. In general, they were healthy women: a minority of them presented a history of sexually-transmitted infections (4%; 6 cases), had a common *Candida* spp. associated disease, were on treatments for systemic hypertension (4%; 7 cases), diabetes mellitus (2%; 4 cases), or other chronic diseases (13%; 22 cases), or reported used drugs like hormonal contraceptives (12%; 20 cases).

These women showed vaginal complains as leucorrhea (34% ;57 cases), pruritus (14%; 23 cases), heat (5%; 8 cases) and dyspareunia in (3%; 6 cases). Remarkably, many of them declared to be asymptomatic (44%; 73), but vaginal speculoscopy showed the opposite. An 88% (147 cases) of them had some kind of vaginal discharge, only 8% (13 cases) presented the "characteristic" vaginal exudates (kind off-white,

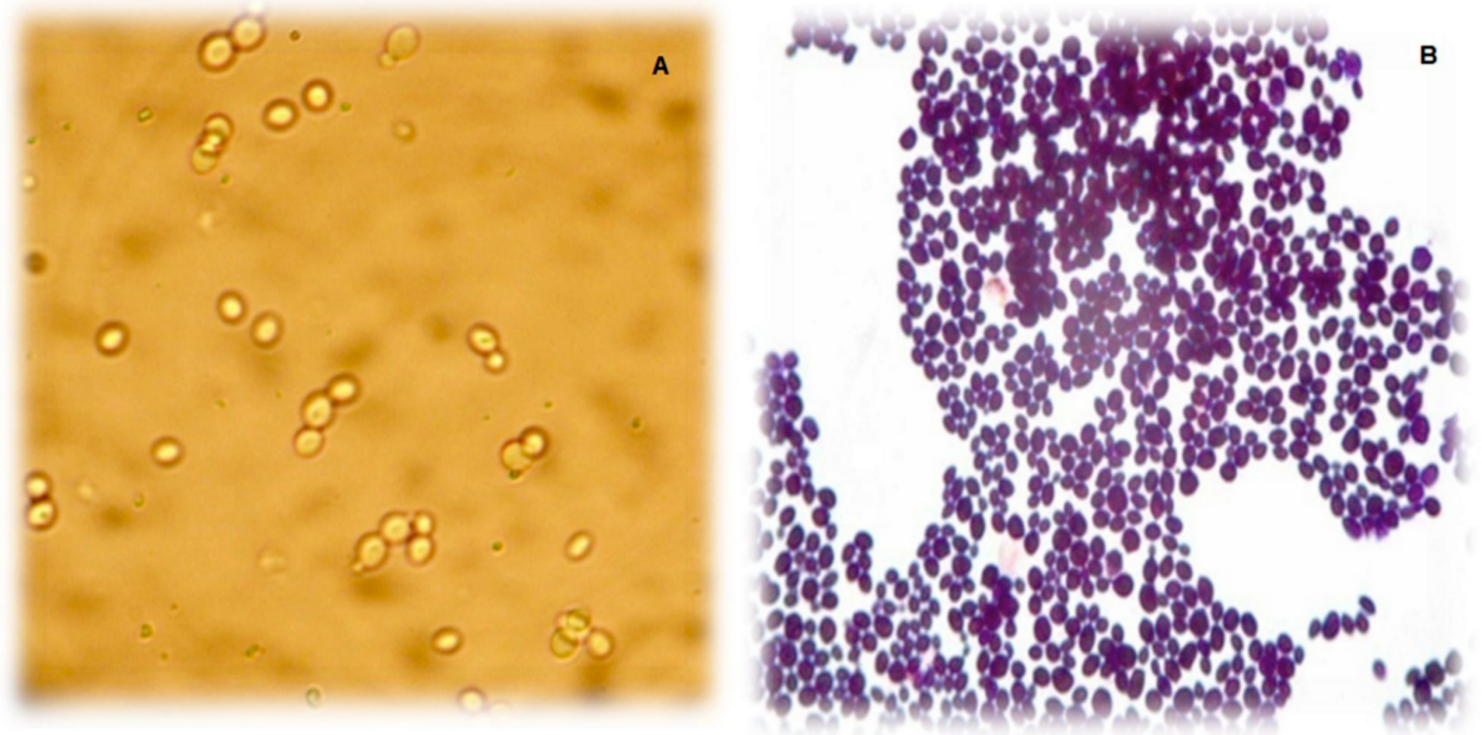

Figure 1. Cellular morphology of Candida spp. A. Yeast stained with Gram technique. B. Yeast, blastoconidia and budding cells.

Table 1. Demographic, socioeconomic and clinical characteristics of women of Tuxtla Gutierrez.

		Studies women n=167		*Candidiasis n= 35*	
Study Variables .		*F*	*%*	*F*	*%*
Age ranges (P=0.5)	15-24	44	26	10	29
	25-34	43	26	11	31
	35-45	80	48	14	40
Residence (OR, 0.9; IC 95%, 0.4-1.9; p= 0.9)	Urban	94	56	19	54
	Rural	73	44	16	46
Occupation (OR, 0.7; IC 95%, 0.4-6.4; p= 0.7)	UW	126	76	28	80
	Sales	27	16	5	14
	Trade	7	4	1	3
	OUW	7	4	1	3
Socioeconomic level (OR, 1.7; IC 95%, 0.4-6.4; p= 0.5)	Low	145	87	32	91
	Medium	22	13	3	9
Asociated disease (OR, 0.7; IC 95%, 0.2-2.2; p= 0.8)	SAH	7	4	0	0
	DM	4	2	2	6
	ID	1	1	1	3
	Other	16	10	2	6
Sex life (OR, 0.7; IC 95%, 0.7-0.8; p= 1)	Active	166	99	35	100
	Passive	1	1	0	0
Number of sexual couple (p= 0.1)	One	115	69	28	80
	Two	39	23	4	11
	Three or more	13	8	3	9
Pregnancy OR, 2.3; IC 95%, 0.8-6.0; p= 0.1	Present	23	14	8	23
History of STI OR, 0.7; IC 95%, 0.2-2.2; p= 0.8	Present	6	4	0	0
Drugs use (OR, 1.6; IC 95%, 0.5-4.5; p= 0.5)	Hormones	20	12	6	17
Sigs and symptoms p= 0.9	Leucorrhea	57	34	12	34
	Vaginal pruritus	23	14	6	17
	Vaginal heat	8	5	0	0
	Dyspareunia	6	3	1	3
Characteristics of vaginal exudates (OR, 1.1; IC 95%, 0.2-4.4; p= 1.0)	Characteristic	13	8	6	17
	No characteristic	134	80	23	66
	Without Leucorrhea	20	12	6	17
Speculoscopy observations (p= 0.9)	Cervical erythema	84	50	23	66
	Vaginal erythema	28	17	3	9
	CVE	26	16	3	9
	AWP	3	2	1	3

UW= Unpaid works, OUW= Other UW, SAH= Systemic Arterial Hypertension, DM= Diabetes mellitus, ID Immune disease, CVE= Cervicovaginal erythema, AWP= Adherent whitish plaques, STI: Sexual Transmitted Infections

semisolid and lumpy of "Cotagge cheese" apparence; refs. 1, 5) and most of them presented "non characteristic" leucorrhea (80%; 134 cases). Additionally, erythema was present in 85% of cases at the cervix (50%; 84 cases), vaginal mucosa (17%; 28 cases), or both areas (16%; 26 cases). Only 2% (3 cases) presented adherent whitish macroscopic plaques, in the vaginal epithelium (Table 1).

Biochemical and microbiological tests

The cellular morphology of the 167 exudates showed Gram staining positive cells (27%; 46 cases) and yeast like cells (19%; 32 cases), and clue cells in the stained frotis (13%; 22 cases; Fig. 1). Amines test and pH greater than 4.5 were positive in 43% (72 cases) of the exudates. Only one exudate was positive to urease test (3%). Biochemical analysis and the related optical microscopy showed neither the sensitivity, nor the specificity to discriminate the clinical relevance (Table 2). All 167 vaginal exudates were grown on YPD solid medium and subjected to biochemical and microbiological tests. Thirty five isolates were positive to *Candida* spp. with characteristics according to Ausubel *et al.* (10). Positive isolates had three different *Candida* spp. presumptive colonial morphologies, as described in Table 3.

Table 2. Comparative results of biochemical tests of Candida isolates.

Tests	*Total exudates (n=167)*		*Exudates (+) Candida* spp. *(n=35)*	*S*	*E*	*PV+*		*PV-*
	F	*%*	*F*	*%*	*%*	*%*	*%*	*%*
pH > 4.5	72	43	27	77	46	58	22	80
Amines (+)	72	43	10	28	29	53	14	74
Urease (+)	1	3	1	3	3	97	20	79
Yeast in fresh	32	19	16	46	43	87	47	85
Yeast Gram (+)	46	27	27	77	63	83	49	89
Clue cells	22	13	4	11	--	--	--	--
Germ tube	9	15	9	26	26	100	100	83

S=Sensibility, E=Specificity, PV= Predictive value

Chromogenic media culture allowed *Candida* species identification, through differential features on solid media. *C. glabrata* (CaTX1, CaTX4, CaTX5, CaTX7, CaTX8, CaTX9, CaTX12, CaTX13, CaTX14, CaTX15, CaTX16, CaTX18, CaTX20, CaTX23, CaTX26, CaTX27, CaTX28, CaTX31, CaTX32, CaTX34 and CaTX35), *C. albicans* (CaTX3, CaTX10, CaTX17, CaTX19, CaTX21, CaTX22, CaTX24, CaTX25, CaTX29 and CaTX30), and *C. krusei* (CaTX6, CaTX11) were identified with CandiSelect ® and CHOROMagar®. The last two isolates were identified as C. *parapsilopsis* (CaTX2 and CaTX33) in CHOROMagar®. These results showed that *Candida glabrata* was the primary causative agent of vaginal candidiasis in low income women of Tuxtla Gutiérrez, Chiapas.

Molecular assays

Primers INT1 and INT2 deduced form *CaYST1* intron sequence produced a 310 pb amplicon from different *C.albicans* laboratory strains (8). PCR amplification seven isolates previously identified as *C. albicans* (CaTX10, CaTX17, CaTX19, CaTX22, CaTX24, CaTX29 and CaTX30) and reference strain CAL1 generated a 310 bp amplicon (Figure 2A). Meanwhile, PCR amplification of *C. glabrata* strains generated a polymorphic pattern. CaTX7, CaTX14, CaTX15, CaTX16, CaTX18, CaTX20, CaTX26, and CaTX27 generated a 350 bp and 100 pb bands. CaTX1, CaTX23, CaTX28, and CaTX32 generate a single 350 bp band and CaTX4, CaTX5, CaTX13, CaTX31, CaTX34, and CaTX35 amplified a single 100 bp band (Fig. 2B). In the case of PCR amplification of CaTX11 (*C. krusei*) and CaTX33 (*C. parasilopsis*) both strains generated a 310 bp single (Figure 2C).

Epidemiological study

Vaginal candidiasis (VC) was identified in 35 women (21%, n = 167) who had a mean age of 30 ± 8.7 years old, range 16-45 years old, and a coefficient of variation of 29%.The highest proportion (40%, 14 cases) of them were located in the range of 35-45 years old. It was found that VC was prevalent among

Table 3. Colonial morphology of Candida spp. isolates

Morphological characteristics of colonies	Kind	Isolates n=35	Fraction (%)
Circular, soft and pasty with yeasty smell from 1 to 3 mm in diameter.	A, B, C	35	100
Smooth, precise borders with white center slightly prominent.	A	32	91
Flat, pale cream color, with irregular borders, and clearly outline dry, fast growing.	B	2	6
Flat, pale cream color, with irregular (borders) and flat and smooth.	C	1	3

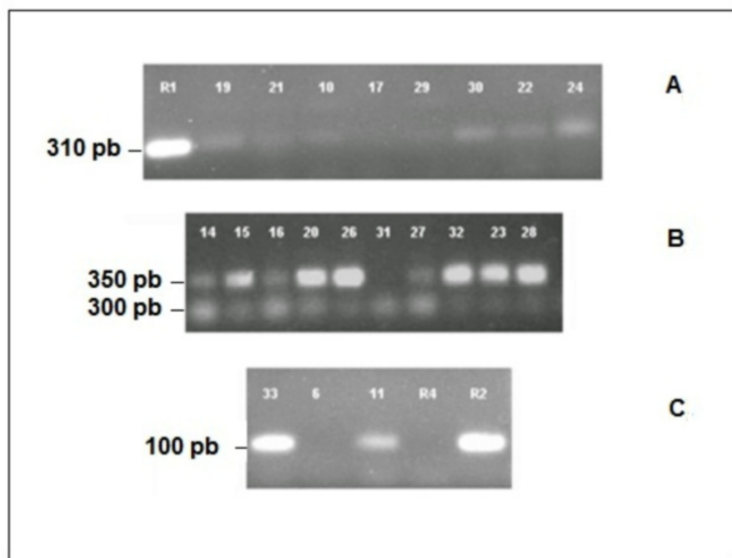

Figure 2. Amplification of INT1- INT2 fragments from genomic DNA of isolates of Candida spp. A: PCR products of C. albicans isolates. Lanes R1: CAL1; 19: CaTX19; 21: CaTX21; 10: CaTX10; 17: CaTX17; 29: CaTX29; 30:CaTX30; 22: CaTX 22 and 24: CaTX 24. B: PCR products of C. glabrata isolates. Lanes 14: CaTX14; 15: CaTX15; 16: CaTX16; 20: CaTX20; 26: CaTX26; 31: CaTX31; 27: CaTX27; 32: CaTX32; 23: CaTX23 and 28: CaTX28. C: PCR products of C. parapsilosis (lane 33: CaTX33 and R4:CPA4); C. krusei (lanes 6: CaTX6 and 11: CaTX11) and C. tropicalis (lane R2:CTR34).

women living in urban areas, with unpaid work and a low socioeconomic status (Table I). Regarding their sexual behavior, all of them were sexually active, 80% (28 cases) had one sexual partner and 23% (8 cases) were pregnant. Diabetes mellitus was prevalent in 6% (2 cases) of women with VC, and 17% (6 cases) of them consumed contraceptives. The main causes of vaginal discomfort were leucorrhoea 34% (12 cases) and vaginal pruritus 17% (6 cases). The 46% (16 cases) did not mention any discomfort. However, the vaginal speculoscopy showed that 83% (29 cases) of them had some kind of vaginal discharge, manifesting the non characteristic leucorrhoea in 66% (23 cases). Likewise, in 84% (29 cases) there was local erythema.

The analysis of the data was obtained with conventional studies in exudates, which were positive for *C. albicans*, (77%; 27 cases) had pH values above 4.5, (28%; 10 cases) resulted in a positive amine test, and 3% (1 case) of the samples were urease positive. Only in 46% (16 cases) of the samples which were positive for *Candida* spp., yeast cells were observed by direct microscopy, and when stained with the Gram technique, (77%; 27 cases) were identified as Gram positive. The latter technique allowed the identification of key cells in 11% (4 cases) of the samples which were positive for *Candida* spp. The 26% (9 cases) of primary isolates corresponding to 90% (9 cases) of those determined as *C. albicans* by biochemical tests, produced germinative tubes (See table 2).

DISCUSSION

Mostly, VC was detected in women who had some of the following factors: age 35-45 years, low socioeconomic status, with use of hormonal contraceptives, diabetes mellitus and pregnancy status. However, an alpha level of significance (X2 0.05) showed that there was not statistical significance to assume these clinical traits had a relationship with the presence of candidiasis. Moreover, the results of this study suggest that to succeed in the infection, the fungi might not require these conditions *a priori*, because candidiasis was found in some women without the above characteristics, even though other authors have documented a close relationship with these factors (1, 6).

The presence of significant alterations in the vaginal ecosystem of women was notable in this study because there is a 43% with a positive amine test and vaginal pH values above 4.5, which according to Casanova *et al.* (1) are indicative of replacement of the normal vaginal microbiota by anaerobic bacteria. Imbalance of few bacteria such as *Dörderleim bacillus* might alter vaginal ecosystem. In addition, 11% (4 cases) of women with VC meet 3 or more criteria for the diagnosis of bacterial vaginosis. According to Amsel (14) bacterial vaginosis showed pH> 4.5, positive amine, the presence of cell contacts, homogeneous vaginal discharge, which indicate the presence of mixed infections, as described by Medina *et al.* (15) and Bucemi *et al.* (3). These changes in the vaginal microenvironment allow for the establishment of VC, because there occur also biochemical, microbiological and physiological changes such as increased epithelial glycogen, alkaline pH, decreasing of the bacilli flora and overcrowding anaerobic flora (16). As shown in Table 2, the statistical indicators used to evaluate the efficiency inherent to conventional diagnostic tests were rather sensitive, and specific for establishing the diagnosis of VC, as mentioned in Baquero *et al*. (8), at least for the germ tube test, which is excellent for discriminating *C. albicans* from other non-albicans species (E = 100%). Therefore, a negative value gives us 83% confidence that the species analyzed could be a non-*albicans* case, and a high probability (VP+ = 100%) that a result with positive value could be infected by *C. albicans*. However, the evidence is inadequate to confirm the non-albicans species (S = 26%).

In agreement with the results obtained in this work, presence of clinical symptoms such as vaginal discharge, vaginal pruritus and heat, not necessarily determine the VC nor the high rate of infection candidiasis (48%) in asymptomatic women (p> 0.05). Medina *et al. (3)* had studied the same behavior in women with these symptoms (including whitish vaginal fluid) could have other types of vaginitis like in the bacterial type (15). Histological changes associated with VC in this study are related to inflammatory processes in the cervicovaginal epithelium that may be correlated to the adhesion and invasiveness capability of *C. albicans* and *C. glabrata,* as described in Castaño *et al.* (17). Whitish plaques adherent to the vaginal epithelium in 1.8% (3) of the women studied were not statistically significant (p> 0.05) although Torres *et al.* (18) related them as a suggestive indication of infection with *Candida* spp.

Species identified in this study were: *C. glabrata* (12.6%), *C. albicans* (6.0%), *C. krusei* (1.2%) and *C. parapsilosis* (1.2%). The species most frequently found was *C. glabrata,* as reports by Iglesias *et al.* (21), Paul *et al.* (22). But these results differ from those obtained in other studies describing *C. albicans* species as the principal organism in infection statistics (19, 20). This transition may suggest that *C. glabrata* incidence can be associated with the use of antifungal azoles for topic use, empirical and indiscriminate chemotherapy treatment or increased use of fluconazole as prophylaxis for enteric route, which could probably determine the presence of recurrent vaginitis (17, 23). VC caused by *C. glabrata* examined in this study is consistent with the low percentage of white plaques adherent to the vaginal mucosa, since this species lacks properties of filamentation. This observation led us to consider *C. glabrata* as a fungus of low virulence. However, its production of proteinases encoded by a family of subtelomeric genes and hydrophobicity of the cell surface,which confers adherence property on it, in comparison to *C. albicans*, and its association with high mortality and the increasing reported cases, allowed us to propose C. glabrata as an emerging pathogen (22, 24). Although *C. glabrata* has higher phylogenetic relationship with *Saccharomyces cerevisiae* rather than *C. albicans*, it shares some features of virulence with the latter. Both funguses have high capacity of adhesion to epithelial cells and the ability to form biofilms that allow them to adapt to different conditions in the host. Besides *C. glabrata* has the ability to develop higher virulence by mutation of subtelomeric genes, homologous to the gene EPA1 (17). Indeed, it can also undergo morphogenetic changes as adaptation to the environment where it grows, creating variations in the colony appearance when growing in culture media supplemented with copper sulfate (25). This behavior could arise in other culture media, as was observed in all three types of colonies obtained in this study. Evidences obtained in this study demonstrated that chromogenic media permits the adequate discrimination between *Candida* species, results consistent with those described by Houang and Yücesoy *et al.* (12). But they can not be conclusive.

Molecular assays offered a particular trend in the population studied. Amplification products were obtained from the isolates of *C. glabrata* (amplicons of 100 and 350 bp), *C. albicans, C. krusei* and *C. parapsilosis* (310 bp amplicons). These results differed from the findings obtained by Baquero *et al*. (8), who obtained amplifications only in *C. albicans* (310 bp), *C. pseudotropicalis* (1,200 bp), *Kluyveromyces marxianus* (1,250 bp) and *C. neoformans* (1,200 bp). These results are consistent

with the hypothesis that there is a significant degree of genetic polymorphism in the intron sequence CaYST1 findings of Baquero *et al*. (8); however, they suggest that these genetic variability results in inconsistent conservation of species in the genus *Candida*. It is prudent to mention that our population of study is part of a genetic mix of various ethnic groups that could influence the molecular findings of this study. However, it should be noted that in her study, Baquero *et al*. (8), presented evidences of alternate tests for confirmatory identification of the non-*albicans* species they used.

Prevalence of *Candida* spp. in the analyzed population was 21.0%, which agrees with the results obtained by Flores *et al.* (26) and Reyna *et al.* (27). Although it was higher when compared to the one observed by Jiménez *et al.* (28) in a population of women with cervical dysplasia in Tuxtla Gutiérrez (15%); who used the germ tube test as the sole study of discrimination of the species *albicans* from non-*albicans*. However, it is necessary to take into account that this test does not identify the 5-10% of *C. albicans* not forming germinative tubes. Moreover, the prevalence obtained in this study was significantly ($p > 0.05$) higher than the prevalence reported in 2007, in Mexico (1%).

Since inclusion of VC condition as part of an epidemiological report is not strictly mandatory, it is possible that the records in the national system of Public Health Institutions contain an underrepresentation of new cases of diseases, leading to a dangerous inaccuracy in the statistical evaluations. The situation is more critical because private medical clinics do not report accurately to the health authorities the morbidity of the population they serve.

CONCLUSIONS

The identification of *Candida* species was done successfully through the isolation of fungi by conventional culture and biochemical tests. Confirmation of the genus was done by PCR analysis using primers INT1 and INT2 and classification of species using chromogenic media. The results of this study allow us to conclude that the prevalence of VC was 21.0%. The isolated species were *C. glabrata, C. albicans, C. krusei* and *C. parapsilosis*. The most frequent species isolated as a causative agent of vaginal infections corresponded to *C. glabrata*. The high frequency of alterations in the vaginal ecosystem of the women studied was risk of vaginitis. Determination of prevalence levels in women relying on common vaginal candidiasis symptoms lacks of reliability because, as demonstrated in this study, a lot of women also have an asymptomatic infection.

Vaginal candidasis is one of the most common infections in women. In Tuxlta Gutiérrez one in five women suffers this infection even when they are asymptomatic. However, in our days public and private medical services lack of accurate clinical, biochemical, microbiological and molecular of tools for diagnostic, that allows an oportune and appropriate treatment in order to improve women health. It is imperative to generate scientific information and combine it with responsible health public policies to improve the quality of life of women in the region of Tuxtla Gutiérrez.

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The inorganic pyrophosphatases of *Taxodium mucronatum* in cone development and flooding stress

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ABSTRACT

Inorganic pyrophosphate (PPi) is produced mainly as a byproduct of anabolism. Therefore the inorganic pyrophosphatase enzymes (PPase; EC 3.6.1.1), responsible for PPi hydrolysis, are essential for the viability of organisms. Different studies on plant PPase have been published, but the functions of these enzymes in the trees are unknown, and, in particular, little is known about the metabolic adaptations of *Taxodium mucronatum* (Ahuehuete). In this paper, the PPase activity of *T. mucronatum* is documented for the first time. The PPase activities were measured in cones and seedlings, and changes in activity of two PPase isoforms were found under flooding conditions. In addition, a partial purification of a PPase from *T. mucronatum* leaves is reported.

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Abbreviations: PPase, soluble inorganic pyrophosphatase; TmPPase, *Taxodium mucronatum* pyrophosphatase; PPi, inorganic pyrophosphate; Pi, inorganic phosphate.

Keywords: Soluble inorganic pyrophosphatases, pyrophosphate, stress, flooding, cone development, *Taxodium mucronatum*, Ahuehuete.

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INTRODUCTION

Inorganic pyrophosphate (PPi, is a compound produced in all living organisms, during the synthesis of macromolecules (DNA, proteins, lipids and complex carbohydrates), and it is also produced in several reactions in the biosynthesis of small metabolites (1). Its hydrolysis is essential because an accumulation of PPi would shift the equilibrium of anabolic reactions and stop growth (2). The recycling of PPi is carried out mainly by inorganic pyrophosphatase enzymes (PPase; EC 3.6.1.1). This activity is ubiquitous in living organisms and catalyzes the irreversible hydrolysis of PPi in two molecules of inorganic phosphate (Pi). However, in comparison with animal cells, several prokaryotic and eukaryotic cells, including plant cells, maintain a high, albeit constant, level of PPi in the cytosol.

In *Escherichia coli*, there is one PPase (3) while in *Sacharomyces cerevisiae* two PPase has been identified, a cytosolic PPase (4) and a mitochondrial PPase (5). In plants, there are several isozymes and the genome of *Arabidopsis thaliana* encodes for six PPase. In this last species, only one PPase is chloroplastic (6) and represents most of all soluble PPase activity (7), while the other five appear to be cytosolic, as inferred from sequence similarity to the two PPase from potato, localized in the cytosol using immunogold electron microscopy (8). Although the presence of 5 cytosolic isoforms may indicate a degree of redundancy in the recycling of PPi, the individual isoforms may have additional individual roles as suggested by a recent work on Pi-starved plants of *Phaseolus vulgaris* (9). In this work, the expression of the three identified PPase changes in response to Pi–starvation, both at the transcriptional and activity levels (9). The PPase activity has been shown to be essential for viability of yeast (5) and bacteria (3) . In plants, several reports highlighted the importance of the PPase. For example, the overexpression the PPase of *E. coli* in the cytosol of transgenic tobacco and potato plants leads to a significant alteration in growth and development (10, 11). In other report, two PPase which are expressed in pollen tubes lead to an inhibition of the growth, when they are phosphorylated in a Ca^{2+}–dependent manner (12).

Plants experience low oxygen concentration due to environmental stress such as soil flooding, or low oxygen diffusion in dense or bulky tissues (13). This condition restricts ATP production and consequently inhibits energy–dependent processes creating a low–energy crisis. Under such condition, ATP production must be optimized and energy consumption must be reduced. Therefore, DNA synthesis, cell division (14), and ribosomal RNA production are reduced dramatically (15). Plants may utilize pyrophosphate (PPi) as an alternative energy donor, particularly when cellular ATP pools become diminished during stresses such as anoxia and nutritional Pi starvation (16) and the use of PPi in some tolerant plants species is similar to that observed in anaerobic Prokaryotes.

There are many reports dealing with the activity and expression of plant PPase enzymes, but to date, the study of these enzymes in trees has been limited, and no information is available regarding *Taxodium mucronatum* (Montezuma bald–cypress, NCBI taxonomy ID: 99812). Little information has been published on this tree's metabolism, and information on the number, structure, function or regulation of the PPase activities in this plant is absent. In fact, to date only 12 amino acid sequences and 20 nucleotide sequences have been deposited in the NCBI sequence databases (http://www.ncbi.nlm.nih.gov/protein/?term=txid99812[Organism:noexp]). *T. mucronatum*, known as Ahuehuete, is endemic to north and central America and is considered a national tree in Mexico. It grows at riversides, where it is exposed to frequent flooding. In this paper, the existence of the PPase activities is documented in *T. mucronatum* for the first time. Changes in PPase activity are reported in cones at five different states of development, and the effect of flooding in the activity of two PPase in young seedlings of this species is documented. Finally, a partial purification of a PPase from *T. mucronatum* is reported.

MATERIALS AND METHODS

Plant material

T. mucronatum seeds were extracted from cones recollected from trees at Mexico city locations. *T. mucronatum* cones were collected at five developmental stages. Seeds were excised from mature cones and disinfected with hypochlorite (2 % w/v) as reported by Wenny and Dumroese (17). Subsequently, the seeds were washed with deionized sterile water to remove the hypochlorite. The seeds were put in sterile wet paper towels inside glass jars. Germination and early growth were allowed to proceed for two weeks at 28ºC, in an incubator chamber, under a 12 h day light cycle. Jars were capped to avoid water loss.

Tissue extraction and activity assay

Cones were frozen with liquid N_2 and ground with mortar and pestle in 3—5 mL of buffer Tris—HCl (see above). The extracts were put in tubes and centrifuged a 16000 ×g 10 min and the soluble fraction was used to measure the activity using native gel electrophoresis. The seedlings of *T. mucronatum* were homogenized using the FastPrep®–24 (MP Biomedicals™) bead-beater equipment in 0.1 M Tris—HCl buffer, pH 7.4, with 3 mM $MgCl_2$, 1 mM EDTA, and 15 % (v/v) glycerol. The extract was centrifuged at 16,000 ×g for 10 min, at 4 ºC and the soluble fraction was applied to SDS–PAGE and native gel electrophoresis.

SDS—PAGE and native electrophoresis was performed as described by (9) and the protein concentration was determinated in microplates with the Bradford assay kit from Bio—Rad™, at 595nm, in a Benchmark microplate reader (Bio-Rad™), using bovine serum albumin as standard. The PPase activity in gels was estimated by densitometric analysis and normalized against the total protein load determined by SDS–PAGE silver staining. Where appropriate, statistical comparisons were make using the Student's t–test. The PPase activity assay was performed as previously described (9).

Flooding stress

Flooding stress test was performed by immersion of the seedling's roots in a vial and filling it with sterile water up to the shoot's base. The seedlings were incubated for variable periods of time (0 to 100 min, at 10 min intervals), at 25 ºC, under natural day light. At the end of the incubation period, the seedlings were extracted as described above.

Partial purification of Taxodium mucronatum *pyrophosphatase*

Ten grams of leaves of *T. mucronatum* were frozen in liquid N_2, ground with a mortar and pestle, and homogenized using in 100 ml of 0.1 M Tris—HCl buffer, pH 7.4, containing 3 mM $MgCl_2$, 1mM EDTA and two tablets of protease inhibitor cocktail (Complete™), at 4ºC. The homogenate was filtered through four layers of cheesecloth, and centrifuged sequentially at 1600, 10000 and 15000 ×g, at 4ºC, recovering the supernatant each time. The use of sequential centrifugation steps aimed at the identification of particulate PPase activity in some cell fraction. The final supernatant was taken to 60% saturation with ammonium sulfate (pH was checked with indicator paper and correct with a small amount of Tris base, when required), allowed to stand at 4 ºC for 1 h, with gentle stirring, and the pellet was separated by centrifugation at 14000 ×g, for 20 min. The supernatant was dialyzed overnight at 4ºC, with gentle stirring, and the sample was passed through a 100 ml column of AG® MP–1M Anion Exchange Resins (Bio-Rad™), and eluted with 0.5 M KCl (in one step), in 0.1 M Tris—HCl buffer, pH 7.4, containing 3 mM $MgCl_2$, 1 mM EDTA. The unbound and column–eluted fractions were further separated by ultrafiltration using 30 kDa Amicon membranes (Millipore™). Both the filtrate and the retentate were analyzed.

RESULTS

Pyrophosphatase activity in cones of *Taxodium mucronatum*

During development of plant tissues, specific biochemical and molecular processes are required and transcriptional and physiological reprogramming pathways are activated (18, 19). In order to examine the changes in PPase activity throughout the development of *T. mucronatum* cones, we sampled the cones from the trees, at five stages of development. In figure 1A, the form, size and color of the intact *T. mucronatum* cones is shown. Figure 1E shows a transversal section of the cones, where their internal morphology and reproductive structures can be appreciated (20). At the initial stages of development (1 and 2), the strobilus presents a thick fruit cover, seed development is not evident to the naked eye, and the tissues are mostly dark green (Fig. 1 E1—E2). At the stage 3 archegonia and teguments become evident (Fig. 1 E3) and at the final developmental stages (4 and 5), the seeds (gametophyte and sporophyte), seed coat and teguments become clear. (Fig. 1 E4—E5) At the last stage, the dark green color of the strobilus was restricted to the external layer (Fig. 1 E5).

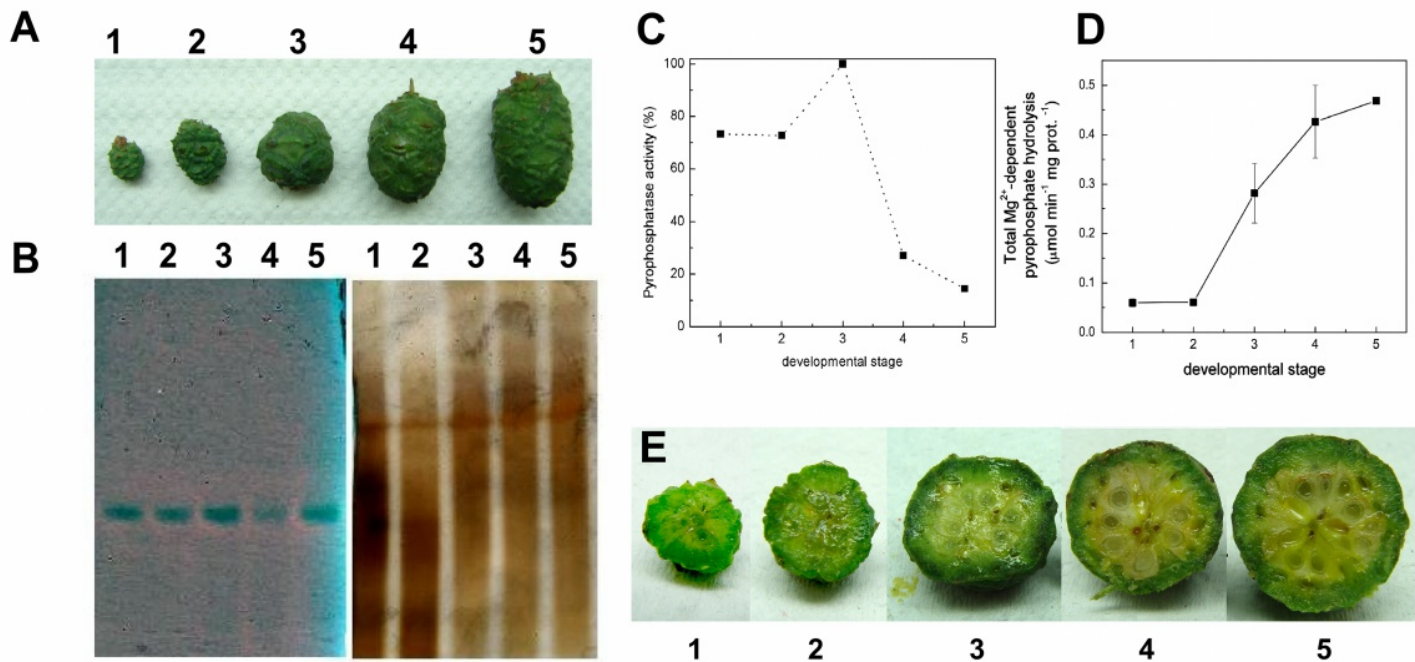

Figure 1. Pyrophosphatase activity in cones of *T. mucronatum*. A) Cone morphology in five developmental stages. B) Zymograms of pyrophosphatase activity. The gel was loaded with 5 µg of total protein. After the run, the gel was halved and the activity was visualized by staining using malachite green (panel left). Protein was visualized with silver (panel right). C) Densitometric analysis of pyrophosphatase activity. The highest activity value (stage three) was taken as 100%, values are normalized using the total density in the corresponding silver-stained SDS-PAGE lane. The dotted line is from spline interpolation. Coefficients of variation were between 15 to 25% (omitted for clarity). D) Total Mg^{2+}-dependent pyrophosphatase activity in crude extracts (error bars are standard deviation). Transversal section of the cones of *T. mucronatum* showing the reproductive structures.

All plants with known genomes encode for more than one PPase, thus the presence of PPase isoforms was determined in zymograms of extracts of *T. mucronatum* cones, which allowed the separation of some isoforms in *P. vulgaris* (9). The gel samples were duplicated in the two halves of the gel, one half was revealed for PPase activity, with malachite green (Fig. 1B, left) and the other half of gel was silver-stained to check protein load (Fig. 1B, right). The normalized densitometric analysis (Fig. 1C) showed a transient increase in the PPase activity as development proceeds with a peak at stage 3, but the differences with stages 1 and 2 were not

statically significant. The activity shows a significant drop towards the end of development, and this drop was statistically significant. In this gel, a mayor band is observed and a subtle smear in the lower part of the gel, which suggest the existence of additional isoforms, either with low expression or low activity, as compared phosphodiesterases and perhaps other activities. Apparently, zymograms are more specific, probably because some activities contributing to the total activity in solution are either lost during the run, or the *in situ* assay conditions do not favor their activity. The higher specificity of the *in situ* assay is also suggested by the

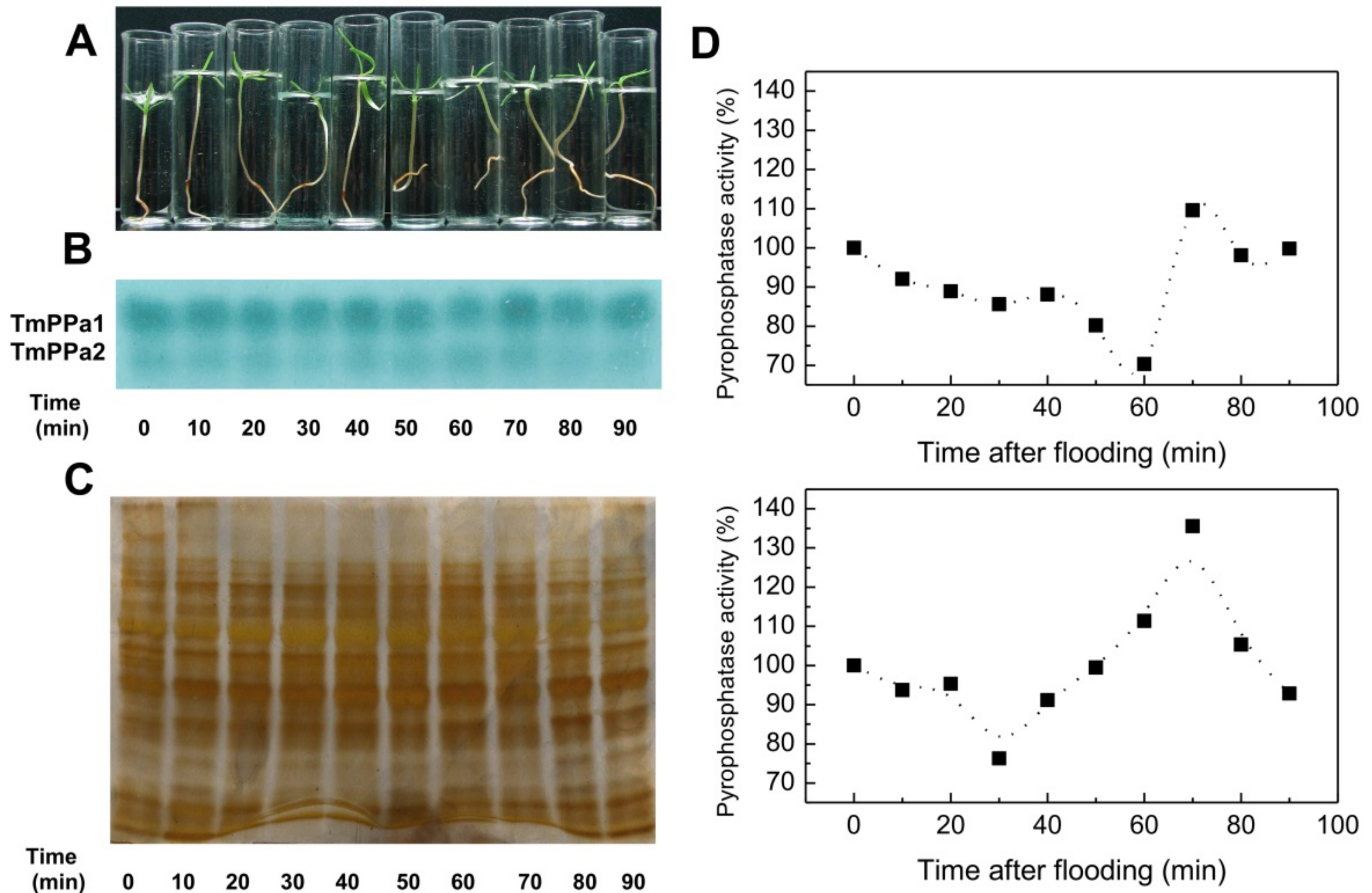

Figure 2. Pyrophosphatase activity changes in *T. mucronatum* seedling under flooding stress. A) Two weeks-old seedlings were incubated under flooding conditions. B) Zymogram of pyrophosphatase activity in crude extracts from seedlings of *T. mucronatum*. C) Silver-stained SDS-PAGE. D) Densitometric analysis of two pyrophosphatase activity bands in (A). TmPPa1 and TmPPa2 normalized against the total protein density of the corresponding silver-stained SDS-PAGE lane. The activity is expressed as percentage of TmPPa1 activity at time zero. Coefficients of variation were between 15 to 25% (omitted for clarity). The dotted line is from spline interpolation.

to the mayor component. Figure 1D shows the total PPase activity in the crude extracts. This *in vitro* activity increases as development advances, reaching an 8-fold increase at stage 5, relative to stage 1, but these measurements may include true PPase, divalent cation activated phosphatases, Nudix enzymes, lack of detectable bands in the gel, in the absence of Mg^{2+}. In contrast, the total activity in solution without Mg^{2+} ranged from 30 % (at stage 5) to 85 % (at stage 1) of the total activity with Mg^{2+} (not shown).

At 10 min intervals the soluble protein was extracted from the whole seedling and the PPase activity was determined using zymograms (Fig. 2B). In contrast to the zymograms from cones (Fig. 1A), where only one band was observed, figure 2B shows two distinct bands present, with different intensity. The bands were named as TmPPa1 and TmPPa2 and their relative band intensity was analyzed by densitometry and normalized with the total protein load observed in silver stained SDS–PAGE. The intensity at zero time of the upper band was considered as 100 % of the activity (Fig. 2D). As shown in figure 2C and D, the zymogram activities do not match the activity measurements in solution. Figure 2D indicated a decrease in the activity in both

Pyrophosphatase activity changes in *Taxodium mucronatum* seedling under flooding stress

Flooding is a frequent environmental stress, it can prevent or reduce the growth of most terrestrial plants, and can result in premature death. Since *T. mucronatum* is tolerant to flooding stress, and continues to growth under this condition, it was of interest to investigate the impact of flooding in the PPase activity of this plant. The flooding condition was replicated in the lab, in two weeks old seedlings, by complete immersion of the plant root in sterile distilled water for up to 100 min (Fig. 2A).

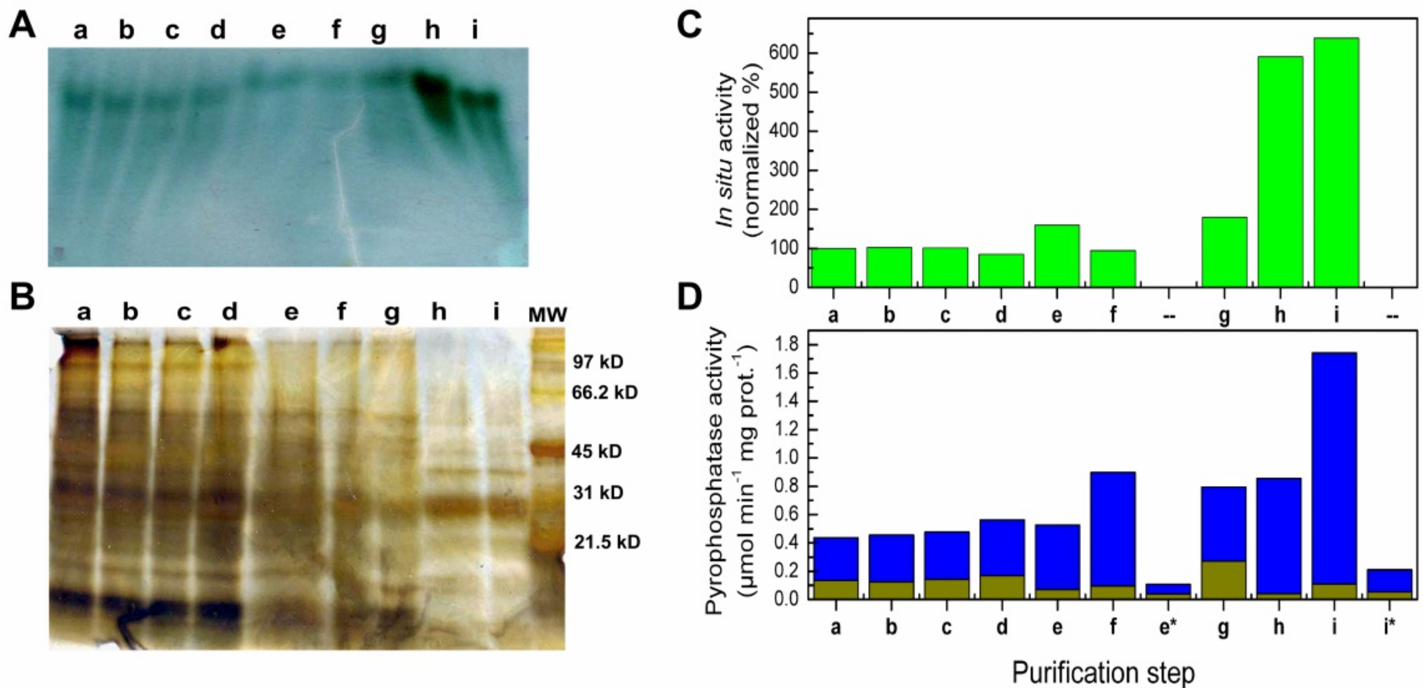

Figure 3. Partial purification of a pyrophosphatase from *T.* mucronatum leaves. (A) Native gel electrophoresis of the purification steps. The activity was visualized by staining with malachite green reagent (B) SDS-PAGE electrophoresis. The protein profile was visualized with silver staining. C) Densitometric analysis of upper band in (A) normalized against the total protein load in (B) of the corresponding lane. Coefficients of variation were between 15 to 25 % (omitted for clarity). (D) In vitro activity of PPi hydrolysis without Mg^{2+} (dark yellow bars) or with 3mM Mg^{2+}, and with 160 µM PPi. Coefficients of variation were between 7 to 18 % (omitted for clarity). The lowercase letters corresponds to different steps of the purification protocol: (a) crude extract, (b) centrifugation 1600 x g, (c) centrifugation 11000 ×g, (d) centrifugation 15000 ×g, (e) 60 % ammonium sulfate supernatant (e*) resuspended pellet from ammonium sulfate precipitation (f) dialyzed supernatant. The dialyzed sample was passed through an anionic resin and the fractions were concentrated by ultra filtration. (g, h) protein bound to the resin and eluted with 0.5 M KCl (one step), (h) filtered through Amicon 30 kDa membranes (g) retained by Amicon 30 kDa membranes, (i, i*) Unbound protein, (i) filtered through Amicon 30 kDa membranes (i*) retained by Amicon 30 kDa membranes. Due to their low activity (e*) and (i*) were not applied to the gel.

PPase during the first 30 min, at this point TmPPa2 started to regain activity, but TmPPa2 activity decreased to reach a minimum at 60 min, and then recovered to almost the same initial level. The drop in activity at 60 min for TmPPase1 and at 30 min for TmPPase2 were only 30 % change but were statistically significant. The increase in activity at 70 min for TmPPase2 was also 35 %, respect zero time activity, but was again statistically significant. The activities of TmPPase1 and 2 at the end of the experiment were not significant from the activities at the starting time, suggesting that an adaptive response could be taking place.

Partial purification of a pyrophosphatase of Taxodium mucronatum.

No genomic or proteomic information is currently available on *T. mucronatum.* The sequences, functions, properties of PPase isozymes present in this plant species are also unknown. A partial purification of a PPase activity was achieved using ammonium sulfate precipitation and ion exchange chromatography. The zymogram of the different preparations throughout the purification process is shown in figure 3A and the silver stained SDS–PAGE in figure 3B. The data in figure 3A indicate the presence on two bands in the crude extract and along the centrifugation steps (a to d and g). Densitometric analysis of the gels was done as before, using the total protein load for normalization, in this case only TmPPa1 was measured (Fig. 3C). The total pyrophosphate hydrolysis was also measured along the purification, both, in the presence or in the absence of 3 mM Mg^{2+} (Fig. 3D). A purification to homogeneity was not achieved, but the data indicate a substantial increase in the *in gel* normalized activity (Fig. 3C) and in the *in vitro* specific activity (Fig. 3D), both from an increase in the activity (Fig. 3A) and from an important reduction in total protein (Fig. 3 B). The activity during the centrifugation steps did not show important changes, indicating that there was no activity in the particulate fractions (attempts to solubilize and measure the activity in the pellets were unsuccessful). The fraction eluted from the column and retained by 30 kDa Amicon membranes had high Mg^{2+}–dependent PPase activity, and very low Mg^{2+}–independent activity. There was also an important level of activity in the unbound protein, but in this case, the fraction with higher activity was in the filtrate from the Amicon membranes. The detection of activity in all these fractions, suggests the existence of multiple isoforms; however, if this was the case, the gels separated these proteins in only two distinct bands. Further attempts to obtain a pure preparation were unsuccessful because the activity was lost gradually at 4 ºC, and more than 90 % of the activity was lost upon freezing.

Some of the PPase activities partially purified may have an absolute requirement for Mg^{2+}, as in its absence, the activity was very low (Fig. 3D, h). However, a more detailed analysis of the kinetic and molecular properties of this protein would require a pure preparation and no further analysis was attempted here.

DISCUSSION

Taxodium mucronatum is an evergreen tree with a size that can easily reach heights of more than 20 m and its life span may exceed 2500 yr. *T. mucronatum* is highly tolerant to flooding stress, but there are no detailed studies of the physiology, biochemistry, proteomics and genomics of this conifer. Since PPi has been proposed as an alternative energy source during some stress conditions, including anoxia (16), it is of interest to investigate the role of pyrophosphatases on *T. mucronatum*.

Many of the events involved in seed formation require an active metabolism (21) and will produce PPi, therefore, PPi–consuming reactions would be required. In figure 1 changes in the PPase activity along cone development were observed and may reflect the level of biosynthetic activity. Unfortunately, the level of metabolic activity along cone development has not been investigated in this tree species, and no further correlations can be established. The activity in solution did not follow the same trend as the activity in zymograms. This reflects the complications of studying these hydrolytic reactions in crude extracts, as many enzymes can interfere with true PPase activity assays. In this sense, the activity in the zymogram may be of more significance.

Recently, we reported the responses of PPase mRNA expression and enzyme activity upon Pi–starvation in *Phaseolus vulgaris* (9) and the evidence provided in that work, suggest a specific role for the PPase isozymes. The present results in *T. mucronatum* also suggest an initial response to flooding stress, in which at least two PPase have a specific role. Under anoxic conditions, the cell energy is limited, and PPi may serve as an alternative source of energy (22), this proposal suggest a possible reduction in PPase activity, since the PPase enzymes seem to play a role during stress responses (9, 23), and experiments under anoxic conditions in the rice coleoptile showed decreased in PPase activity (24). The recovery of both TmPPase activity bands at the end of the flooding stress experiment suggests an adaptive response, consistent with the ability of this tree species to tolerate frequent flooding, but also suggest a role for PPase enzymes during the transition to the new metabolic state. The data presented in this work also reveal the existence of at least two different PPase isozymes in *T. mucronatum* tissues. In addition, other isoforms may exist, which were not resolved in the zymograms. In addition, the existence of multiple PPase isoforms appears to be extensive to trees. A BLAST search in the *Populus tricocarpa* genome (http://www.phytozome.net/) revealed the existence of at least 5 genes (gb accesions ABK93489.1, ABK93990.1, ABK94595.1, ABK94842.1, ABK95078.1) with high similarity to *A. thaliana* PPase. However, *P. tricocarpa* belongs to the Magnoliophyta group, while *T. mucronatum* belongs to Coniferophyta, implying an important phylogenetic separation.

CONCLUSION

The results in this paper reveal changes in the *Taxodium mucronatum* PPase activity in two unrelated processes: during cone development and under flooding stress. These changes are consistent with an important role of PPase activity during plant growth and development. This work gives evidence for at least two PPase isoforms in *T. mucronatum* and highlights the importance of the PPase activity during conifer tree development and stress adaptation. In our opinion, the metabolism and molecular physiology of this species deserves more studies.

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