Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section B: Environmental Biotechnology

CODEN (USA): JCBPAT Research Article

Influence of Yellow Azo Dye on the Expression Profile of Phenoloxidases of *Pleurotus ostreatus* Grown in Submerged Fermentation

Rubén Díaz[2], Berenice Nava-Galicia[1], Gerardo Díaz-Godínez[2], Martha Dolores Bibbins-Martínez[1].

[1]Centro de Investigación en Biotecnología Aplicada, Instituto Politécnico Nacional, México.
[2]Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, México.

Abstract: *Pleurotus ostreatus* is a white rot fungus capable of degrading many xenobiotic and recalcitrant compounds due to their ability to produce a nonspecific enzyme system able to catalyze the oxidation of many types of organic compounds including textile dyes. Several studies had demonstrated the influence of the structure of dyes on the patterns of expression of phenoloxidases, produced by this fungus during the process of growth and dye oxidation. The objective of this research was to study by RT-PCR the temporal expression of five phenoloxidases genes (1 Po-DyP, 4 Po-DyP, 2 Po-MnP, 3 Po-MnP y 4 Po-MnP) of *Pleurotus ostreatus* grown in submerged fermentation in the presence of yellow azo dye (4-amino-1,1 '-azobenzene 3,4'-disulfonic acid mono-sodium salt). This dye induced the expression of phenoloxidases genes however, the expression level fluctuated in each monitored growth phase. The highest induction was observed for 4 Po-DyP and 4 Po-MnP. Patterns generated for each peroxidase gene, suggest that the 4 Po-DyP in conjunction with 4 Po-MnP genes contribute to the decolourization of the dyes. Furthermore enzymatic and molecular studies of dye peroxidase produced by *Pleurotus ostreatus* could contribute to have enzyme selection criteria leading to the development of effective bioremediation methods.

Key words: *Pleurotus ostreatus*, phenoloxidases, expression, genes, submerged fermentation**.**

INTRODUCTION

The genus *Pleurotus* is a group of ligninolytic fungi that grow and develop in many different environments, are edible with a high nutritional value and the presence of biomolecules with therapeutic properties is suggested also various biotechnological applications[1]. *Pleurotus* fungi produce multiple enzymes, of which the most representative are the phenoloxidases as laccases, manganese peroxidases, versatile peroxidases and dye peroxidase, which allow you to use different ligninolytic substrates to grow[2].

These fungi are easily adapted to different growth conditions including submerged cultures; has been reported to degrade pollutants and phenolic compounds produced in different industries such as textiles and paper, among others. This degradation of xenobiotic compounds is attributed mainly to enzymes phenoloxidases[3]. Versatile peroxidase enzyme (VP) shares catalytic and structural properties with lignin peroxidase (LiP) and manganese peroxidase (MnP), it is able to directly oxidize low redox potential substrates, including phenols, as well as high redox potential dyes that are oxidized by LiP only in the presence of some mediator. VP presents both Mn^{2+} binding site and the catalytic tryptophan[4].

On the other hand, dye peroxidases (DyP) are heme peroxidases that exhibit significant catalytic versatility due to its ability to bleach dyes, including high redox potential anthraquinone derivatives not oxidized by other peroxidases.

Dueñas *et al*.[5] by an in silico analysis determined the presence of four putative genes coding for VP enzymes. Two of which seem to be allelic variants of *Pleurotus ostreatus* MnP1 and MnP2, in addition up to four putative genes coding DyP enzymes were also determine. Castanera *et al*.[2] evaluated the expression profile of laccase genes from different strains of *Pleurotus ostreatus* and they observed that the activity and expression level of these enzymes were dependent on the strain and the growth medium either solid or submerged fermentation.

Diaz *et al.*[1] evaluated the expression profile of five genes of *Pleurotus ostreatus* laccases in submerged fermentation developed different initial pH, where they observed that the expression of genes depends on the initial conditions of development, suggesting the presence of transcription factors activated by pH change in the culture medium.

In this study, the effect of yellow azo dye (4-amino-1,1 '-azobenzene 3,4'-disulfonic acid mono-sodium salt) on expression of five phenoloxidases genes was observed (1 Po-DyP, 4 Po-DyP, 2 Po-MnP, 3 Po-MnP y 4 Po-MnP) of *Pleurotus ostreatus* grown in submerged fermentation (SmF).

METHODS

Microorganism: A strain of *Pleurotus ostreatus* from the American Type Culture Collection (ATCC 32783) (Manassas, Virginia, U.S.A.) was used.

Culture conditions: Mycelial plugs (4 mm diameter) taken from the periphery of colonies of *Pleurotus ostreatus* grown for 7 d at 25 °C in Petri dishes containing potato dextrose agar were used as inoculum. A liquid medium, previously optimized for this fungus in SmF[6], was prepared containing (in g/L): glucose, 10; yeast extract, 5; KH_2PO_4, 0.6; $MgSO_4$-$7H_2O$, 0.5; K_2HPO_4, 0.4; $CuSO_4$-$5H_2O$, 0.25; $FeSO_4$-$7H_2O$, 0.05; $MnSO_4$, 0.05; $ZnSO_4$-$7H_2O$, 0.001.

Two fermentation by triplicate were performed, each was adjusted the initial pH at 6.5, using 0.1 M HCl. Flasks of 150 mL containing 50 mL of culture medium were inoculated with three mycelial plugs each.

The cultures were incubated at 25°C for 23 days on a rotary shaker at 120 rpm (12). For biomass the samples were taken every 24 h after the third day of growth, and for expression patterns were obtained at 168, 192, 216, 240, 264, 288, 312 and 336 h of fermentation. Gene expression was observed by RT-PCR[7].

Biomass evaluation

The biomass (X) was determined as difference of dry weight (g/L).

Assay of biomass X = X(t) was done using the Velhurst-Pearl or logistic equation,

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{max}}\right]X$$

Where μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when dX/dt= 0 for $X > 0$. The solution of equation 1 is as follows;

$$X = \frac{X_{max}}{1 + Ce^{-\mu t}}$$

Where, $C = (X_{max} - X_0)/X_0$, and $X = X_0$; the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using a non-linear least square-fitting program "Solver" (Excel, Microsoft)[8].

Total RNA Isolation and RT-PCR: A RT-PCR semi-quantitative was used to study phenoloxidases genes expression of *Pleurotus ostreatus*. RNA was isolated from frozen mycelium produced at different fermentation times (see culture conditions section), using the TRIZOL (Invitrogen) extraction and was spectrophotometrically quantified by determining the optical density at $OD_{260/280}$. RNA was treated with DNAse I free of RNAse (Invitrogen) and then resuspended in 20 μL of diethylpyrocarbonate-treated water.

cDNA synthesis was performed using oligosdT and Moloney *murine Leukaemia* virus reverse transcriptase (M-MuLV-RT; Fermentas) according to the manufacturer's instructions. The RT reaction mixture product (1 μg) and 10 ρmol of specific primers were mixed for specific PCR amplification using the Kit Accses Quick™ (PROMEGA™). Denaturation conditions were 94 °C for 3 min.

The program included 35 cycles of 94°C for 40 s, 56°C for 40 s and 72°C for 50 s. Constitutive expression of glyceraldehyde-3-phosphate dehydrogenase (gpd) was tested as housekeeping. For the design of isoenzyme phenoloxidases specific primers (Table 1), the open *primer-blast* software was used

http://genome.jgi.doe.gov/PleosPC15_2/PleosPC15_2.home.html. Complete sequences of mRNAs of phenoloxidases (1 Po-DyP, 4 Po-DyP, 2 Po-MnP, 3 Po-MnP y 4 Po-MnP) reported in the Gene bank: http://genome.jgi.doe.gov/cgi-bin/disp Transcript?db=PleosPC15_2&id=1096331&useCoords=1were used. Complete sequences of mRNAs of phenoloxidases(1 Po-DyP, 4 Po-DyP, 2 Po-MnP, 3 Po-MnP y 4 Po-MnP) reported in the Gene bank: http://genome.jgi.doe.gov/cgi-bin/dispTranscript?db=PleosPC15_2& id=1096331&useCoords=1were used.

Table 1: Specific primers of phenoloxidases genes of *Pleurotus ostreatus* grown in SmF with and without yellow azo dye.

Access Number (JGI)	Genes	Primer´s name	Sequence (5´-3´)
62271	estExt_fgenesh1_pm.C_90223	1 Po-DyP	Fw GCTGGAGGAGATTCAAGGAG Rv AGTGCAGACCATTTGAGCAG
1069077	estExt_Genewise1Plus.C_111427	4 Po-DyP	Fw GGTATCTCCAACCCTGCTGT Rv CGAGGAAGTCGTCAAACTCA
1096331	estExt_fgenesh1_pg.C_040032	2 Po-MnP	Fw CGTTACCAACTGTCCTGGTG Rv CCAGACAACCTCGACAGAGA
199491	fjr_genemark.7107_g	3 Po-MnP	Fw GTCGTCTGGCTCCTTTCTTC Rv AAGGAAGTTCCACGGAGTTG
199491	fjr_genemark.7107_g	4 Po-MnP	Fw TATCGCTCGTCACAACATCA Rv GAAAGGCTCTGGGACAAGAC
GU062704.1 AB690874.1	Gliceraldehyde-3-phosphate-deshydrogenase	gpd	Fw TCTGCGGTGTTAACCTTGAGTCGT Rv TGGTAGCGTGGATGGTGCTCATTA

RESULTS

Figure 1 shows X of *Pleurotus ostreatus* grown in SmF without dye (a) and in the presence of azo yellow dye (b) at initial pH 6.5 of development, μ values of 0.020 and 0. 016 h^{-1} respectively, were observed. The fungus can grow and develop in the presence of compounds such as textile dyes without significantly altering their growth.

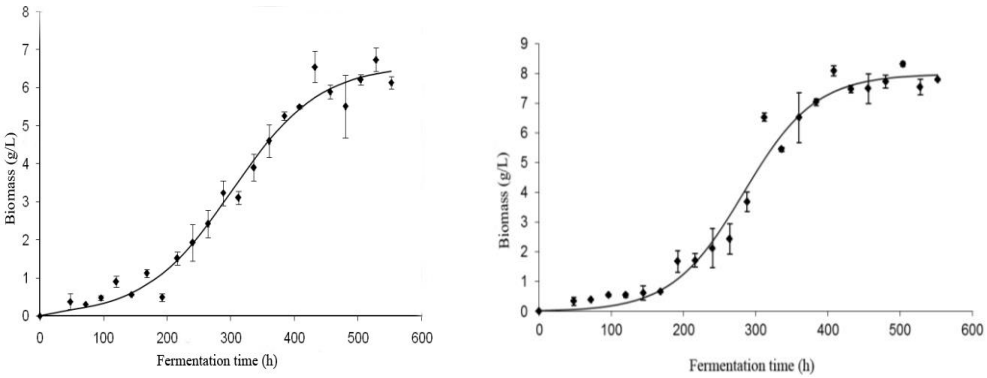


Fig.1: *Pleurotus ostreatus* grown in SmF without dye (a) and in the presence of azo yellow dye (b).

Figures 2 and 3 show the extraction of total RNA from mycelia of *Pleurotus ostreatus* at sampling times, for SmF without dye (a) and added with yellow azo dye (b). Figures 2b, 2c, 3b and 3c show the total RNA with concentration setting of 300 ng/μL and in presence of DNAse I. Table 2 shows the concentration of total RNA for each point of the fermentations performed.

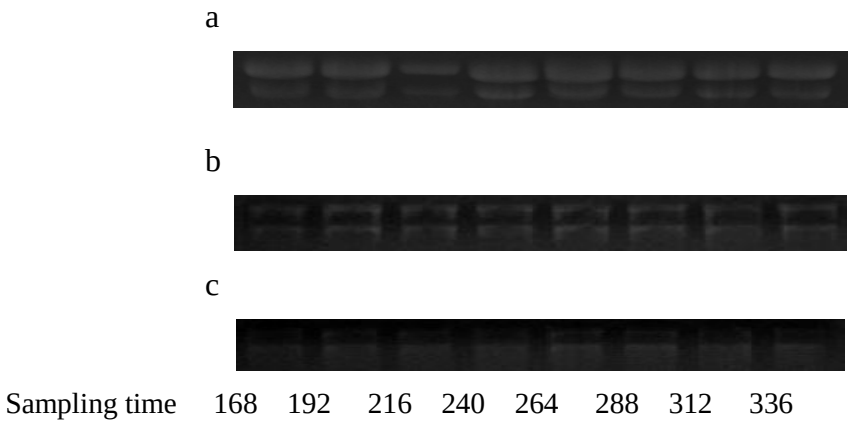


Fig. 2: Profile of total RNA (a), set at 300 ng/µL (b) and treatment with DNAse (c), from mycelium of *Pleurotus ostreatus* grown in SmF without dye.

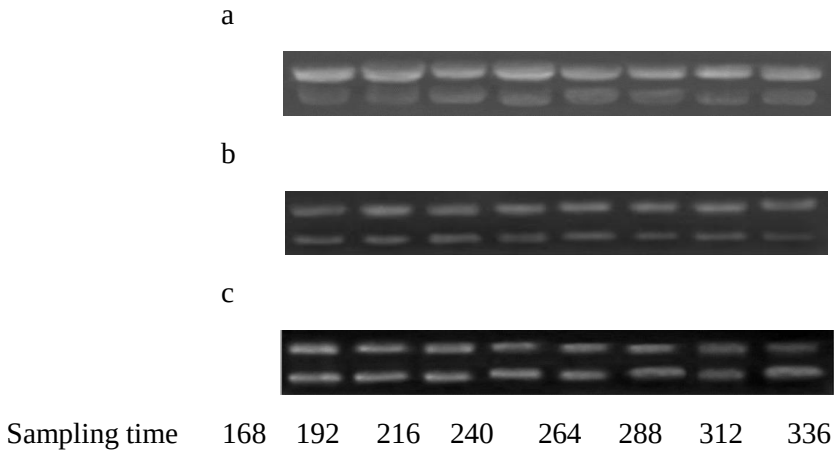


Fig. 3: Profile of total RNA (a), set at 300 ng/µL (b) and treatment with DNAse (c), from mycelium of *Pleurotus ostreatus* grown in SmF added with yellow azo dye.

The expression patterns were determined by RT-PCR for genes 1 Po-DyP, 4 Po-DyP, 2 Po-MnP, 3 Po-MnP and 4 Po-MnP as seen in Figure 4, the constitutive gene gpd (glyceraldehyde 3-phosphate dehydrogenase) was maintained throughout the fermentation.

In the fermentation without dye, only 1 Po-DyP, 4 Po-DyP and 3 Po-MnP genes were expressed, although the gene with the highest expression under these growth conditions was 1 Po-DyP, followed by 3 Po-MnP. The constitutive gene was observed in all evaluated times.

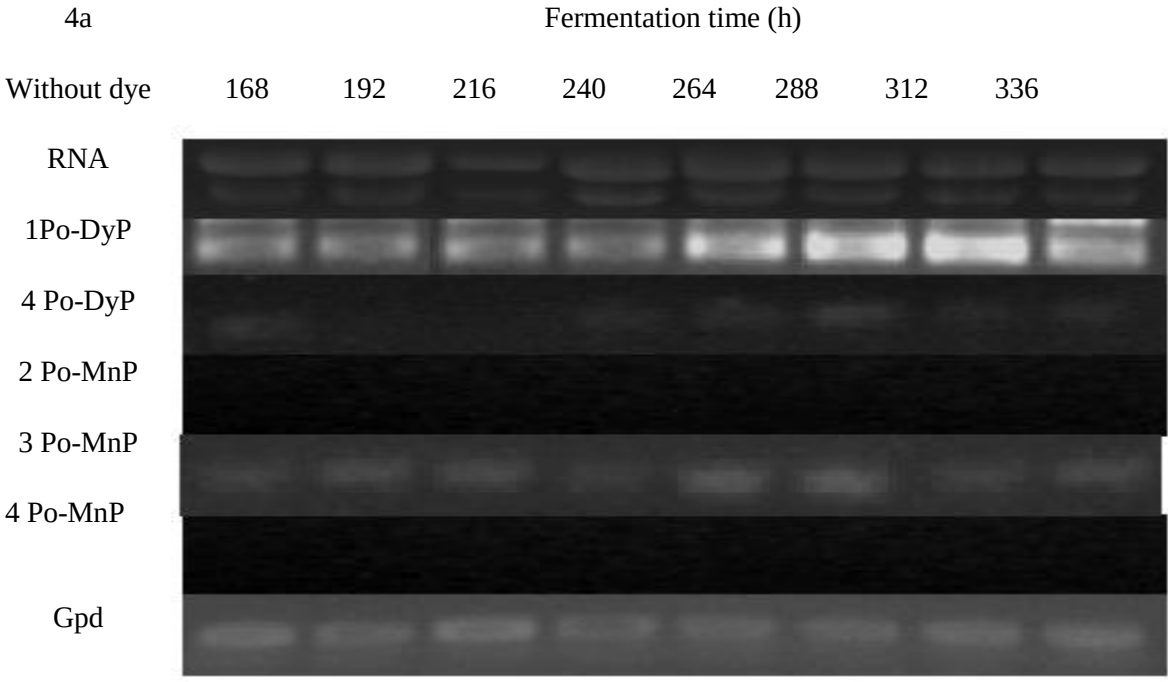
In the fermentation with dye, expression of 1 Po-DyP gene in the early times of exponential growth phase was observed, suggesting that this gene has a higher expression in the organism at adaptation stage. Furthermore the 4 Po-DyP gene was observed in all times of fermentation.

In the fermentation with dye, expression of 1 Po-DyP gene in the early times of exponential growth phase was observed, suggesting that this gene has a higher expression in the organism at adaptation stage. Furthermore the 4 Po-DyP gene was observed in all times of fermentation.

Table 2: Concentration of total RNA obtained from macerated mycelium of *Pleurotus ostreatus* grown in SmF.

Fermentation time (h)	Concentration of total RNA (ng/µL) without dye	Concentration of total RNA (ng/µL) with dye
168	614.1	3097.4
192	1061.6	8842.5
216	911.1	1301.9
240	767.6	9472.4
264	710.0	11509.6
288	1394.4	9109.9
312	787.2	2942.9
336	1313.4	2985.2

2 Po-MnP and 4 Po-MnP genes were expressed with higher level at exponential growth phase and 3 Po-MnP gene showed very low expression, maybe by the repression induced by dye.4 Po-DyP and 4 de Po-MnP genes were expressed at high level in presence of dye. In general, 1 Po-DyP and 3 Po-MnP genes were expressed mainly in the fermentation without dye and 4 Po-DyP, 2 Po-MnP and 4 Po-MnP genes were expressed predominantly in the fermentation with dye.



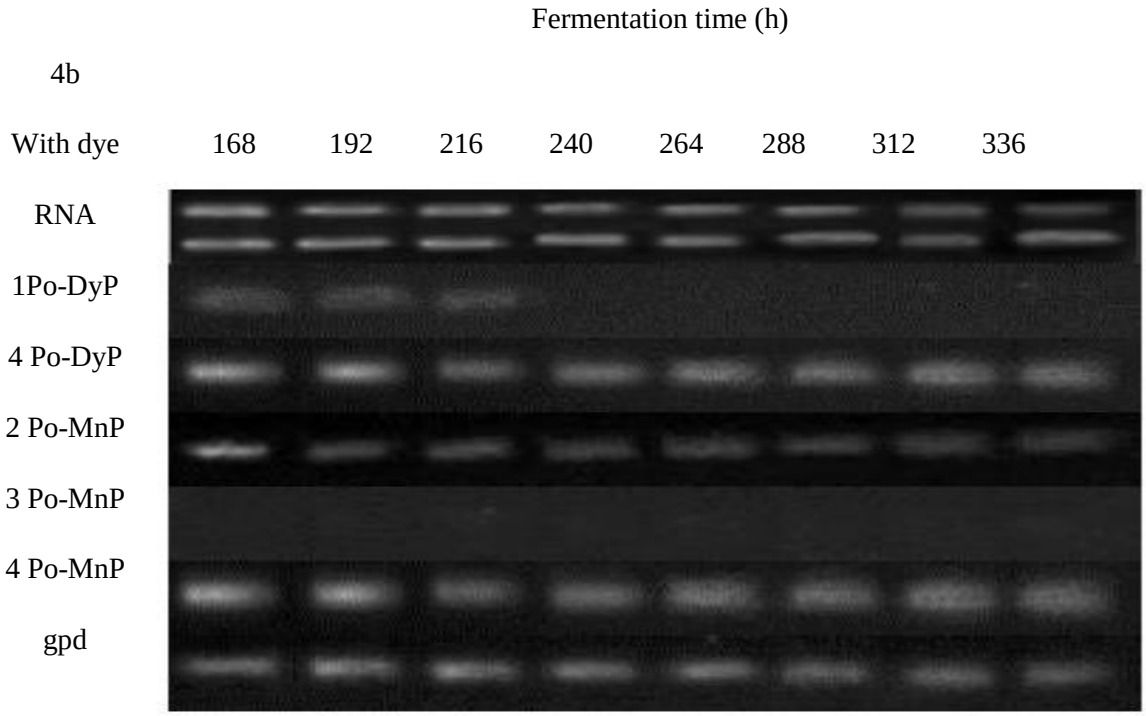


Fig. 4: Expression profile of genes: 1 Po-DyP, 4 Po-DyP, 2 Po-MnP 3 Po-MnP 4 Po-MnP and gpd in SmF without dye (4a) and in the presence of dye (4b).

CONCLUSIONS

The presence of xenobiotics in the culture medium is an important factor which regulates the expression of the phenoloxidases genes in addition to having an effect on the activity and number of isoenzymes produced. These results contribute with the understanding of the regulation of the expression of the phenoloxidases genes.

ACKNOWLEDGEMENTS

Thanks to CIBA-IPN Tlaxcala (project CONACYT CB. No. 134348) and to Universidad Autónoma de Tlaxcala (project CACyPI-UATX-2014) for supporting this research.

REFERENCES

1. R. Díaz, M. Téllez-Téllez, C. Sánchez, M.D Bibbins-Martínez, G. Díaz-Godínez, J. Soriano-Santos. Influence of initial pH of the growing medium on the activity, production and genes expression profiles of laccase of *Pleurotus ostreatus* in submerged fermentations. *Electron. J. Biotechnol.*vol., 2013, 16 (4): fulltext 6.
2. R. Castanera, G. Pérez, O. Omarini, M. Alfaro, A.G. Pisabarro, V. Faraco, A. Amore, L. Ramírez. Transcriptional and enzymatic profiling of *Pleurotus ostreatus* laccase genes in

submerged and solid-state fermentation cultures. *Appl. Environ. Microbiol.*vol., 2012, 78 (11): 4037-4045.

3. M. Téllez-Téllez, G. Díaz-Godínez, M.B. Aguilar, C. Sánchez, F. Fernández. Description of a laccase gene from *Pleurotus ostreatus* expressed under submerged fermentation conditions. *Bioresources.*vol., 2012, 7(2): 2038-2050.
4. F.J. Ruiz-Dueñas, E. Fernández, M.J. Martínez, A.T. Martíne. *Pleurotus ostreatus* heme peroxidases: An in silico analysis from the genome sequence to the enzyme molecular structure. *C. R. Biol*. Vol., 2011, (334): 795-805.
5. F.J. Ruiz-Dueñas, M. Morales, E. García, Y. Miki, M.J. Martínez, A.T. Martínez. Substrate oxidation sites in versatile peroxidase and otherbasidiomycete peroxidases. *J. Exp. Bot*. vol., 2009, 60(2): 441-452.
6. M. Téllez-Téllez, F.J. Fernández, A.M. Montiel-González, C. Sánchez,G. Díaz-Godínez. Growth and laccase production by *Pleurotus ostreatus* in submerged and solid-state fermentation. *Appl. Microbiol. Biotechnol.*vol., 2008, (81): 675-679.
7. R. Díaz, S. Alonso, C. Sánchez, A. Tomasini, M.D. Bibbins-Martínez, G. Díaz-Godínez. Characterization of the growth and laccase activity of strains of *Pleurotus ostreatus* in submerged fermentation. *Bioresources*. vol., 2011, 6(1):282-290.
8. G. Díaz-Godínez, J. Soriano-Santos, C. Augur, G. Viniegra-González. Exopectinases produced by *Aspergillus niger* in solid state and submerged fermentation: a comparative study. J. *Ind. Microbiol. Biotechnol.*vol., 2001, (26): 271-275.

*** Corresponding author: Rubén Díaz**

Centro de Investigación en Ciencias Biológicas, UATx México.
rdiazgod@hotmail.com