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African Journal of Biotechnology

*Full Length Research Paper*

# Characterization of fluorescent *Pseudomonas* from *Oryza sativa* L. rhizosphere with antagonistic activity against *Pyricularia oryzae* (SACC.)

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**The aim of this work was to select and characterize native Cuban fluorescent *Pseudomonas* from the rhizosphere of *Oryza sativa* with *in vitro* and *in vivo* antagonistic activity against *Pyricularia oryzae*. A total of 200 isolates were recovered with the typical growth and cultural characteristics of fluorescent *Pseudomonas*. The results showed that 12.5% of total isolates were capable of inhibiting mycelial growth, with different levels of inhibition between strains. Six of them (AI03, AI05, AI08, AJ01, AJ13 and AJ29) were able to reduce disease severity and incidence *in vivo* whereas only four (AI05, AJ13, AJ01 and AI08) showed efficient control of *P. oryzae* in greenhouse conditions. *Pseudomonas fluorescens* AI05 and *Pseudomonas putida* AJ13 were able to inhibit *in vitro* the mycelial growth of *P. oryzae* and to reduce symptom severity of *Pyricularia* infection. The production of lytic enzymes, siderophores, hydrogen cyanide (HCN), as well as the detection of genes encoding antibiotics and bacterial motility were also assessed for both strains. They were able to fix nitrogen, produce indolic compounds and to solubilize Pi. These results demonstrate the potential use of *P. fluorescens* AI05 and *P. putida* AJ13 as a biocontrol agent for the protection of rice plants from *P. oryzae* infection.**

**Key words:** Biocontrol, rice blast, *Pyricularia*, *Pseudomonas*, plant growth-promoting bacteria.

## INTRODUCTION

Rice (Oryza sativa L.) is an important crop for human consumption. This cereal is the staple food for over half the world population and is considered the world's largest by the size of the area where it is grown and the number of people who depend on their crop (IRRI, 2002). In Cuba, rice is an important part in the daily diet of the population and current per capita consumption is among the highest in Latin America, contributing 20% of daily calories consumed (Cárdenas et al., 2007). The area planted with rice in Cuba is approximately 138 455 ha and the production was estimated to be around 250 000 tons per year (MINAG, 2006).

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Controlling diseases that may attack the crop is of great importance to increase productivity in rice growing areas. In particular, fungi have been described as causal agents of various rice diseases (Herrera, 2003). Rice blast, caused by *Pyricularia oryzae* (teleomorph = *Magnaporthe oryzae*) is one of the most important diseases, due to its worldwide distribution, destructiveness and high degree of pathogenicity (Thuan et al., 2006; Rodríguez et al., 2007). Rice blast can cause up to 100% in yield reduction, depending on the cultivar susceptibility, cropping technology and climatic conditions (Prabhu et al., 2009, Magar et al., 2015). Although infections are mostly reported to occur in leaves and stems, seeds and roots may also be infected by *P. oryzae*, using the typical developmental processes of root-infecting fungi (Sesma and Osbourn, 2004).

In Cuba, rice blast is the most relevant disease in rice crop and its incidence is closely related to environmental conditions, such as high relative humidity and temperature (Rodríguez et al., 2007; Almaguer-Chavez et al., 2012). Application of chemical inputs for rice blast control and prevention is not only ineffective, but also hazardous to the environment (Padovani et al., 2006). On the other hand, recent studies have linked nitrogen fertilization with an increase in rice blast severity (Sester et al., 2014).

Plant growth-promoting bacteria (PGPB) could be a good alternative for plant disease management and for reducing the amount of chemical fertilizers applied to rice crop (Adesemoye et al., 2009), as well as a means to cut down production cost (Gupta et al., 2015; Liu et al., 2018). Within PGPB, fluorescent *Pseudomonas* are often described as excellent plant growth promotion and biocontrol agents (Höfte and Altier, 2010; Pathma et al., 2011; Ghirardi et al., 2012). The main microbial mechanisms involved in control of pathogens include direct antagonism, through the production of antimicrobial metabolites (antibiotics, cyclic lipopeptides, volatile compounds, siderophores and a wide variety of lytic enzymes) and competitive exclusion of other microbial groups (Pathma et al., 2011). Competition for nutrients is another mechanism that contributes to plant protection by PGPB (Acebo-Guerrero et al., 2015).

The efficiency of plant growth-promoting rhizobacteria (PGPR) as a biocontrol strategy to fight against fungal diseases in rice has been documented (Filippi et al., 2011; Sharifi-Noori et al., 2015; Prasanna Kumar et al., 2017). These potential biocontrol agents have been mainly focused on bacteria of the genera *Bacillus* (Chaiharn et al., 2009; Rojas-Badía et al., 2011) and *Pseudomonas* (De Vleesschauwer et al., 2006; Hernández-Rodríguez et al., 2010). Fluorescent *Pseudomonas* are one of the microbial groups widely used as bacterial antagonists of *P. oryzae* (Gohel and Chauhan, 2015; Sharifi-Noori et al., 2015). However, these reports are often limited to the assessment of *in vitro* antagonistic activity and lack a thorough identification and physiological characterization of the antagonists. In addition, to achieve an efficient bacterial product, native strains of each edaphoclimatic regions must be selected.

The aims of this work were to select and characterize fluorescent *Pseudomonas* from the rhizosphere of *O. sativa* L. with antagonistic activity against *P. oryzae* (SACC.), and to evaluate their *in vivo* biocontrol efficiency.

## MATERIALS AND METHODS

### Isolation and screening of *in vitro* antagonistic activity against *P. oryzae*

The isolation of fluorescent *Pseudomonas* was done using the Microcosm model (Kabir et al., 1995) from rice rhizosphere samples. The rhizosphere samples were taken from 15-day-old plants (*O. sativa* L. cv. J-104) sown in field that had never been biofertilized. A soil sample was analyzed by the Chemical Analysis Laboratory from the National Agricultural Science Institute according to Paneque (2000). Some of the agrochemical characteristics of the soil were the following: pH 7.2, organic matter percentage 3.92%, phosphorus 125 ppm, potassium 0.76 cmol·kg$^{-1}$, calcium 28.5 cmol·kg$^{-1}$, magnesium 3.5 cmol·kg$^{-1}$).

All the bacterial isolates were screened for *in vitro* antagonism against *P. oryzae.* To determine *in vitro* antagonistic activity, five strains of *P. oryzae* (AAM-275, AAM-361, AAM-403, AAM-425 and AAM-903) previously isolated from the aerial ecosystem of rice fields by Almaguer-Chavez et al. (2012) that belonged to the Fungi Culture Collection of the Faculty of Biology of the University of Havana were used in this study. The fungal strains were cultured in Potato Dextrose Agar (PDA; Merck, Germany) for 5 days at 28°C. The selected bacterial isolates were grown in King B (KB) broth (Merck, Germany) for 24 h at 28°C at 200 rpm. The cell concentration was adjusted to $10^8$ cfu·mL$^{-1}$ and 100 µL of each bacterial culture was spread on 9-cm-diameter Petri dishes containing KB Agar (Merck, Germany) and straightaway, a 5-mm plug from the leading edge of a 5-days-old fungal culture was placed in the centre of each Petri dish. The control was set by inoculating only the fungus in KB Agar. The plates were incubated for 7 days at 28°C. The fungal inhibition was scored by measuring radial growth of the fungus (in mm) in every plate and the inhibition percentage was calculated through a comparison with the control plate, according to Bashan et al. (1996). The experiment was repeated three times with five replicates per treatment. For each isolate, an inhibitory efficiency index (IEI) was calculated against all 5 fungal strains as the sum of the inhibition frequency of each isolate of the fungal strains and the average inhibition for each bacterial strain (Hernández-Rodríguez et al., 2010). The isolates with an IEI higher than 1.0 were selected for further studies.

### *In vivo* evaluation of antagonistic activity of selected isolates

The bioassay was conducted on *O. sativa* cv. J-104. For disinfection, seeds were surface decontaminated with calcium hypochlorite according to Hernández-Rodríguez et al. (2008). Some seeds were placed in a Nutrient Agar plate to confirm the disinfection process. P. oryzae AAM-275 was selected for the assays in plants, with the intention to assess if the antagonistic isolates were able to control the most common strain pathogen in

the rice agroecosystem (Almaguer-Chávez et al., 2012).

The fungal inoculum was prepared from Potato Dextrose Broth (PDB; Merck, Germany) liquid cultures incubated at 30°C for 7 days in intervals of light/darkness (12 h/12 h) at 250 rpm. The cultures were then centrifuged and the supernatant containing the spores was kept for further use. Part of the soil was infected by mixing it thoroughly with the fungal inoculum adjusted to a final concentration of $10^5$ spores/g of soil. The bacterial inocula were prepared by culturing the isolates overnight in KB Broth (30°C, 250 rpm) and adjusting the cell density to $10^8$ cfu·mL$^{-1}$ and they were then inoculated on the seeds through their immersion in the culture broth for 45 min. Then the following treatments were established: T1, non-inoculated seeds in uninfected soil control; T2, non-inoculated seeds in fungus-infected soil control; T3 to T14, seeds inoculated with the selected bacteria in the fungus-infected soil. The plants were kept at 30°C, with a 16 h light photoperiod. The antagonistic effect was evaluated 21 days after seed germination. A completely randomized design was established with nine replicates per treatment and three repetitions of the experiment. Fungal suppression was based on observations of symptoms of fungal disease in the roots, since Sesma and Osbourn (2004) demonstrated that *P. oryzae* is able to infect rice roots using the typical developmental processes of root-infecting fungi. The disease incidence was based on the percentage of roots with disease symptoms in nine randomly selected rice plants. Disease severity was assessed using the following scale (Bigiramana et al., 2000): scale 1, no symptoms shown; scale 3, infected roots up to 1%; scale 5, infected roots up to 5%; scale 7, infected roots up to 10%; scale 9, more than 25% of the roots exhibited disease symptoms and the seeds were severely damaged. Disease severity and control efficacy were calculated as follows: Disease severity = [Σ(The number of diseased plants in this index × Disease index) / (Total number of plants investigated × The highest disease index)] × 100%. Control efficacy = [(Disease severity of control × Disease severity of treated group) / Disease severity of control] × 100% (Yang et al., 2009).

### *In planta* disease severity assay

The strains that were effective in the control of the disease symptoms in the *in vivo* preliminary test were selected to perform the experiment in greenhouse conditions. Disinfected rice seeds from cv. J-104 were used. Bacterial and fungal inocula were prepared and the inoculation of bacteria in the seeds was carried out as described earlier. Then they were planted in 30-cm-diameter pots in a greenhouse at 30°C, with a photoperiod of 16 h of light. The fungus was inoculated by foliar spraying of a dilution of $10^5$ spores·mL$^{-1}$ when plants were 60 days old. Treatments of 20 replicates comprising one plant per pot were established. The treatments were: T1, non-inoculated control; T2, fungus-inoculated control; T3-T8, seeds inoculated with the selected bacterial strains and then inoculated with the fungal strain.

Plants were arranged in a completely randomized block and watered three times a week with sterile, distilled water. The percentage of plants showing the characteristic blast ellipsoidal lesions on leaves was determined 7 days post-inoculation with the fungal strain (Ribot et al., 2008). Then the reduction of disease symptoms was determined as follows: the percentage of plants showing disease symptoms was evaluated, considering the fungus-inoculated control as 100%, and the resulting value of the subtraction from this 100% and the percentage obtained with the rest of the treatments was considered to be the reduction of disease symptoms for each treatment (Hernández-Rodríguez et al., 2008). The experiment was repeated three times. The best performing strains were selected to continue their further characterization.

### Molecular characterization of the selected isolates

For the most promising strains, partial regions of the *16S rRNA* were sequenced, using the primers 27f and 1492r (Table 1). Amplification was performed, with the following temperature program: initial denaturing at 95°C for 5 min, 30 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by a final extension step of 10 min at 72°C. Polymerase chain reaction (PCR) products were visualized by electrophoresis in agarose gel (1.5%). PCR products were cloned using the TOPO TA Cloning Kit®, according to the manufacturer's instructions (Invitrogen Life Technologies SA). The selected clones (three per strain) were sequenced by GATC Biotech® (Germany).

Phylogenetic analysis of *16S rRNA* gene sequences was carried out using these isolates and the *Pseudomonas* type strains with the most similar sequences retrieved from GenBank. Sequence alignment was carried out using the program MEGA 6 (Tamura et al., 2013) and corrected manually. A maximum likelihood tree was used to build an individual tree for 16srDNA with the models that best fitted the data, and confidence analysis was undertaken using 1000 bootstrap replicates.

### Physiological characterization of selected isolates

Lipase activity was detected according to Poritsanos (2005) in Luria Bertani (LB; Merck, Germany) agar medium supplemented with Tween 80 (2% v/v) and $CaCl_2$ (0.22 g.L$^{-1}$). Protease activity was detected in Nutrient Agar supplemented with 2.5% (w/v) skimmed milk (Nestlé) according to Perneel et al. (2007).

Pyoverdine production was quantified according to Meyer and Abdallah (1978), using overnight cultures of the bacteria in Casamino Acid (CA) Broth (Bacto Casamino Acid, BD, 5 g.L$^{-1}$; $K_2HPO_4$ 1.18 g L$^{-1}$; $MgSO_4.7H_2O$ 0.25 g L$^{-1}$) (28°C at 170 rev min$^{-1}$).

The ability to produce hydrogen cyanide was detected in Tryptic Soy Agar (Merck, Germany) amended with glycine (4.4% w/v), using the picrate/$Na_2CO_3$ saturated filter paper, fixed to the underside of Petri dish lids, which were sealed with parafilm before incubation at 28°C for 96 h (Kremer and Souissi, 2001).

The screening for phlD, prnC, phzCD and pltB (involved in the production of the antibiotics 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), phenazines (PHZ), pyoluteorin (PLT), respectively) was carried out by PCR, according to Mavrodi et al. (2001) and Calderón et al. (2013). Primers are listed in Table 1. Antibiotic production was confirmed by TLC, as described elsewhere (Whistler et al., 2000; Cazorla et al., 2006). Briefly, bacterial cultures (15 ml) of 4 days in King B broth at 28°C without shaking were centrifuged at 3800 g for 15 min. The supernatants were extracted twice with 5 ml ethyl acetate, left to dry out and resuspended in 100 ml of methanol. The metabolites were detected by thin layer chromatography using silica RP-18F254S TLC plates (Merck AG, Germany). *Pseudomonas protegens* Pf-5 was used as control for PLT, PRN, and DAPG; *Pseudomonas chlororaphis* 30 to 84 for PHZ.

Additional analyses were carried out using high-pressure liquid chromatography (HPLC) and mass spectra (MS) to identify bioactive compounds by comparison with the UV spectra and molecular weight of reference compounds. Selected strains were grown in Casamino Acids Agar (CAA) for 96 h at 28°C (seven plates per strain). Agar (with bacteria) was removed from the plates, placed in glass bottles and 300 mL of ethyl acetate were added. Extraction was carried out for 3 h while shaking at room temperature and the organic phase was transferred to a round-bottom glass flask and flash evaporated, and the residue was dissolved in 1 ml of HPLC-grade methanol for the analyses. Analytical HPLC was performed by using a Waters apparatus equipped with a 626 pump, a 626

**Table 1.** Primers used in this study.

| Primer | Sequence (5'-3') | Amplicon size (bp) | Amplified gene | Annealing PCR temperature (°C) | Reference |
|---|---|---|---|---|---|
| 27f<br>1492 | ACTCCTACGGGAGGCAG<br>GGCGTCTGTACAAGGCCCGG | ~1500 | *16s rDNA* | 55 | Lane (1991) |
| Phl2a<br>Phl2b | GAGGACGTCGAAGACCACCA<br>ACCGCAGCATCGTGTATGAG | 745 | *phlD* | 58 | Mavrodi et al. (2001) |
| PrnCf<br>PrnCr | CCACAAGCCCGGCCAGGAGC<br>GAGAAGAGCGGGTCGATGAAGCC | 719 | *prnC* | 58 | Mavrodi et al. (2001) |
| PHZ1<br>PHZ2 | GGCGACATGGTCAACGG<br>CGGCTGGCGGCGTATTC | 1400 | *phzCD* | 56 | Delaney et al. (2001) |
| PltBf<br>PltBr | CGGAGCATGGACCCCCAGC<br>GTGCCCGATATTGGTCTTGACCGAG | 790 | *pltB* | 58 | Mavrodi et al. (2001) |
| 30-84/1(F)<br>30-84/2(R) | CAGTTCATCCGGCGGGCTGCA<br>CCCGTTTCAGTAAGTCTTCCATGATGCG | 1200 | *phzX* | 58 | Mavrodi et al. (1998) |

controller and a 996-photodiode array detector. Samples were analysed on an HPLC apparatus (Waters 600 system) coupled to both UV (Waters 2487 detector) and mass (Micromass Waters VG Quattro II mass spectrometer) detectors. A BIO Wide Pore C-18 column (250 × 4.6 mm; 5 µm) was used and the solvent elution consisted of a linear gradient of water and acetonitrile (from 5 to 100% acetonitrile in 30 min) at a flow rate of 1 ml/min. After UV detection at 215 and 254 nm, the column elute was split (LC Packings splitter), and 0.1 $ml.min^{-1}$ was directed to the mass spectrometer fitted with an ESI interface. For mass detection, analyte ionization was achieved by using the positive electrospray mode. The ESI parameters used were: nebulizing gas ($N_2$, 20 $l.h^{-1}$), drying gas ($N_2$, 250 $l.h^{-1}$), source temperature (80°C), cone voltage (35 V) and capillary voltage (35 kV). The total ion current scanning was from m/z 115 to 1000 with 1 s/scan. Nitrogenase activity was assessed by the acetylene reduction assay (ARA), described by Boddey et al. (1990). The bacteria were grown in 15-ml bottles containing 5 ml of semi-solid Nfb medium without pH indicator and capped with cotton stoppers. These flasks were incubated at 30°C for 48 h. After the formation of the film, cotton stoppers were removed aseptically and replaced with sterile pierceable rubber stoppers (Suba·Seal, Sigma-Aldrich, USA). Immediately after, 1 ml of air was taken from each bottle with a syringe and 1 ml of acetylene was injected in the bottles and they were incubated at 30°C for an hour. To assess the concentration of ethylene in the flasks, 0.5 ml of the gas phase was injected into a gas chromatograph with flame ionization (Perkin Elmer model F11).

Indole production was analyzed by the microplate method (Sarwar and Kremer, 1995). Bacteria were grown for 42 h in 20 ml of DYGS broth supplemented with L-tryptophan (200 $µg·ml^{-1}$) in the dark, at 33°C and with 150 rpm. Aliquots of 1 ml were centrifuged at 10,000 rpm for 15 min and 150 µl of the supernatant were mixed with 100 µl of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 49 ml of 35% perchloric acid) in U-type 96-well plates. The samples remained in the dark for 30 min at room temperature. The absorbance ($A_{540}$) was measured with a microplate reader (Labsystemiems reader MF, Labsystem). The quantification of indole compounds was achieved using a calibration curve prepared with serial dilutions of IAA standards. The results were expressed in $µg·ml^{-1}$ of IAA per unit of protein, using 3 replicates for each strain.

The ability of the strains to solubilize Pi was tested on NBRIP plates containing 5% tricalcium phosphate (Nautiyal, 1999). The diazotrophic *Gluconacetobacter diazotrophicus* strain $PAL5^T$ (BR 11281) (Maheshkumar et al., 1999) was used as a positive control. The assay was carried out using a randomized design with three replicates. Four 20-µl drops of cell suspension (log-phase) were inoculated on the surface of NBRIP Agar plates and incubated for 15 days at 30°C. The diameter of the solubilization halo (translucent area around the colony) and the colony diameter were used to calculate the solubilization index (SI), according to Kumar and Narula (1999).

The quantification of soluble P was carried out with the phosphomolybdenum blue method according to Chen et al. (2006). The *G. diazotrophicus* strain $PAL5^T$ was utilized as a positive control. The strains were grown in liquid DYGS medium (Rodrigues et al., 2006) for 24 h at 30°C and 150 rpm. The cultures were adjusted to 0.9 to 1 ($OD_{600}$) with saline solution and 5 mL was used to inoculate 250 mL Erlenmeyer flasks containing 45 mL NBRIP medium supplemented with 0.05% $Ca_3(PO_4)_2$ (tricalcium phosphate) as a phosphorus source. The flasks were incubated in a shaker at 30°C and 150 rpm. The experiment was carried out using a randomized design with three replicates. Samples were taken 5 days after inoculation. Changes in the pH of the culture medium as well as the bacterial population, measured by the microdrop technique (Spencer and Ragout, 2001) were monitored during the growth period.

Swarming and twitching motilities were tested in LB medium (Merck, Germany) with Agar concentrations of 0.6 and 1%, respectively, according to Rasamiravaka et al. (2013), with slight modifications. The inoculum was 5 µl of an overnight culture of the strains in KB broth (28°C at 170 rev $min^{-1}$) diluted to an $OD_{600}$ of 1. To assess swarming, the inoculum was placed on the Agar surface and swarming motility was recorded as the diameter of bacterial growth in mm after 48 h of incubation at 28°C and any diameter

higher than twofold the inoculation diameter (5 mm) was considered positive. For twitching, the inoculum was deposited in between the Agar surface and the bottom of the plate. Twitching was recorded as the diameter of the stained area and any bacterial strain that had a detectable twitching zone upon visible inspection was scored as positive. Three replicates were established by strain and the experiment was repeated three times.

#### Statistical analysis

Normality and homogeneity of variance tests were carried out for all the data. Data were processed with the analysis of variance (ANOVA) and means were separated with Tukey's honest significance test (plant growth data) and Fisher's Protected Least Significant Difference (LSD) (disease severity, control efficacy, percentage of infected leaves per plant and disease reduction). Kruskall-Wallis' ANOVA and Mann-Whitney U test were performed for the data that did not fulfill the normality criteria to assess significant differences ($P<0.05$). A representative repetition of each experiment was used for the figures. Standard deviation was calculated and indicated as vertical bars in the figures. All tests were performed using the 7 software STATISTICA 8.0 (Stat Soft, Inc., Tulsa, OK, USA).

## RESULTS

### Selection of the isolates with *in vitro* antagonistic activity against *P. oryzae*

In this study, from the 200 isolates that showed the typical characteristics of fluorescent *Pseudomonas*, 25 (representing 12.5% of the isolates) were capable of inhibiting mycelial growth of at least one of the five fungal strains (Figure 1). However, the inhibition varied depending on the fungal strain the bacteria were faced with. When *P. oryzae* AAM-275 was tested (Figure 1A), it was shown that 19 of the isolates were capable of inhibiting the mycelial growth, from 54 to 84%. *P. oryzae* AAM-903 and AAM-403 were inhibited by 15 of the isolates, from 27 to 100% and 28 to 100% of mycelial growth inhibition, respectively (Figure 1B and C). *P. oryzae* AAM-425 was inhibited by 11 isolates, with 44 to 84% of mycelial growth inhibition (Figure 1D). The isolates showed less antagonistic effect against *P. oryzae* AAM-361, since only 10 of them were capable of inhibiting fungal growth from 36 to 89% (Figure 1E). The inhibitory efficiency index for each isolate was calculated and the isolates AJ01, AI05, AJ13, AJ30, AI08, AJ28, AJ29, AI03, AI07, AJ26, AJ25 and AI02 were selected, since their index values were higher than 1.0.

### *In vivo* antagonistic activity of selected isolates against *P. oryzae*

The incidence and severity of P. oryzae AAM-275 in 21-days-old control plants were of 100 and 78% respectively. When the rhizobacteria were applied, only six of them were able to reduce disease severity and incidence to some extent (Table 2). The disease incidence ranged from 11 to 33% and disease severity from 11 to 27%, achieving the best results with the strains AI05, AJ01 and AJ13, according to the results of the control efficacy (Table 2).

### Disease severity is reduced in leaves when the selected isolates are applied

Four of the isolates showed efficient control of *P. oryzae* in greenhouse conditions, while the others did not (data not shown). The percentage of infected leaves per plant was considerably lower in all the treatments inoculated with rhizobacteria, as opposed to the control infected with the fungus (43.27 to 30.17%) (Table 3). All the strains exerted biocontrol over *P. oryzae*, as shown by the disease reduction percentage in the rhizobacteria-treated plants (28.23 to 41.33%). According to this research, the strains AI05 and AJ13, AJ01 and AI08 can be used for biocontrol of *P. oryzae* in rice. The strains AI05 and AJ13 were selected for further studies, since these strains were also able to stimulate plant growth in field conditions (data not shown).

### The selected isolates were identified as *Pseudomonas putida* and *Pseudomonas fluorescens*

The obtained sequences (GenBank access no. HQ446870 and HQ446871) were compared to the retrieved sequences from GenBank. According to the sequences of the *16s rDNA* gene, the strains AJ13 belonged in the *Ps. putida* cluster and AI05 is in *P. fluorescens* cluster (Figure 2).

### Physiological characterization of the selected isolates

Some traits that could be involved in biocontrol and/or plant growth promoting activity were tested and the results are shown in Table 4. The strains AI05 and AJ13 were positive for exoprotease and lipase activities, piochelin, hydrogen cyanide (HCN) and pyoluteorin production. When pyoverdine production was quantified, AJ13 produced almost twice as much pyoverdines as AI05.

Antibiotic genes were identified by PCR and their corresponding products were detected by TLC. In both strains, a 790-bp *pltB* fragment was amplified, suggesting that they produce pyoluteorin. The detection of a 719-bp (*prnC*) and a 1200-bp (*phzX*) bands were amplified for AJ13 but not for AI05, indicating that AJ13 could produce pyrrolnitrin and phenazine-1-carboxylate.

When the extracts of AI05 and AJ13 strains in CAA

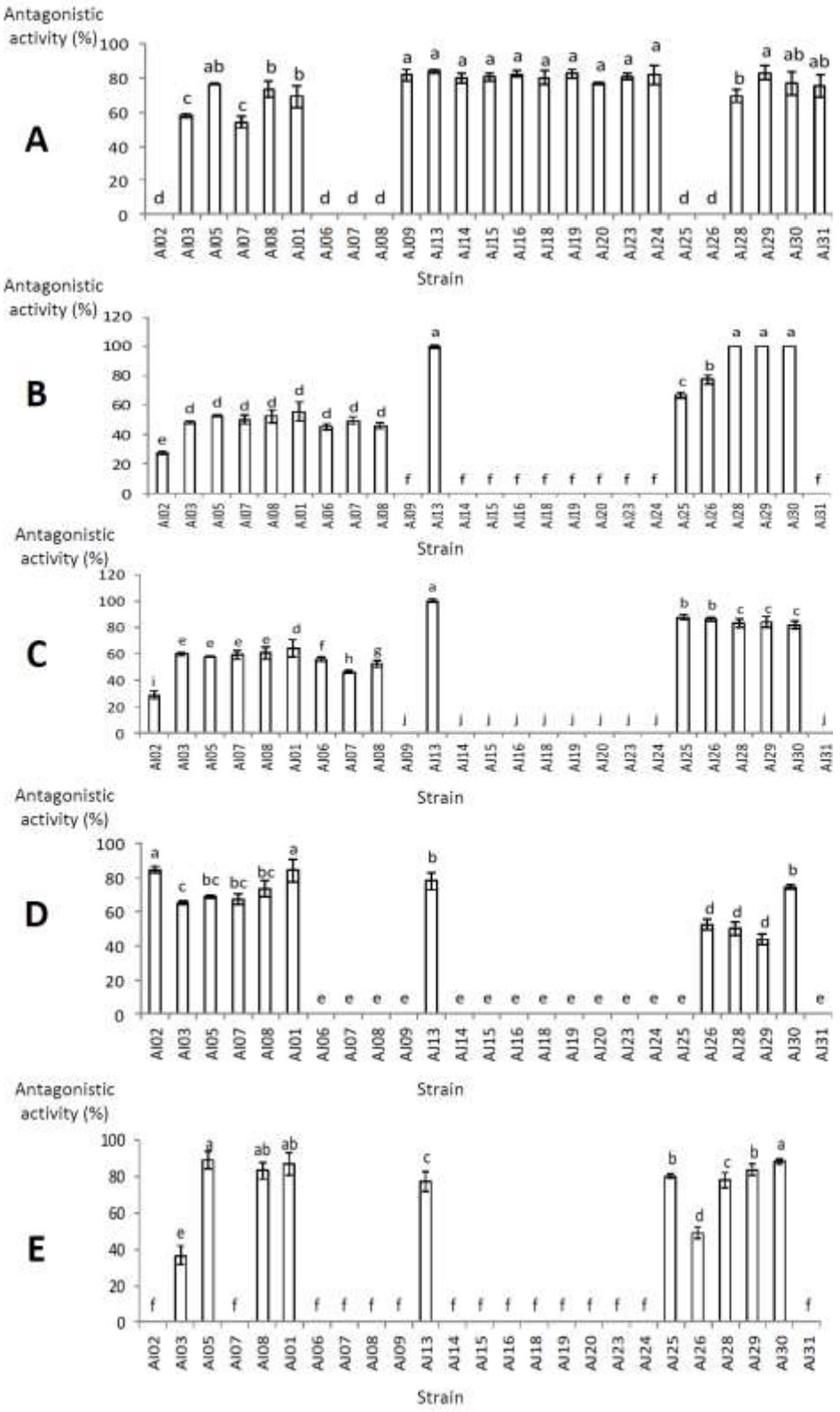


**Figure 1.** Antagonistic effect of bacterial isolates against *P. oryzae*. Percentage of inhibition of mycelial growth of fungal strains. **(A)** AAM275; **(B)** AAM903; **(C)** AAM403; **(D)** AAM425; **(E)** AAM361. Non common letters suggest significant diferences according to Bonferroni correction (I = Standard deviation of the mean).

**Table 2.** Control of rice blast by rhizobacteria in 21-days-old rice plants.

| Treatment | Disease incidence (%) | Disease severity (%) | Control efficacy (%) |
|---|---|---|---|
| NIC | 0[e] | 0[d] | - |
| FIC | 100[a] | 78[a] | - |
| FS + AI03 | 33[b] | 27[b] | 65[c] |
| FS + AI05 | 11[d] | 11[c] | 86[a] |
| FS + AI08 | 22[c] | 22[b] | 72[b] |
| FS + AJ01 | 33[b] | 11[c] | 86[a] |
| FS + AJ13 | 33[b] | 13[c] | 83[a] |
| FS + AJ29 | 33[b] | 22[b] | 72[b] |

NIC, Non-inoculated control; FIC, fungus-inoculated control; FS, fungus-inoculated soil. Values in columns followed by similar letters were not significantly different according to Fisher's protected LSD test (P = 0.05).

**Table 3.** Ability of potential antagonistic rhizobacteria to suppress *P. oryzae* in 60-days-old rice plants in greenhouse study.

| Treatment | Infected leaves per plant (%) | Disease reduction (%) |
|---|---|---|
| NIC | 25.00[e] | - |
| FIC | 71.50[a] | 0[d] |
| FI + AJ01 | 38.50[c] | 33.00[b] |
| FI + AI05 | 30.52[d] | 40.98[a] |
| FI + AJ13 | 30.17[d] | 41.33[a] |
| FI + AI08 | 43.27[b] | 28.23[c] |

NIC, Non-inoculated control; FIC: fungus-inoculated control; FI, fungus-inoculated. Values in columns followed by similar letters were not significantly different according to Fisher's protected LSD test (P = 0.05).

Agar were analysed through HPLC-MS, the results showed that both strains were able to produce pyoverdines (1160 Da) and different isoforms of pyochelin (324 Da). Additionally, the production of ornibactin (734 Da) was detected for the strain *P. fluorescens* AI05.

The analysis showed that both strains were able to fix nitrogen, produce indolic compounds and to solubilize Pi when grown on NBRIP (pH 7.0) Agar medium, as indicated by the presence of a visible halo that was used to calculate a solubilization index (SI). The strains AI05 and AJ13 showed maximum Pi solubilization at 325 and 348 $mg.L^{-1}$, respectively. AI05 and AJ13 exhibit swarming motility, since their diameters were significantly higher (81.2 ± 0.17 and 45.1 ± 0.14, respectively) than the inoculation diameter (5 mm). However, AJ13 showed the highest diameter (5.65 ± 0.03 mm) in twitching assay.

## DISCUSSION

In the present study, of the 200 isolates of fluorescent *Pseudomonas* evaluated, 25 of them showed *in vitro* antagonistic activity against *P. oryzae*. Although in comparison with other studies, the number of recovered antagonists may be consider as high (Acebo-Guerrero et al., 2015); there are several factors that may contribute to the frequency of antagonistic bacteria, such as the soil type, plant species and environmental conditions (Lugtenberg and Kamilova, 2009; Filippi et al., 2011).

This research revealed that these Cuban native strains of fluorescent *Pseudomonas* from rice rhizosphere have *in vitro* antagonistic activity, although only four of them (AI05, AJ13, AJ01 and AI08) are able to control *P. oryzae* in greenhouse conditions. In the preliminary *in vitro* dual culture experiments, the antagonistic activity of the isolates was assessed against five strains of *P. oryzae* (AAM-275, AAM-361, AAM-403, AAM-425 and AAM-903), showing a differential antagonistic effect of the rhizobacteria against the phytopathogenic strains. These results may be explained regarding the differences between the strains within species and also the specificity of the different physiological biovars of a pathogen (Almaguer-Chavez et al., 2012), that correspond with different levels of resistance to an antagonist (Karthikeyan and Gnanamanickam, 2008). Jaiganesh et

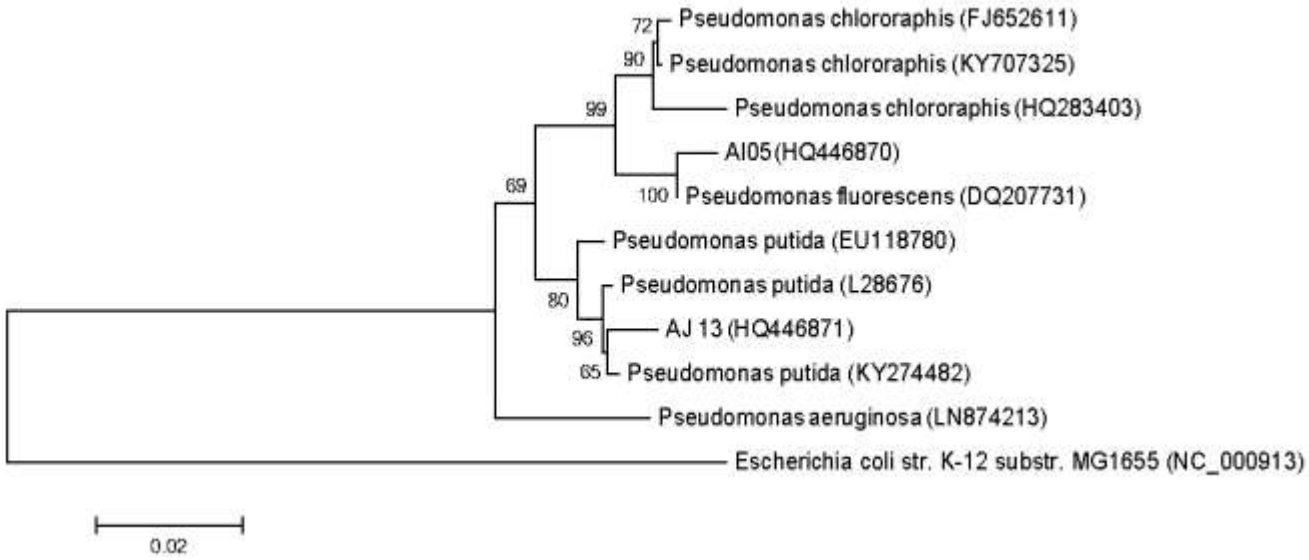


**Figure 2.** Phylogenetic tree of the isolates and strains of *Pseudomonas* species inferred from *16S rRNA* gene sequences. Numbers at the nodes are percentage bootstrap values based on 1000 resampled datasets, with only bootstrap values ≥50% shown. The *16S rRNA* gene sequence of *E. coli* K12 (NC000913) was used as outgroup. The evolutionary distances were computed using the Kimura 2-parameter method and the rate variation among sites was modelled with a gamma distribution. The scale bar indicates a genetic distance of 0.005 nt substitutions per site.

**Table 4.** Overview of the distribution of bacterial metabolite production and motility among the antagonistic *Pseudomonas* strains.

| Characteristics | Strains | |
|---|---|---|
| | AI05 | AJ13 |
| **Lipase** | + | + |
| Protease | + | + |
| Pyoverdines | 0.15 ± 0.09 | 0.33 ± 0.09 |
| Pyochelin | + | + |
| Ornibactin | + | - |
| HCN | + | + |
| | | |
| **Antibiotic production*** | | |
| PHZ | - | + |
| PLT | + | + |
| PRN | - | + |
| ARA (nmol $C_2H_2$/mg protein/h[a]) | 68.9 ± 0.09 | 63.5 ± 0.11 |
| Indole production (µg/mL) | 9.11± 1.09 | 12.61 ± 1.06 |
| Solubilisation Index (S.I.) Medium NBRIP (pH 7.0) | 2.88 ± 0.03 | 2.42 ± 0.13 |
| Soluble-P (mg.$L^{-1}$) | 325 ± 4.68 | 348 ± 2.47 |
| | | |
| **Motility** | | |
| Swarming** | 81.2 ± 0.17 | 45.1 ± 0.14 |
| Twitching*** | 0.25 ± 0.01 | 5.65 ± 0.03 |

+, positive reaction; -, negative reaction. Non-common letters indicate significant differences according to Tukey test ($P < 0.05$). PHZ, Phenacines; PLT: pyoluteorin; PRN, pyrrolnitrin. *The antibiotic genes were detected by PCR and the antibiotic production was detected by TLC. **Value represents the diameter of the swarming area (the bacterial growth diameter minus the inoculation diameter (5 mm)). ***Value represents diameter of the stained area (mm) _ standard deviation.

al. (2007) suggested that when an interaction is established between the antagonists and phytopathogens, there is a set of strain-dependent responses, the biocontrol activity being the sum of various mechanisms.

In this work, two strains (*P. fluorescens* AI05 and *P. putida* AJ13) isolated from *O. sativa* showed potential to be used as biocontrol agents, since they were able to inhibit *in vitro* the mycelial growth of *P. oryzae* and to reduce symptom severity of *Pyricularia* infection. These strains also have the ability to promote plant growth under field conditions. *Pseudomonas* has been described as highly adaptable in several ecosystems, prevailing in some of them and largely known as a plant growth promoting agent (Kang et al., 2014; Heng et al., 2017), as a biocontrol agent (Kumar et al., 2015; Sun et al., 2017), as well as a bioremediating agent (Nelson et al., 2002; Zhang et al., 2016).

The most commonly reported mechanisms of biocontrol by *Pseudomonas* include production of antibiotics, hydrogen cyanide, lytic exoenzymes (Mishra and Arora, 2017), cyclic lipopeptides (Flury et al., 2017), competition for nutrients and niches (Saber et al., 2015), competition for iron mediated by siderophores (Ahmad et al., 2008; Buataité et al., 2017) and induced systemic resistance (Kumar et al., 2015). In this particular study, the strains *P. putida* AJ13 and *P. fluorescens* AI05 were able to produce siderophores, such as pyoverdine and pyochelin and in the case of AJ13, the production of ornibactin was also detected. Siderophore production could have played a major role in antagonistic activity, since the King B medium is iron-limited, which favors bacterial siderophore production and iron uptake over fungal siderophores, and has been reported as a desirable trait for the biocontrol of phytopathogens (Cornelis, 2010).

Fluorescent *Pseudomonas* produce an array of extracellular metabolites with antifungal activity (Park et al., 2011). In this study, AJ13 and AI05 have potentialities to produce metabolites that have been reported as involved in biocontrol activity, such as pyoluteorin for both strains and for AJ13, the additional production of pyrrolnitrin and phenazine-1-carboxylate was predicted. The production of these metabolites and the siderophores could partially explain the beneficial effects observed in greenhouse conditions, although other studies are needed to confirm their involvement in this particular plant-microorganism interaction.

In addition to the production of antagonistic metabolites, *Pseudomonas* have been reported to stimulate plant growth by increasing the availability and uptake of mineral nutrients or by enhancing root growth and morphology *via* the production of phytohormones such as auxins (Ghirardi et al., 2012; Mehnaz, 2013). In this study, both AJ13 and AI05 were able to produce auxins and to fix atmospheric nitrogen which suggests that they may be excellent plant growth promoting agents. In this context, with the application of these rhizobacteria as a microbial inoculant, a greater contribution of atmospheric nitrogen to the plant is achieved, which reduces the use of chemical products. These reductions in the application of nitrogenous fertilizers could affect the physiology of *P. oryzae* that needs this element for its own nutrition (Agrios, 2015), which would cause a decrease in the concentrations of this phytopathogen and a lower incidence of the disease in field conditions (Sester et al., 2014). At the same time, these bacteria release metabolites in the rhizosphere that contribute to the ecological balance of the soil. However, the demonstration of this hypothesis will require future research.

This study reports for the first time the ability of the strains AI05 and AJ13 to produce siderophores and to solubilize inorganic phosphate. In this sense, a recent study by Estrada et al. (2013) related the *in vitro* fixation of nitrogen and the solubilization of inorganic phosphorus with the ability to promote plant growth in rice for *Bacillus* and *Herbaspirillum* strains. On the other hand, the production of auxins by *Pseudomonas* has also been related to plant stress alleviation (Egamberdieva and Lugtenberg, 2014).

These results demonstrate that rizosphere bacteria can be used to reduce the damage caused by *P. oryzae* in *O. sativa* L. Specifically, the isolates *P. putida* AJ13 and *P. fluorescens* AI05 showed promising potentialities for biocontrol and plant growth promotion. However, there was a difference in behavior between the strains, which suggest that the mechanisms of action against *P. oryzae* are probably different.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENT

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*Full Length Research Paper*

# Chromium (III) and its effects on soil microbial activities and phytoremediation potentials of *Arachis hypogea* and *Vigna unguiculata*

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**The aim of this research was to evaluate the impact of chromium (III) nitrate on soil microbial activities and growth performance and phytoremediation potentials of two staple leguminous crops, namely cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogea*). Pristine sandy loam soil samples were polluted with nitrate salts of chromium (III) at four different levels (50, 100, 200 and 400 mg/kg) in triplicates. There was a significant ($P < 0.05$) retarding effect of this metal on the study parameters. A consistent decrease in the total bacterial count in response to increase in dosage of the metal salt was observed. Chromium was also observed to significantly ($P<0.05$) affect the microbial metabolism as indicated by the decline in microbial respiration shown by the lowering of $CO_2$ evolution in the test samples. There was a reduction in the general growth performance of the two test plants treated with different levels of chromium when compared with the control. Phytoaccumulation experiment showed that only cowpea roots accumulated the pollutant from the 400 mg/kg treated soil, with no metal salt presence in aerial parts of the plants. This chromium-removal potential demonstrated by cowpea makes it a better candidate than groundnut for the phytoremediation of chromium-contaminated soils.**

**Key words:** Chromium (III) nitrate, microbial activities, cowpea, groundnut, phytoremediation.

## INTRODUCTION

Chromium (Cr) is one of the naturally occurring elements found in rocks, soils, and in volcanic dust and gases. It occurs in two main forms in the environment, namely, chromium (III) and chromium (VI). Chromium is a toxic non-essential metal for microorganisms and plants. In plants, it reduces productivity by the imposition of chronic diseases, loss of chlorophyll and protein contents (Ma et al., 2016). Hexavalent chromium ($Cr^{6+}$) is more toxic than trivalent chromium ($Cr^{3+}$) (Saha et al., 2011). The trivalent form is a trace mineral important in human nutrition; in large doses however it can be harmful to health. Chromium (III) may react with carboxyl and sulfhydryl groups of enzymes causing alterations in their structure and activities. It also modifies DNA polymerase and other enzyme activities as a result of the displacement of magnesium ions by chromium ions (Snow, 1994).

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Hexavalent chromium has been found to be carcinogenic. It causes mutation through the generation of free radicals as it is reduced to lower oxidation states in biological systems (Kadiiska et al., 1994). Chromium (VI) is a product of some industrial processes and can be found in such products as paints, dyes, tannery chemicals, wood preservatives, anti-corrosion agents, etc. Chromium occurs in neutral or alkaline soils in the trivalent form ($Cr^{3+}$), which has low solubility and mobility. Shanker et al. (2005) observed that chromium impact on the physiological development of plants depends on the metal speciation, which is responsible for its mobilization, uptake and subsequent toxicity in the plant system. Heavy metal species commonly found in the soils as a result of human activities include, copper (Cu), zinc (Zn), nickel (Ni), lead (Pb), cadmium (Cd), cobalt (Co), mercury (Hg), chromium (Cr), arsenic (As). etc. Generally, metals are not degradable and so these heavy metals can persist in the environment indefinitely (Walker et al., 2003). Some of them act as micronutrients in small concentrations for the development of living organisms, but when bioaccumulated over time they become toxic to life.

The risk associated with polluted soils is contamination of the food chain. When plants grow on polluted soils, they become potential threats to human and animal health. Plants may also have their growth sharply reduced by high levels of toxic elements in their tissues, causing a decrease in crop yields and further economic loss. Uptake and accumulation of a number of metals by plants are affected by pH, clay content, organic matter content, cation exchange capacity, nutrient balance, mobility of the heavy metal species and soil moisture and temperature (Sauve et al., 1997).

Microorganisms, namely, bacteria, fungi, protozoa and algae coexist in the soil especially within the rhizosphere region. Some such as plant growth promoting rhizobacteria (PGPR), phosphorus solubilizing bacteria, mycorrhizal helping bacteria (MHB) and arbuscular mycorrhizal fungi (AMF) in the rhizosphere of plants growing on trace metal contaminated soils play an important role in phytoremediation (Khan, 2005; Ahemad, 2015; Stambulaka et al., 2018). These microorganisms can survive and serve as effective metal sequestering and growth-promoting bioinoculants for plants in metal stressed soils (Rajkumar and Freitas, 2008). They mitigate the toxic effects of these heavy metals on plants through secretion of acids, proteins, phytoantibiotics and other chemicals (Denton, 2007). In phytoremediation, a plant can be classified as an accumulator, excluder or an indicator according to the concentration of metals found in its tissue (Baker, 1981). Harnessing the phyto-remediation potentials of legumes is currently being advocated, though this move has been criticized in some quarters due to the belief that this might lead to health challenges and food scarcity, especially in the developing world such as Nigeria where these plants serve as staple foods.

The growing increase in heavy metals pollution of the soil occasioned by industrialization, brings with it a concomitant concern for plants and microbial safety, considering their significant roles in the ecosystem. This research was therefore carried out to evaluate the impact of chromium (III) on soil microbial activities, growth performance and phytoremediation potentials of *Arachis hypogea* (groundnut) and *Vigna unguiculata* (cowpea).

## MATERIALS AND METHODS

### Study area and sample collection

This study was carried out in Nsukka, Southeastern Nigeria. Soil samples were collected from the Plant Science and Biotechnology Garden, University of Nigeria, Nsukka at a depth of within 0 to 15 cm. Viable seeds of both cowpea and groundnut were purchased from Ogige market in Nsukka metropolis and stored at room temperature for 24 h. Seed viability testing was carried out using floatation technique. Analytical grade of chromium (III) nitrate salt ($Cr(NO_3)_3.9H_2O$) was used.

### Soil analysis

Physical and chemical properties of the soil samples were determined. Particle size was determined using the Boyoucos Hydrometer Method of Gee and Bauder (1987). Potential of hydrogen (pH) was analyzed according to Black (1965). Percentage organic matter, phosphorus, nitrogen, cation exchange capacity and soil moisture were also determined using the methods of Black (1965).

### Determination of the effects of chromium on soil bacterial population

Pristine sandy loam soil was air-dried, sieved and dispensed in 100 g weights into twelve 250 ml conical flasks placed in four groups; each group comprising three flasks. Each triplicate group was polluted with one of four different levels (50, 100, 200, and 400 mg) of $Cr(NO_3)_3$. A control experiment made up of three unpolluted soil samples were also set up. The conical flasks were watered periodically to sustain the microorganisms. Bacterial analysis was done using 1.0 g of soil collected from each flask at one weekly interval over a period of four weeks. The population of viable bacterial cells in each soil sample was determined by a ten-fold serial dilution and spread plating technique as described by Wistreich (1997).

### Determination of the effects of chromium on soil microbial respiration

Fifty grams of each polluted soil sample was weighed in duplicate into kliner jars and three unpolluted soil samples served as control. Sterile water was carefully sprinkled on the soil up to 60% water holding capacity to make it moist. In each jar containing the soil, a vial with 15 ml of already prepared 0.05 M NaOH was carefully placed at the centre. Three empty kliner jars, each containing a vial with 15 ml of 0.05 M NaOH, were used as blanks. The tops of the jars were greased properly to prevent the escape of $CO_2$ and were tightly capped and incubated at room temperature. At weekly

intervals, the vials were removed and 3 ml of 20% $BaCl_2$ was added. Three drops of phenol red indicator were subsequently added and then titrated using 0.05 M HCl until a colourless end point was observed. This was repeated for four weeks.

### Calculation of results

The rate of respiration was calculated by the following relationship:

$CO_2$ (mg)/SW/T = (Vo - V) × 1.1 / DWT

Where, SW is the amount of soil dry weight in grams, T is the incubation time in hours, Vo is the total volume of HCl used for titration. V is the volume of HCl used for the soil sample, DWT is the dry weight of 1 g moist soil and 1.1 is the conversion factor (1 ml 0.05 NaOH equals 1.1 mg $CO_2$).

### Planting experiments

After the soil samples have been air dried and homogenized, they were sieved and dispensed in 3 kg weights into 24 (20 cm deep × 18 cm diameter) plastic pots, each perforated at the bottom. Each of the four different levels of Cr $(NO_3)_3$ $9H_2O$ (50, 100, 200 and 400 mg/kg) was used in triplicates to pollute the soil in the pots. The pots were kept in a green house and allowed to stabilize for seven days to compensate for the disturbance caused by sampling and sieving (Baath, 1998), and also to ease gradual distribution of the metal (Cr) in the soil. Soil samples which received no metal pollutants were prepared and kept as control. Seven days after soil pollution, four viable seeds each of groundnut or cowpea were planted in each plastic pot. The pots were watered with 200 ml of sterilized water every four days for eight weeks. Over-watering was avoided as much as possible to prevent water logging and leaching of the metal salt. The germination percentage and growth rates were monitored and recorded. Seeds were considered germinated when the radial reached a length of 1 mm. The germination percentage was calculated as:

$$Germination\ percentage = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} \times 100$$

Length of shoot was measured with the help of a scale and reading was taken from both test plants and the control. This was done on a weekly interval. At the end of the planting experiment which lasted for eight weeks, the plants were gently uprooted and the following measurements carried out:

### Root length

This was done by measuring the root length of the legume after harvesting to determine the length in relation to the control.

### Nodulation

The number of nodules formed on each plant in each treatment was carefully counted and recorded.

### Weight of plant

The uprooted plants were washed with distilled water. After air-drying, the wet weight was measured. This was followed by oven-drying at 105°C for 24 h to determine the dry weight.

### Metal uptake potential

At the end of the weighing process, the plants were separated into root and shoot and pulverized using laboratory milling machine. The pulverized samples were digested using $HCL/HNO_3$ (3:1 v/v) and the metal uptake determined using an Atomic Absorption Spectro-photometer (FS 240 AA Agilent Technology). The bioconcentration factor (BCF) and translocation factor (TF) were calculated. The BCF is the ratio of metal concentration in the roots to that in the soil or water and TF is the ratio of metal concentration in the shoots to that of the roots (Malik et al., 2010).

$$BCF = \frac{Concentration\ in\ root\ (\frac{mg}{g})}{Concentration\ in\ soil\ (\frac{mg}{g})}\ and\ TF = \frac{Concentration\ in\ shoot}{Concentration\ in\ root}$$

Plants are categorized as phytoextractor when TF > 1 (Fitz and Wenzel, 2002) and as phytostabilizer when BCF > 1 and TF < 1, respectively (Mendez and Maier, 2008).

### Statistical analysis

All data were subjected to analysis of variance (ANOVA) using SPSS version 16 and reported as mean ± standard deviation (SD). Statistical value at $p < 0.05$ was considered to be significant.

## RESULTS

### Soil physicochemical analysis

Selected physico-chemical characteristics of the soil are shown in Table 1. From the analysis, the soil was classified as sandy loam with a moisture content of 6.2%, pH 6.3, organic matter of 1.98 and cation exchange capacity (CEC) of 8.0. The pH is considered an important parameter because it affects the availability of trace metals in the soil.

### Effects of chromium on the germination, growth performance and phytoremediation potentials of *V. unguiculata*

Table 2 shows the effects of chromium on germination time, germination percentage, nodulation, weight and root length, metal uptake along with BCF and TF potentials of *V. unguiculata*.

The result presented in Table 2 shows that at a treatment level of 400 mg chromium/kg of soil, a prolonged germination time (9±1 days) was observed unlike the control (4±1 days). Germination time increased with increase in chromium dosage. In both control and 50 mg/kg treated soil, all the cowpea seeds sown in each pot germinated whereas in the other levels, there was a concomitant reduction in the number of germinated seeds as the dose increased. The uptake of chromium by cowpea was also found to be concentration dependent. The root was seen to be a better site for chromium

**Table 1.** Soil physicochemical properties.

| Parameter | Result |
|---|---|
| **Particle size analysis (%)** | |
| (a) Clay | 12 |
| (b) Silt | 4 |
| (c) Fine sand | 27 |
| (d) Coarse sand | 57 |
| Textural class | Sandy loam |
| pH | 6.3 |
| Organic matter (%) | 1.98 |
| Nitrogen (%) | 0.126 |
| Cation exchange capacity (CEC; me/100 g) | 8.00 |
| Phosphorus (ppm) | 11.19 |
| Moisture (%) | 6.2 |

**Table 2.** Effects of chromium on germination, general vegetative growth and metal uptake potentials of *Vigna unguiculata*.

| Parameter | 50 mg | 100 mg | 200 mg | 400 mg | Control |
|---|---|---|---|---|---|
| Germination time (days) | 4.5±1.29 | 6.5±1.29 | 7±1 | 9±1 | 4±1 |
| Germination percentage (%) | 100 | 92 | 83.33 | 41.67 | 100 |
| Shoot metal uptake (mg/kg) | - | - | - | - | - |
| Root metal uptake (mg/kg) | - | - | - | 0.578±0.004 | - |
| Bioconcentration factor (mg/kg) | - | - | - | 6.31 | - |
| Translocation factor (mg/kg) | - | - | - | - | - |
| Nodulation | 31.67±1.53 | 24.00±1.00 | 13.33±1.53 | 6.33±1.53 | 41.06±0.42 |
| Wet weight (mg) | 3.16±0.06 | 1.93±0.15 | 1.7±0.26 | 1.33±0.15 | 2.9±0.10 |
| Dry weight (mg) | 1.73±0.15 | 1.48±0.07 | 0.96±0.15 | 0.88±0.15 | 2.05±0.05 |
| Root length (cm) | 14.8±0.31 | 12.8±0.3 | 11.4±0.21 | 10.5±0.61 | 13.0±0.75 |

(-)Not observed.

accumulation than the shoot, though at 400 mg/kg treated soil only.

### Effects of chromium on the germination, growth performance and phytoremediation potentials of *A. hypogea*

Table 3 shows the effects of chromium on germination time, germination percentage, nodulation, weight and root length, metal uptake along with BCF and TF potentials of *A. hypogea.*

A prolonged germination time of 8.75±1.7days was observed unlike the control (4±1 days). In both control and 50 mg/kg treated soil, all the groundnut seeds sown in each pot germinated as observed in cowpea. However, there was a reduction in the number of germinated seeds as the dose increased. Chromium accumulation was not observed in both the shoot and root parts of the plant. Nodulation as well as wet and dry weights were dose dependent.

### Effects of chromium on microbial activities and shoot growth of the legumes

Figures 1 and 2 show the effects of different levels of chromium on the shoot lenghts of cowpea and groundnut, respectively. Chromium exhibited a dose-dependent retardative effect on the shoot growth of the legumes. This dose-dependent retardation was also observed on the bacterial growth as well as on microbial respiration (Figures 3 and 4).

## DISCUSSION

The observed dose-dependent inhibitory effect of chromium on soil bacterial population is in conformity with

**Table 3.** Effect of chromium on germination, general vegetative growth and metal uptake potentials of *Arachis hypogea*.

| Parameter | 50 mg | 100 mg | 200 mg | 400 mg | Control |
|---|---|---|---|---|---|
| Germination time (days) | 4.5±1.29 | 5.5±1.29 | 7±1 | 8.75±1.7 | 4±1 |
| Germination percentage (%) | 100 | 91.76 | 66.76 | 41.47 | 100 |
| Shoot metal uptake (mg/kg) | - | - | - | - | - |
| Root metal uptake (mg/kg) | - | - | - | - | - |
| Bioconcentration factor (BCF) | - | - | - | - | - |
| Translocation factor (TF) | - | - | - | - | - |
| Nodulation | 50.33±1.53 | 41.33±0.57 | 30.33±1.53 | 20.33±1.53 | 65.33±1.53 |
| Wet weight (mg) | 5.433±0.25 | 4.46±0.15 | 3.36±0.25 | 2.93±0.12 | 6.33±0.15 |
| Dry weight (mg) | 3.27±0.15 | 2.86±0.15 | 2.13±0.15 | 1.76±0.15 | 4.56±0.12 |
| Root length (cm) | 13.33±1.15 | 12.33±1.26 | 9.53±0.75 | 8.73±0.862 | 13.1±1.21 |

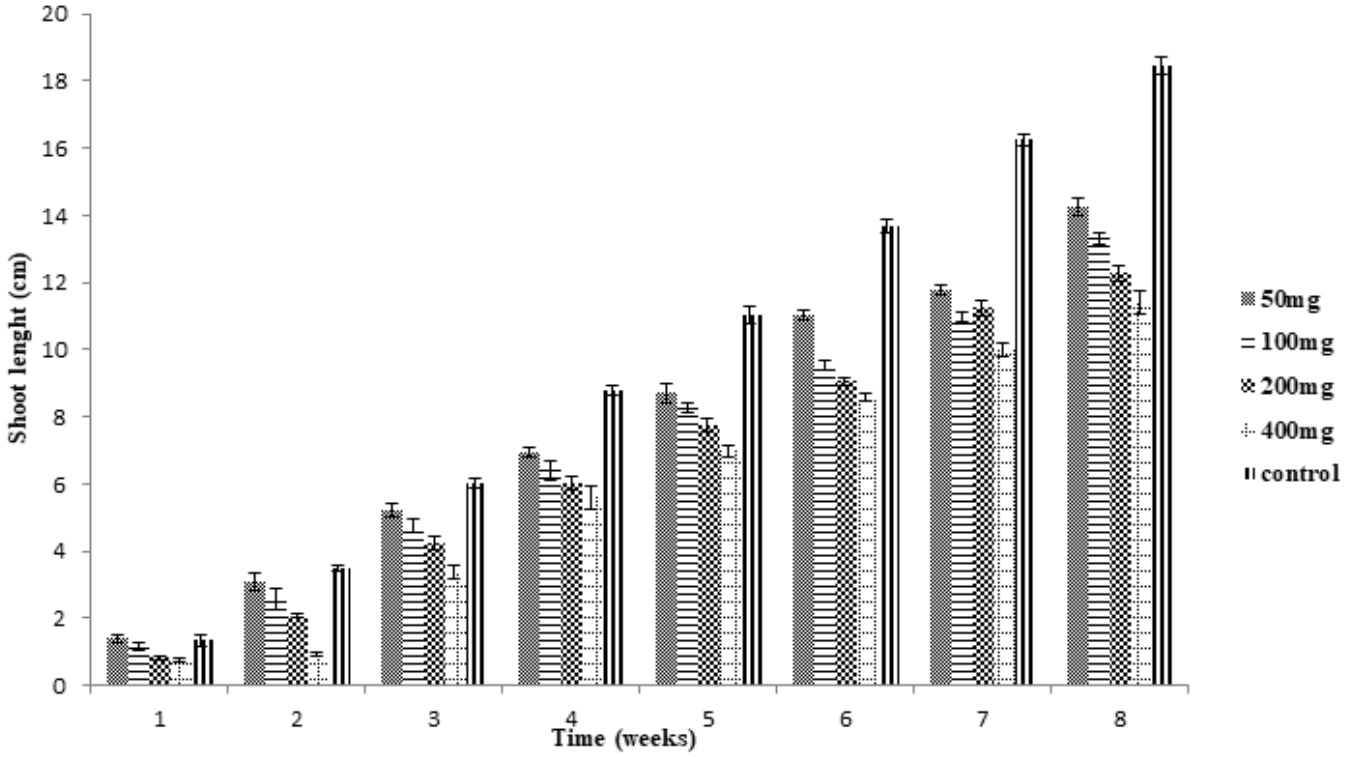


**Figure 1.** Effects of different levels of chromium on the shoot length of cowpea grown in sandy loam soil.

the report of Wani et al. (2008) who stated in their work (though not specific to chromium) that metals have serious effects on both soil bacterial count and plant growth promoting rhizobacteria. The deleterious effect of chromium on soil bacterial population was found to increase with increase in levels of pollution. The higher the metal dose, the more significant ($P < 0.05$) the retardative effects on the total bacterial count. Ghorbani et al. (2002) in their study identified a reduction in microbial biomass as a result of heavy metal ecotoxicity in soil environment. Once a rise in soil metal build-up occurs, it becomes uninhabitable for microbial communities and unsuitable for crop production, thus inhibiting the growth and activities of various groups of microorganisms including symbiotic nitrogen fixers such as *Rhizobium leguminosarum*, *Mesorhizobium ciceri*, *Bradyrhizobium* species and *Sinorhizobium.*

Results of this study show that chromium contamination of soils has an inhibitory effect on carbon (iv) oxide evolution by microorganisms and this effect was observed to be concentration-dependent. Sethi and Gupta (2009) essayed the opinion that heavy metals are

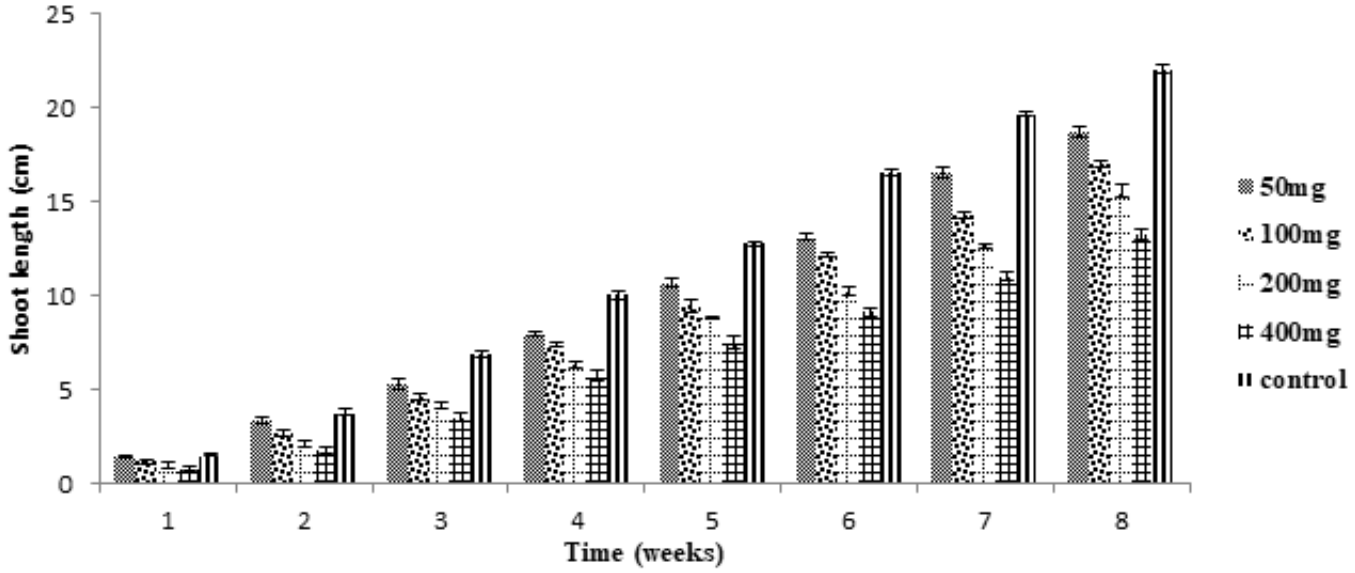


**Figure 2.** Effect of different levels of chromium on the shoot length of groundnut grown in sandy loam soil.

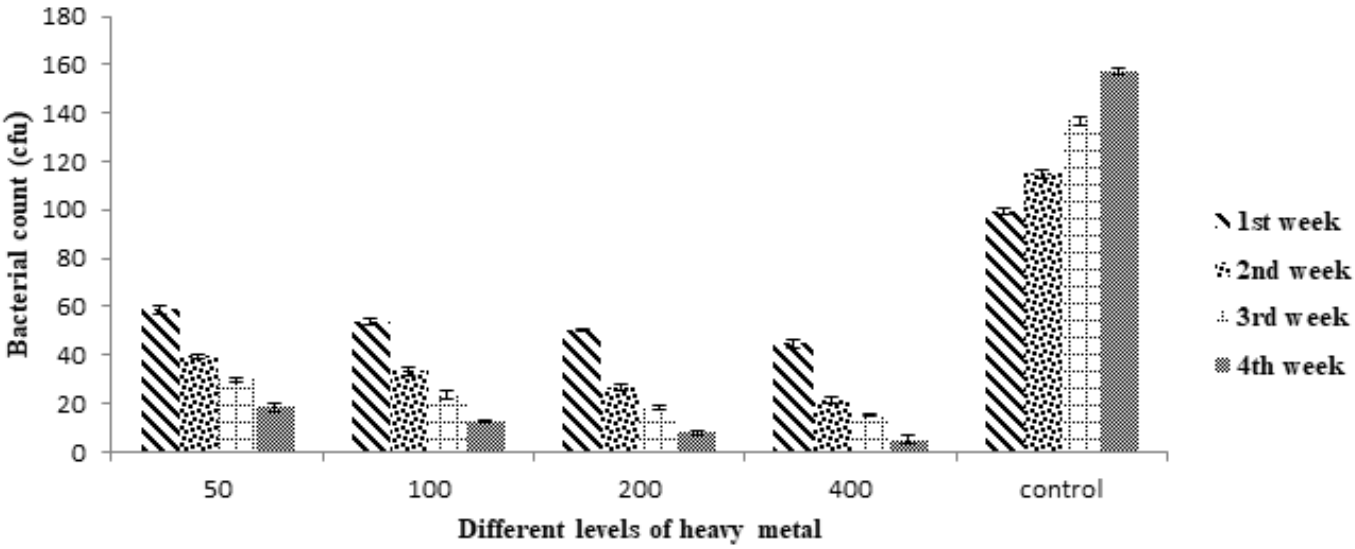


**Figure 3.** Effect of different levels of chromium on bacterial population.

deleterious to microbial metabolism and that their effects might be more detrimental with rise in the level or dose of application. Soil respiration is a useful indicator for determining soil health. Microorganisms domiciled in the soil play a key role in the mineralization of nutrients, decomposition of organic matter and degradation/ transformation of toxic compounds.

Seed germination is the first visible evidence of plant growth. It is regulated by a number of physical and physiological processes. The investigation on the impact of chromium on the growth and development of the two legumes (*A. hypogea* and *V. unguiculata*) revealed an adverse effect on the plants general growth performance. Reports have shown that soil metal contents affect the growth and physiology of plants (Luilo and Othman, 2006; Trinh et al., 2014). The reduced germination (germination time and percentage) in both plants (cowpea and groundnut) as observed in the present finding could be attributed to metal toxicity. From the results it was observed that chromium had a more adverse effect on the germination and growth of groundnut than cowpea. This shows that cowpea probably has a higher intrinsic resistance to chromium than groundnut. Pandey et al. (2005) reported that growth

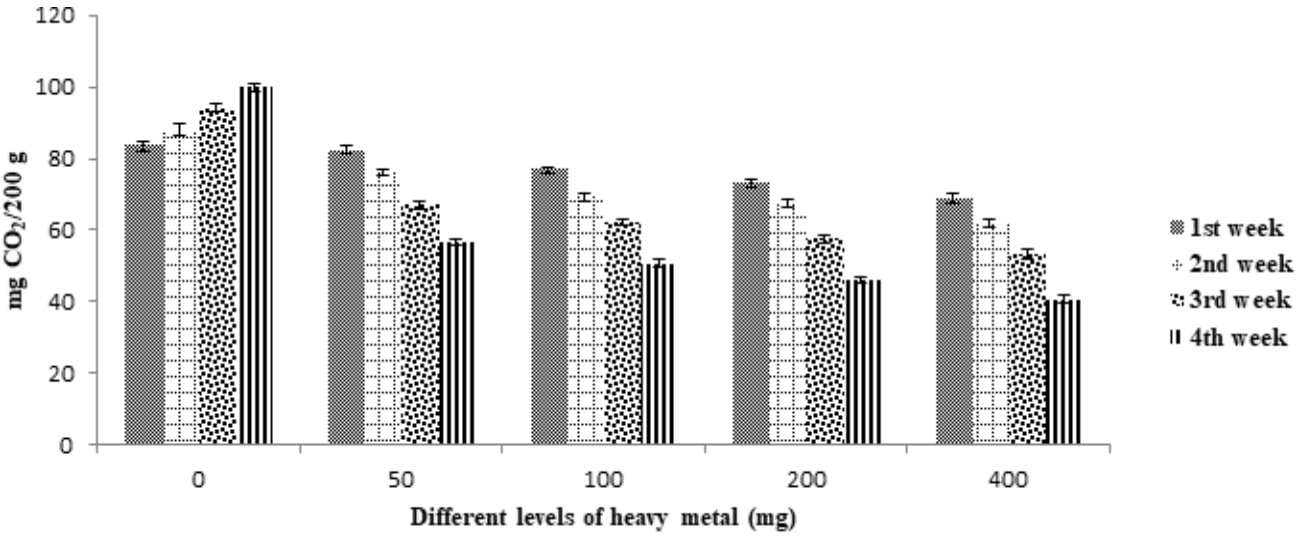


**Figure 4.** Effects of different levels of chromium on microbial respiration.

inhibition of plants exposed to chromium might be due to the generation of free radicals and reactive oxygen species (ROS), which pose constant oxidative damage by degrading important cellular components. Shafiq et al. (2008) attributed it to accelerated breakdown of stored nutrients in seeds and alteration of selective permeability properties of cell membranes.

The observed decrease in the shoot lengths of the legumes could be the result of chromium toxicity which caused alteration in root cell functions leading to reduced nutrient uptake and water mobility. In a relevant work, Diwan et al. (2010) reported that reduction in shoot length caused by chromium stress may be due to its accumulation in the root region, or absence of its translocation from roots to other tissues, thus causing increase in chromium concentration in roots and inhibiting shoot development. Also Hu et al. (2015) suggested that damage of chloroplasts and possible reduction in photosynthesis could lead to significant reduction in shoot lengths of *Crambe sativa* and *Eruca sativa* when grown in chromium (VI) polluted soil.

The reduction in the number of nodules in the legumes planted in chromium (III)-polluted soil could also be the result of heavy metal toxicity. Ibekwe et al. (1996) similarly opined that the toxic effect of metals on the root hairs or rhizobia might be responsible for reduced nodulation when they examined alfalfa plant under zinc and cadmium stress. Manier et al. (2009) stated that the nodulation index of white clover could serve as a suitable bioindicator of increased heavy metal toxicity in soil. It is also possible that the chromium (III) inhibited nitrogenase activity and photosynthesis leading to retardation in nodulation and overall growth stagnation.

The observed decrease in the biomass of cowpea and groundnut is also an index of chromium phytotoxicity. Similar discovery on decreased biomass production in legumes was also reported as a direct toxic effect of chromium polluted tannery effluent used for irrigation (Santos et al., 2011). Klimek-Kopyra et al. (2015) in their recent work observed that increase in heavy metals pollution limit the longitudinal growth and biomass of roots with a corresponding reduction in nodule formation in field pea and spring vetch grown in contaminated soil.

Results from the present research show that the two plants used in the study were not able to facilitate the mobility of chromium (III) to the shoot region in all levels of treatment. However, a trace amount was observed to be domiciled within the root region in cowpea; in groundnut no metal presence was detected in the roots indicating a low mobility potential of chromium in the two plants. It could be that the two plants have barriers against chromium (III) transport or lack mechanisms for its transport from root to shoot. This is explained by the fact that the two forms of chromium, Cr (III) and Cr (VI) play no role in plants metabolism (Shanker et al., 2005) and nutrient uptake. The ability of plants to tolerate and take up heavy metals is useful for their classification for phytoremediation purposes (Yoon et al., 2006). The two legumes demonstrated low mobility for chromium. Kleiman and Cogliatti (1998) in their work with other plant species also reported a low mobility of chromium due to some mechanism that hindered its transportation.

## Conclusion

This work has brought the following to limelight:

(1) The retardative effects of chromium (III) on soil microbial population and metabolism.
(2) The inhibitory effects of chromium (III) on the germination and general growth performance of

groundnut and cowpea.
(3) The low translocation potentials for chromium exhibited by groundnut and cowpea. Cowpea however demonstrated a better phytoremediation capability than groundnut for soil heavily polluted with chromium (III) nitrate.

There is need for more research to delve into the mechanisms responsible for the higher resistance to chromium (III) toxicity exhibited by cowpea over groundnut. Understanding this could help enhance and engineer this trait for a more effective phytoremediation of environments contaminated with chromium (III) compounds.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Phytochemical and proximate composition of cucumber (*Cucumis sativus*) fruit from Nsukka, Nigeria

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**Cucumber (*Cucumis sativus* L.) is very common, cultivated throughout the world and often eaten as a raw vegetable without cooking. In this study, the phytochemical and proximate compositions of cucumber were investigated. Quantitative phytochemical analysis of the homogenate of *C. sativus* fruit showed that reducing sugars (574.36 ± 3.88 mg/g) was highest amount when compared to other phytochemicals, alkaloids (2.22 ± 0.96 mg/g) and flavonoids (2.14 ± 0.56 mg/g) were moderately present, while cyanogenic glycoside (0.21 ± 0.13 mg/g) was the lowest in quantity. Proximate analysis showed that *C. sativus* fruit contained the following - fibre (1.02 ± 0.01%), moisture (94.2 ± 0.08%), protein (3.01 ± 0.07%), lipid (0.55 ± 0.13%), carbohydrate (0.28 ± 0.09%) and ash (0.94 ± 0.24%) contents.**

**Key words:** Phytochemicals, *Cucumis sativus,* proximate constituents.

## INTRODUCTION

Phytochemicals are secondary metabolites produced by plants. These products are biologically active, naturally occurring chemicals in various parts of a plant, providing health benefits for humans further than those attributed to macronutrients and micronutrients. Their functions are diverse and include provision of strength to plants, attraction of insects for pollination and feeding, while some are simply waste products (Ibegbulem et al*.,* 2003). They give plants colour, flavour, and smell and are part of plants' natural defence system, protecting plants' cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attacks (Ejele and Akujobi, 2011). These compounds have been linked to human health by contributing to protection against degenerative diseases (Dandjesso et al*.*, 2012). Phytochemicals are present in varieties of plants utilized as important components of both human and animal diets. These include fruits, seeds, herbs and vegetables (Okwu, 2005). Different mechanisms have been suggested for the action of phytochemicals. They may act as antioxidants, or modulate gene expression and signal transduction pathways (Dandjesso et al*.*, 2012). Phytochemicals may be used as chemotherapeutic or chemopreventive agents (Paolo et al., 1991). They are formed during the plant normal metabolic processes. The medicinal values of a plant lie in its constituent phytochemicals, which produce

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the definite physiological actions on human body. The most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Iwu, 2000).

Cucumber (*Cucumis sativus)* fruit is a widely cultivated plant in the gourd family of Cucurbitaceae, which also includes important crops such as melon, water melon and squash (Vivek et al., 2017). The plant has large leaves that form a canopy over the fruit. The fruit of the cucumber is roughly cylindrical, elongated with tapered ends, and may be as large as 60 cm (24 inches) long and 10 cm (3.9 inches) in diameter. Having an enclosed seed and developing from flowers, botanically speaking, cucumber can be classified as an accessory fruit (Huang et al*.,* 2009). There is increased consumption of *C. sativus* fruits possibly because of their high nutritional value. The nutritional compositions of *C. sativus* include protein, fat and carbohydrate as primary metabolites; along with dietary fibre which is important for the digestive system. *C. sativus* contains some essential vitamins and anti-oxidants which are effective in human health (Grubben and Denton, 2004; Wang et al., 2007). *C. sativus* is used for jaundice, bleeding disorders and anuria; while its seeds are highly nourishing (Gogte, 2000). Till date, the present study on *C. sativus* represents variety of pharmacological activities like anticancer, anthelmentic, antimicrobial, hypolipidemic, antiulcer, analgesic and antioxidant (Dhande et al., 2013). It is believed that *C. sativus* seed has flavonoid, tannin, terpenoids and some phytochemicals (Kumar et al., 2010). Despite the acclaimed presence of those phytochemicals, to the best of our knowledge, the phytochemical and proximate compositions of the whole fruit (homogenate) is yet to be empirically established. Here, the phytochemical and proximate compositions, along with the potential pharmaceutical function of the whole *C. sativus* L. fruit were examined, highlighted and shown as homogenate.

## MATERIALS AND METHODS

### Plant material

Cucumber (*C. sativus*) fruits were purchased from Nsukka Main Market, Nsukka, Enugu State, Nigeria and were identified by Mr. Alfred Ozioko of Bioresources Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu. The fruits were washed under running water, homogenized with Kenwood high speed blender and used for analysis without further dilution.

### Qualitative phytochemical analysis

The qualitative phytochemical analyses of the fruits of *C. sativus* were carried out according to the methods of Harborne (1998) and Trease and Evans (2002). Quantitative determination of tannin was conducted using spectrophotometric determination method described by Gupta and Verma (2010). The total phenol content of *C. sativus* fruit was determined using a spectrophotometric method of Wolfe et al*.* (2003). Cyanogenic glycoside was determined using alkaline picrate method as described by Harborne (1998).

Spectrophotometric determination of glycoside content was carried out with a method described by Quasheesh (1937). The flavonoid content was estimated using ferric chloride colorimetric method of Mattila and Kumpulairen (2002). Saponin content was quantitatively estimated by spectrometric determination method of Uematsu et al*.* (2000). Determination of alkaloid content was carried out by the method described by Harborne (1998). The amount of steroid was determined by the method described by Edeoga et al*.* (2005). Quantitative determination of reducing sugars was carried out using Folin and Wu method (1920). Resin content was determined quantitatively by the UV absorption method of Harborne (1998). Quantitative estimation of terpenoid content was carried out using oxidation method of Harborne (1998). Anthocyanin content was estimated quantitatively with pH differentiation method of Harborne (1998). Chlorophyll content was determined using Harborne (1998) method.

$$\text{Chlorophyll b}\left(\frac{\text{mg}}{\text{g}}\right)=\frac{(19.3\times\text{Absorbance @ 645})-3.6\times(\text{Absorbance @ 663})}{1000\times\text{W}}\times\text{V}$$

$$\text{Chlorophyll a}\left(\frac{\text{mg}}{\text{g}}\right)=\frac{(12.3\times\text{Absorbance @ 663})-0.86\times(\text{Absorbance @ 645})}{1000\times\text{W}}\times\text{V}$$

Where, V = Volume of solvent, and W = Weight of homogenate.

### Proximate analysis

The proximate analysis of the homogenate of *C. sativus* fruits for moisture, ash, fat and carbohydrate were determined as described by AOAC (2000). The concentration of crude protein and fibre were determined using methods described by Pearson (1976). All determinations were done in triplicates and the results were expressed as means of percent values on dry weight basis.

### Statistical analysis

Each experiment was repeated three times, and the results were presented as means and standard deviation.

## RESULTS AND DISCUSSION

As shown in Table 1, bioactive compounds such as steroids, terpenoids, glycosides and resins were found in relatively high concentrations; saponins, alkaloids and flavonoids were present in moderate concentrations, while tannins were slightly present. Table 2 shows quantitatively, the phytochemical composition of *C. sativus* fruit homogenate. Bioactive compounds such as reducing sugars were found to be in highest amount (574 ± 3.88 mg/g) relatively compared to other phytochemicals as shown in Table 2. Alkaloids and flavonoids that were moderately present were found in the concentration range of 2.22 ± 0.96 and 2.14 ± 0.56 mg/g respectively. The concentration range of cyanogenic glycosides: 0.21 ± 0.13 mg/g was very low. The proximate analyses of the homogenate of *C. sativus* fruit showed the presence of moisture, crude protein, ash and crude fibre with values shown in Table 3. The homogenate of *C. sativus* fruit had high concentrations of moisture (94.6 ± 0.08%).

**Table 1.** Qualitative phytochemical constituents of the homogenate of *Cucumis sativus* fruit.

| Phytochemical | Relative presence |
|---|---|
| Alkaloids | ++ |
| Glycosides | +++ |
| Steroids | +++ |
| Saponins | ++ |
| Tannins | + |
| Flavonoids | ++ |
| Terpenoids | +++ |
| Resins | +++ |

+ = Slightly present; ++ = moderately present; +++ = highly present.

**Table 2.** Quantitative phytochemical constituents of the homogenate of *Cucumis sativus* fruit.

| Phytochemicals | Composition (mg/g) |
|---|---|
| Tannins | 1.26 ± 0.07 |
| Polyphenols | 8.51 ± 0.50 |
| Phenols | 7.72 ± 0.50 |
| Cyanogenic glycosides | 0.21 ±0.13 |
| Anthocyanins | 1.21 ± 0.39 |
| Glycosides | 32.23 ± 0.41 |
| Reducing sugars | 574.36 ± 3.88 |
| Saponins | 2.01 ± 0.08 |
| Alkaloids | 2.22 ± 0.96 |
| Flavonoids | 2.14 ± 0.56 |
| Terpenoids | 26.27 ± 1.37 |
| Steroids | 11.69 ± 1.80 |
| Resins | 50.70 ± 8.82 |
| Chlorophyll a | 4.49 ± 0.03 |
| Chlorophyll b | 12.09 ± 0.04 |

Values indicate Mean ± SD (n = 3).

Proximate analyses also revealed ash as being very low (1.07 ± 0.24%).

This study reveals the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals such as terpenoids, reducing sugar, glycosides, resins, flavonoids, alkaloids, phenols, saponins, steroids and tannins were present in the homogenate of cucumber fruits (Table 1). Bioactive compounds such as reducing sugars were found to be in highest amount (574 ± 3.88 mg/g) relatively compared to other phytochemicals as shown in Table 2. Alkaloids and flavonoids that were moderately present were found in the concentration range of 2.22 ± 0.96 and 2.14 ± 0.56 mg/g, respectively. The concentration range of cyanogenic glycosides (0.21 ± 0.13 mg/g) was very low. The presence of flavonoids in cucumber fruit homogenate suggests that the fruit homogenate has the ability to

**Table 3.** Proximate composition of the homogenate of *Cucumis sativus* fruit.

| Constituent | Percentage |
|---|---|
| Moisture | 94.2 ± 0.08 |
| Crude protein | 3.01 ± 0.07 |
| Crude fibre | 1.02 ± 0.01 |
| Ash | 0.94 ± 0.24 |
| Lipid | 0.55 ± 0.13 |
| Carbohydrate | 0.28 ± 0.09 |

Values indicate mean percentage ± SD (n = 3).

scavenge free radicals as they are the chief sources of antioxidant (Singh Gill et al., 2010; Egbung et al., 2013) in plants which have been known to play some role in free radical scavenging. The antioxidant activity of the phenolics, tannins, flavonoid compounds are attributed to their redox properties which can act as reducing agents, hydrogen donors and singlet oxygen quenchers (Gulcin et al., 2007; Andreia et al., 2013). Polyphenolics having hydroxyl groups are very important plant constituents which can protect the body from different types of oxidative stress (Jing et al*.,* 2010; Anoop and Bindu, 2015) such as $CCl_4$ induced hepatotoxicity. Epidemiologic studies recommend that coronary heart disease is opposed by dietary flavonoids (Wadood et al*.,* 2013). Saponins detected in the fruit, is a known anti-nutritional factor, which reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intra-lumenal physiochemical interactions (Shi et al*.,* 2004; Agbafor et al., 2015). It has been reported to have hypocholesterolemic effects; hence it is useful in human diet in controlling cholesterol levels (James et al*.,* 2010) and may aid in lessening the metabolic burden that would have been placed on the liver during metabolism. The homogenate of *C. sativus* fruits show trace amount of tannins which have been reported to possess some medicinal properties (Ekeanyanwu et al*.,* 2010). Its wound healing properties, which include anti-inflammatory, analgesic (Ayinde et al*.,* 2007) and antioxidant properties (Okwu and Okwu, 2004) have been reported; although they (tannins) are anti-nutrients (Doss et al*.,* 2011). Ibrahim et al. (2014) reported anti-microbial effects of tannins through membrane disruption, binding to proteins, adhesions and enzyme inhibition. This result is in line with the findings of Liener (1994) who stated that lower concentrations of tannins in plants are found to be desirable for human and animal consumption. It could be the reduced amounts of tannins in the homogenate of the fruit that enhanced the protective property rather than the side effects. The phytochemical screening result of this study is contrary to the report of Jony and Roksana (2012) who reported the absence of flavonoids in the ethanol extract of *C. sativus.* Perhaps also, flavonoids were not detected as a result of the

extraction method used, as Kumar et al. (2010) reported the presence of flavonoids in the aqueous extract, thus correlating the findings of this investigation. The homogenate of *C. sativus* fruit also revealed the presence of significant amount of chlorophylls a and b. Chlorophyll is important in many plant metabolic functions such as growth and respiration. It is used in medicinal preparation for treating anaemia and hypertension as a healing agent and in oral hygiene (Fischman, 1997; Mujoriya and Bodla, 2012), indicating that *C. sativus* can be used to reduce bad breath and as healing agent.

The homogenate of *C. sativus* fruit also revealed the presence of alkaloids. Plants having alkaloids are used in medicines for reducing headache and fever. These are attributed to their antibacterial and analgesic properties (Pietta, 2000; Sotiroudis et al., 2010). Alkaloids have been reported to act as central nervous system stimulant (Abubakar et al., 2015). Also, they possess antispasmodic, antifungal, anti-fibrogenic effects (Ibraheem and Maimoko, 2014). Terpenoids detected in the fruit are reported to have anti-inflammatory (Olorunju et al., 2012), anti-viral, anti-malarial, inhibition of cholesterol synthesis (Njagi et al., 2015) and anti-bacterial properties (Wadood et al., 2013). The significant amount of terpenoids and alkaloids from this study show that *C. sativus* fruit homogenate could be recommended as an effective source of anti-bacterial agent. The proximate analyses show that the homogenate of *C. sativus* fruits have high concentration of moisture and relative amount of fibre, crude protein, ash, lipid and carbohydrate. Dietary fibre helps to reduce the chance of gastro intestinal problems such as constipation and diarrhoea by increasing the weight, size and wetness of stool (Weickert and Pfeiffer, 2008; Aina et al., 2012). Plant fibres are long chain carbohydrates (polysaccharides) that are indigestible by the digestive enzymes of human gastro intestinal tract (GIT). They help to keep the digestive system healthy and also aid and speed up the excretion of waste materials from the body (Weickert and Pfeiffer, 2008). The recommended daily allowance of fibre for a healthy adult is 20-25 g/day (American Dietetic Association, 2005). The result of this study is in line with the report that *C. sativus* is useful in fighting constipation, as the fibre content helps to overcome the hypotonic which aids constipation (Yohanna, 2013). The low concentration of lipid obtained for the fruit homogenate suggests that its regular incorporation and consumption in the diet is healthy for people on low fat diet. The result on the high concentration of moisture agrees with the report of Aina et al. (2012) that fleshy fruits have high percentage of moisture which aids in digestion and acts as a solvent in chemical reactions in the body system. The high moisture concentration is in accordance with the report of Egan et al. (1981) and Okoye (2013) which showed the moisture content of *C. sativus* as 96.4 and 97.8%, respectively. The appreciable amount of ash recorded from the study (Table 3) shows that *C. sativus* fruit homogenate could be recommended as effective sources of mineral nutrients.

## Conclusion

Cucumber (*C. sativus*) fruit is a source of the secondary metabolites, that is, alkaloids, flavonoids, terpenoids, tannins, saponins, steroids, phenols, glycosides, reducing sugars, etc. Cucumber fruit may play vital role in preventing various diseases such as inflammation, bacterial infection, lipid peroxidation, fever, constipation, etc. The anti-inflammatory, anti-bacterial, antioxidant, analgesic and anti-constipation may be due to the presence of the above mentioned phytochemicals especially flavonoid (2.14 ± 0.56 mg/g), alkaloids (2.22 ± 0.96 mg/g) and proximate constituents. Thus, it is expected that the important phytochemical properties and proximate compositions identified in this study in the homogenate of cucumber fruit will be helpful in the copping of different diseases.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# *Trichoderma atroviride* 102C1: A promising mutant strain for the production of a β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities using agroindustrial by-products

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**Holocellulolytic accessory enzymes are very important in assisting hydrolysis of biomass. The use of these enzymes in the enzymatic hydrolysis of plant biomass is very important for obtaining building blocks in the concept of biorefinery. In previous studies, the mutant strain *Trichoderma atroviride* 102C1 was tested for production of endoglucanases, FPases and endoxylanases. This study aimed at evaluating the efficiency in holocellulolytic accessories enzymes production (β-glucosidase, β-xylosidase and α-L-arabinofuranosidase) by *Trichoderma atroviride* 102C1 using different lignocelluloses biomass as substrates. Accessory enzymes production was carried out in Erlenmeyer flasks containing Mandels salt medium, supplemented with different concentrations of sugarcane bagasse (SCB) and corn steep liquor (CSL), according to a Central Composite Rotational Design (CCRD). The fermentation system was incubated under agitation for 2 days / 28°C. For pH and temperature profile studies, a new CCRD was carried out. The best condition common to all enzymes, 55.4 U.mL$^{-1}$ (β-glucosidase), 10.8 U.mL$^{-1}$ (β-xylosidase) and 143.23 U.mL$^{-1}$ (α-L-arabinofuranosidase), was observed when 2.5% (w/v) sugarcane bagasse (SCB) and 1.26% (w/v) of corn steep liquor (CSL) were used. All enzymes presented acidophilic characteristic in two different temperatures (44 and 55°C). The optimal profile characteristic for β-glucosidase and β-xylosidase activities were pH 5.0 and 3.0, respectively, both at 55°C, while for α-L-arabinofuranosidase it was pH 3.6 at 44°C. This study demonstrated the potential of *T. atroviride* 102C1 to produce three important holocellulolytic accessory enzymes in the presence of SCB and CSL, suggesting its use for enzymatic hydrolysis of lignocellulosic biomass.**

**Key words:** *Trichoderma atroviride* 102C1, holocellulolytic enzymes, sugarcane bagasse, corn steep liquor.

## INTRODUCTION

Plant cell walls are a source of renewable carbon present in nature as cellulose, hemicelluloses and lignin. Cellulose is an abundant linear polymer worldwide and is composed of glucose residues linked by β-1,4-glucosidic bonds. Hemicellulose is a branched heteropolymer of pentoses and/or hexoses and various types of uronic acids residues linked by β-1,4 / β-1,3 glucosidic bonds. The term hollocellulose comprises the cellulose and hemicelluloses of plant cell wall linked together (Gottschalk et al., 2010; Zampieri et al., 2013).

Worldwide attention has focused on the major biotechnological uses of the carbohydrates in agro-industrial by-products, as biomass syrups that have sugars with five or six carbons (derived from xylan and glucan). They can be used as carbon sources in industrial fermentations producing antibiotics, industrial enzymes, and bulk chemicals, including ethanol. For these purposes, however, the polysaccharides in the biomass must first be hydrolyzed (Gottschalk et al., 2010; Shinozaki et al., 2015).

The complete hydrolysis of polysaccharide fraction of lignocellulose biomass requires the cooperative action of several enzymes. Endo-1,4-β-D-glucanases (EC 3.2.1.4) which hydrolyze the cellulose polymer internally, exposing reducing and non-reducing ends, and exo-1,4-β-D-glucanases (3.2.1.91) which act on the reducing and non-reducing ends, releasing cellobiose and cellooligosaccharides. The complete hydrolysis of cellulose is finalized through the action of β-glucosidase (E.C. 3.2.1.21), which cleaves cellooligosaccharides and cellobiose to glucose (Zampieri et al., 2013; Singhania et al., 2013). Considering the hemicellulose fraction, endo-1,4-β-D-endoxylanases (EC 3.2.1.8) can degrade randomly the xylan portion of the polymer, releasing xylooligosaccharides. Synergistically, β-xylosidase (E.C. 3.2.1.37) release D-xylose residues from xylooligosaccharides and xylobiose (Terrasan et al., 2010; Kirikyali and Connerton, 2014). Due to the structural complexity of the hemicelluloses, other enzymes are also required for the hydrolysis of that polymer, as acetyl-xylan esterase (E.C. 3.1.1.72) and a-L-arabinofuranosidase (E.C. 3.2.1.55), which are able to release α-L-1,2-, α-L-1,3- and α-L-1,5-arabinofuranosyl side chains of arabinoxylans, arabinans and arabinogalactans (Temer et al., 2014; Shinozaki et al., 2015).

*Trichoderma atroviride* 676, isolated from Amazon forest soil, was capable to produce cellulases and endoxylanases when agro-industrial by-products were used as substrates (Grigorevski-Lima et al., 2013). After mutation procedures in this strain, a new mutant, *T. atroviride* 102C1, producer of high titers of endoglucanase, exoglucanase and endoxylanase was obtained (Oliveira et al., 2014). The objective of the present study was to investigate the production of some hollocellulolytic accessory enzymes of *T. atroviride* 102C1, including β-glucosidase, β-xylosidase and α-L-arabinofuranosidase, using submerged fermentation and, as main substrates, sugarcane bagasse *in natura* and corn steep liquor.

## MATERIALS AND METHODS

### Maintenance of microorganism

Mutant strain *T. atroviride* 102C1 was obtained from the wild strain *T. atroviride* 676 (Grigorevski-Lima et al., 2013), after successive exposures to U.V. radiation and nitrosoguanidine, according to Oliveira et al. (2014). Spore suspensions were prepared according to Hopwood et al. (Hopwood et al., 1985), after cultivation at 28°C for 7 days in potato dextrose agar medium (Hanada et al., 2002). Spores were maintained in 20% (v/v) glycerol at −20°C. Concentration of the spore suspension was determined in a Neubauer chamber.

### Enzyme production

Enzyme production was performed in submerged fermentation in Erlenmeyer flasks (125 ml) containing 25 ml of a modified culture medium (Mandels and Weber, 1969), in $g.L^{-1}$: urea, 0.3; $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $CoCl_2.6H_2O$, 0.02; $MnSO_4.4H_2O$, 0.016; and $ZnSO_4.7H_2O$, 0.014 supplemented with sugarcane bagasse *in natura* (SCB) and corn steep liquor (CSL) at different concentrations, according to experimental design $2^2$ central composite rotational design (CCRD). Response surface methodology (RSM) was used as a tool for the optimization of SCB and CSL concentrations (independent variables) in the range indicated in Table 1. Enzymes activities, β-glucosidase, β-xylosidase and α-L-arabinofuranosidase ($U \cdot mL^{-1}$) were the dependent variables. Medium start pH was adjusted to 4.8. The growth media were inoculated with 25 µl of a spore suspension ($10^{11}$ spores$\cdot mL^{-1}$) and incubated at 28°C, under agitation (200 rpm). Based on preliminary tests, a two days period was chosen. After this, the whole content of a shake flask was filtered through a glass microfiber filter (Whatman GF/A) in duplicate, and the culture supernatants obtained were used to determine the all enzymatic assays.

### Enzymatic assays

β-Glucosidase (BGU), β-xylosidase (BXU) and α-L-arabinofuranosidase (ARF) activities were determined using standard IUPAC procedures and expressed in international units(IU), by release of *p*-nitrophenol obtained by hydrolysis of the were substrates (Rasmussen et al., 2001). BGU and BXU activities

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**Table 1.** Values of the independent variables ($X_1$ = sugarcane bagasse *in natura*; $X_2$ = corn steep liquor) of the CCRD 2², showing the statistically values for producing β-glucosidase, β-xylosidase and α-L-arabinofuranosidase by *T. atroviride* 102C1

| Assay | Coded setting levels | | Actual levels (%) (w/v) | | β-glucosidase ($U.mL^{-1}$)* | β-xylosidase ($U.mL^{-1}$)* | α-L-arabinofuranosidase ($U.mL^{-1}$)* |
|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_1$ | $X_2$ | | | |
| 1 | -1 | -1 | 1.5 | 0.3 | 28.32 | 1.01 | 70.30 |
| 2 | +1 | -1 | 3.5 | 0.3 | 33.60 | 1.79 | 127.23 |
| 3 | -1 | +1 | 1.5 | 1.1 | 38.25 | 10.92 | 114.36 |
| 4 | +1 | +1 | 3.5 | 1.1 | 54.59 | 2.21 | 159.24 |
| 5 | -1.41 | 0 | 1.09 | 0.7 | 42.05 | 3.18 | 99.18 |
| 6 | +1.41 | 0 | 3.91 | 0.7 | 41.01 | 2.18 | 157.43 |
| 7 | 0 | -1.41 | 2.5 | 0.15 | 26.11 | 1.69 | 86.30 |
| 8 | 0 | +1.41 | 2.5 | 1.26 | 55.38 | 10.75 | 143.23 |
| 9 | 0 | 0 | 2.5 | 0.7 | 46.60 | 2.36 | 126.73 |
| 10 | 0 | 0 | 2.5 | 0.7 | 45.74 | 2.08 | 130.20 |
| 11 | 0 | 0 | 2.5 | 0.7 | 49.11 | 1.97 | 130.69 |

* Values are based on Mean ± SD of 2 individual observations.

determined by mixing 50 µl of the enzyme preparation to 100 µl of *p*-nitrophenyl-β-D-glucopyranoside or *p*-nitrophenyl-β-D-xilopyranoside (10 mM) in 200 µl of 100 mM sodium acetate buffer, pH 5.0 at 50°C for 10 min, respectively, supplemented with 650 µl of distilled water. ARF activity was determined by mixing 100 µl of the enzyme preparation to 100 µl of *p*-nitrophenyl-α-L-arabinofuranoside (10 mM) in 200 µl of 100 mM sodium acetate buffer, pH 5.0 at 50°C for 10 min, supplemented with 600 µl of distilled water. After incubation time, the enzyme reaction was stopped by addition of 500 µl of $Na_2CO_3$ (1.0 M), pH 10.0 and reading in spectrophotometer at 420 nm. One unit of BGU, BXU and ARF (IU) corresponded to formation of 1.0 µmol of *p*-nitrophenol at 50°C per minute. The results were analyzed using Statistica Statsoft 7.0®.

**Determination of physico-chemical properties of enzymes: pH, temperature, thermal stability and metal ions effect on enzymatic activities**

Temperature and pH effect on BGU, BXU and ARF activities was investigated using standard assay methods at various temperatures and pH range, according to CCRD 2². In the 12 experiments which were carried out, the temperature ranged from 40 to 70°C and the pH values from 3.0 to 7.0 as shown in Table 3. Citrate buffer (50 mM) was used for pH 3.0, 3.6 and 5.0 and citrate-phosphate (50 mM) for pH 6.4 and 7.0. Statistical analysis was performed using the software Statistica Statsoft 7.0®. Temperature stability range was determined by incubating the crude enzyme at 50 and 70°C. Residual enzyme activity was determined at different time interval up to 8 h. Influence of sodium, calcium, potassium, manganese, and barium ions in the chloride form, and copper, magnesium, zinc, and iron in the sulfate form, on the BGU, BXU and ARF activities were performed by the addition of the relevant salts at 2 mM final concentration in the enzyme activity assay using the previously determined optimal conditions for pH and temperature.

## RESULTS

### Enzyme production

In this study the mutant strain *T. atroviride* 102C1 have produced some holocellulolytic accessory enzymes when using sugarcane bagasse (SCB) and corn steep liquor (CSL) as substrates in submerged fermentation. The use of RSM and CCRD tools for the optimization resulted in enzyme activities accumulation in the range of 26.11 to 55.38 $U.mL^{-1}$ for β-glucosidase, 1.01 to 10.75 $U.mL^{-1}$ for β-xylosidase and 70.30 to 159.24 $U.mL^{-1}$ for α-L-arabinofuranosidase after two days fermentation (Table 1). The fitted response surface for the production of the three enzymes is given in Figure 1 and, as can be seen, best conditions for each one, were different. For β-glucosidase, the best enzyme production (55.38 $U.mL^{-1}$) was observed when 2.5% (w/v) SCB and 1.26% (w/v) CSL were used. For β-xylosidase (10.92 $U.mL^{-1}$) best concentrations were 1.5% (w/v) SCB and 1.1% (w/v) CSL, and for α-L-arabinofuranosidase (159.24 $U.mL^{-1}$), 3.5% (w/v) SCB and 0.3% (w/v) CSL. However, for studying the BGU, BXU and ARF concomitantly, the run 8 [2.5% (w/v) SCB and 1.26% (w/v) CSL] was considered as the best for further analysis. Under these conditions the enzyme activities were 55.38 $U.mL^{-1}$ (BGU), 10.75 $U.mL^{-1}$ (BXU) and 143.23 $U.mL^{-1}$ (ARF). The relevant regression equations, resulting from the analysis of variance (ANOVA) (Table 2) have shown β-glucosidase, β-xylosidase and α-L-arabinofuranosidase production as a function of the codified values of SCB and CSL. The equations that represented a suitable model for β-glucosidase ($Y_{BGU}$), β-xylosidase ($Y_{BXU}$) and α-L-arabinofuranosidase ($Y_{ARF}$) production are given in:

$$Y_{BGU} = 47.15 + 9.04*CSL + 2.7*SCB*CSL - 3.42*SCB^2 - 3.82*CSL^2 + 5.4*SCB*CSL^2 \quad (1)$$

$$Y_{BXU} = 2.25 + 2.89*CSL - 2.37*SCB*CSL + 1.9*CSL^2 - 1.98*SCB*CSL^2 \quad (2)$$

$$Y_{ARF} = 127.9 + 23.02*SCB + 19.57*CSL - 7.75*CSL^2 \quad (3)$$

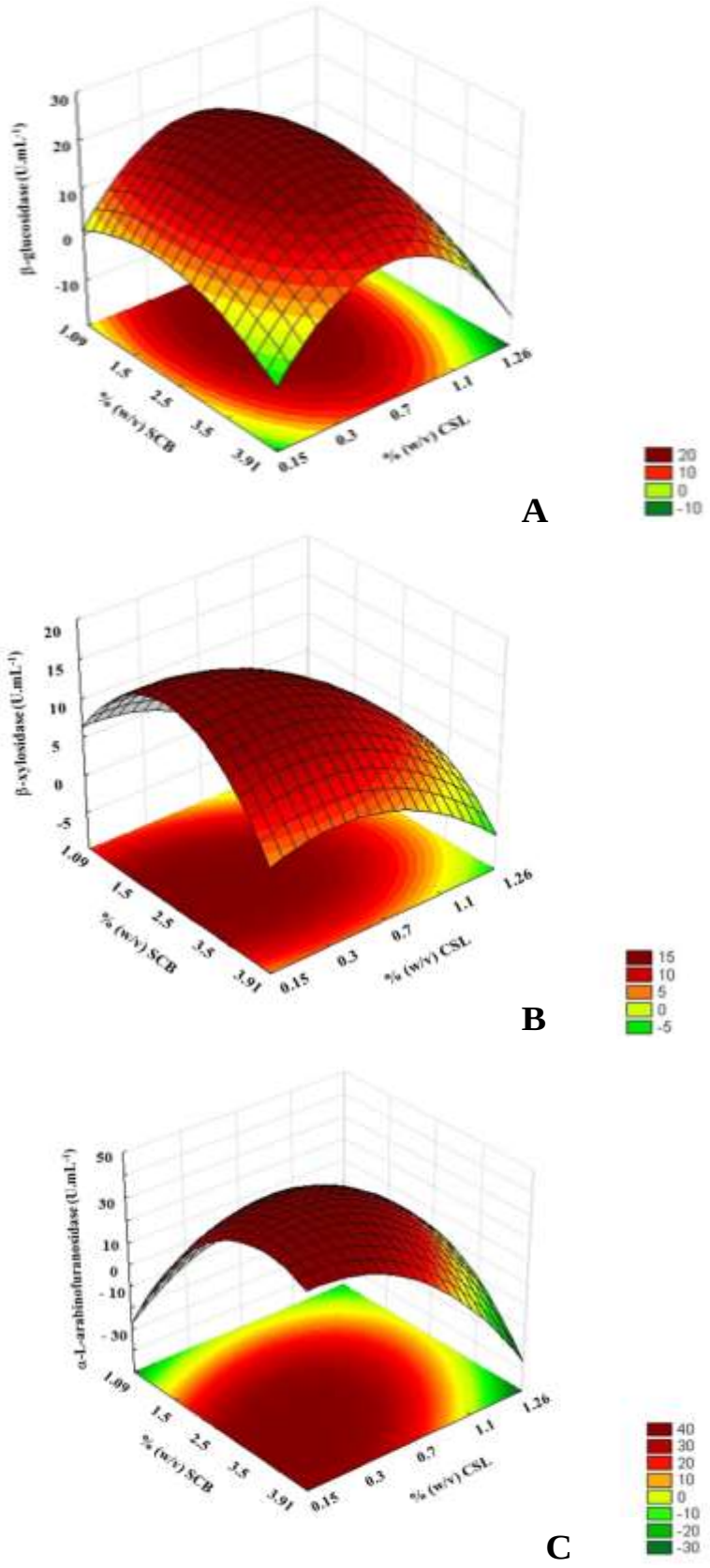


**Figure 1.** Response surface on β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) production by *T. atroviride* 102C1 using SCB and CSL concentrations as the independent variables.

**Table 2.** Statistical ANOVA for the model of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities by *T. atroviride* 102C1 at different concentrations of SCB and CSL values.

| Enzyme | Source of variation | Sum of squares | Degrees of freedom | Mean squares | F value | Value (prob > )[b] |
|---|---|---|---|---|---|---|
| β-glucosidase | Model | 916.14 | 5 | 183.23 | 28.32 | 0.0011 |
| | Residual | 32.37 | 5 | 6.47 | | |
| | Lack of Fit | 26.25 | 3 | 8.75 | 2.86 | 0.2697 |
| | Pure Error | 6.12 | 2 | 3.06 | | |
| | Total | 948.51 | 10 | | | |
| β-xylosidase | Model | 127.61 | 4 | 31.90 | 93.82 | <0.0001 |
| | Residual | 2.03 | 6 | 0.34 | | |
| | Lack of Fit | 1.90 | 4 | 0.48 | 7.41 | 0.1224 |
| | Pure Error | 0.13 | 2 | 0.064 | | |
| | Total | 129.64 | 10 | | | |
| α-L-arabinofuranosidase | Model | 7,676.96 | 3 | 2,558.99 | 133.28 | <0.0001 |
| | Residual | 134.41 | 7 | 19.20 | | |
| | Lack of Fit | 125.10 | 5 | 25.02 | 5.37 | 0.1643 |
| | Pure Error | 9.31 | 2 | 4.66 | | |
| | Total | 7,811.37 | 10 | | | |

[b] Statistically significant at 95% of confidence level; $R^2$= 0.967 (β-glucosidase); $R^2$= 0.984 (β-xylosidase); $R^2$= 0.982 (α-L-arabinofuranosidase).

The model $F$ values of 28.32, 93.82 and 133.28 imply that the models are significant at a high confidence level. The probability value was also very low (<0.05) indicating the significance of the model.

## Determination of physico-chemical properties of enzymes

With respect to pH and temperature effects, the maximum enzyme accumulation were, as expected, influenced by pH and temperature. According to CCRD, the analysis of resulting surface response plots revealed that maximal β-glucosidase relative activity (Figure 2A) occurred in pH range of 4.5-5.5 and temperature of 50-60°C, while the maximal β-xylosidase (Figure 2B) and α-L-arabinofuranosidase (Figure 2C) relative activities occurred in pH range of 3.0-4.0 and temperature of 50-60°C / 40-50°C, respectively. So, characterization of the crude enzyme showed that the best values for enzyme production from *T. atroviride* 102C1 were: 55°C and pH 5.0 for BGU, 55°C and pH 3.0 for BXU, and 44°C and 3.6 for ARF (Table 3). These results indicate an acidic condition favoring all enzymes activities, whereas best temperature varied between 44 and 55°C (Table 3). Considering the concomitant production of the enzymes, one should choose pH 5.0 and temperature of 55°C, for best results (assay number 10, Table 3).

The model was tested for adequacy by ANOVA (Table 4). The model $F$ values of 11.64 (BGU), 45.21 (BXU) and 14.68 (ARF) indicates that the model is significant at a high confidence level. The probability $P$ value was also very low (<0.05) indicating the significance of the model. The coefficient of determination obtained for β-glucosidase ($R^2$=0.8845), β-xylosidase ($R^2$=0.9540) and α-L-arabinofuranosidase ($R^2$=0.9070) indicates that 88.45% (BGU), 95.40% (BXU) and 90.70% (ARF) of the variability of the responses can be explained by the model. The regression equations, obtained after the ANOVA, demonstrated enzymes activities as a function of the codified values of pH and temperature. The equation that represented a suitable model for β-glucosidase relative activity (Y) is given in:

$$Y_{BGU} = 95.80 - 12.01*pH + 2.56*T - 45.38*pH^2 - 17.82*T^2 - 8.25*pH*T \quad (4)$$

$$Y_{BXU} = 86.18 - 30.46*pH - 16.15*pH^2 - 33.83*T^2 - 7.37*pH*T \quad (5)$$

$$Y_{ARF} = 45.87 - 25.03*pH - 15.28*T - 18.22*T^2 + 21.83*pH*T \quad (6)$$

Thermal stability constitutes also an important property when studying the industrial importance of an enzyme. The results of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase thermal stability are shown in Figure 3. When the enzyme crude extract was incubated at 60°C, the relative activities of β-glucosidase (Figure 3A) and β-xylosidase (Figure 3B) decreased around 50% within 30 min, however, a strong decreased (95%) was

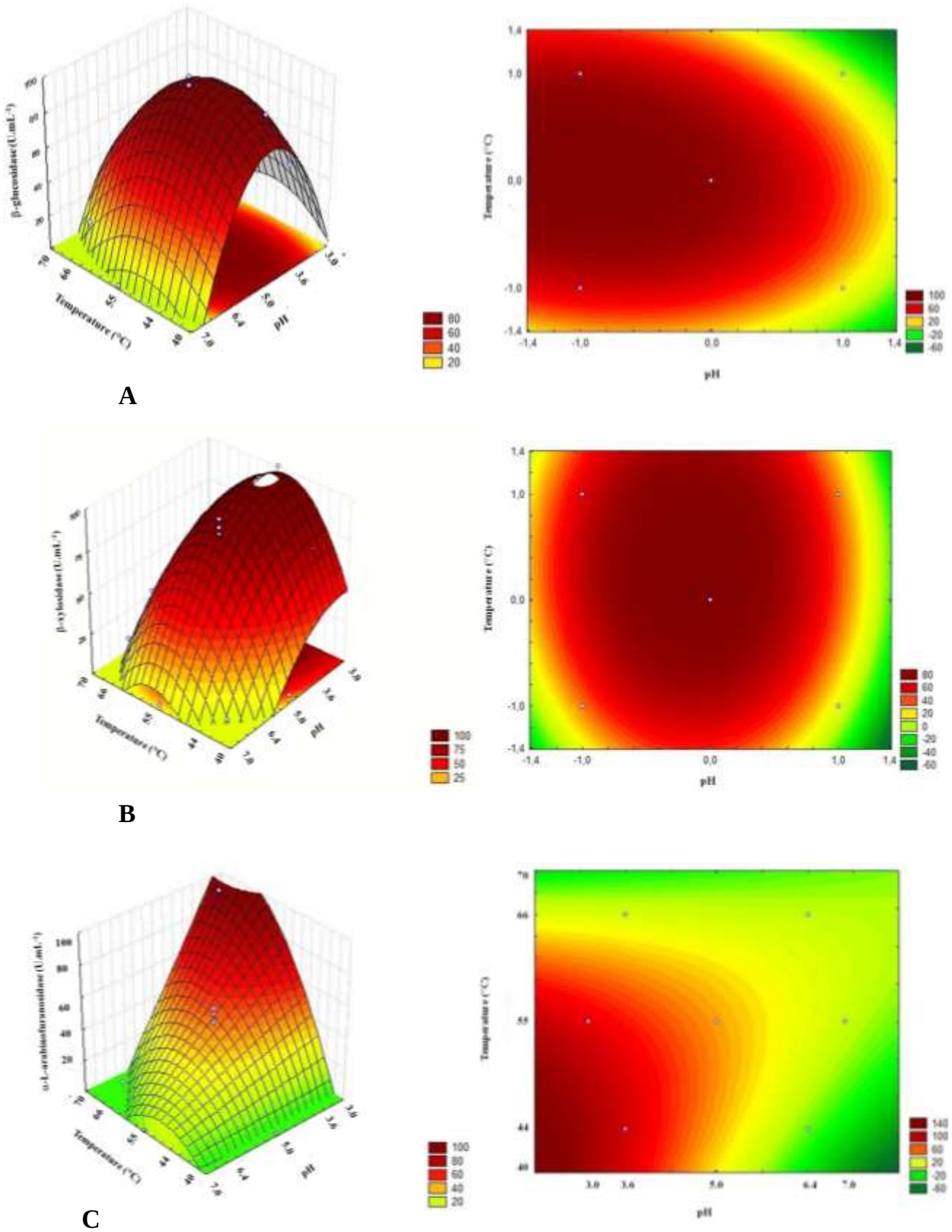


**Figure 2.** Response surface for *T. atroviride* 102C1 β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) activities by using pH and temperature values as the independent variables.

**Table 3.** Values of the independent variables [$X_1$ = pH; $X_2$ = temperature (°C)] of the CCRD $2^2$, showing the statistically values for producing β-glucosidase, β-xylosidase and α-L-arabinofuranosidase by *T. atroviride* 102C1

| Assay | Coded setting levels | | Actual levels (%) (w/v) | | β-glucosidase (Relative Activity %)* | β-xylosidase (Relative Activity %)* | α-L-arabinofuranosidase (Relative Activity %)* |
|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_1$ | $X_2$ | | | |
| 1 | -1 | -1 | 3.6 | 44 | 29.61 | 59.73 | 100 |
| 2 | +1 | -1 | 6.4 | 44 | 15.52 | 21.28 | 7.05 |
| 3 | -1 | +1 | 3.6 | 66 | 49.20 | 70.89 | 6.13 |
| 4 | +1 | +1 | 6.4 | 66 | 2.00 | 3.01 | 0.49 |
| 5 | -1.41 | 0 | 3.0 | 55 | 26.39 | 100 | 72.12 |
| 6 | +1.41 | 0 | 7.0 | 55 | 1.89 | 3.12 | 0.44 |
| 7 | 0 | -1.41 | 5.0 | 40 | 38.33 | 27.58 | 16.49 |
| 8 | 0 | +1.41 | 5.0 | 70 | 99.61 | 5.19 | 1.25 |
| 9 | 0 | 0 | 5.0 | 55 | 92.49 | 82.01 | 52.27 |
| 10 | 0 | 0 | 5.0 | 55 | 100.0 | 90.73 | 47.22 |
| 11 | 0 | 0 | 5.0 | 55 | 94.72 | 85.91 | 55.77 |

* Values are based on Mean ± SD of 2 individual observations.

**Table 4.** Statistical ANOVA for the model of β-glucosidase, β-xylosidase and α-L-arabinofuranosidase relative activities at different levels of pH and temperature values.

| Enzyme | Source of variation | Sum of squares | Degrees of freedom | Mean squares | F value | Value (prob > )[b] |
|---|---|---|---|---|---|---|
| β-glucosidase | Model | 13,892.1 | 4 | 3,473.03 | 11.64 | 0.0054 |
| | Residual | 1,790.90 | 6 | 298.48 | | |
| | Lack of Fit | 1,761.17 | 4 | 440.29 | 29.62 | 0.0329 |
| | Pure Error | 29.73 | 2 | | | |
| | Total | 15,683.0 | 10 | | | |
| β-xylosidase | Model | 14,267.89 | 4 | 3,566.97 | 45.21 | 0.0001 |
| | Residual | 473.32 | 6 | 78.89 | | |
| | Lack of Fit | 435.34 | 4 | 108.84 | 5.73 | 0.1540 |
| | Pure Error | 37.98 | 2 | 18.99 | | |
| | Total | 14,741.21 | 10 | | | |
| α-L-arabinofuranosidase | Model | 10,799.6 | 4 | 2,699.9 | 14.68 | 0.0029 |
| | Residual | 1,103.59 | 6 | 183.93 | | |
| | Lack of Fit | 1,066.64 | 4 | 266.66 | 14.43 | 0.0658 |
| | Pure Error | 36.95 | 2 | 18.47 | | |
| | Total | 11,903.19 | 10 | | | |

[b] Statistically significant at 95% of confidence level; $R^2$= 0.8858 (β-glucosidase); $R^2$= 0.9806 (β-xylosidase); $R^2$= 0.9073 (α-L-arabinofuranosidase).

observed for α-L-arabinofuranosidase. When tests were performed at 50°C the β-glucosidase relative activity decreased 35% after 30 min and remained stable up to 8 h incubation, whereas for α-L-arabinofuranosidase a decrease of more than 80% after 1 h of incubation was observed (Figure 3C). Inversely β-xylosidase relative activity was practically not influenced by this temperature, being completely stable even after 8 h incubation.

The effect of metal ions on enzymes activities from *T. atroviride* 102C1 was also studied (Table 5). In general the tested ions have caused a marked inhibition on enzyme relative activities. The presence of $Ba^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Ca^{2+}$ $Zn^{2+}$ and $Mn^{2+}$ have totally inhibited the three enzymes. The exceptions were for ion $K^+$, $Na^+$ and $Mg^{+2}$. Ion $K^+$ had no effect on β-xylosidase, but caused a decrease of 40 and 82% on β-glucosidase and α-L-

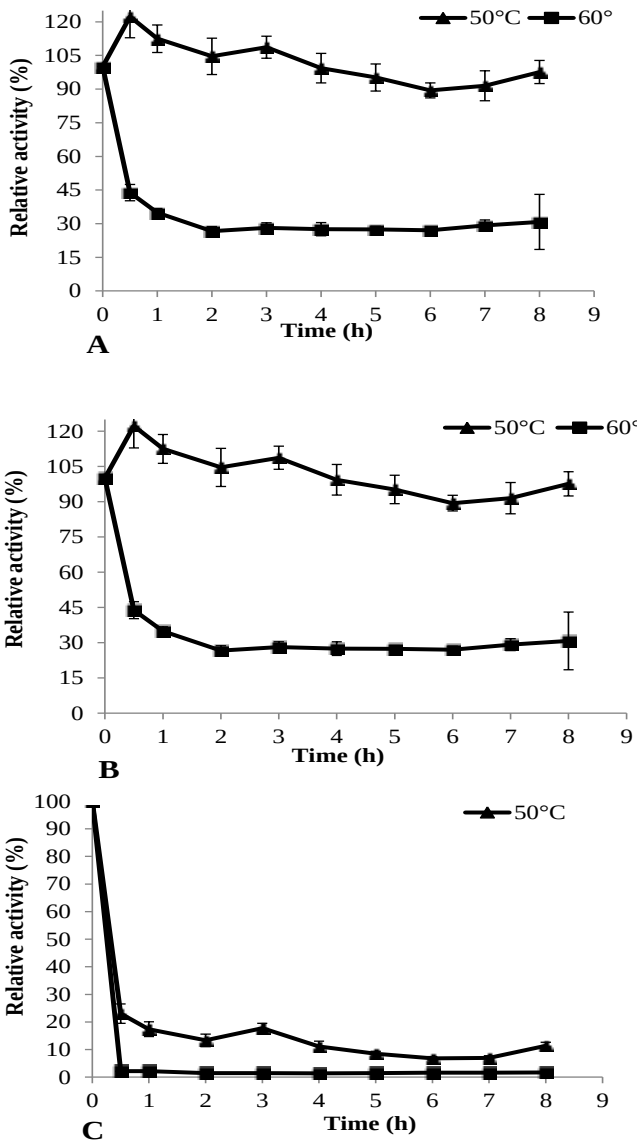


**Figure 3.** Thermal stability of *T. atroviride* 102C1 β-glucosidase (A), β-xylosidase (B) and α-L-arabinofuranosidase (C) activities at 60°C (filled square) and 50°C (filled triangle). Residual activity is expressed as a percentage of the original activity. Error bars represent one standard deviation of each experimental point ($n$ = 3).

arabinofuranosidase relative activities, respectively. Ion $Na^+$ has caused the inhibition of only 9% on β-xylosidase, but of 54% on β-glucosidase, and 81% in α-L-arabinofuranosidase. Also effect of $Mg^{2+}$ on β-xylosidase and β-glucosidase relative activities was very strong, but on α-L-arabinofuranosidase a decreased of only 30% was observed.

## DISCUSSION

β-glucosidase, β-xylosidase and α-L-arabinofuranosidases are important accessory enzymes in the process of complete degradation of lignocellulosic materials. The α-L-arabinofuranosidases, in particular, participate in the initiation of the degradation process releasing specific side chains of hemicellulose polymers, whereas the β-glucosidases and β-xylosidases have important functions in the later stages of the degradation process releasing the fermentable sugars, glucose and xylose, respectively.

Data concerning β-glucosidase and β-xylosidase accumulation by *Trichoderma* species are scarce in literature. Most studies were performed using

**Table 5.** Effect of different metallic ions on β-glucosidase, β-xylosidase and α-L-arabinofuranosidase activities of the supernatant of *T. atroviride* 102C1.

| Ion [a] | Relative activity (%) [b] | | |
|---|---|---|---|
| | β-glucosidase | β-xylosidase | α-L-arabinofuranosidase |
| Control | 100 | 100 | 100 |
| $Cu^{2+}$ | 0.0 | 0.0 | 0.0 |
| $Mg^{2+}$ | 0.1 | 0.4 | 72.0 |
| $Fe^{2+}$ | 0.0 | 0.0 | 0.0 |
| $Ca^{2+}$ | 0.0 | 0.0 | 0.0 |
| $Mn^{2+}$ | 0.0 | 0.0 | 0.0 |
| $Zn^{2+}$ | 0.0 | 0.0 | 0.0 |
| $K^{+}$ | 60.0 | 103.0 | 18.1 |
| $Na^{+}$ | 56.0 | 91.0 | 19.0 |
| $Ba^{2+}$ | 0.0 | 0.0 | 0.0 |

[a] The final concentration in the reaction mixture was 2 mM.
[b] Relative activity is expressed as a percentage of Control.

*Trichoderma reesei* and *T. atroviride* however, some studies using other fungal species have been described. Also, in general, differently from our research, the enzymes production has been studied in separate. Considering the *Trichoderma* genus, a mutant strain of *T. reesei* RUT-C30 (ATCC 56765) was studied by Gottschalk et al. (2010). They described a very low titer of β-glucosidase (0.15 $U.mL^{-1}$) when the strain was grown in the presence of lactose, after 5 days fermentation. An interesting result was obtained when an heterologous expression of a β-glucosidase gene from *Penicillium decumbens* was inserted in *T. reesei* RUT-C30, the maximal activity observed being improved from 4.4 to 34.3 $U.mL^{-1}$, when using 2% (w/v) wheat bran and 3% (w/v) microcrystalline cellulose, after 7 days fermentation (Ma et al., 2011). As to *T. atroviride,* Kovács et al. (2008), developed a mutants strain, F-1505, which was considered the best mutant for β-glucosidase activity, endoglucanase activity and filter paper cellulase activity (FPase). The maximal activity for β-glucosidase (11.7 $U.mL^{-1}$) was observed when pretreated sugarcane bagasse was used, after 3 days fermentation. Also, in previous studies, our group verified a very low β-glucosidase production (0.17 $U.mL^{-1}$) in sugarcane bagasse, after 4 days fermentation, by *T. atroviride* 676, the wild strain of mutant *T. atroviride* 102C1 (Grigorevski-Lima et al., 2013). The results here obtained, of 55.4 $U.mL^{-1}$ of β-glucosidase for *T. atroviride* 102C1 is favorable.

Other fungal species have also been studied for β-glucosidase production, some with very high titres, such as *Aspergillus niger* strain NII 08121 which was cultivate using 1.0% (w/v) wheat bran for 4 days, a maximal β-glucosidase activity of 1,400.0 $U.mL^{-1}$ being observed (Singhania et al., 2011). Also Aliyah et al. (2017) observed a maximal β-glucosidase activity (91.67 and 85.01 $U.mL^{-1}$) when *A. niger* was cultivate using sugarcane bagasse and corn cob for 6 days, respectively. However, *Aspergillus* and *Penicillium* species, are considered greater β-glucosidase producers (Zampieri et al. 2013).

Studies on β-xylosidase production by *Trichoderma* strains such as *Aspergillus*, *Penicillium* and *Talaromyces* have shown very low results, compared to those here described, were values of 10.8 $U.mL^{-1}$ were obtained. *Trichoderma reesei* RUT C30, for instance, when grown on cellulose 1% (w/v) for 7 days, have shown maximal enzyme activity of 0.25 $U.mL^{-1}$ (Jiang et al., 2011). *Trichoderma virens* CTGxAviL, also grown on α-cellulose + xylan beechwood, after 3 days, have produced activities of 0.38 $U.mL^{-1}$ (Tarayre et al., 2015). Other fungal strains, as *Aspergillus awamori* (Paredes et al., 2015), for instance, have also shown very low results when grown on agro-industrial residues.

However, some studies are comparable with ours, such as those using *Lichtheimia ramose* by Garcia et al. (2015) which have shown a maximal β-xylosidase activity (11.57 $U.mL^{-1}$) in the presence of wheat bran, after 4 days fermentation. *Ceratocystis fimbriata* RM 35 when grown on wheat bran for 7 days was also able to produce 14.40 $U.mL^{-1}$ of β-xylosidase (Martins et al., 2018). Even though, our results were obtained after 48 hrs, which can be considered a great advantage.

Concerning AFR, as far as we are concerned, there are no studies in literature describing this enzyme activity for *Trichoderma* strains. However, some studies have shown a low production by other fungi. *Penicillium janczewskii*, for instance, was tested first with oat spelt xylan, during 7 days fermentation, producing 0.8 $U.mL^{-1}$, and then, using 1% (w/v) brewer's spent grain and 1% (w/v) orange waste after 10 days, with maximal values of 0.7 $U.mL^{-1}$ (Temer et al., 2014). In the same way, production of ARF by *A. awamori* 2B.361 U2/1 was low, of 0.7 $U.mL^{-1}$, when using media containing wheat bran, after 5 fermentation

days (Paredes et al., 2015). Other fungal strains, such as *Acremonium zeae* EA0802 and *Talaromyces thermophilus*, gave also very low values of α-L-arabinofuranosidase, when grown in oat spelts xylan (0.045 $U.mL^{-1}$) after 18 days (Almeida et al., 2011), and wheat bran (0.85 $U.mL^{-1}$) after 100 h (Guerfali et al., 2011). Our strain, *T. atroviride* 102C1, was able to produce 143.23 $U.mL^{-1}$, which, in comparison, is a very high titre.

Considering the production of the three enzymes concomitantly, the results obtained in the present research are quite promising. The mutant *T. atroviride* 102C1 have produced high titers of β-glucosidase (55.38 $U.mL^{-1}$), considering a *Trichoderma* species, good titres of β-xylosidase (10.75 $U.mL^{-1}$) and also high titres of α-L-arabinofuranosidase (143.23 $U.mL^{-1}$), at the same time, using very low cost residues of agro-industry, namely sugarcane bagasse and corn steep liquor, after only 2 days fermentation. There are few reports in the literature which study the production of the three enzymes concomitantly by fungi strain, as was done in the present work. Paredes et al. (2015), observed the production of the three enzymes by *A. awamori* 2B.361 U2/1 in the presence of 5 $g.L^{-1}$ xylan in growth medium at initial pH 5.0. In these conditions, the fungus produced 46 $U.mL^{-1}$ of xylanase (EC 3.2.1.8), 3.3 $U.mL^{-1}$ of β-glucosidase EC 3.2.1.21), 0.24 $U.mL^{-1}$ of β-xylosidase (EC 3.2.1.37), and 0.6 $U.mL^{-1}$ of α-L-arabinofuranosidase (EC 3.2.1.55) in the supernatant.

Data from literature have shown that, in general, BGU activities produced by different fungi were also acidic with optimal temperatures from 54 to 70°C. *Trichoderma harzianum* IOC-4038 showed as optimal conditions for enzyme relative activity pH 5.0 and 54°C (Castro et al., 2010). Bonfa et al. (2018) observed a maximal relative activity at pH 5.0 and 60ºC from thermophilic fungus *Myceliophthora thermophila* M.7.7. *A. niger* NII-08121 showed an acidophilic and thermophilic profile, with pH 4.8 and 70°C (Singhania et al., 2011).

Concerning BXU, it is common to observed in literature an acidophilic and thermophilic profile, with optima of pH and temperature around 4.0-5.0, and between 65-70°C, as observed for *Aspergillus* (Díaz-Malváez et al., 2013; Wakiyama et al., 2008) and *Ceratocystis* strains (Martins et al., 2018). Terrasan et al. (2011) observed the best condition for β-xylosidase when the enzymatic extract was incubated at 75°C and pH 5.0 from *P. janczewskii*. These results concerning optimal temperature are compatible with those showed by *T. atroviride* 102C1, which showed a maximal enzyme relative activity at 55°C. However, concerning pH, the best pH was more acidic (3.0), close to that observed by Knob and Carmona (2009) for *Penicillium sclerotiorum*, which have shown an even lower optimum pH, 2.5, and an optimum of temperature at 60°C. As for ARF data on literature are scarce. Enzyme relative activity from *P. janczewskii* was considered acidophilic and thermophilic, with best production at pH 4.0 and 60°C (Temer et al. 2014) whereas Guerfali et al. (2011) have found optimal ARF activity from *Talaromyces thermophilus* on more mesophilic conditions, pH, 6.0-7.0 at 55°C. In the present study, ARF from *T. atroviride* 102C1, was acidophilic for best pH, 3.6, and mesophilic for best temperature, 44°C.

There are some studies in literature which report inhibitory effects of some metal ions on β-glucosidases activities, mostly decreasing the enzyme relative activity by several degrees. For instance, Bonfa et al. (2018) observed a decreased of 30% for $Na^{2+}$, 50% for $Mn^{2+}$, 100% for $Fe^{2+}$ and 37% for $Zn^{2+}$ on BGU enzymatic relative activity from *My. thermophila* M.7.7, but a different result was observed for $Ba^{2+}$ and $Mg^{2+}$, where no inhibition was detected. Some studies have reported the apparent activation of fungal BXU by $Mn^{2+}$ and $Ca^{2+}$, suggesting that these ions activate and protect the active site (Yang et al., 2014; Pereira et al., 2015; Martins et al., 2018). Terrasan et al. (2011) observed a slight inhibition of β-xylosidase from *P. janczewskii* by $Mn^{2+}$ (17%) and $Ca^{2+}$ (21%), whereas Bonfa et al. (2018), have shown an inhibition of 50% by $Mn^{2+}$ for the β-xylosidase from crude extract from *M. thermophila* M.7.7. Concerning ARF, Pereira et al. (2015) showed the metal ions $Zn^{2+}$ and $Co^{2+}$ as potential inhibitors of the α-L-arabinofuranosidase from *Penicillium chrysogenum* while Yang et al. (2015) observed that $Fe^{2+}$, $Na^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ had no effect, but $Ca^{2+}$ (19%), $K^{+}$ (42%) and $Co^{2+}$ (75%) inhibited the enzyme relative activity from *Alicyclobacillus* spp. A4. As one can seen, all these results from literature are compatible to the results obtained for *T. atroviride* 102C1.

## Conclusion

As a conclusion of our study, we can say that the accessory enzymes here studied, produced by the mutant fungus *T. atroviride* 102C1, can be interesting for various industrial applications. It is important to stress that the three enzymes were obtained concomitantly, in only two days, in expressive amounts. Furthermore, the use of sugarcane bagasse and corn steep liquor, the main energy sources for microbial growth and enzymes production, allows for optimum use of these low cost agriculture residues. Activity at high temperatures is interesting, considering the fungus is mesophilic and can be very important for a future application in processes that are carried out at high temperatures. By this way, the environment will be highly favored due to the use of this system by the bioenergy plants for biofuel production, especially the production of bioethanol in Brazil, increasing sustainability and generating less environmental pollutants.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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