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African Journal of Biotechnology

*Full Length Research Paper*

# Detection of virulence genes and antibiogram of Shiga toxin-producing *Escherichia coli* O157:H7 from slaughtered cattle and abattoir effluent in Zaria, Nigeria

**Chukwudi Emelogu[1], Samuel Mailafia[1]*, James Agbo Ameh[1], Remigius Chinaka Njoku[2], Bridget Adah Jessica[1] and Martha Echioda-Ogbole[1]**

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**Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a formidable human pathogen causing food-borne diseases worldwide. The present study investigates virulence genes and determines the antimicrobial characteristics of STEC O157:H7 isolates from faecal and effluent samples at Zaria abattoir in Zaria. PCR assays were used to detect the virulence genes of STEC O157:H7 isolates. The isolates that produced *stx* genes were subjected to antibiotic susceptibility testing using Kirby Bauer disc diffusion technique. Out of the 13 STEC O157:H7 isolates evaluated, *E. coli* 16SrRNA gene was detected in 12 isolates (92.3%) and the distribution of the virulence genes showed that of the 12 *E. coli* 16SrRNA gene positive isolates, 10 (83.3%) had $stx_1$ gene, 2 (16.7%) produced $stx_2$ gene, 2 (16.7%) harbored both $stx_1$ and $stx_2$ genes, and 2 (16.7%) lacked *stx* genes. Similarly, 8.3% had *hlyA* gene, 58.3% were positive for *fliC* gene and none produced *eaeA* gene. The ten isolates that produced *stx* genes were highly susceptible to Imipenem 100%, Ciprofloxacin 100%, and Ofloxacin 100% while strong resistance was observed against Cefepime 90.0%, Streptomycin 70.0% and Ampicillin 70.0% and Tetracycline 60.0%. Also, 80.0% of the ten *stx* genes positive isolates exhibited Multiple Antibiotic Resistance (MAR), with the Multiple Antibiotic Resistance Index (MARI) ranging from 0.083 to 0.67. 60.0% exhibited Multiple Drug Resistance (MDR) traits. These high MAR and MDR values suggest that there may be abuse of antibiotics in the study area. Thus, good hygienic practices should be adopted in abattoirs to prevent the transmission of STEC O157:H7 pathogens responsible for many foodborne diseases in humans.**

**Key words:** Abattoir, effluent, virulence genes, antibiotic susceptibility, Shiga toxin-producing *Escherichia coli* (STEC).

## INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a foodborne pathogen that causes diseases by producing Shiga toxins (Pruimboom-Brees et al., 2000). These cytotoxins inhibit protein synthesis which results in necrotic or apoptotic cell death in humans and animals (Pruimboom-Brees et al., 2000; Iwu et al., 2021). Shiga

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toxin-mediated vascular damages in colon and kidney have been reported to cause bloody diarrhea, gastroenteritis, enterocolitis, cystitis, hemorrhagic colitis (HC) and haemolytic-uremic syndrome (HUS) (Wang et al., 2002; Bitrus et al., 2011; Brandelli et al., 2015). HUS is characterized by haemorrhagic anaemia and thromobocytopenia, which cause kidney infections, neurological issues and acute renal failure cases in children (Bitrus et al., 2011). Epidemiological data of human infection showed that the HUS caused by STEC is on the increase, that is, from 3 to 5% globally, with the highest mortality rate in children of about 2 years old (Loconsole et al., 2020).

Domestic and wild ruminants have been identified as reservoirs of STEC O157:H7. Cattle are important carriers of STEC O157:H7 (Nadya et al., 2016). Feces of clinically normal cattle are important source of STEC O157:H7. Cattle spread infection by shedding STEC O157:H7 pathogen in their feces. Similarly, abattoir effluent is sewage discharge flowing out of a slaughterhouse into the environment and usually consists of wastewater, blood, animal body fluids, faeces, dirt, meats, soaps and detergents to mention but a few. One of the problems with abattoir effluent is that it harbours STEC O157:H7. This slaughterhouse waste is eventually discharged into the environment and therefore poses a serious threat to air, water, soil quality, microorganisms, humans, and animals if not well-treated (Bamidele and Okoya, 2015).

STEC O157:H7 pathogen is associated with various virulence genes that promote its colonization of the host and determine the ability of the pathogen to multiply within the host cells or cause diseases. Some of these virulence genes include Shiga toxin 1 and 2 (encoded by $stx_1$, $stx_2$), mucus-activatable Shiga toxin 2d genotype (encoded by $stx_{2d}$), enterohemolysin (encoded by *hlyA*, also known as *ehxA*) and intimin (encoded by *eaeA*). Apart from genes coding for virulence, there are identification or specific genes of STEC O157:H7 such as $rfbE_{O157}$, which codes the GDP perosamine synthetase ($rfb_{O157}$), O-antigen specific for STEC O157:H7; flagellin (encoded by $fliC_{H7}$), H-antigen specific for *E. coli* O157:H7, which is responsible for producing H7 flagellum of STEC O157:H7 serotype and *uidA*, which encodes β-glucuronidase. Other genes associated with STEC O157:H7 include the genes for the cytotoxic necrotizing factors, heat-labile toxin, heat-stable toxin, enteroinvasive toxin, and the enteroaggregative protein (Wang et al., 2002; Al-Ajmi et al., 2020).

The emergence of antibiotic resistance has become a global threat, with serious adverse effect on the economy and public health. Antibiotic resistance can occur naturally, but it is usually caused by misuse of antibiotics in humans and animals. Antibiotics are not approved therapeutic drugs for treatment of human infections caused by STEC O157:H7 because of easy transmission of resistance genes horizontally to other pathogens in hosts and environment (Moses et al., 2018). Disposal of abattoir effluents serves as an important vehicle for community wide dissemination of antibiotic resistance of STEC O157:H7 strains. Person to person transmission of STEC O157:H7 is responsible for the epidemiology and maintenance of the disease in a population. Therefore, the present study was designed to determine virulence genes present in STEC O157:H7 isolates using Polymerase Chain Reaction and conduct antibiogram on STEC O157:H7 isolates using Kirby diffusion test. The study exposed the occurrence of STEC O157:H7 pathogen in slaughtered cattle and effluent at Zaria abattoir. The research findings added information to the baseline data and stimulated prompt Public Health action in preventing and controlling the spread of the pathogen.

## METHODOLOGY

### Collection of bacterial isolates

A total of 13 pre-identified STEC O157:H7 isolates were used in the present study. The isolates obtained from this study were sourced from reference specimens stored in the microbiology laboratory of the Faculty of Veterinary Medicine, University of Abuja. The isolates were subjected to rigorous microbiological growth protocols involving growth on broth media, selective media, differential media, preliminary biochemical tests and confirmation using Microbact$^{TM}$ GNB 24 E; it was identified to be STEC using serology, verotoxin assay, and molecular identification of *stx* gene.

These isolates comprised 8 and 5 isolates from slaughtered cattle faeces and abattoir effluent waste samples collected at Zaria abattoir. The isolates had been in storage for less than a month and were then transferred to Molecular Laboratory of National Veterinary Research Institute (NVRI), Vom, Nigeria for further studies.

### DNA extraction

Overnight culture of bacterial cells was used for this investigation. The genomic DNA of STEC O157:H7 was extracted and purified using commercially available DNA extraction kit, the qiagen kit {QIAamp DNA Mini Kit (250), Catalogue number 51306; Qiagen} for Gram-negative organisms, according to the manufacturer's instructions, and the total genomic DNA was determined by running on 1.0% agarose gel.

### Polymerase chain reaction (PCR) amplification of 16S rRNA genes

The number colonies of STEC O157:H7 isolates were determined by plating serial dilutions of cultures on agar plates. The isolates were vortexed for 30 s and then added directly to PCR for amplification of the 16S rRNA genes. DNA of STEC O157:H7 isolates were purified using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and used as a PCR template. PCR amplification of 16S rRNA genes was performed using the 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) containing the 27F/1492R primer set (**Figure 1**) and LongAmp$^{TM}$ Taq 29 Master Mix (New England Biolabs, Ipswich, MA, USA). Amplification was conducted using an Applied Biosystems Veriti$^{TM}$ Thermal Cycler (Thermo Fischer Scientific, Waltham, MA, USA)

**Table 1.** List of primers sequences, amplicon sizes and target genes.

| Primer set | Primer | Sequence (5′- 3′) | Target genes | Size of amplicon (bp) | References |
|---|---|---|---|---|---|
| A | E16S-F<br>E16S-R | CCCCCTGGACGAAGACTGAC<br>ACCGCTGGCAACAAAGGATA | *16SrRNA* | 401 | Wang et al. (2002) |
| B | Stx1-F<br>Stx1-R | GAAGAGTCCGTGGGATTACG<br>AGCGATGCAGCTATTA | $stx_1$ | 130 | Bitrus et al. (2011) |
| C | Stx2-F<br>Stx2-R | TTAACCACACCCACGGCAGT<br>GCTCTGGATGCATCTCTGGT | $stx_2$ | 346 | Bitrus et al. (2011) |
| D | HylA-F<br>HylA-R | AGCTGCAAGTGCGGGTCTG<br>TACGGGTTATGCCTGCAAGTTCAC | *EHEC hylA* | 569 | Wang et al. (2002) |
| E | FliC-F<br>FliC-R | TACCATCGCAAAAGCAACTCC<br>GTCGGCAACGTTAGTGATACC | $fliC_{H7}$ | 247 | Wang et al. (2002) |
| F | EAE-F<br>EAE-R | GCAAATTTAGGTGCGGGTCAGCGTT<br>GGCTCAATTTGCTGAGACCACGGTT | *eaeA* | 494 | Bitrus et al. (2011) |

Source: Wang et al. (2002) and Bitrus et al. (2011).

with the following PCR conditions initial denaturation at 95°C for 3 min, 25 cycles of 95°C for 20 s, 55°C for 30 s, and 65°C for 2 min, followed by a final extension at 65°C for 5 min (Kai et al., 2019). *E. coli* O157:H7 strain EDL 933 (EDL 933) was used as a positive control and the following primers were used: forward, 5'-CCCCCTGGACGAAGACTGAC-3'; and reverse, 5'-ACCGCTGGCAACAAAGGATA-3' (Bitrus et al., 2011).

**PCR amplification of Shiga-toxin genes**

$Stx_1$ and $Stx_2$ primers sets were used to detect $Stx_1$ and $Stx_2$ genes (Bitrus et al., 2011). The PCR reaction mixture was a total of 25 µl containing 5.0 µl DNA extract, 2.5 µl of 10 × buffer (Fermentas®), 0.5 µl of 10 mM dNTPs mix (Fermentas®), 0.5 µl of 20 µM primers (Table 1), 1.5 µl of 25 mM $MgCl_2$ and 2.5 U of Taq Polymerase (Fermentas®). PCR amplification was performed in GeneAmp 9700 (Applied Biosystems) with initial denaturation at 94°C for 5 min. This was followed by 40 cycles of denaturation at 94°C for 1 min; annealing at 53°C for $Stx_1$ and at 55°C for $Stx_2$ for 1 min; and extension at 72°C for 1 min. Ten microlitres of the PCR product were electrophoresed in an agarose gel (1.5%) containing 5 µl of 10 mg/ml ethidium bromide at 80 V for 60 min. 50 bp DNA marker (Fementas) was used as molecular size marker. DNA amplifications were examined under UV transilluminator and results documented using Gel Documentation System (Synegene®) (Bitrus et al., 2011).

**Multiplex PCR amplification of *fliC, hlyA* and *eaeA* genes**

A set of primer mixture was used to run the Multiplex PCR (Table 1). The reagents mixture was as follows: 5 µl of genomic DNA extract was used as template with $Mg^{2+}$ free 2.5 µl 10x PCR reaction buffer (Fermentas®); 0.5 µl of 10 mM dNTPs; 1.5 µl of 25 mM $MgCl_2$ and 2.5 units of Taq polymerase (Fementas®). The set contained 0.6 µM *hlyA*, 0.4 µM of *fliC* and 0.75 µM *eaeA* primers. PCR amplification was performed in GeneAmp 9700 (Applied Biosystems) with initial denaturation at 95°C for 8 min. This was followed by 30 cycles of denaturation at 95°C for 30 s; annealing at 58°C for 30 s; and extension at 72°C for 30 s. Final extension was at 72°C for 7 min. Ten microlitres of the PCR product were electrophoresed in an agarose gel (1.5%) containing 5 µl of 10 mg/ml ethidium bromide at 80 v for 60 min. 50 bp DNA marker (Fementas®) was used as molecular size marker. DNA amplifications were examined under UV transilluminator (Sigma) and results documented using Gel Documentation System (Synegene®) (Bitrus et al., 2011).

**Antibiotic susceptibility testing**

Kirby Bauer disc diffusion test was performed to screen STEC O157: H7 isolates for *in vitro* antimicrobial susceptibility using the protocol in CLSI (2020). Mueller-Hinton agar was prepared according to the manufacturer’s instructions. The medium was cooled to 45-50°C and poured into plates. Plates could set on a level surface to a depth of approximately 4 mm. When the agar has solidified, plates could dry before use. An 18 to 24 h old broth culture of the *E. coli* isolate was standardized by diluting to 0.5 Mcfarland’s standard. A sterile swab stick was inserted into the standardized STEC O157: H7 inoculum, drained to remove excess inoculum load, and inoculated by spreading on the surface of prepared Mueller-Hinton agar plate. After this, the inoculated Mueller-Hinton agar plate could dry for a few minutes at room temperature with the lid closed. After the agar surface has dried for few minutes, antibiotic impregnated discs (Oxoid, UK) of known concentrations: Ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 20/10 µg), cefepime (FEP, 30 µg), ofloxacin (OFX, 5 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GM, 10 µg), imipenem (IPM, 10 µg), streptomycin (S, 10 µg), and tetracycline (TE, 30 µg) were carefully applied on the inoculated

**Table 2.** List of antimicrobial categories, antimicrobial agents, disc contents, and zone diameters breakpoints (nearest whole millimeters).

| S/N | Antimicrobial categories | Antimicrobial agents | Disc contents (µg) | Zone diameters breakpoints (mm) | | | |
|---|---|---|---|---|---|---|---|
| | | | | S | SDD | I | R |
| 1 | Aminoglycosides | Gentamicin | 15 | ≥ 15 | - | 13-14 | ≤ 12 |
| | | Streptomycin | 10 | ≥15 | - | 12-14 | ≤ 11 |
| 2 | β-lactam combination agents | Amoxicillins-Clavulanic acid | 20/10 | ≥ 18 | - | 14-17 | ≤ 13 |
| 3 | Carbapenems | Imipenem | 10 | ≥ 23 | - | 20-22 | ≤ 19 |
| 4 | Cephems | Cefepime, | 30 | ≥ 25 | 19-24 | - | ≤ 18 |
| | | Ceftazidime | 30 | ≥21 | - | 18-20 | ≤ 17 |
| | | Ceftriaxone | 30 | ≥23 | - | 20-22 | ≤ 19 |
| 5 | Penicillins | Ampicillin | 10 | ≥17 | - | 14-16 | ≤ 13 |
| 6 | Phenicols | Chloramphenicol | 30 | ≥18 | - | 13-17 | ≤ 12 |
| 7 | Quinolones and Fluoroquinolones | Ciprofloxacin | 5 | ≥26 | - | 22-25 | ≤ 21 |
| | | Ofloxacin | 5 | ≥16 | - | 13-15 | ≤12 |
| 8 | Tetracyclines | Tetracycline | 30 | ≥15 | - | 12-14 | ≤11 |

I=Intermediate, R=Resistance, S=Susceptibility, SDD=Susceptible-Dose-Dependent.
Source: CLSI (2020).

Mueller-Hinton agar plates using sterile forceps. The plates were then incubated at 37°C for 24 h. After incubation, the diameters of the zones of inhibition were measured with a ruler to the nearest millimeter and recorded. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) strains were used as controls in each assay. The results were recorded as resistant, intermediate and susceptible according to the guidelines of Clinical Laboratory Standards Institute criteria for Enterobacteriaceae (Table 2) (Moses et al., 2018; Ayandele et al., 2020; CLSI, 2020).

### Determination of multiple antibiotic resistance (MAR) and multiple drug resistance (MDR)

MAR of the STEC O157:H7 isolates was calculated as antimicrobial resistance to two or more antibiotics while multiple antibiotic resistance indices (MARI) were calculated using the standard method (Moses et al., 2018; Ayandele et al., 2020). This was calculated as the number of antibiotics to which the tested isolate was resistant to (a), divided by the total number of antibiotics that was tested on the isolates (b). It was calculated using its formula as: MARI = a/b.

MDR of the STEC O157:H7 isolates was calculated using the standard method (Ibrahim et al., 2012). MDR was of STEC O157:H7 taken as antimicrobial resistance to three or more antimicrobial classes or categories. Antimicrobial categories are classifications of antimicrobial agents based on their mode of action and specific to target organisms (Table 2) (Ibrahim et al., 2012).

### Statistical analyses

Descriptive statistics such as tables, figures, charts and frequencies were used in data analyses.

## RESULTS

### PCR amplification of 16S rRNA

The results of PCR amplification of 16Sr RNA genes (Table 1 and Figure 1) showed that out of thirteen STEC O157:H7 isolates investigated, 12 (92.3%) harboured 16Sr RNA gene. The analysis of the results showed that out of the twelve positive isolates, 7 (87.5%) were from faecal sources while 5 (100.0%) were from effluents samples.

### PCR amplification of Shiga-toxin genes

The PCR results (Table 1 and Figures 2 and 3) indicated that STEC O157:H7 harboured the $stx_1$ 10 (83.3%) and $stx_2$ 2 (16.7%) genes, respectively. Of the ten isolates that possessed $stx_1$ gene, 6 (85.7%) were from faecal samples and 4 (80.0%) originated from effluent samples. Similarly, 1 (14.3%) of the isolates that harboured $stx_2$ genes was from faecal samples while the remaining 1 (20.0%) was from the effluent samples.

### Multiplex PCR amplification of *fliC, hlyA* and *eaeA* genes

The PCR results (Table 1 and Figure 4) revealed that

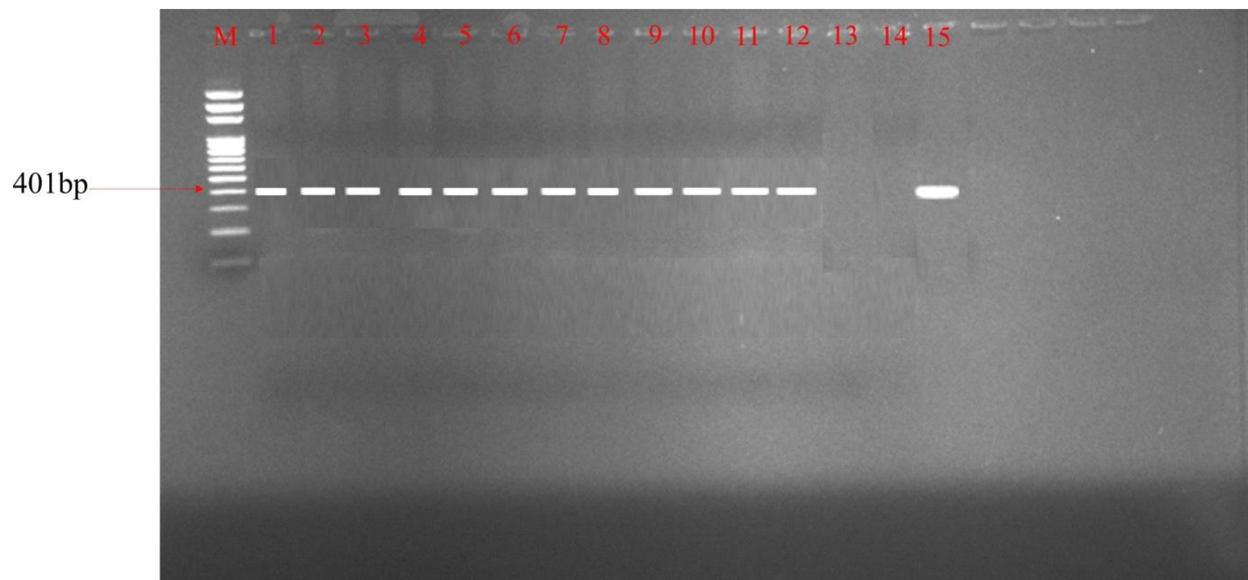


**Figure 1.** Electrophoretogram showing PCR detection of *16SrRNA* gene in STEC O157:H7 isolates. Lane M is 401 bp DNA marker/ladder. Lanes 1-12 show the amplified product *16SrRNA* gene in STEC O157:H7 isolates recovered in this study. Lane 13 shows negative amplification for *16SrRNA* gene. Lane 14 is negative control (nuclease free water). Lane 15 is positive control (E16S primers set that were used to amplify *E. coli* 16S rRNA gene).

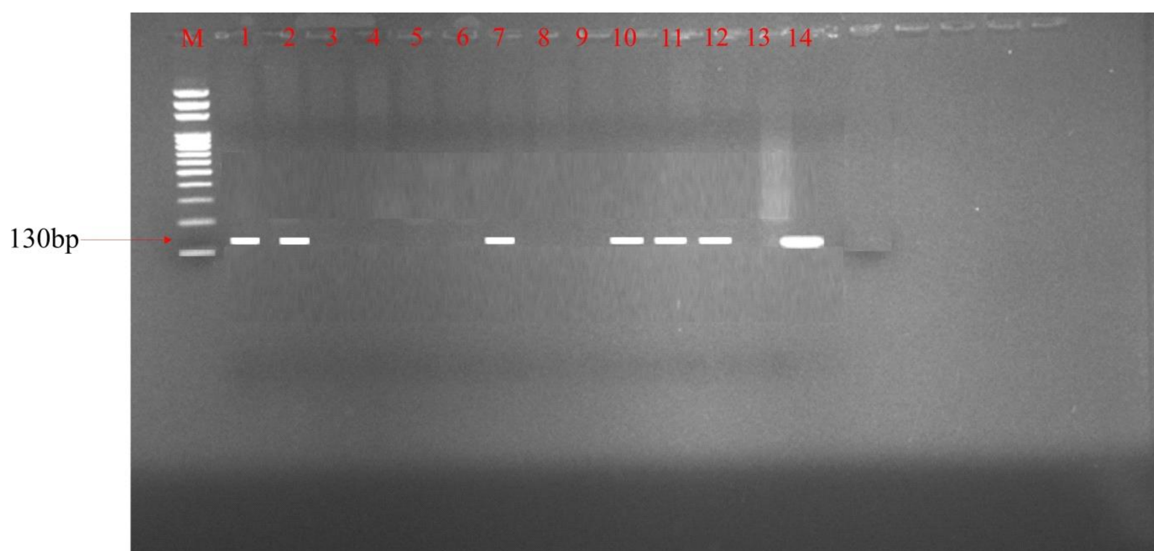


**Figure 2.** Electrophoretogram showing PCR detection of $stx_1$ gene in STEC O157:H7 isolates. Lane M is 130 bp DNA marker/ladder. Lanes 1-2, 7, 10-12 show the amplified product $stx_1$ gene in STEC O157:H7 isolates recovered in this study. Lanes 3-6, 8-9 show negative amplification for $stx_1$ gene. Lane 13 is negative control (nuclease free water). Lane 14 is positive control (EDL 933 primers set used to amplify $stx_1$ gene).

STEC O157:H7 harboured the *fliC* 7 (58.3) and *hlyA* 1 (8.3%) genes, respectively but lacked *eaeA* gene. The analysis of the results indicated that out of seven isolates that had *fliC* genes, 5 (71.4%) originated from faecal sources while 2 (40.0%) emanated from effluent samples. Also, the lone isolate that harboured *hlyA* gene was from effluent samples.

**Distribution of virulence genes in faecal and effluent samples**

Out of the twelve (12) isolates analysed for the presence of virulence genes, 7 (53.8%) were from faecal samples while 5 (38.5%) were from effluent samples. 10 (83.3%) had $stx_1$ gene comprising 6 (85.7%) from faecal samples

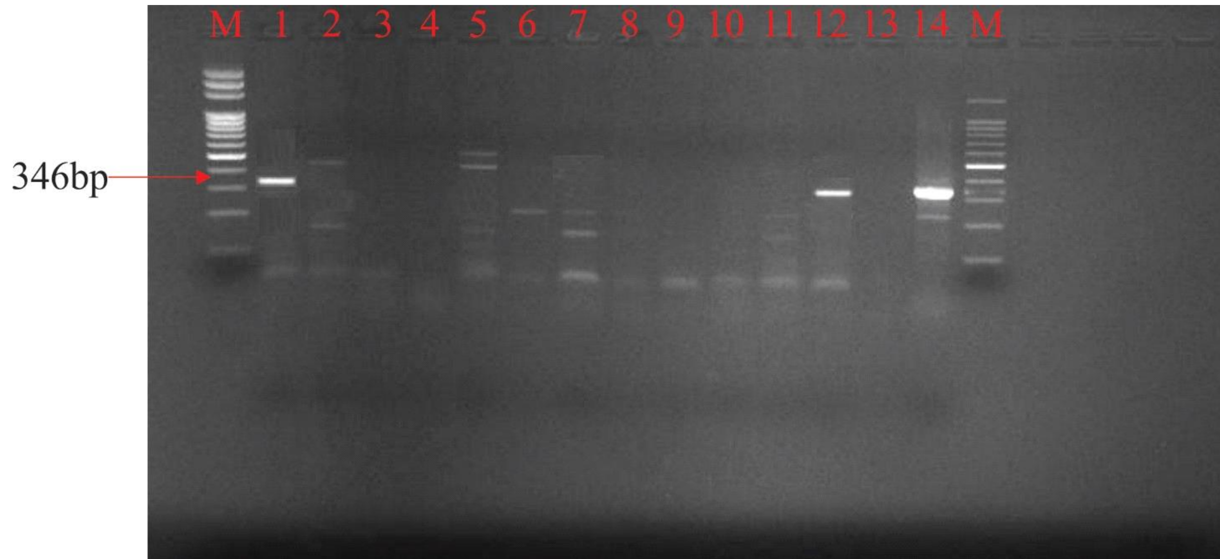


**Figure 3.** Electrophoretogram showing PCR detection of *stx*$_2$ gene in STEC O157:H7 isolates. Lane M is 346 bp DNA marker/ladder. Lanes 1&12 show the amplified product *stx*$_2$ gene in STEC O157:H7 isolates recovered in this study. Lanes 2-11 show amplification for *stx*$_2$ gene. Lane 13 is negative control (nuclease free water). Lane 14 is positive control (EDL 933 primers set used to amplify *stx*$_2$ gene).

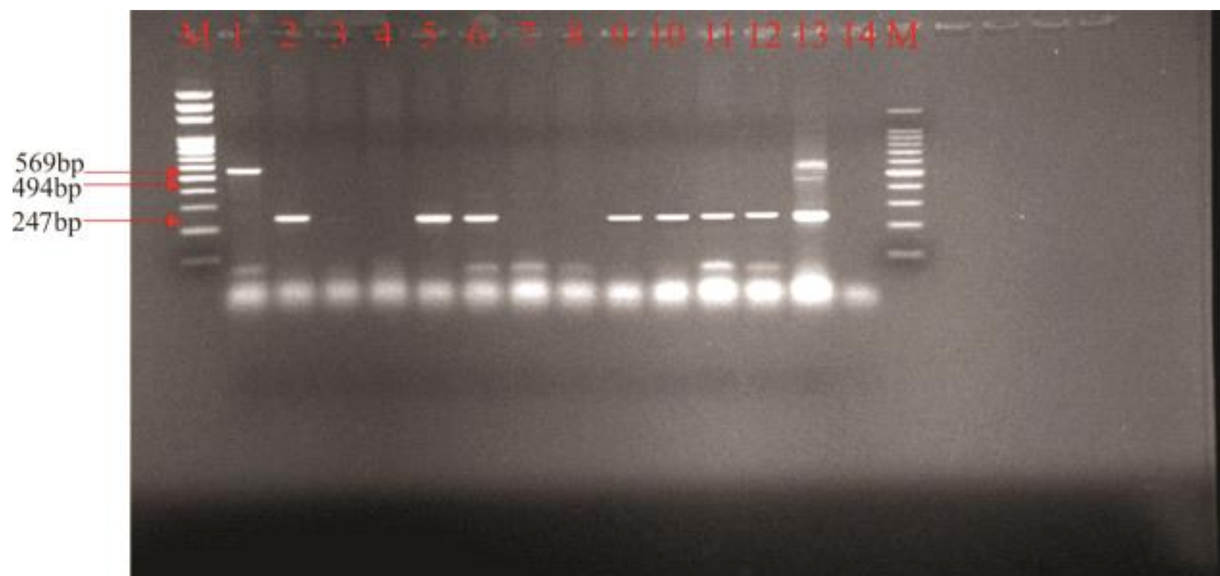


**Figure 4**. Electrophoretogram showing PCR detection of *fliC, hlyA* and *eaeA* genes in STEC O157:H7 isolates. Lane M contains 247bp, 494bp and 569bp DNA markers/ladders. Lane 2, 5-6, 9-12 and Lane 1 show the amplified products of *fliC* and *hlyA* genes respectively in STEC O157:H7 isolates recovered in this study. Lanes 1, 3-4, 7-8; 2-12 and 1-12 indicates negative amplification for *fliC, hlyA* and *eaeA* genes respectively. Lane 13 is the negative control (nuclease free water). Lane 14 is positive control (EDL 933 primers set used to amplify *fliC, hlyA* and *eaeA* genes).

and 4 (80.0%) from effluent samples. 2 (16.7%) lacked *stx*$_1$ gene. Also, 2 (16.7%) out of the twelve isolates were positive for *stx*$_2$ gene composing of 1 (14.3%) isolate from faecal and 1 (20.0%) from effluent samples. 2 (16.7%) harboured both *stx*$_1$ and *stx*$_2$ genes comprising 1 (14.3%) from faecal sample and 1 (20.0%) from effluent samples. 10 (83.3%) had *stx* genes which constituted of 6 (85.7%) faecal samples and 4 (80.0%) effluent samples while 2 (16.7%) lacked *stx* genes making up of 1 (14.3%) faecal sample and 1 (20.0%) effluent sample (Table 3).

**Table 3.** Percentage distribution of virulence genes in faecal and effluent samples.

| Genes tested | No. tested faeces | No +ve faeces (%) | No. tested effluent | No +ve effluent (%) | Total no. tested | Total No +ve (%) |
|---|---|---|---|---|---|---|
| *stx1* | 7 | 6 (85.7) | 5 | 4 (80.0) | 12 | 10 (83.3) |
| *stx2* | 7 | 1 (14.3) | 5 | 1 (20.0) | 12 | 2 (16.7) |
| *fliC* | 7 | 5 (71.4) | 5 | 2 (40.0) | 12 | 7 (58.3) |
| *hlyA* | 7 | 0 | 5 | 1 (20.0) | 12 | 1 (8.3) |
| *eaeA* | 7 | 0 | 5 | 0 | 12 | 0 |

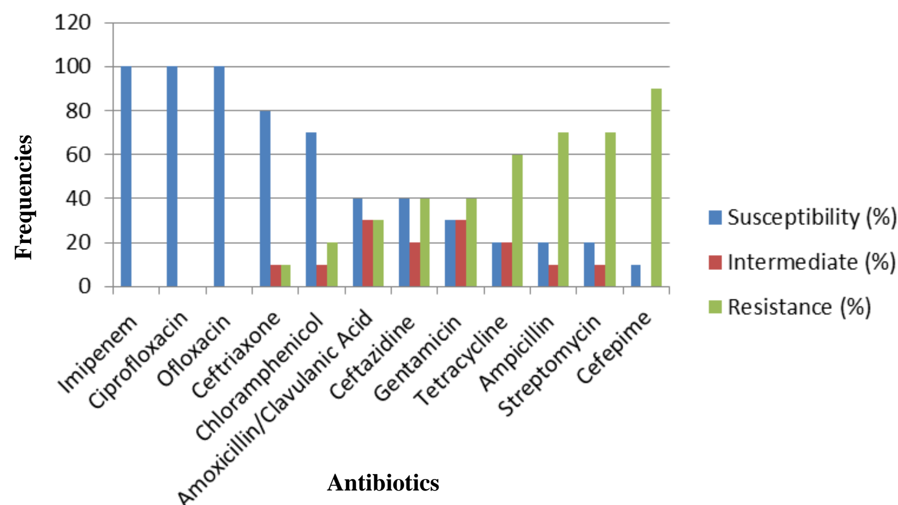


**Figure 5.** Comparison of sensitivity patterns of STEC O157:H7 isolates from faecal and effluent to different antibiotics.

Interestingly, 7 (58.3%) of the twelve STEC O157:H7 isolates investigated harboured *fliC* gene, comprising 5 (71.4%) from faecal samples and 2 (40.0%) from effluent samples. Furthermore, 1 (8.3%) of the twelve isolates possessed *hlyA* genes which was detected from effluent samples 1 (20.0%). No *hlyA* gene was detected from faecal samples. 11 (91.7%) were negative for *hlyA* gene. However, no *eaeA* gene was detected in all the STEC O157:H7 isolates investigated (Table 3).

## Antimicrobial susceptibility profile of *E. coli* 0157:H7 isolates

The results of the antibiotic susceptibility studies (Figure 5) revealed that all the ten STEC O157:H7 isolates were highly susceptible to Imipenem (100.0%), Ciprofloxacin (100.0%) and Ofloxacin (100.0%). Further analysis of results showed that the isolates exhibited the following susceptibilities to other antimicrobial agents: Ceftriaxone (80.0%), Chloramphenicol (70.0%), Amoxicillin/Clavulanic acid (40.0%), Ceftazidime (40.0%), Gentamicin (30.0%), Tetracycline (20%), Ampicillin (20%), Streptomycin (20%), and Cefepime (10%). High level of resistance was observed against Cefepime (90.0%), Streptomycin (70.0%), Ampicillin (70.0%) and Tetracycline (60%). The level of resistance showed by other antibiotics was Gentamicin (40%), Ceftazidime (40%), Amoxicillin/ Clavulanic acid (30.0%), Chloramphenicol (20%) and Ceftriaxone (10.0%). Furthermore, intermediate susceptibilities were observed in Tetracyclic (40.0%), Amoxicillin/Clavulanic acid (30.0%), Gentamicin (30%), Ceftazidime (20.0%), Ampicillin (10%), Chloramphenicol (10%), Streptomycin (10%) and Ceftriaxone (10.0%).

## Resistance patterns, MAR, and MDR of STEC O157:H7 isolates

The ten STEC O157:H7 isolates showed ten distinct susceptibility patterns to the twelve antibiotics (Table 4). Eight (80.0%) out of the ten STEC O157:H7 isolates exhibited MAR as they were resistant to at least two different antibiotics. Only 2 (20.0%) out of the ten STEC O157:H7 isolates did not exhibit MAR as they were resistant to only one antibiotics drug. The average MARI

**Table 4.** Resistance patterns, MARI and MDR of STEC O157:H7 isolates.

| S/N | Isolate code | Resistance patterns | Mean diameter of growth inhibition (mm) ± S.E.M | | | | |
|---|---|---|---|---|---|---|---|
| | | | NR (n=12) | MAR (%) | MARI | NACR (n=8) | MDR (%) |
| 1 | E1 | FEP | 1 ± 0.0 | - | 0.083 | 1 ± 0.0 | - |
| 2 | E44 | C AMC CAZ TE FEP S AMP GM | 8 ± 2.0 | + | 0.667 | 6 ± 0.5 | + |
| 3 | E68 | CAZ TE CRO GM S AMP FEP | 7 ± 1.5 | + | 0.583 | 3 ± 0.2 | + |
| 4 | E212 | AMP | 1 ± 0.0 | - | 0.083 | 1 ± 0.0 | - |
| 5 | F155 | TE C S FEP AMP | 5 ± 0.1 | + | 0.417 | 5 ± 0.1 | + |
| 6 | F175 | AMC CAZ GM TE S AMP FEP | 7 ± 1.5 | + | 0.583 | 5 ± 0.1 | + |
| 7 | F209 | S FEP | 2 ± 0.4 | + | 0.167 | 2 0.4± | - |
| 8 | F230 | GM CAZ AMC TE S FEP AMP | 7 ± 1.5 | + | 0.583 | 5 ± 0.1 | + |
| 9 | F234 | AMP S FEP | 3 ± 0.2 | + | 0.250 | 3 ± 0.2 | + |
| 10 | F279 | TE FEP | 2 ± 0.4 | + | 0.167 | 2 ± 0.4 | - |
| | | | | 8 (80.0) | 0.358 | | 6 (60.0) |

NR= Number resistance, NACR= number antibiotic category resistance, AMC=amoxicillin/clavulanic acid; AMP=ampicillin; C=chloramphenicol; CAZ=ceftazidine; CIP=ciprofloxacin; CRO=ceftriaxone; FEP=cefepime; GM=gentamicin; IPM=imipenem; OFX=ofloxacin; S=streptomycin; TE= tetracycline.

value of the STEC O157:H7 isolates was 0.358. The MARI of STEC O157:H7 isolates ranged from 0.083 to 0.667 (Table 4). 6 (60.0%) out of the ten STEC O157:H7 isolates exhibited multi-drug resistance traits as they were resistant to at least one antibiotic in three different antimicrobial categories. 4 (40.0%) out of the ten STEC O157:H7 isolates did not exhibit multi-drug resistance traits as they did not resist at least one antibiotics drug in three different antimicrobial categories (Table 4).

## DISCUSSION

Bacterial identification is commonly performed in environmental samples using culturing methods. Recently, PCR amplification of 16S rRNA gene has been recognized as an effective method of bacterial identification. The use of 16S rRNA gene sequences as a common genetic marker for bacterial phylogenic and taxonomic studies is gaining momentum. The reasons may be the presence of 16S rRNA gene in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution), and the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Manaka et al., 2017).

The present study also revealed an overall prevalence rate of 92.3% for identification of STEC O157:H7 using PCR amplification of 16SrRNA genes. These findings suggest that 92.3% of isolates were members of the genus *Escherichia*. This observation collaborates well with the reports of some previous studies (Manaka et al., 2017). Furthermore, the results of this study also revealed higher sensitivity (92.3%) to detect specific bacterial genus than usual cultural method. However, the results of this study did not distinguish closely related species. These findings are in accordance with Manaka et al. (2017) that reported several limitations associated with PCR amplification of the 16S rRNA. Obviously, closely related species might be difficult to distinguish using PCR amplification of 16S rRNA gene, and the identification of bacteria to a species level might be inaccurate. Furthermore, the antimicrobial susceptibility of bacterial and fungal strains could not be detected using PCR amplification of 16S rRNA gene.

The results of this study revealed the presence of both $stx_1$ (83.3%) and $stx_2$ (16.7%) genes in STEC O157:H7 isolates. The presence of either or both genes has been implicated in the ability of STEC strains to cause serious infections in humans. STEC strains producing $stx_2$ only have been shown to be more commonly associated with serious human diseases such as HUS, than those producing $stx_1$ alone or $stx_1$ and $stx_2$ possibly because the level of transcription of $stx_2$ *in vivo* is higher than that of $stx_1$ (Bitrus et al., 2011). STEC cause infections such as gastroenteritis, haemorrhagic colitis (HC), HUS and renal failure in children by production of Shiga-toxins (Sharaf and Shabana, 2017).

In this study, $stx_1$ gene was detected more than $stx_2$ gene in the isolates (83.3% versus 16.7%, respectively). Similar observations were reported previously in Nigeria and Turkey (Bitrus et al., 2011; Ferreira et al., 2015). However, results of this study contradicted the reports of many other previous researchers where $stx_2$ gene was detected more than $stx_1$ (Wang et al., 2002). In contrast, this study did not corroborate well with the reports of many previous studies where no $stx_1$ was detected in any of the samples (Al-Ajmi et al., 2020). These discrepancies might be due to differences in samples, sources of collection, geographical locations and laboratory techniques used for detection of STEC O157:H7 isolates

(Pradel et al., 2000). Furthermore, the results of this study showed that the distribution of $stx_1$ gene detection was higher in cattle feces (85.7%) than in effluent water samples (80.0%), which was in accordance with the results of several previous studies (Kalender and Kilic, 2016).

Using multiplex PCR primer set conceived for detection of flagellin (*fliC*), hemolysin (*hlyA*) and intimin (*eaeA*) genes (Bitrus et al., 2011); two virulence genes, *fliC* (58.3%) and *hlyA* (8.3%) were detected in the STEC O157:H7 isolates studied. High distribution of H-7 specific flagellar protein, $fliC_{H7}$ gene observed in this study was in correlation with the results obtained by previous studies in China, Iraq, Greece and South Africa (Wang et al., 2002; Makhubalo et al., 2016; Alzubaidy, 2019). The presence of $fliC_{H7}$ gene in large proportion (58.3%) of the *E. coli* O157 isolates indicated that many of these isolates were genetically H7 with flagellum antigens as detected in serotyping tests. In contrast, the findings of this study were not consistent with the results of several earlier studies where no $fliC_{H7}$ gene was identified (Ferreira et al., 2015).

The low *hlyA* gene detection (8.3%) encountered in this study may be ascribed to different sources and pathogenesis of STEC O157:H7. These findings are in accordance with earlier studies performed in China (Wang et al., 2002). In the same vein, several earlier studies reported relatively high distribution of *hlyA* gene among STEC O157:H7 isolates. This may also be related to the variability of *hlyA* gene among *E. coli* strains. However, the result of this study is inconsistent with some previous investigations in Nigeria where no hemolysin (*hlyA*) gene was detected (Oloyede et al., 2016). The lone isolate that possessed *hlyA* genotype was isolated from effluent waste. This may suggest that STEC O157:H7 isolates without *hlyA* gene may possess reduced pathogenicity or may even be nonpathogenic in humans (Wang et al., 2002).

However, *eaeA* was not detected in the DNA of the twelve isolates. This report tallies with Kalin et al. (2012) in which all other virulence genes were detected except the *eaeA* gene. However, this result was not in agreement with reports of some previous studies in Turkey and Ethiopia which all the isolates harbored *eaeA* and other virulence genes (Kalender and Kilic, 2016). Intimin (eae gene product), has been recognized as the gene responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing lesions in the intestinal mucosas. However, a significant number of human STEC isolates, including those from patients with HC and HUS, lacked eaeA, showing that intimin is not important for human virulence (Bitrus et al., 2011). The differences observed in the distribution virulence genes in the DNA of the twelve isolates may be due to differences in geographical locations and laboratory techniques used for the detection of virulence genes (Adamu, 2014).

The antimicrobial susceptibility test results of STEC O157:H7 strains isolated from faecal and effluent sources showed the highest level of susceptibility for Imipenem, Ciprofloxacin and Ofloxacin (100% each). These were closely followed by Ceftriaxone and Chloramphenicol with susceptibility rates of 80.0 and 70.0%, respectively. This observation agrees with a previous study in Nigeria (Moses et al., 2018). However, these findings were not in conformity with previous studies conducted by Atnafie et al. (2017) and Ayandele et al. (2020). The differences observed may be due to differences in geographical locations and laboratory techniques used for the detection of antimicrobial susceptibility.

Interestingly, the highest level of resistance was observed against Cefepime (90%), Streptomycin (70%), Ampicillin (70%), Tetracycline (60%), and Amoxicillin (95.8%). The results of this study are in conformity with the findings of previous studies. The resistance rates recorded in this study are higher than the results of Moses et al. (2018) and lower than the results of Atnafie et al. (2017) and Tadesse et al. (2018). High level of resistance to Cefepime was not in consistence with the reports of previous studies in Nigerian (Moses et al., 2018; Ayandele et al., 2020); however, high level of resistance to Streptomycin agrees with a previous study in Nigeria (Atnafie et al., 2017).

The different trends of resistance patterns as observed in this study did not conform with reports of the previous studies conducted in Abakaliki, Southeast Nigeria and Lagos, Southwest, Nigeria where some isolates with more than one similar resistance patterns were observed in majority of the *E. coli* and *Salmonella* isolates evaluated, respectively (Akinyemi et al., 2018; Moses et al., 2018). Investigation on MAR showed that 80.0% of the ten STEC O157:H7 isolates exhibited multiple antibiotic resistances as they were resistant to at least two different antibiotics. The MARI of the STEC O157:H7 isolates in this study ranged from 0.083 to 0.667. Data obtained in this study however showed that the highest MARI of STEC O157: H7 isolates was 0.667. The findings of this present study are similar to the results of previous studies in Nigeria (Moses et al., 2018; Ayandele et al., 2020). Moses et al. (2018) and Ayandele et al. (2020) reported highest MARI value of 0.335 and 1.00, respectively. The optimal multiple antibiotic resistance of STEC O157:H7 isolates in this study may be attributed to robust control of antibiotics usage in livestock production in the area covered by this study. 60% of STEC O157:H7 isolates exhibited multi-drug resistance traits as they were resistant to one or more antibiotics in more than three different antimicrobial categories and were regarded as multidrug resistance strains. Multiple drug resistance has become an important trait of many microorganisms especially the human pathogens (Adenaike et al., 2016). Results of this study are similar to those of Subramani and Vignesh (2012) of which 50.0% of the isolates tested in their study exhibited

multidrug resistance attribute. Data obtained in this study however is much higher than those of Moses et al. (2018) of which 16.0 and 28.6%, respectively of the isolates tested in their studies exhibited multidrug resistance traits, suggesting the existence of lower frequency of MDR strains in their study areas. The differences observed in the multi-drug resistance of the isolates investigated in this study may be due to differences in geographical locations, samples and laboratory techniques. The higher multidrug resistance of Shiga-toxin producing *E. coli* O157:H7 isolates in this study may be attributed to the existence of greater frequency of MDR strains in the area covered by this study.

## Conclusion

This study suggests that slaughtered cattle faeces and abattoir effluents are sources of contamination with STEC O157:H7 and non-STEC O157:H7. Our study exposed the presence of virulence genes such as $stx_1$ (83.3%), $stx_2$ (16.7%), *hlyA* (58.3%) and *fliC* (8.3%) genes. It also revealed that the pathogens lacked *eaeA* genes. Our results suggest that Imipenem (100.0%), Ciprofloxacin (100.0%), Ofloxacin (100.0%), Ceftriaxone (80.0%) and Chloramphenicol (70.0%) demonstrated high antibiotic susceptibility to STEC O157:H7 pathogens; while Cefepime (10.0%) is no longer effective. STEC O157:H7 isolates exhibited relatively high MAR (80.0%) and MDR (60.0%) traits, respectively. The presence of MDR pathogens indicates serious public health concern and signals possible outbreak of foodborne infections. Therefore, coordinated efforts are needed to safeguard the populace against this pathogen and to ensure proper use of antibiotics both in veterinary and human treatment.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Systematic studies on tetra SL hybrid hen technology and rearing for chicken welfare

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**In Serbia, there are 4 to 6 genotypes of hens used for producing eggs for consumption, including hybrid Tetra SL. The proper rearing of commercial flocks is critical for timely laying, intensity of laying capacity, number of laid eggs, and period of hen usage. The aim of this paper is to analyze the results of rearing the offspring of hybrid Tetra SL, from one-day-old chickens to eighteen-week-olds, with a review of the conditions to which domestic producers must adjust in order to meet the European Union standards for laying hens breeding systems. At the beginning of rearing period, 9500 heads were put into two objects, totaling 19000 one-day-old chickens. A completely identical rearing technology was applied in these two identical objects. The chickens were of the same age and line hybrid, and the same manufacturer's equipment was used, resulting in equal costs. The chickens were debeaked on the first and tenth day, respectively. The comparative method was used. The breeder adhered to the technological norms recommended by the hybrid selectioner. A special attention was paid to increasing chicken body mass and vitality from the beginning until the end of rearing (18th week).**

**Key words:** Poultry, hybrid Tetra SL, offspring rearing, mortality, 18th week chicken, EU standards.

## INTRODUCTION

Rearing poultry, from brooding eggs to final products (meat and eggs for consumption), used to be one single process even in different rearing systems (Živković et al., 1991), while today each stage of this type of production is done separately and as such, is a main industry, that is, a direction in production. In this way, today we have defined norms for the requirements for housing, appropriate feed, micro-climate (ambient) conditions, and health protection (Miljković, 2006). The goal is to achieve as large production and as good product quality as possible, taking into consideration that these are high quality products for human nutrition, but with gradual decrease and complete abolishment of keeping animals in cages (ECI, 2020).

Serbian poultry producers keep laying hens in cages which are very remote from European standards and

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animal well-being. All the so-called 'commercial' hybrids ensure exceptionally high production (Mitrović, 1996; Bogosavljević-Bošković and Mitrović, 2005; Milošević and Perić, 2011). They are primarily reared in closed premises and fed with ready-made, complete forage mixtures exclusively. EU laws are quite different from domestic legislation when it comes to the housing of laying hens. In 2010, the Rulebook on requirements for animal well-being was passed in Serbia, dealing with space for animals, rooms, and equipment in the objects in which animals are kept, bred and put into circulation for production purposes, way of keeping, breeding and circulation of certain animal species and categories, and the content and way of keeping register on animals ("Sl. glasnik RS" br. 6/2010 i 57/2014). Serbia provided a year and a half period to the producers to adjust to new regulations. Since they have not adjusted, the issue was postponed until December 2020, which is the deadline the producers were not ready to meet once again. In order for all poultry farms to adjust to new requirements, it takes between 50 and 60 million euros investment per year, according to the estimates of Serbian Chamber of Commerce, so the new deadline is 2023.

A selectioner in their leaflets gives performances related to production properties and quality of each hybrid, with certain waranties. In order for the offspring rearing to be successful, certain fixed requirements must be met. Health status, premises temperature, work organisation are just a few of the factors that have a capacity to change genetic potential. Tetra SL hybrid is very adaptable concerning harmonization of egg size and colour, and it should be noted here that maximum performances are, however, only possible in controlled environmental conditions.

Tetra SL is the most popular brown egg layer, which has outstanding resistance against climatic and management conditions, the highest livability on the market and an excellent egg quality. These properties make her a market leader in many European countries. Concerning feed conversion rate, she is competitive with any other brown egg layers on the market (https://www.winmixsoft.com/en/blog/item/tetra).

In our country, this hen hybrid (Tetra SL) is not reared in significant numbers (though the numbers have been rising in recent years), unlike hybrids Isa Brown or Lohmann Brown, which are also light hybrids for the production of eggs of coloured shell. Accordingly, Škorić (2006) and Mitrović et al. (2015) point out that in our country, 4 to 6 different genotypes are reared for the production of eggs for consumption and rearing system has a great influence on them.

It is completely clear that the following things are dependant on the success in this very production stage and proper rearing: timely laying, laying capacity intensity, number of laid eggs, and the period of laying hen, that is, commercial flock and usage (Pavlovski, 2006; Khatibi et al., 2021).

It is for these very reasons that this hybrid was taken for the analysis and monitoring of achieved results in appropriate conditions on the agricultural property of Mr Miodrag Stojanovic in the village of Kosančić.

The aim of this paper is to look at only one producition stage, that is, the analysis of the technology and results of Tetra SL hybrid hen offspring rearing, from one-day-old chicks to eighteen-week-olds.

## MATERIALS AND METHODS

On the agricultural property of Mr Miodrag Stojanovic in the village of Kosančić, the offspring of laying hens of light line hybrid Tetra SL was reared from the first day of life until the 18th week. The offspring rearing was conducted in the first half of 2019.

At the beginning of rearing, 9500 one-day-old chickens were moved into two objects, which is a total of 19000 heads. The two objects were identical and a completely identical rearing technology was applied in both of them. The inputs were the same, that is, the chickens were of the same age and same line hybrid and the equipment produced by the same manufacturer was used. Consequently, the cost levels were equal.

One-day-old chickens of light line hybrid Tetra SL were moved into previously prepared objects suitably furnished for this type of production. Appropriately allocated objects, according to the EU standards, have 0.85 m$^2$ of space for each laying hen, 0.6 m$^2$ of useful space with a nest with protective curtain where they can freely lay eggs, beds should be made of artificial grass and sand so that they can peck, blunt their beaks, spread wings, so that the most natural conditions for animals well-being are met (Council Directive, 1999/74/EZ; Council Directive 1998/58/EZ). Different minimal standards apply for non-member states (Directive 1988/166/EZ). During the period of rearing, the technology of rearing, feeding, and health protection (immunoprophylaxis) recommended by the selectioner (www.babolnatetra.com) of the hen hybrid was used. The offspring was reared on the floor with deep mat. The population density during offspring rearing was on a suitable level (approximately 12 heads/m$^2$ of floor surface), which depended on head age, that is, rearing stage. It must be noted that chicken were accommodated in one quarter of the object until the end of third week. From the fourth week onward, the offspring was reared on the whole production surface of the object.

Throughout the rearing process, the offspring was fed at their own will (*ad libitum*). Three full forage mixtures were used, with various participation of individual nutrients and various chemical composition.

In some periods of rearing laying hens, individual control measurings of body mass were conducted. During each measurinig, a minimum of 2% of the total number of heads in the object were measured, selected by the random sample method. The mentioned control measurings were conducted early in the morning before the feed was distributed.

In addition to the body mass, the mortality and removals from production were also monitored during rearing. The deaths of offspring were registered daily and then calculated per week and shown in absolute and relative values.

In each control measuring of heads and for the whole period of offspring rearing, the common variation and statistical indicators were calculated: arithmetic mean – average ($\bar{x}$), arithmetic mean error ($S\bar{x}$), standard deviation (S) and coefficient of variation (CV).

**The technology of rearing hen offspring**

Rearing poultry hybrids sets the ground for industrial poultry keeping and production. The process of obtaining good quality commercial hybrid is a long and complex one, which is why it inherently requires high expertise. Commercial poultry flocks are specially created for egg production, and heterosis effect is fully expressed in hybrids. Hybrids for egg production compared to pure poultry breeds have stronger average laying capacity, larger egg mass, better feed usage and calmer temperament. When appropriately reared, that is, when basic technological procedures are adequately applied, laying hens of commercial flock lay eggs at the end of 19th and beginning of 20th week already. Since heritability coefficient for the majority of reproductive and production properties in poultry is pretty low (around 30%), production capabilites are more dependant on rearing conditions, nutrition and health protection during production process, especially in the early period. The application of appropriate technology and compliance with it have a direct impact on the commercial effect.

For the production of eggs for consumption in our country, exclusively foreign hybrids are used (there are no domestic ones). All the hybrids are of various origin and name (commercial name), and Isabrown, Tetra SL, Hisex Brown, Harco, Shever, Hisex White, Pure Line and others (Mitrović, 1996; Bogosavljević-Bošković and Mitrović, 2005; Milošević and Perić, 2011) are reared most frequently and in largest numbers.

In light line hybrids, the period of rearing lasts 18 weeks. On the farming household in the village of Kosančić, the rearing of offspring until the 18th week is done in two completely identical objects. During rearing, the following conditions were provided:

(1) appropriate housing conditions - objects;
(2) optimal micro-climatic conditions in the object;
(3) adequate nourishment and hydration;
(4) appropriate health protection;
(5) regular organization of the production cycle.

In order to breed poultry successfully, it is necessary to provide a proper housing for it in the first place (Milošević, 2006; England and Ruhnke, 2020).

A good hennery (object) should:

(1) Protect the poultry against cold, too much heat, moisture and various pests or predators;
(2) Enable an easy maintenance of hygiene, it should be dry, lighted and ventilated;
(3) Enable a proper equipment positioning and rational space usage.

This technology is supported by research done by Milisits et al. (2021).

The spread of both alternative and non-cage laying hen housing systems and the more forceful European refusal of beak trimming generate new problems in commercial egg production. The hybrid layers, which have been genetically selected under cage housing conditions for more decades, have lively temperament, are more susceptible for feather pecking and, in more cases, they are expressly aggressive, which led to permanent conflict situations in the large group keeping systems. Therefore, the omission of beak trimming could lead to an increased risk for feather pecking and consequently to a risk for increased mortality in the hen house by using the current commercial layers. Therefore, changes in the live weight, plumage and body condition, egg production, and mortality of different Tetra pure line non-beak-trimmed laying hens were compared during the egg-production period in the current study, where the plumage condition was considered as an indicator trait for feather pecking.

When Serbia joins the European Union, new problems of keeping and breeding laying hens will appear. The transitional shock could significantly impact the economy of production, so if Serbian poultry keepers do not harmonize their cages with the EU standards, it is questionable whether they will be able to work in this industry in the future. As a consequence of change, it can be expected that farm capacity will drop (Babić et al., 2015). Joining the EU brings the implementation of all its standards for Serbia, which includes regulations that regulate the keeping of laying hens. It is important to point out that within the EU member states, there are a divergence and different approaches to the problem of cage keeping of laying hens (Rodić et al., 2023).

European consumers have adopted a progressive and proactive approach in order to ensure animal well-being and food safety. Regional instructions and the political standards which indisputably affect the public opinion can determine which way of keeping is acceptable and will ensure the desired well-being and production results that meet the arranged requirements (Matković et al., 2007).

## RESULTS AND DISCUSSION

Each poultry production stage is characterized by its own specificities. In this manner, the production of eggs for consumption is a very complex production process which can be divided into two stages: rearing the offspring of laying hens and using the commercial flock. Bearing in mind that the breeding of commercial flock is the final stage in the production of eggs for consumption, all the producers (breeders) of these flocks must pay a special attention to the period of offspring rearing. Special attention should be paid to offspring debeaking. Debeaking one-day-old chickens using infrared rays (in object I) and ten-day-old chickens (in object II) caused the difference in mortality, that is, of egg mortality, and it is known that flock welfare is judged according to mortality. Debeaking is performed by professional staff at the age of 1 to 10 days (Hester and Shea-Moore, 2003), which was done in this case too, only at different age. It is common to debeak one quarter or one third of beak, leaving approximately 2 mm to the nostrils (Sandilands and Savory, 2002; Singh, 2021). Debeaking is only a partial removal of beak tip and it is a routine method that can be repeated between the 8 and 12th week. It is typically performed using infrared technology (infrared beak trimming) or thermal cauteries (hot blades). Infrared light is used to damage the beak so that the tip falls off (https://poultry.extension.org/articles/poultry-behavior/beak-trimming-of-poultry/). This method directs a strong source of heat into the inner tissues of the beak and within a week or two the tips of the upper and lower beak die and fall off, leaving the bird with a shorter beak and blunt tip. While infra-red beak trimming (IRBT) of day-old chicks was recognised by the Farm Animal Welfare Committee as not causing any pain to birds, successive governments have always indicated they were keen to introduce a ban (https://www.poultryworld.net/poultry/

beak-trimming -the-way-ahead-for-the-uk/).

In addition to preventing feather pecking and canibalism, another reason for applyling debeaking is a reduced food scattering, reduced food intake and poor growth. However, chickens and later hens, must adjust to the new beak shape. The ability to consume food is reduced after debeaking, so authors think that it is necessary to do it as soon as possible in our context (before the 1st day) for the purposes of faster adjustment.

Recently, in 2017, a company Roxell (https://www.roxell.com) in Hanover used ethology and data about the ways poultry feeds to design special feeders using which poultry performs the debeaking themselves. This is when for the first time a high quality and permanent solution for new trends and regulations on poultry welfare was presented, with the concept of Natural Beak Smoothing which has been used in some welfare states but very rarely in Serbia due to economical reasons.

It is exactly this period that is the most important and most sensitive stage of such a complex production process (cycle) because in this period, to put it roughly, a good quality laying hen is formed through proper rearing of offspring. For all the these reasons, a special attention should be paid to the proper growth (development) of heads, ambiental conditions, nutrition, health protection and body mass control, all this to the aim of producing a larger number (lower percent of mortality and removals) of healthy and vital commercial laying hens.

## Offspring mortality during rearing

In addition to body mass (growth), mortality and removals were also monitored during the rearing of offspring of the laying hens which produce eggs for consumption. The data are shown in Table 1 in absolute and relative values per week, that is, for the total period of rearing.

The data in Table 1 show that in both objects the same number of one-day-old chickens was put, 9500 heads per object. At the end of the rearing period, there were 9311 pullets in object I (mortality 1.99%) and 9221 laying hens in object II (mortality 2.94%). On top of that, 279 heads died and were removed in object II, which means the mortality was 0.95% higher compared to object I in which 189 pullets died and were removed.

Observed per weeks, mortality and removal were higher in object II, except in 12th week (0.07%), 16th week (0.06%) and 18th week (0.05%) compared to object I. The hypothesis is that the cause of higher mortality in object II is later debeaking (on the 10th day) because the food scattering was probably larger, food consumption was smaller and adjustment to feeding after debeaking lasted longer. Also, it is clearly visible from the data that the highest mortality and removal happened in the first weeks in both objects. However, the mortality of offspring of laying hens in both objects was within the limits of technological norms (www.babolnatetra.com). More recent research (Milisits et al., 2021) shows that laying hens should be of good health and with low mortality rates in order to keep egg production on a high level. The same authors (Milisits et al., 2021) point to the bad impact of debeaking on laying capacity itself and mutual pecking in contemporary housing systems, which is a bad influence on their welfare. Therefore, some of the measures introduced over the last decade should be reconsidered.

## Body mass of laying hens in the period of rearing

For objective and subjective reasons, the control measurings of offspring (11 measurings in total) in defined periods of rearing, the body mass of heads selected by random sample method was controlled. It included a smaller number of heads (120 heads) out of projected number (2% = 190 heads), but even with that, the conducted measurings were not enough to analyse achieved results. However, with the application of the proper nutrition regime and proper selection, it was the idea for the offspring to have during rearing a body mass that corresponds to this genotype and production direction, which was mostly achieved on the farm (object I).

The trend of average of offspring body mass from day 1 until week 18 of rearing and variabilities are shown in Table 2.

The data from Table 2 show that the average body mass of offspring during rearing was in certain periods in accordance with technological norms. Also, the data show that the flock reared in object I was more homogenous than the flock from object II. This is confirmed by the calculated coefficients of variation, which were higher in heads reared in object II, both in one-day-old chickens just moved in and in pullets during rearing. Besides, at the end of the rearing period, pullets from object I had a higher body mass (1435.69 g) than heads from object II (1371.56 g), which is more favourable, that is, closer, to the values of technological norms (www.babolnatetra.com) of the examined hen hybrid. Also, later debeaking can be the cause of smaller average body mass in hens in object II because, earlier, chickens grew faster and had better food consumption. There are many factors that can have an influence on severe feather pecking behaviour: the feed composition such as reduced content and disproportion of minerals (Ca and P); the climate; the size of the group and the type of light in the house, to name a few. All of these factors can also influence one another. However, since all of them were identical in the present research, it can be concluded that chicken age at the moment of debeaking had an impact on mortality and body mass

**Table 1.** Mortality and removals of heads during rearing.

| Age | Object | At the beginning of week | At the end of week | Mortality | |
|---|---|---|---|---|---|
| | | | | Heads | % |
| 1st week | I | 9500 | 9458 | 42 | 0.44 |
| | II | 9500 | 9449 | 51 | 0.54 |
| 2nd week | I | 9458 | 9441 | 17 | 0.18 |
| | II | 9449 | 9411 | 38 | 0.40 |
| 3rd week | I | 9441 | 9422 | 19 | 0.20 |
| | II | 9411 | 9380 | 31 | 0.33 |
| 4th week | I | 9422 | 9412 | 10 | 0.11 |
| | II | 9380 | 9360 | 20 | 0.21 |
| 5th week | I | 9412 | 9400 | 12 | 0.13 |
| | II | 9360 | 9343 | 17 | 0.18 |
| 6th week | I | 9400 | 9389 | 11 | 0.12 |
| | II | 9343 | 9325 | 18 | 0.19 |
| 7th week | I | 9389 | 9379 | 10 | 0.11 |
| | II | 9325 | 9313 | 12 | 0.13 |
| 8th week | I | 9379 | 9371 | 8 | 0.08 |
| | II | 9313 | 9302 | 11 | 0.12 |
| 9th week | I | 9371 | 9363 | 8 | 0.08 |
| | II | 9302 | 9290 | 12 | 0.13 |
| 10th week | I | 9363 | 9357 | 6 | 0.06 |
| | II | 9290 | 9281 | 9 | 0.10 |
| 11th week | I | 9357 | 9350 | 7 | 0.07 |
| | II | 9281 | 9272 | 9 | 0.10 |
| 12th week | I | 9350 | 9343 | 7 | 0.07 |
| | II | 9272 | 9265 | 7 | 0.07 |
| 13th week | I | 9343 | 9337 | 6 | 0.06 |
| | II | 9265 | 9257 | 8 | 0.09 |
| 14th week | I | 9337 | 9332 | 5 | 0.05 |
| | II | 9257 | 9248 | 9 | 0.10 |
| 15th week | I | 9332 | 9327 | 5 | 0.05 |
| | II | 9248 | 9241 | 7 | 0.08 |
| 16th week | I | 9327 | 9321 | 6 | 0.06 |
| | II | 9241 | 9235 | 6 | 0.06 |
| 17th week | I | 9321 | 9316 | 5 | 0.05 |
| | II | 9235 | 9226 | 9 | 0.10 |

**Table 1.** Contd.

| | | | | | |
|---|---|---|---|---|---|
| 18th week | I | 9316 | 9311 | 5 | 0.05 |
| | II | 9226 | 9221 | 5 | 0.05 |
| **Total** | I | / | / | 189 | 1.99 |
| | II | / | / | 279 | 2.94 |

**Table 2.** Average values and variabilities of offspring body mass (g).

| Age | Object | $\bar{x}$ | $S_x$ | SD | VC |
|---|---|---|---|---|---|
| 1st day | I | 42.00 | 0.32 | 3.46 | 8.24 |
| | II | 40.00 | 0.34 | 3.77 | 9.42 |
| 29th day | I | 266.10 | 1.96 | 21.51 | 8.08 |
| | II | 202.18 | 2.47 | 27.10 | 13.40 |
| 36th day | I | 364.20 | 2.34 | 25.65 | 7.04 |
| | II | 305.58 | 2.45 | 26.82 | 8.78 |
| 43rd day | I | 484.50 | 2.84 | 31.11 | 6.42 |
| | II | 407.67 | 3.50 | 38.34 | 9.40 |
| 58th day | I | 642.70 | 2.96 | 32.43 | 5.05 |
| | II | 611.80 | 3.14 | 34.41 | 5.62 |
| 66th day | I | 723.00 | 3.52 | 38.55 | 5.33 |
| | II | 655.10 | 3.71 | 40.65 | 6.20 |
| 75th day | I | 796.07 | 3.37 | 36.93 | 4.64 |
| | II | 747.70 | 3.52 | 38.52 | 8.11 |
| 92nd day | I | 1236.90 | 4.68 | 51.28 | 4.15 |
| | II | 1160.94 | 5.88 | 64.41 | 5.55 |
| 109th day | I | 1307.60 | 5.72 | 62.67 | 4.80 |
| | II | 1241.65 | 5.95 | 65.14 | 5.25 |
| 119th day | I | 1388.70 | 4.15 | 45.46 | 3.27 |
| | II | 1314.33 | 5.55 | 60.74 | 4.62 |
| 126th day | I | 1435.69 | 5.29 | 57.88 | 4.03 |
| | II | 1371.56 | 6.09 | 66.72 | 4.86 |

during rearing.

## Conclusion

Over the last decade, 4 to 6 different genotypes, including the hybrid Tetra SL, have been bred in our country in order to obtain eggs for consumption. Bearing in mind the fact that the success in this production stage and the proper breeding of commercial flocks are critical for timely laying, laying capacity intensity, number of laid eggs, period of laying hens and commercial flock usage, the main goal of the paper was to analyse the technology and results of this hybrid offspring rearing from day 1 until

week 18, using comparative method.

Viewed as a whole, it can be concluded that the analyzed flock at the farm of Mr Miodrag Stojanović in the village of Kosančić produced significant results. However, regardless of the achieved results during offspring rearing, it is necessary to pay more attention to regular (weekly) growth/body mass control and keep a precise and neat register of food consumption (Kabir and Islam, 2021). The implementation of alternative systems of production can bring into question the economy of production, taking into consideration the mutual independence of fixed costs on the number of animals. It can be argued that it would be useful to direct a 'general recommendation' to producers, which would be based on the experiences of states which were last to join the European Union and to make a deal on the relation, producers - state - EU - producers, with necessary consideration of interests of all interested parties.

It is particularly important to highlight the fact that the behavioural problems of feather pecking and cannibalism seem to occur both in intensive farming systems and in organic and hobby poultry farms. Scientists are looking into ways to improve chicken welfare by finding alternative measures to combat injurious pecking behaviour. Because egg laying strains of chicken can be kept in smaller group sizes in caged systems, cannibalism is reduced, leading to a lowered trend in mortality as compared to non-cage systems. Good breeding programme could reduce the problem. The scientists also indicated that selecting for social behaviour would lead to a reduction in feather pecking. If stress and anxiety could also be removed then the likelihood of the birds engaging in this excessive behaviour would also be reduced.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Effects of seed coat pigmentation on germination characteristics and antioxidant properties of *Argyrolobium uniflorum* ((Decne.) Jaub. & Spach) in southern Tunisia

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**Seeds of *Argyrolobium uniflorum* (Fabaceae), a spontaneous plant in arid and semi-arid regions of Tunisia, showed a color variability in their seed coats (green, orange, yellow and brown). The influence of the color of seed coat on germination and phytochemical seed composition was evaluated. Germination tests were carried out and the results obtained show that the applied chemical treatment (sulfuric acid) improved germination, independently of the color tested. In addition, this plant germinated at a temperature range of 10 to 30°C, where each color requires a suitable temperature. Brown seeds have the highest percentage of germination. Total polyphenolic contents of different colors of seeds were studied using three solvents. The yellow seeds TPC was found higher than that of the green, brown and orange colors, with the highest values in acetone extract reaching 7.98 and 8.68 mg EAG $g^{-1}$ MS. However, the level of antioxidant activity estimated by DPPH ($EC_{50}$ = 0.24 to 1.84 mg / ml) also showed that the yellow seeds exhibit the lowest value ($EC_{50}$ = 0.24 mg / ml). The highest values of flavonoids were observed in the seed brown with values 3 mg EC $g^{-1}$ MS. Germination associate with the colors of coat of seeds were to be a good process to improve the phenolic content and antioxidant activity of *A. uniflorum* seeds.**

**Key words:** *Argyrolobium uniflorum*, germination, colors, antioxidant activity.

## INTRODUCTION

*Argyrolobium uniflorum* ((Decne.) Jaub. & Spach), a pluriannual herbaceous legume is from North Africa

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(Dekak et al., 2018) and is a pastoral legume that is widely distributed in arid and semi-arid regions in Tunisia (Ferchichi, 1990). It is considered the most promising complement to highly palatable native Werker (Visser et al., 2011). *A. uniflorum* plays an important role in the maintenance of soil fertility, soil coverage and dune stability (Ferchichi, 1990).

In Tunisia, *A. uniflorum* is a species of wide ecological amplitude (Jauffret and Visser, 2003). It is distributed from the semi-arid to the upper Saharan, with a rainfall range of 50 to 600 mm (Chaieb and Boukhris, 1998). Seeds of *A. uniflorum* are represented as a pod up to 3 cm by 3 mm with 6 to 10 seeds. At maturity, the pod opens at two valves each carrying half the seeds (Pottier-Alepatite, 1979; Neffati, 2008). This taxon is cleistogamous and its seed is highly polymorphic (Visser et al., 2011). The seeds are multicolored (yellow, orange, brown and green) very uniform, hard, containing the albumen. The weight of thousand seeds of this species is 1.750 g. It can produce many seeds. The number of one gram can reach 800 to 1000 seeds, with an average of 8 to 9 seeds per pod (Pottier-alepatite, 1979; Neffati, 2008). The propagation seems possible only by sowing (Neffati, 1994).

Successful establishment of plants largely depends on successful germination. However, the presence of hard seed coat is characteristic feature of the most of legume species. Dormancy imposed by a hard seed coat is a part of seed survival strategy in many species (Werker, 1981; Kelly et al., 1992; Bhatt et al., 2016). Several factors such as high soil temperature soil, microbial attack, temperature fluctuations, humid atmospheric conditions and passage through the digestive system of animal scan contribute to increasing coat permeability of legumes and therefore, increase seed coat germination percentage (Baskin et al., 2000; Bhatt et al., 2016).

Polyphenols are a large and diverse group of phytochemicals in plants (Harborne and Williams, 2000). It was reported that polyphenols had strong antioxidant activities associated with their ability to scavenge free radicals, break radical chain reactions and chelate metals (Kaisoon et al., 2011). Today, polyphenols are considered to be the most important dietary antioxidants due to their high antioxidant capacity and presence in plant foods (Kardum et al., 2014). In arid regions of Tunisia many natural antioxidants have already been isolated from different seeds of the plants, *Astragalus gombiformis* (Teyeb et al., 2012) and *Calligonum* species (Dhief et al., 2013). To the researchers' knowledge, there are no study relating the properties of the polyphenols in *A. uniflorum* in arid regions of Tunisia. It could be suspected to study the germination responses of *A. uniflorum* to temperature in relation to the pigmentation affecting their seeds and evaluate their total polyphenolic content and compare the effects of different extraction solvents on these potentials.

## MATERIALS AND METHODS

### Seed collection and storage

Seeds of *A. uniflorum* used in study were collected in May 2012 from plants growing on Benguerdane (33° 17' 35N) (10° 46' 47 E). After collection, the seeds were stored at 20 and 30°C relative humidity in Seed Bank of Laboratory of Pastoral Ecosystems and Valorization of Spontaneous Plants, Institute of Arid Regions (Médenine, Tunisia), prior to testing and experiments began in December 2016.

### *Seed viability test*

Tetrazolium test was used to determine seed viability; about 25 seeds with four replicates were taken from each color of seeds according to Tommasi et al. (2006). The isolated embryos (25) were incubated for 1 h in 5 mL of a solution of 1% (w/v) 2, 3, 5 triphenyl tetrazolium chloride (TTC) in phosphate buffer 50 mM, pH 7.3 for 3 to 4 h at 25°C in darkness. Seeds of which the embryo exhibited no overall carmine red staining were scored as non-viable. Finally, the viability percentage (VP) was calculated as the number of viable embryos/total number of embryos × 100.

### *Germination under laboratory conditions*

The effects of temperature on seed germination were assessed in incubator at 10, 15, 20 25 and 30°) (Lumincube II analysis, Belgium, MLR- 350, Sanyo, Japan) in the dark and in sterile Petri dishes of 9 cm diameter fitted with two layers of Whatman N°1 filter paper moistened with 5 ml of distilled water, and four replicates of 25 seeds in each level of treatment. The Petri dishes were observed every two days for further 16 days to monitor the number of germinating seeds. Germination was considered to be the incidence of radical protrusion (> 2 mm) (Kulkarni et al., 2007).

### Seed pretreatment

Although seeds varied apparently in color (Figure 1), they received the same sulphuric acid treatment. Seeds were immersed in 70% sulphuric acid for one hour and then rinsed thoroughly in running water for 2 to 3 min and with distilled water, after sterilized seeds had dried.

### Germination characteristics

Three germination characteristics were determined; final germination percentage, cumulative germination percentage which is the cumulative number of germination seeds counted daily and time to reach 50% germination ($T_{50}$). It was estimated according to the formulae of Coolbear et al. (1984) modified by Farooq et al. (2005).

$$T_{50} = t_i + ((N/2 - n_i))^* (t_j - t_i)) / (n_j - n_i)$$

where N is the final number of germination and $n_i$ and $n_j$ the cumulative number of seeds germinated by adjacent counts at time $t_i$ and $t_j$ when $n_i < N/2 < n_i$.

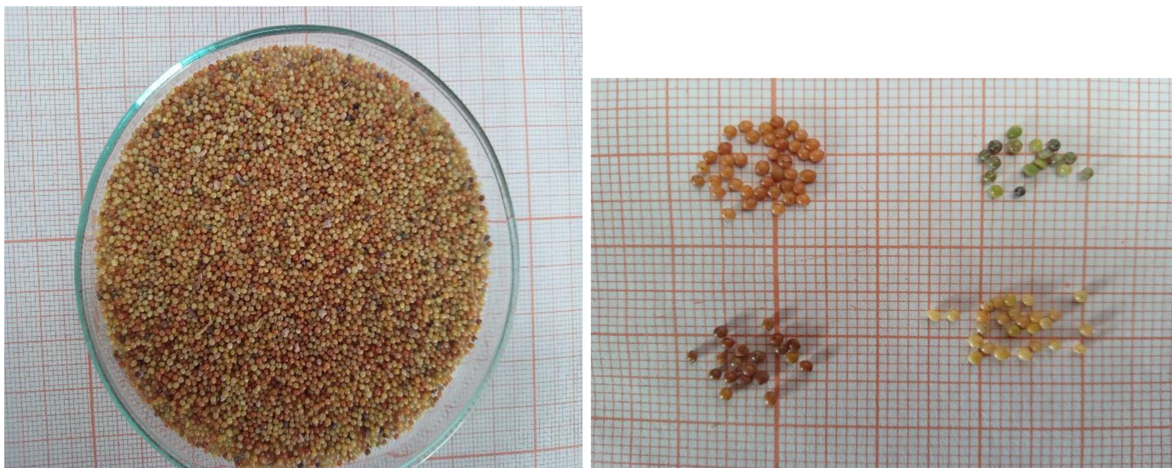

**Figure 1.** Phenotype of four representative seeds of each colored *A. uniflorum*. O (orange seeds), Y (yellow seeds), G (green seeds), B (brown seeds).

#### Preparation of seeds extract

Seeds of different colors (yellow, orange, green and brown) are reduced to powder. Polyphenols and flavonoids were extracted using three different solvents; ethanol, acetone and methanol mixed with acetone. A 0.1 g of each sample of different colors of seeds is dissolved in 1 ml of 70% ethanol, 70% acetone or methanol/water (50: 50); v/v). The extracts were centrifuged at 10.000 g for 1 0 min. At the end, the extracts from ethanol and acetone were recovered. However, the first supernatant from methanol extraction is kept and was added in 1 ml of 70% acetone. A second supernatant is obtained following another centrifugation for 1 h. The two supernatants are stored at -20°C overnight for use.

#### Extraction of polyphenolic compounds

The polyphenol content of the different colors of seeds was determined by the method of Singleton and Rossi (1965) using Folin- Ciocalteau. A volume of 150 µmol of each extract was mixed with 2400 µmol of distilled water, 150 µmol of Folin- Ciocalteau reagent and 300 µmol of 2% $Na_2CO_3$. The solution was adjusted to a final volume of 3 mL, with distilled water and mixed vigorously. After incubation at room temperature 50°C for 2 h, the absorbance was read at 725 nm using a Milton Roy 601 UV- vis spectrophotometer. A calibration curve with gallic acid at different concentration of 0, 0. 01, 0.025 and 0.1 mg/ml. The polyphenols contents are reported in mg gallic acid equivalents per g of dry matter (mg EAG/g DM).

#### Total flavonoïd content

The total flavonoid was extracted by spectrophotometrically with aluminum chloride using the method of Zhuang (1992). The method was based on the formation of a complex flavonoïd aluminium having maximum absorbance at 415 nm. Firstly, 300 µL of each sample were adjusted with 600 µL of distilled water, 45 µL de $NaNO_2$ (5%) and 45 µmol of 10% methanolic aluminium chloride $ALCL_3$ solution. After 2 min of a stand at room temperature, the absorbance was measured at 415 nm using spectrophotometer. Total flavonoid contents were calculated as quercetin equivalents (RE) from a calibration 0.005, 0.01, 0.05, 0.1 and 0.2 mg/ml and expressed as mg RE/100 g DW. All measurements were performed in triplicate.

#### Diphenyl radical scavenging activity assay (DPPH)

DPPH (2,2-diphenyl-1-pycrilhydrazil hydrate) radical scavenging activity was determined according to the method of Amarowicz et al. (2004) that is slightly modified by Yesil-Celiktas et al. (2007). A lot of 100 mg of seed FW was homogenized with 2 mL of methanol and centrifuged at 9,000 g for 15 min. To 100 µL of extract, 2.9 mL of 0.1 mM methanolic solution of DPPH was added. The contents were stirred vigorously and then left to stand at room temperature for 30 min in dark. Decrease in colorization was measured spectrophotometrically at 517 nm. The radical scavenging activity (RSA) was calculated using the equation:

$$RSA\ (\%) = 100 \times (1 - AE/AD)$$

where, AE represent the absorbance of the solution containing antioxidant extract and AD is the absorbance of the DPPH solution. All measurements were done in triplicates.

## RESULTS

### Seed viability test

The viability test shows that the brown seeds of *A. uniflorum* recorded the highest percentage of viability (98%) compared to those of yellow color seeds and orange color seeds (97%). Indeed, the seeds of green color presented the low percentage of viability (96%).

### Seed germination

The germination responses of the different colors of *A.*

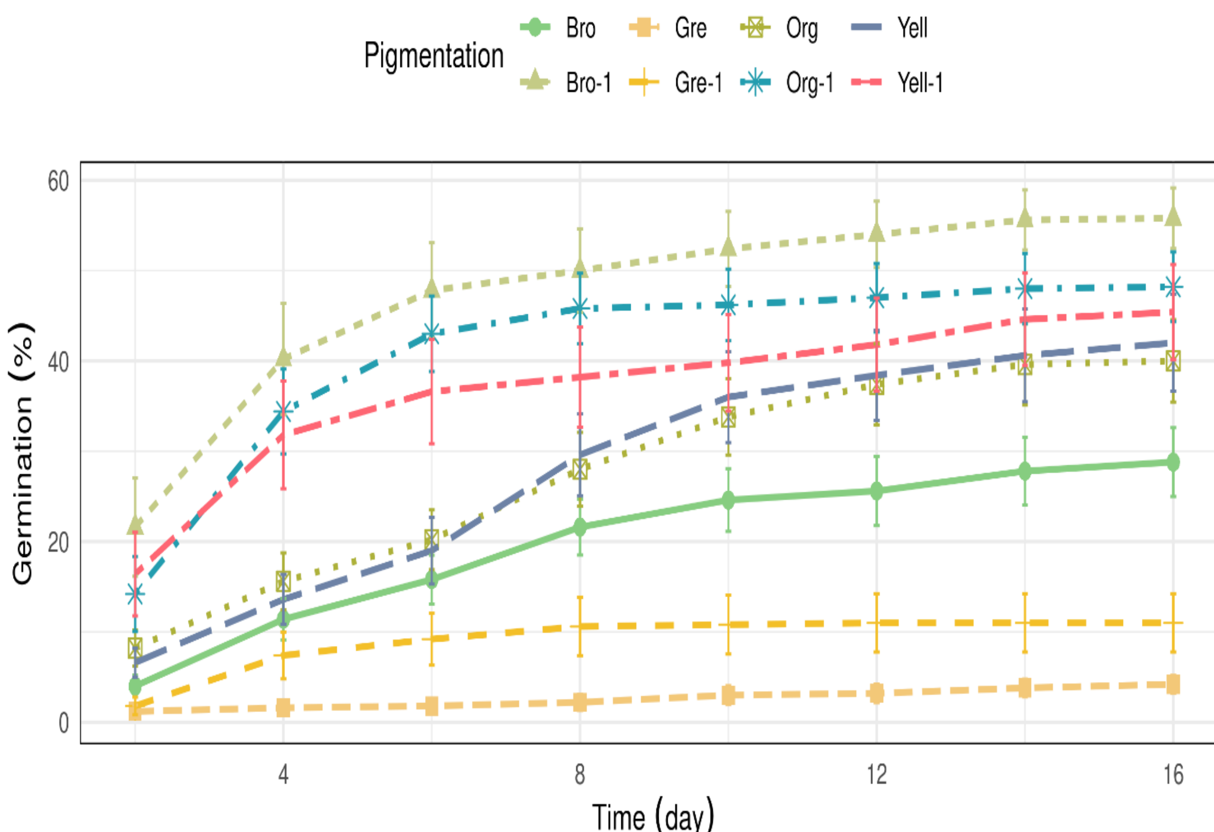


**Figure 2.** Cumulative germination percentage of withness and treaty of four colors ((A) orange, (B) yellow, (C)green, (D) brown) of *A. uniflorum* seeds for 16 days at different temperatures (10, 15, 20, 25 and 30°C) (n=4).

*uniflorum* (control and treated) to a wide range of temperatures (10 to 30°C) are illustrated in Figure 2. At these temperatures, the final germination percentage of different colors of seeds (control and treated) were higher than 50%. Incubation temperature from 10° to 30°C was suitable for germination of *A. uniflorum*. The germination percentage of the control seeds of the different colors presented more or less variable rates. This percentage varying between 0 and 56%, is recorded in green and orange seeds respectively. The delay of germination (DG) decreased with increasing temperature; it varied between 2 and 4 days in all control seeds. The study recorded only 2 days for temperatures 20, 25 and 15°C and it was delayed for 4 days at 10°C.

The final germination percentage of treated seeds of different colors ranging between 4 and 72%, recorded respectively in treated green seeds and yellow seeds. The delay of germination (DG) varied between 2 and 6 days respectively in treated orange seeds and brown seeds and between 0 to 8 days respectively in yellow and green seeds. Figure 3 illustrated that the germination percentage of treated seeds of *A. uniflorum* of all colors remains between 15 and 20°C with a percentage that did not exceed 70%. At 10°C the germination percentage is low; 0% was recorded in green seeds color at all temperatures tested (10 to 30°C) the treated brown seeds exhibited high germination percentage recorded at 25°C.

The curves relating to the mean MTG germination time (Figure 5) show that the germination rate of the treated and control seeds decreases as one moves away from the temperatures allowing the highest germination rates to be obtained and that this reduction in speed is generally greater for the lower temperatures than for the higher temperatures.

Temperature was significantly affected the percentage of germination of the treated and control seeds of all the colors ($P<0.001$) and showed a non-significant effect at the 5% on delay of germination for all the seeds colors (Table 1). Two-way ANOVA (Table 2) indicated a highly significant effect shows that in all the colors of the seeds tested the effect of the treatment on the percentage of germination of the different colors of seeds ($P_{0,003}$) and on delay of germination.

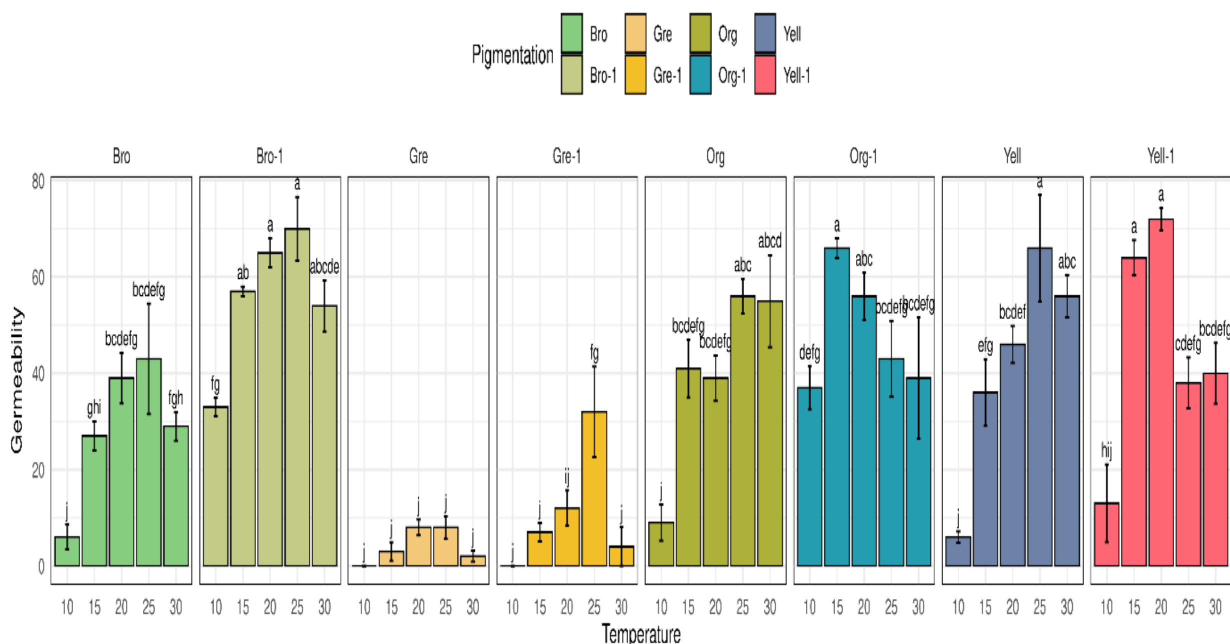


**Figure 3.** Seeds germination of different colors of *A. uniflorum* withness and treaty; O (orange seeds), Y (yellow seeds), G (green seeds), B (brown seeds) at different temperatures of incubation (10, 20, 25 and 30°C).

**Extraction of polyphenolic compounds**

The TPC of *A. uniflorum* seeds of different colors (brown, yellow, green and orange) using the three solvents as shown in Figure 4. The results indicated that the highest amount of total phenolic compounds (7.98 and 8.68 mg EAG $g^{-1}$ MS) is obtained for the acetone extracts by comparison with the other extracts (ethanol or mixture of methanol: acetone) (Figure 5). In the different seed colors, the polyphenol contents obtained from the ethanoic extracts vary between 6.92 and 7.42 mg EAG $g^{-1}$ MS. Nevertheless, these values remain low (6.79 and 7.49 mg EAG $g^{-1}$ MS) from the methanol: acetone extracts and those in all the different colors of seeds. It thus appears that the yellow color of seeds is the richest in polyphenols.

**Total flavonoid content**

Analysis of the results in Figure 4 shows that the green seeds have the highest content of flavonoids compared to the other colors tested. The lowest content was registered in the orange seeds. This figure shows the importance of the ethanoic extract fraction registered in the seed yellow and seed orange shows the extracts similar values in the order of 2.5 mg EC $g^{-1}$ MS, while the seed brown color reaches slightly higher values (3 mg EC $g^{-1}$ MS). In the case of acetone extracts, the flavonoid contents are similar for orange, yellow and brown seeds (3 and 4 mg EC $g^{-1}$ DM). Furthermore, the contents of the methanoic extracts are all less than 2 mg EC $g^{-1}$ DM. As a result, acetone followed by ethanol are the best solvents for extracting flavonoids compared to methanol.

**Diphenyl radical scavenging activity assay (DPPH)**

The antioxidant activities of different colors of seeds of *A. uniflorum* extracts were assessed and confirmed using one functional analytical method based on the anti-radical activity DPPH (Figure 6). The results illustrated in this figure show that the fraction of the acetone extract exhibits the higher activity than other extracts with $EC_{50}$ values varying from 0.24 to 1.84 mg/ml (Figure 7). It should be noted that the acetone extracts from the yellow seeds have the lowest $EC_{50}$ concentration (0.24 mg / ml). The solvent effect allowing to order the extracts as follows: acetone> ethanol> methanol.

**DISCUSSION**

These studies investigated that all the colors of seed coat of *A. uniflorum* were able to germinate over in range of temperatures varied from 15 to 30°C. Similar germination

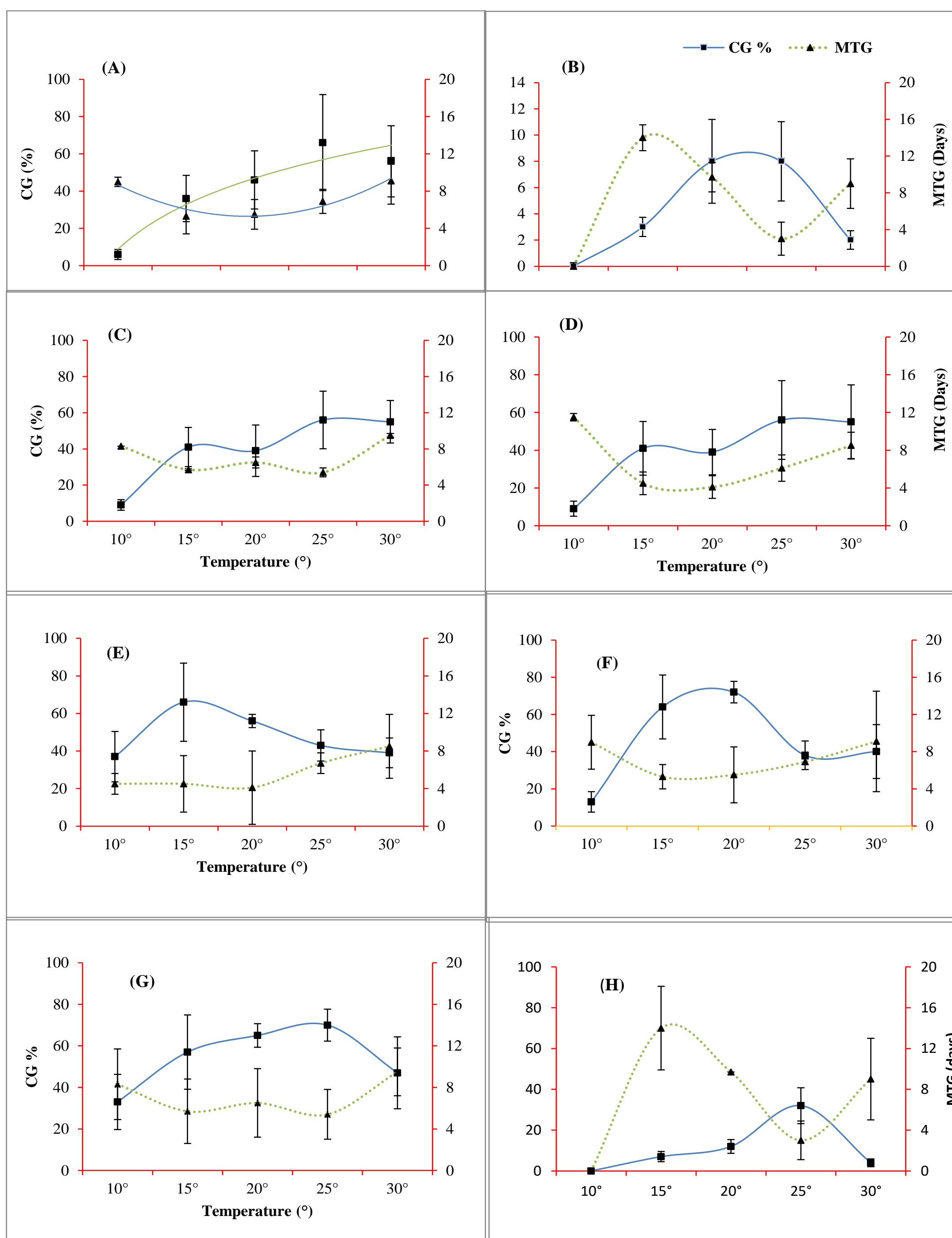


**Figure 4.** Variation of the cumulative germination percentage (CG) of four colors (A) yellow, (B) green, (C) brown, (D) orange) of withness and ((E) yellow, (F) green, (G) brown, (H) orange) of treated seeds of *A. uniflorum* and the speed of germination (MTG, days) according to the different temperatures (10, 15, 20, 25 and 30°C).

capability was observed in different southern Tunisia species (*Phragmites australis*, *Diplotaxis harra*, *Lotus creticus* and *Ziziphus lotus*) (Tlig et al, 2008; Gorai et al., 2009; Rejili et al., 2010; Zammouri and Neffati, 2021). The optimum germination in brown and green colored seeds are recorded at the temperature 25°C, it was 30°C

**Table 1.** Analysis of variance (two way-ANOVA) of the effects of temperature on germination percentage and delay of germination of different colors (green, yellow, brown and orange) of seeds (control and treated) of *A. uniflorum*.

| Variable | df | MS | P level |
|---|---|---|---|
| GP | 3 | 801,440 | 0.000*** |
| DG | 3 | 3,492 | 0.784$^{NS}$ |

GP: Germination percentage; DG: Delay of germination. *** P<0.001; NS: Not significant.

**Table 2.** Analysis of variance (two way-ANOVA) of the effect of treatment (control or treated) on germination percentage and delay of germination of different colors of seeds (green, yellow, brown and orange) of *A. uniflorum*.

| Variable | df | MS | P level |
|---|---|---|---|
| GP | 1 | 327,756 | 0.003*** |
| DG | 1 | 65,025 | 0.009*** |

GP: Germination percentage; DG: Delay of germination. *** P<0.001

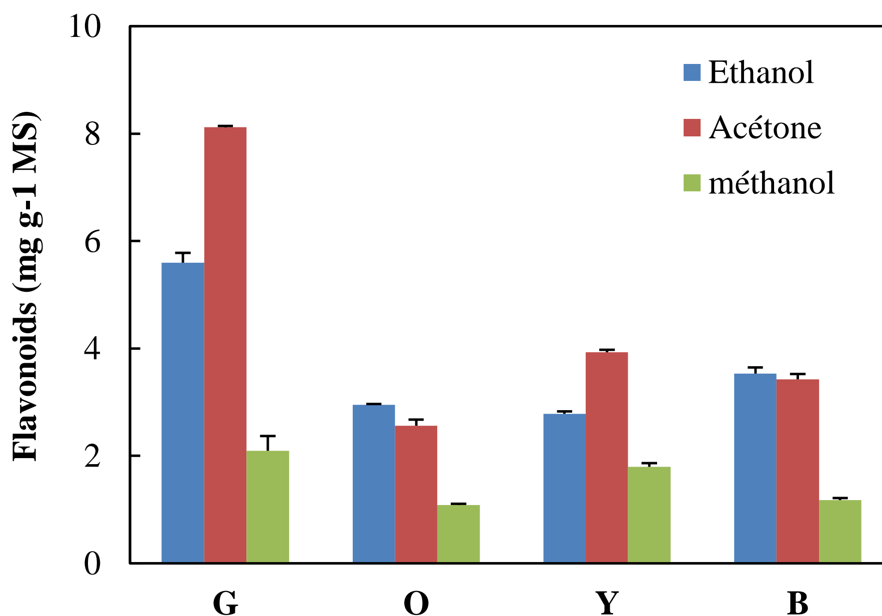


**Figure 5.** Total flavonoid extracts in the different colors of seeds of *A. uniflorum*.

for the yellow seeds and we recorded a thermal optimum 20°C for the orange colored seeds (Figure 4). Further, the study demonstrated that the recorded germination higher capacity did not exceed 70% for all the colors of the seeds treated. Similarly, variable germination capacities were recorded between the treated seeds and the control. Nevertheless, this germination percentage did not exceed 56% for the control.

It is noted that the treated seeds of brown color have a significantly higher germination percentage compared to the other seeds (yellow, orange and green). Nevertheless, the allowed value of germination in control seeds (not

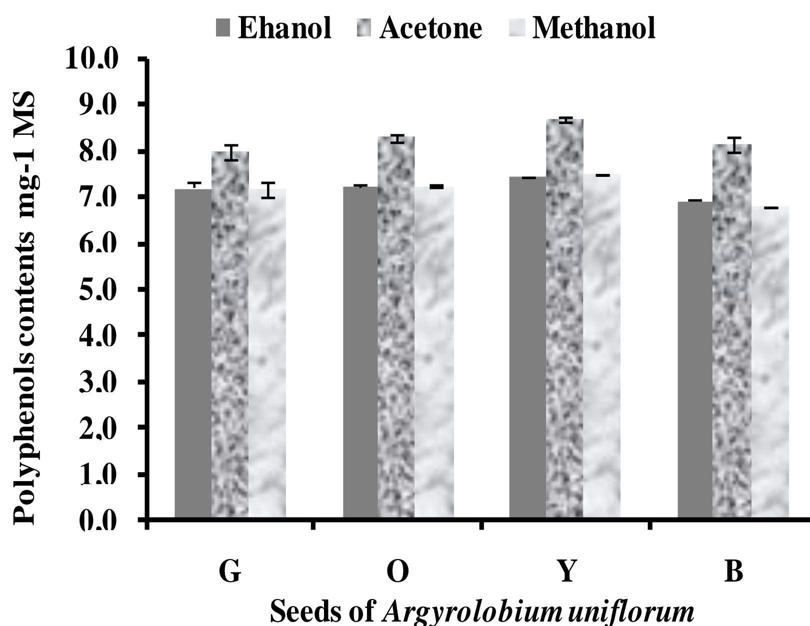


**Figure 6.** Total polyphenolic content (TPC) of different colors of seeds of *A. uniflorum* expressed as mg catechol equivalent/100 g dry materiel. For extraction, three different solvents were used.

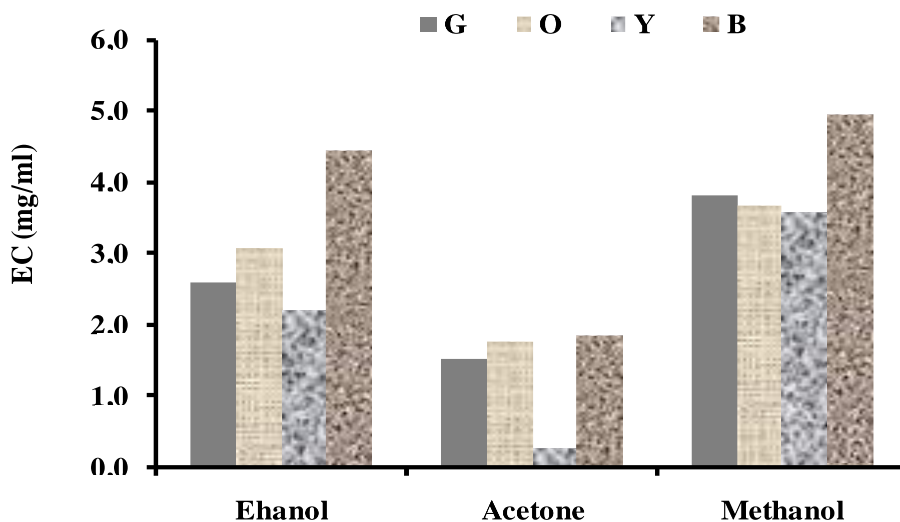


**Figure 7.** Antioxidant activity of the different colors of seeds of *A. uniflorum*: G (green seeds), O (orange seeds), Y (yellow seeds) and B (brown seeds).

treated) is considered a typical behavior of Mediterranean plants, with optimal germination occurring at temperatures ranging between 15 and 20°C (Baskin and Baskin, 1998). In contrast, low germination percentage of seeds associated with physiological dormancy was observed in *A. uniflorum* as well as in different species Fabaceae (Gama-Arachchige et al., 2013), this dormancy has been described by Gómez-Maqueo et al. (2021) in many species of this family. The results obtained demonstrate the capital role of sulfuric acid in removing the inhibition of the seed coat. In fact, immersing the seeds for 1 h in 70% sulfuric acid makes it possible to

obtain the highest germination rate and a reduction in the average germination time.

Under controlled conditions (laboratory) germination of the different colors of seeds of *A. uniflorum* are higher at temperatures ranging from 15 to 30°C and that the low germination capacities are recorded at 10°C. This result shows the importance of dormancy for the survival of this species, which maintained high viability even after the storage period (5 years). Moreover, these seeds are orthodox, which persist for long periods in *ex situ* seed banks, it can be quiescent or dormant, and they have very scarce metabolic activity that is activated to germinate in the appropriate environmental conditions, mainly determined by the availability of the water and by the temperature (Gómez-Maqueo et al., 2021).

Previous studies on the activity antioxidant properties obtained from *A. uniflorum* revealed that it displays antioxidant activity. These results are in accordance with the findings of other authors concerning phenolic contents and antioxidant activity in valuable species in arid Tunisian regions such *Lotus creticus* L. (Mahmoudi et al., 2020), *Periploca angustifolia* Labill. (Abdellaoui et al., 2013), *Polygonum equisitiforme* (Mahmoudi et al., 2019), and *Allium roseum* L. (Najjaa et al., 2011).

The data analysis reveals that the yellow seeds of *A. uniflorum* presented a high rate of polyphenols which have been correlated with their high percentage of germinated seeds (38 to 72%) and a phenolic content ($R_2$=0.868). Many researchers showed the role of phenolic compounds in maintaining the viability of seeds. The results are similar to those reported by Abdellaoui et al. (2013) on *Periploca laevigata* L. seeds stored from one to 15 years showing that germination capacity was strongly and positively correlated with amounts of total phenolic compounds. Bailly (2004) has suggested that the dormancy can be alleviated with oxidants, which can oxidize the phenolic compounds present in the seed envelopes, and may allow improved oxygenation of the embryo during seed imbibition. It can also cause cracking in the coat of hard seeds, thus facilitating their imbibition (Chien and Lin, 1994).

Reports on flavonoids possessing various biological activities such as anti-inflammatory, wound healing, antiulcer, hepatoprotective, anticancer, neuroprotective, antibacterial, antidiabetic and antithrombotic are available in the literature. Oxidative stress and antioxidant defense mechanism may also be a contributing as they are linked with inflammatory conditions (Pietta, 2000; Nayeem et al., 2022).

In this study, the reduction activity of the DPPH free radicals in the ethanoic extract of *A. uniflorum* seeds, estimated by the concentration of inhibition ($IC_{50}$) varies between 2.5 and 4 µg/ml. These ranges of variation were higher than those reported to *A. gombiformis* seeds (76.41 ± 3.72 µg/ml) and (from 4.71 mg QE/g DR to 72.79 mg QE/g DR) in seeds of *Capparis spinosa* (Lekmine et al., 2020; Tlili et al., 2015). Moreover, the DPPH scavenging activity of *A. uniflorum* seeds showed a small change with the color of coats of seeds.

Flavonoids are among the most important phenolic compounds that may exert several biological activities including antioxidant, antiviral, anti-allergic, anti-inflammatory, antimicrobial, antitumor, hepatoprotective and vasculoprotective (Bruneton, 1999; Seyoum et al., 2006). These properties were largely attributed to the antioxidant potential of flavonoids, ensuring free-radical scavenging activity and protection against oxidative stress (Xu et al., 2007). The remarkably high level of flavonoids ensures *A. uniflorum* seeds as a considerable therapeutic value promoting their use in nutritional and pharmaceutical industries.

Therefore, antioxidant compounds and enzymes have been widely considered as being of particular importance for the completion of germination. The antioxidant compounds α-tocopherol (Simontacchi et al., 1993, 2003; Yang et al., 2001), flavonoids and phenolics (Simontacchi et al., 1993; Andarwulan et al., 1999; Yang et al., 2001) increase during germination. In this study, the DPPH free radicals in the acetone extract of *A. uniflorum* seeds, estimated by the concentration of inhibition ($EC_{50}$) varies between varying from 0.24 to 1.84 mg/ml. These ranges of variation were higher than those reported to *Calicotome. villosa* (16.5 and 22.0 µg/ml). These ranges of variation were higher than those reported to *Retama raetam* (25.16 µg/ml) and *Prosopis farcta* (455 µg/ml) (Boughalleb et al., 2019; Tlili et al., 2015). Moreover, the DPPH scavenging activity of *A. uniflorum* seeds showed a small change with the colors of coats of seeds. The results showed that *A. uniflorum* seeds, due to their phenolic content and high antioxidant activity, could be a prospective source of natural bioactive molecules that could replace synthetic antioxidants, but at the same time, they may point to a source of easily accessible natural antioxidants that could be used as a forage with an important palatability factor influencing yield quantity and quality.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Screening of tuberculosis in highly exposed children in the population of Cameroon central region

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**Although the treatment for tuberculosis (TB) is available nowadays, it remains a real concern, especially since cases in children are often detected late and underreported. Studies have shown that elevated serum levels of C-reactive protein (CRP) are associated with TB in adults. This study aims to assess the CRP levels among the pediatric cases at Jamot Hospital (Yaoundé) and Meguemessi community in Cameroon. This was a cross-sectional study that enrolled 97 participants aged 2 to 15 years between 2020 and 2023. Sputum was tested for active tuberculosis using Gene-x-pert and culture. Blood was collected to measure the CRP concentration levels using nephelometry and to screen for latent tuberculosis infection using Interferon Gamma Released Assay (IGRA). Amongst the 97 participants there were 7 confirmed cases of active TB, 27 cases of latent TB infection, and 44 healthy individuals (HI). The mean CRP levels for active TB, latent tuberculosis infected (LTBI), and HI were 110.81 ± 74.96, 7.76 ± 15.33 and 9.27 ± 15.57 mg/L, respectively. High levels of CRP were associated with active tuberculosis ($p < 0.001$). Serum CRP levels can provide valuable insight into the severity of the disease, making it a useful marker for early diagnosis, preventive therapy orientation and treatment of TB.**

**Key words:** Active tuberculosis, latent tuberculosis infection, acute phase marker, C-reactive protein, contact children, central region, Cameroon.

## INTRODUCTION

Tuberculosis continues to pose a significant public health threat worldwide, with an estimated 10.6 million new cases reported in 2021 (WHO, 2022). The burden of pediatric tuberculosis is substantial, with the World Health Organization (WHO) estimating that approximately 1.2 million children under the age of 15 fell ill with tuberculosis globally in 2021, resulting in approximately 200,000 deaths (WHO, 2022). Children aged 0-4 years, and those aged 5-14 years living with HIV, who live in the same household with an index tuberculosis patient are at an increased risk of progression to active tuberculosis disease, once infected (Fox et al., 2013; Du Preez et al., 2022). Furthermore, these children are more susceptible to severe forms of the disease, such as tuberculosis meningitis, and have a higher risk of mortality than other age groups (Du Preez et al., 2022; Marais et al., 2004). Household contact management (HCM) targets both pathways to improve tuberculosis control. It involves systematic tuberculosis screening for household contacts of patients newly diagnosed with tuberculosis (index patients) to identify and treat additional co-prevalent disease. Additionally, it provides tuberculosis-preventive treatment (TPT) to decrease tuberculosis risk in those without disease. Although only a minority of tuberculosis transmissions are believed to occur within households (Martinez et al., 2019), household contacts remain at a high risk of tuberculosis infection and disease in many settings (Velen et al., 2021). The WHO recommends systematic screening for tuberculosis in household contacts of individuals diagnosed with tuberculosis and tuberculosis preventive treatment initiation if eligible, with priority given to children under the age of 5 years and 5-14 years living with HIV (WHO, 2022). However, tuberculosis child contact management has not been routinely or effectively implemented in resource-limited settings due to multiple obstacles including healthcare system-related barriers such as infrastructure and human resources (Hwang et al., 2011; Tesfaye et al., 2020). Families also face several challenges in bringing children to the healthcare facility, including, among other things, the burden of travel, financial challenges, and transport costs (Szkwarko et al., 2020; Ayakaka et al., 2017). Active contact investigation at the community and household level, a key element of the family-centered care concept, is considered a critical intervention for enhancing both case finding and provision of TPT among children and adolescents (Yuen et al., 2022). It is difficult to collect sputum samples from infants and young children. In addition, the diagnostic tests used to detect mycobacteria in sputum are less likely to produce a positive result, which is explained by the fact that children are more likely to have tuberculosis caused by a smaller number of bacteria. Therefore, C-reactive protein is a promising acute-phase marker for the screening of active tuberculosis in an endemic setting.

CRP is a very sensitive marker for the acute phase response but cannot be used as specific diagnostic tool because of its non-specificity. Nevertheless, measurement of CRP in a patient's serum can provide useful information to clinician, as it is used as a marker of inflammation (Kashyap et al., 2020). In children with tuberculosis, serial estimation of CRP levels can provide a clue to the response of antitubercular therapy. Studies have shown that CRP levels rise with the onset of infection and fall significantly with clinical improvement, returning to normal levels when the inflammatory reaction subsides. It appears to be a suitable indicator of disease activity and if its level does not fall within 3-6 months of therapy, the patient should be reassessed to rule out progressive tuberculosis or treatment failure. Further evaluation of CRP by semi-quantitative, latex agglutination technique is quite rapid, giving result in 15 to 20 min and the test can be done in small laboratory and even in rural areas lacking newer technology. The objective of this study was to assess serum levels of CRP among the highly exposed pediatric population to tuberculosis infected individuals in the Centre region in Cameroon.

## MATERIALS AND METHODS

### Study design and participants

A prospective study was conducted among children aged 2 to 15 years old at Jamot reference hospital (Yaounde) and Meguemessi rural community (Akonolinga Health District) in the Centre Region, Cameroon between December 2020 and August 2023. Children aged two to fifteen years old were eligible for enrolment. Participants were grouped into three categories:

(i) The group of latent tuberculosis infected (LTBI) children, confirmed positive for whole blood Interferon Gamma Release Assay (IGRA), the Quanti FERON TB-Gold in Tube (GFT-GIT) (QIAGEN, Strasse 1, 40724 Hilden, Germany) and negative for TB symptoms. They were identified by liaising a pulmonary TB patient

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(household contact) at tuberculosis treatment unit from Jamot reference hospital (urban area);
(ii) The second category was healthy individual (HI) randomly recruited in the Meguemessi rural community (Akonolinga Health District), negative for IGRAs;
(iii) The third category were children with confirmed active tuberculosis (ATB) recruited from the Jamot hospital before the beginning of treatment or within one week of anti-tuberculosis drug initiation. This third category were children with at least two of the following clinical manifestations (a) a cough for at least two weeks; (b) unexplained weight loss or failure to thrive; (c) unexplained diminished playfulness for at least 1 week; (d) an abnormal chest-X Ray (CXR). All ATB children were found positive by GeneXpert MTB/RIF and culture. All participating children underwent a CRP testing.

#### Clinical procedures

Demographic and clinical questionnaires were administered and standard TB evaluation including a physical exam was performed, as well as CXR and microbiology testing (children presenting clinical characteristic of TB). Expectoration specimen was collected for molecular testing with Xpert MTB/RIF Ultra test (Cepheid), acid fast bacilli (AFB) smear microscopy and AFB culture. In addition, 5 mL of blood was collected in the heparin tube for later diagnosis of latent tuberculosis infection, excluding children with identified TB signs. Volumes of 2 to 3 mL of blood were collected from each of the participants. After centrifugation, serum was separated and stored at -60°C until further use to CRP levels determination. The test was performed using commercially available high sensitivity C-reactive protein assay (Genrui Biotech, Shenzhen, China). The decision regarding initiation of preventive therapy or anti-TB treatment was made by TB clinic staff in accordance with Cameroon National Guideline (PNLT, 2019).

#### TB testing procedure

All tests were performed at the Centre Pasteur of Cameroon laboratory in accordance with standard protocols [Reference]. GeneXpert, microscopy, culture was performed on solid media: Löwenstein Jensen (LJ) and Löwenstein Jensen containing 0.4% of sodium pyruvate solution (Narvaiz de Kantor et al., 1998; Niobe-Eyangoh et al., 2003; Assam et al., 2013; Koro et al., 2016).

#### LTBI testing

LTBI was performed by Interferon Gamma Released Assay (IGRA) according to the manufacturer instruction, QuantiFERON TB Gold in Tube (QIAGEN, Strasse 1, 40724 Hilden, Germany) and positivity was defined as an interferon gamma concentration (IFN-$\gamma$) equal to or higher to 0.35 IU/mL.

#### C-reactive protein (CRP) testing procedure

The concentration of CRP was measured by nephelometry with PA54 protein analyzer and CRP Detection Kit Reagent (Genrui Biotech Inc, Shenzhen, China). The test's detection range was 3 to 300 mg/L.

#### Ethical considerations

Written informed parental consent was obtained for all children enrolled in the study as well as assent for participants 12 years and older. The study was approved by the Human Health Research Council Ethics Committee of Centre Regional Cameroon (EC N°1903/CRERSHC/2020), the Jamot Hospital authority and the Akonolinga Health District authority.

#### Statistical analysis

The statistical analysis was conducted using R software version 4.1.3 (R Core Team, 2021). Demographic and clinical characteristics were summarized using descriptive statistical analysis. The differences in CRP markers were analysed among active TB, LTBI, and healthy individuals (HI), and 95% confidence intervals were calculated. Non parametric tests as the Kruskal-Wallis test was performed to compare the three groups (Guo et al., 2013), and the Mann-Whitney U test (Guo et al., 2013) was used for comparisons between two groups. Additionally, a receiver operator characteristic (ROC) curve analysis (Xavier et al., 2011) was performed to verify the clinical utility of the results and to determine the cut-off value, specificity, and sensitivity of the assays (Shi et al., 2022; Kang et al., 2022). The statistical significance was considered for p-values less than 0.05. All data obtained and reported here were treated anonymously by the investigators.

## RESULTS

### Demographic and clinical characteristics of the study participants

A total of 97 children were enrolled into the study. Of this study participants, 44 (45.4%) were healthy control; 7 (7.2%) were confirmed TB patients, among which, 1 case was of extra pulmonary TB; 46 (47.4%) were children contact of pulmonary TB cases in household. From 46 children contact to index case, 27 (58.2%) had latent TB. Age group 2 to 5 years old (40.2%) were highly represented as well, and the female gender was most represented (54.6%). The detailed characteristics of the study participants are shown in Table 1.

### Quantitative CRP analysis results for the active TB, LTBI and healthy individuals

The mean CRP values for the active TB, LTBI, and healthy individuals (HI) were 110.81 ± 74.96, 7.76 ± 15.33, and 9.27 ± 15.57 mg/L, respectively (Table 2). Individuals with active tuberculosis (ATB) exhibited significantly higher CRP levels compared to both HI and those with LTBI (Chi square = 20.434, $p < 0.001$, df = 2). ATB had significantly higher CRP serum level than LTBI ($p < 0.001$). A similar result was obtained when ATB was compared to HI ($p < 0.001$). Notably, CRP levels in LTBI individuals were not significantly different from those in

**Table 1.** Clinical characteristics of the study subjects.

| Variable | Totals (%) | Total ATB cases (%) | Total TB contact cases (%) | Total healthy individuals (%) |
|---|---|---|---|---|
| **Age group (years)** | | | | |
| [2-5] | 39 (40.2) | 1 (14.3) | 20 (43.5) | 18 (40.9) |
| [6-10] | 32 (33) | 2 (28.6) | 14 (30.4) | 16 (36.4) |
| [11-15] | 26 (26.8) | 4 (57.1) | 12 (26.1) | 10 (22.7) |
| **Gender** | | | | |
| Female | 53 (54.6) | 5 (71.4) | 25 (54.3) | 23 (52.3) |
| Male | 44 (45.4) | 2 (28.6) | 21 (45.7) | 21 (47.7) |
| **AFB microscopy** | | | | |
| Negative | 1 (16.7) | 1 (16.7) | NA | NA |
| Positive | 5 (83.3) | 5 (83.3) | NA | NA |
| **AFB culture** | | | | |
| Negative | 0 (0) | 0 (0) | NA | NA |
| Positive | 6 (100) | 6 (100) | NA | NA |
| **MTB_PCR** | | | | |
| Négative | 0 (0) | 0 (0) | NA | NA |
| Positive | 6 (100) | 6 (100) | NA | NA |
| **IGRA_Test** | | | | |
| Negative | 62 (68.9) | NA | 19 (41.3) | 43 (97.7) |
| Positive | 28 (31.1) | NA | 27 (58.7) | 1 (2.3) |

NA: Not applicable (not done). MTB: Mycobacterium tuberculosis, IGRA: Interferon Gamma Release Assay, AFB: Acid Fast Bacilli, PCR: Polymerase Chain Reaction.

the healthy group (p = 0.4035) (Figure 1 and Table 2). These findings suggest a strong association between active tuberculosis and high systemic inflammation, as indicated by CRP, while latent infection appears to have minimal impact on CRP levels.

## An analysis of the receiver operator characteristic (ROC) curve based on the results of CRP

The ROC curve analysis was performed to assess the clinical usefulness of the CRP results. The Area under the receiver operating characteristic curve (AUC) for CRP was found to be 94.7% (95% CI: 87.5 - 100) (Figure 2 and Table 3). The best cut-off value of 3.291 mg/L was obtained. The optimum cut-off point was calculated where sum of sensitivity and specificity was maximum (sensitivity 100%; specificity 80.0%). Thus, AUC of serum CRP levels was found to significantly discriminate between ATB and healthy children (p < 0.001). At a threshold of 0.5 mg/L, CRP has lower sensitivity (71.4%) but higher specificity (100%). This means that the test may not detect all cases of TB, resulting in false negatives, but it will accurately identify all healthy individuals, avoiding false positives.

To investigate the relevance of the CRP, the area under the receiver operating characteristic curve (AUC), the sensitivity and the specificity will be analysed (Table 3).

## DISCUSSION

A biomarker-based triage test that can be performed in primary care clinics to assess the outcome of household TB+-contact is needed for children. This test should be rapid and efficient. Although CRP has shown promise for TB triage among adults living with HIV and correlates with other host gene expression signatures for TB diagnosis (Yoon et al., 2017; Södersten et al., 2021), we

**Table 2.** Acute-phase protein levels in serum samples between ATB (active tuberculosis), LTBI (latent tuberculosis infected), and HI (healthy individual).

| N (number of participants) C-reactive protein (mg/L) | ATB 7 | LTBI 27 | HI 43 |
|---|---|---|---|
| Median | 152.42 | 3 | 3 |
| Mean | 110.81 | 7.76 | 9.27 |
| SD | *74.96* | *15.33* | *15.45* |

**SD**: Standard deviation (n=77).

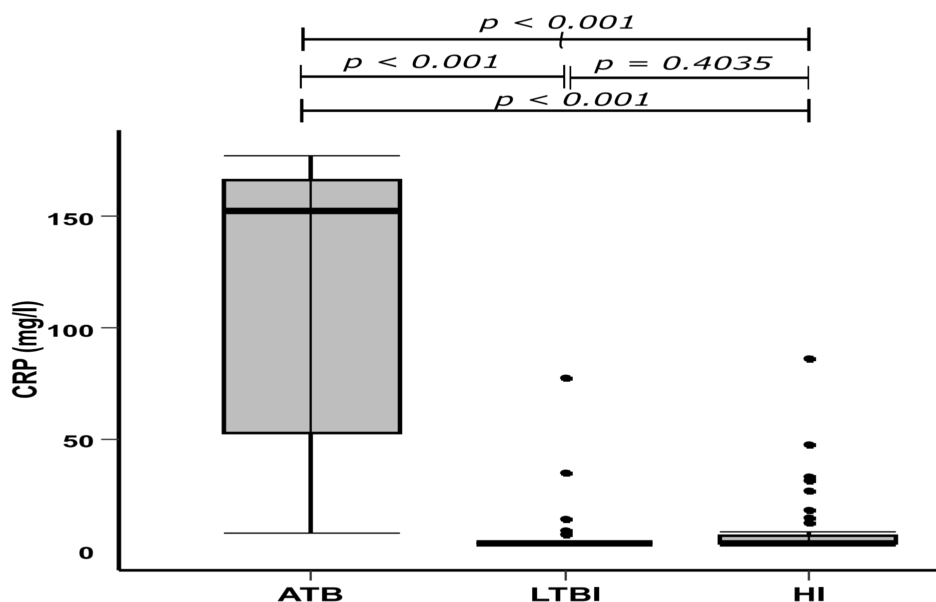


**Figure 1.** Comparison of C reactive proteins (CRP) between the different groups of patients studied: active tuberculosis (ATB), latent tuberculosis infected (LTBI) children, and healthy individual (HI).

found that CRP levels had limited performance in discriminating between TB disease and LTBI in children. Our findings indicate that CRP has a limited role in triaging latent TB Infection in children at the point of care. In 2012, WHO first recommended Household Contact Management for children under 5 years old and people living with HIV (WHO, 2012). This recommendation was later expanded to include all child contacts under 15 years old (WHO, 2018). However, global coverage of TPT in children younger than 5 years remains low. As of the end of 2022, only 55% of the target of 4 million set for the 2018 to 2022 period by the UN General Assembly high-level meeting on tuberculosis had been reached according to WHO (2023).

This study included mostly female children, with 40.2% under the age of 5. Of the total, 47.4% were children who had been in contact with pulmonary TB cases in their household, 45.4% were healthy controls, and 7.2% were confirmed TB patients. Among the 46 children who had been in contact with the index case, 58.2% had latent TB. It is crucial to manage TB effectively to differentiate between LTBI and active TB and to identify the appropriate anti-TB treatment. Currently, either the TST or IGRA test is used to diagnose latent tuberculosis infection (LTBI) (Sharma et al., 2017). It is important to note that a single test can lead to erroneous diagnoses of both LTBI and active TB (Mamishi et al., 2019).

CRP values for individuals with active TB, LTBI, and without TB (healthy individuals (HI)) were 110.81 ± 74.96, 7.76 ± 15.33, and 9.27 ± 15.57 mg/L, respectively. CRP levels were notably high in children with confirmed TB. This high CRP level is a profile of post-primary TB (De Beer et al., 1984). Generally, in primary paediatric TB, the CRP level is very low (Zitrin, 1960; Albuquerque et

**Table 3.** The diagnostic utility of CRP marker for tuberculosis (ATB).

| Parameter | AUC% (95% CI) | Cut-off value | Sensitivity % (95% CI) | Specificity % (95% CI) | *P*-value |
|---|---|---|---|---|---|
| **CRP** | 94.7 (987.5-100) | 3.2 | 100 (100-100) | 80 (70-88.7) | <0.001 |

CI: Confidence interval.

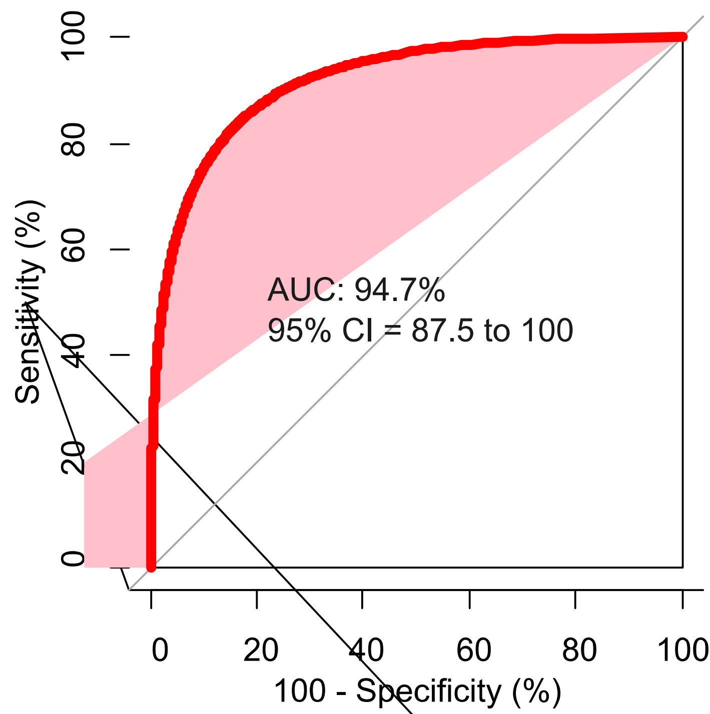


**Figure 2.** ROC curve analysis for serum levels of CRP among the ATB participants and healthy individuals.

al., 2019; De Beer et al., 1984). The studies that have shown CRP elevation in paediatric TB have mainly involved older children who are more likely to have post-primary TB (Herlina et al., 2011; Kashyap et al., 2020). This study suggests that higher CRP levels in children with TB may be related to post-primary disease rather than primary TB, as is the case in younger children. Similar observation were made in a study conducted in South Africa which compared CRP levels in children with primary TB, adolescents and adults with no lung destruction, and adults with post-primary TB with lung destruction (De Beer et al., 1984). During primary tuberculosis, *M. tuberculosis* spreads through the lymphatic system and is less likely to cause extensive lung damage and cavitation (Starke and Donald, 2016), resulting in a less robust acute phase response. It is important to note that the immune response to TB differs between children and adults (Basu et al., 2019), which may affect CRP levels.

The ROC curve analysis was performed to assess the clinical usefulness of the CRP results. The area under the receiver operating characteristic curve (AUC) for CRP was found to be 94.7% (95% CI: 87.5 - 100). The data showed a significant increase in CRP values ($p < 0.0001$, AUC = 94.7%) in the active TB group compared to the LTBI group and healthy individuals. Although numerous studies have been conducted, a gold standard that can differentiate between active TB, LTBI and healthy individuals groups does not exist. This study thus aimed to compare the CRP levels in serum samples to differentiate between active TB, LTBI and healthy individual groups and ultimately identify as biomarkers that can be used to differentiate LTBI from active TB.

However, this study presents some limitations. Firstly, the sample size was small, due to missed appointments caused by poor network coverage and the unavailability

of children's caregivers. To validate the significance of the results reported in this study and to improve the accuracy of discriminating between the active tuberculosis and latent tuberculosis-infection groups, a study with a larger number of contact cases and a larger population of children under 15 years of age is necessary.

## Conclusion

In Cameroon a significant number of pediatric TB cases and LTBI remain undiagnosed due to the lack of a gold standard. Tuberculosis is often diagnosed based on clinical and radiological criteria along with medical history. Although CRP is a non-specific marker of inflammation, it can significantly differentiate between pediatric TB cases and healthy controls. The use of this tool may be incorporated into the algorithms for diagnosing pediatric tuberculosis, along with clinical history, microbiology, radiology, and tuberculin skin tests. It has the potential to be an important tool in ruling out tuberculosis in children.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Genetic diversity of *Fusarium* isolates infecting potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) crops in Zimbabwe

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**Potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) are important crops that contribute significantly to Zimbabwe's economic development. However, they are attacked by *Fusarium* species leading to reduced yields, quality and profitability for farmers. A study was conducted to investigate the diversity of *Fusarium* spp. causing wilt and rots in both crops in Zimbabwe. Plants displaying symptoms of wilting, root and tuber rots were collected during nationwide disease surveys. *Fusarium* spp. were isolated and characterized morphologically, using pathogenicity tests and DNA sequencing targeting the internal transcribed spacer (ITS) region. Morphological characterization revealed the presence of *Fusarium* spp. that produced brown, grey, white and pink colonies on PDA. They also produced conidia of different shapes. All the *Fusarium* isolates induced some rotting, yellowing, and wilting in potatoes. Seven *Fusarium* spp., namely *Fusarium falciforme, Fusarium foetens, Fusarium fujikuroi, Fusarium perseae, Fusarium longifundum, Fusarium nygamai*, and *Fusarium chlamydosporum,* were identified through DNA sequencing. Furthermore, *F. falciforme* was most (41.67%) prevalent on tobacco, while *F. foetens* (25%) and *F. fujikuroi* (12.5%) were detected in both crops. This study highlighted the wide diversity of *Fusarium* spp. infecting potatoes and tobacco in Zimbabwe thus providing a base for developing management strategies against the pathogens. The 24 partial ITS sequences deposited in the GenBank database will be used in future studies to understand the diversity of *Fusarium* spp.**

**Key words:** *Fusarium*, Internal-Transcribed-Spacer Region, mycotoxins, potato, tobacco, Zimbabwe.

## INTRODUCTION

The family Solanaceae contains economically important crops like tobacco (*Nicotiana tabacum* L.) and potato (*Solanum tuberosum* L.) that are widely grown in Zimbabwe. In the 2021-2022 farming season, Zimbabwean farmers produced 211 million kilograms of flue-cured tobacco worth US$650 million and over 475

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000 metric tonnes of potatoes worth over US$390 million. However, several abiotic and biotic factors can significantly reduce the yield and quality of both crops. Amongst the biotic factors are ascomycetous fungi belonging to the genus *Fusarium* (Aoki et al., 2014).

*Fusarium* species' infection of potatoes and tobacco can result in root and tuber rots and plant wilting. In tobacco, the pathogen induces foliage yellowing that precedes wilting, while sudden wilting can occur without any foliage yellowing (Ntui et al., 2011). If *Fusarium*-infected potato tubers are planted, they can rot, leading to reduced crop stand in the field. Tuber rots can also occur in storage, giving rise to potato dry rot, a major postharvest disease worldwide (Stefanczyk et al., 2016). Some *Fusarium* spp. produce mycotoxins such as beauvericin, moniliformin, fumonisins, trichothecenes and zearalenone that can contaminate potatoes, making them unsuitable for human and livestock consumption (Nicholson et al., 2004; Wu, 2007; Mohd Zainudin et al., 2008; Stepien, 2013).

The genus *Fusarium* includes over 400 phylogenetically distinct species nested within 23 species complexes. Six species complexes, namely *Fusarium fujikuroi* species complex (FFSC), *Fusarium incarnatum-equiseti* species complex (FIESC), *Fusarium oxysporum* species complex (FOSC), *Fusarium sambucinum* species complex (FSAMSC), *Fusarium solani* species complex (FSSC), and *Fusarium tricinctum* species complex (FTSC), are of particular interest to plant pathologists as they account for approximately 75% of the known genealogically exclusive phylospecies (O'Donnell et al., 2022). Some *Fusarium* spp. are highly pathogenic on crops while others are weak saprophytes (Peters et al., 2008).

The range and identity of *Fusarium* spp. that infect crops vary depending on geographical location and time of year (Dongzhen et al., 2020; Azil et al., 2021), with some highly pathogenic on crops and others acting as weak saprophytes (Peters et al., 2008). *F. oxysporum* f. sp. *nicotianae* is commonly reported in many tobacco-growing countries. In Michigan, USA, *F. oxysporum* was identified as the primary agent responsible for potato dry rot among 11 species (Gachango et al., 2012). The number of *Fusarium* spp. associated with potato dry rot is increasing globally, with at least 17 species reported by Dongzhen et al. (2020). This upsurge in reported species may stem from recent improvements in disease diagnostics, allowing for more precise identification and characterization of *Fusarium* spp. infecting crops worldwide.

In recent times, the incidence of *Fusarium*-induced disease cases has increased in Zimbabwe. According to records at the Kutsaga Research Station Plant Clinic, 45% of potato and tobacco plant samples brought in for diagnostics in the 2021-2022 farming season were infected with *Fusarium* spp. However, morphological characterization that is used for diagnoses at the clinic is time-consuming and limited. Therefore, robust DNA-based studies are required for precise and speedy pathogen identification and characterization.

Molecular assays are a powerful tool for inter- and intra-species identification of fungal species (Pinaria et al., 2015; Chehri, 2016). Anonymous markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and single sequence repeats (SSR) (Baysal et al., 2010) and sequence-specific markers like the internal transcribed spacer (ITS), intergenic spacer (IGS), transposons and translation elongation factor-1α (TEF-1α), are widely employed in fungal diagnostics. Spacer regions, such as ITS and IGS, are targeted to identify genetic variability of various *Fusarium* spp., and polymorphism in these regions is more stable and reliable than in anonymous markers (Gashgari and Gherbawy, 2013).

This study aimed at investigating the diversity of *Fusarium* spp. associated with wilts and rots in potato and tobacco crops in Zimbabwe, using morphological and pathogenicity assays alongside DNA sequencing targeting the ITS region. Accurate pathogen characterization is critical in developing and implementing effective disease management strategies for improved crop yield and quality.

## MATERIALS AND METHODS

### Pathogen isolation

*Fusarium* spp. were isolated from tobacco and potato plant samples that were collected during disease surveys and submissions made by growers to the Kutsaga Research Station Plant Clinic in the 2021-2022 farming season. Symptomatic plant materials consisting of stems, leaves, roots and tubers were surface sterilized with 1% sodium hypochlorite for 2 min, blot-dried on filter paper and plated on chloramphenicol-amended potato dextrose agar (PDA) media. After incubation at 24 ± 2°C for 5 days, mycelial growth was observed, and pure *Fusarium* cultures obtained through sub-culturing. The cultures were stored on agar slants in a refrigerator until use. A total of 24 *Fusarium* isolates were used in this study.

### Morphological identification

The colours and patterns of fungal colonies that developed on PDA after 5 days of incubation were observed and recorded. Slide cultures of *Fusarium* isolates were prepared, and the mycelia and spores were examined under the ZEISS Primo Star compound microscope [Manufacturer, City/Country] at 400X magnification.

### Pathogenicity tests

#### *In vitro pathogenicity test*

The experiment was conducted following the procedure described by Gashgari and Gherbawy (2013). The study was designed as a one-factor experiment in a completely randomized design with six replications. Uniform-sized healthy-looking potato tubers (cultivar Diamond) were selected, washed in water to remove excess soil, and surface-sterilized in 1% sodium hypochlorite. The tubers were

then air-dried, and wounded with a sterile 5 mm diameter cork borer to a depth of 5 mm. Then, a 5 mm diameter agar plug containing actively-growing *Fusarium* isolates' mycelium was placed into the wound, and sealed with the excised plug of tuber tissue. The control treatment consisted of potato tubers that were inoculated with plain PDA. The inoculated tubers were wrapped in black polyethylene plastic and incubated at 24°C for three weeks. Disease development was measured by cutting the tubers longitudinally from the point of inoculation and measuring the depth of internal necrosis at 7, 14, and 21 days after inoculation.

### *Preparation of Fusarium inoculum for in vivo pathogenicity tests*

To prepare conidial suspensions, conidia from *Fusarium* cultures grown on PDA for 21 days at 18°C were scrapped into sterile distilled water. Mycelia and spores were retrieved by aseptically scraping the PDA surface and transferred into flasks containing 200 ml sterile distilled water. The inoculum was sieved to remove the mycelia and the conidia present in the supernatant were adjusted to a concentration of $10^6$ conidia $ml^{-1}$ by dilution.

### *In vivo pathogenicity test*

Individual *Fusarium* isolates were evaluated for their pathogenicity on potato plants that were grown in 15 cm diameter plastic pots in a greenhouse. The experiment was designed as a one-factor experiment using a completely randomized design with five replications. Three weeks after emergence, plants were inoculated by pouring 5 ml of a fungal inoculum suspension ($10^6$ conidia $ml^{-1}$) into the soil at the base of the potato plants in the pots. The control treatment was inoculated with distilled water. Disease progress was assessed at two-week intervals for six weeks by counting and recording the number of yellowing leaves against the total number of leaves per plant. Additionally, for root disease progress assessment, plants were uprooted, and the tubers and roots were scored for discolorations, using the scale where: 0 = No visible disease symptom; 1 = Less than 15% of roots affected; 2 = 15-35% of roots affected; 3 = 36-49% of roots affected; 4 = 50-74% of roots affected; and 5 = more than 75% of roots affected as well as plants which no longer had any roots at all.

### Molecular characterization of *Fusarium* wilt isolates

### *Fungal DNA extraction*

DNA was extracted from the *Fusarium* isolates using the modified Cetyltrimethyl ammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987). Firstly, a 3% CTAB solution was heated in a microwave for at least 2 min to dissolve it. Then, the solution was added into a 2 mL Eppendorf tube and vortexed rigorously. Thereafter, the contents were incubated in a shaking incubator for 1 h at 125 rpm and 65°C. Afterward, the tubes were centrifuged for 10 min at 12,500 rpm for 10 min to sediment the debris. The supernatant was collected and an equal volume of chloroform was added, followed by another centrifugation step. The aqueous phase was collected and, to it an equal volume of chloroform was added, followed by another centrifugation step at 12,500 rpm for 10 min. Next, 1% CTAB was added to the upper aqueous phase and incubated at ambient temperature for 1 h. The tubes were again centrifuged for 10 min at 12,500 rpm and the supernatant was discarded. The now visible pellet was treated with 1 M CsCl and isopropanol and incubated. The solution was centrifuged, and supernatant discarded. The pellet was then precipitated using isopropanol, washed in 70% ethanol, and finally dissolved in ultra-pure water. DNA quality and quantity were assessed for using a spectrophotometer (BioDrop®, UK).

### *ITS region amplification and sequencing of Fusarium isolates*

The genomic DNA samples were sent to Inqaba Laboratories (Pty) Ltd. (Pretoria, South Africa) for amplification of the ITS-rDNA region using primers ITS1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) (White et al., 1990). The amplification reaction was performed using 2 µl of DNA template, 5 µl of 10X Standard Taq Reaction Buffer, 3 µl forward primer, 3 µl reverse primer, 1 µl of 10 mM dNTPs, and 4 µl of Taq DNA Polymerase and 32 µl of nuclease-free water, in a total reaction volume of 50 µl. The cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles denaturation at 94°C for 30 s, annealing at 54°C for 1 min extension at 72°C for 2 min and a final extension step at 72°C for 7 min. A negative control was included in the PCR amplifications. The amplified fragments were analyzed by 1.5% agarose gel electrophoresis, and the amplicons were excised and purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). The purified amplicons were then sequenced in both directions on the ABI Big Dye Terminator Sequencing Kit (v3.1) and read with an ABI13730xl capillary sequencer (Applied Biosystems, Foster City, CA, USA).

### Data analysis

The lesion diameter depths of inoculated potatoes were analyzed using one-way analysis of variance (ANOVA) using Genstat. When significant differences were detected, means were separated using the least significant difference (LSD) at 5% probability level.

For molecular data, consensus sequences were generated from the forward and reverse sequences of the *Fusarium* isolates using the BioEdit software. The consensus sequence for each isolate was blasted into the MEGA 6.0 programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences of the *Fusarium* isolates reported in this study have been deposited into the GenBank under the accession numbers shown in Table 2.

The Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969) was used to infer the evolutionary history of isolates generated in this study. Partial ITS sequences of *Fusarium* spp. reported from other parts of the world were obtained from GenBank and used for comparison using MEGA 6.0. The data from a ClustalW alignment of the ITS were used as input for the phylogenetic tree construction using MEGA 6.0 software (Tamura et al., 2013). The bootstrap consensus tree inferred 1000 replicates were taken to represent the evolutionary history of the analyzed taxa. *Colletotrichum sojae* (NR 158358.1) was used as the outgroup.

## RESULTS

### Morphological characteristics of the *Fusarium* isolates

The *Fusarium* isolates produced different colony colours when cultured on PDA as shown in Figure 1a to f. Some isolates produced white mycelia with concentric rings. Others produced white, creamy white, pink, and gray colourations. The isolates had typically septate hyphal strands and produced canoe-shaped, rice-shaped, and

**Table 1.** Pathogenicity of *Fusarium* isolates, as measured by first and second assessments' means of the lesion depths (mm) of the inoculated potato tubers. The lesion depths were measured at 7, 14 and 21 days after inoculation.

| Isolate | Lesion depth (mm) (mean ± SD) | | |
|---|---|---|---|
| | 1st assessment | 2nd assessment | 3rd assessment |
| Control | 16.33 ± 3.37 | 17.53 ± 2.27 | 8.07[a] ± 1.60 |
| *Fusarium perseae* | 13.67 ± 4.10 | 14.17 ± 3.13 | 13.10[ab] ± 3.41 |
| *Fusarium foetens* | 14.27 ± 3.58 | 14.60 ± 0.12 | 14.80[bc] ± 4.51 |
| *Fusarium nygamai* | 12.97 ± 4.35 | 13.67 ± 3.42 | 14.95[bc] ± 1.62 |
| *Fusarium longifundum* | 12.60 ± 4.04 | 14.92 ± 2.17 | 16.80[bc] ± 3.36 |
| *Fusarium fujikuroi* | 14.25 ± 3.13 | 14.38 ± 2.18 | 12.50[ab] ± 2.05 |
| *Fusarium falciforme* | 18.40 ± 0.72 | 18.82 ± 0.10 | 19.60[c] ± 2.27 |
| *Fusarium chlamydosporum* | 11.68 ± 5.71 | 13.33 ± 4.52 | 8.47[a] ± 2.12 |
| P- value | 0.529 | 0.467 | 0.004 |
| S.E.D. | 2.90 | 2.61 | 2.49 |
| LSD | 6.00 | 6.38 | 5.17 |
| CV (%) | 24.80 | 20.08 | 22.00 |

*Lesion depths followed by the same letter in a column are not statistically different at p = 0.05.

bullet-shaped conidia (Figure 1g to i).

## Pathogenicity tests

### *In vitro tests*

Some rotting were observed in the potato tubers that were inoculated with PDA plugs containing mycelia of different *Fusarium* isolates (Figure 2). No significant differences ($p = 0.529$) were observed in the lesion depths of the potato tubers in the first and second assessments. Significant differences ($p = 0.004$) were observed in the third assessment with *Fusarium chlamydosporum* (8.47 ± 2.12 mm) having the smallest lesion depth while *F. falciforme* (19.6 ± 2.27 mm) had the biggest lesion depth (Table 1).

### *In vivo tests*

After inoculation with the fungal isolates, the potato plants were observed daily to follow disease progress. There were no significant differences ($p > 0.05$) in disease development in the potato plants that were inoculated with the *Fusarium* isolates, as measured by the amount of wilting and yellowing of leaves. Symptoms of wilting and leaf yellowing were first observed on the lower leaves of the plant at 12 days after inoculation. In some plants, the wilting and yellowing occurred on one side of the plant. As the disease progressed, there was leaf curling (Figure 3). By the fifth week (that is, three weeks after inoculation), most potato plants had wilted and died. The control plants showed no symptoms of wilting and/or yellowing.

## Molecular characterization of *Fusarium* isolates obtained from tobacco and potato plants in Zimbabwe

Blastn analysis revealed that ten isolates (41.67%) were most closely related to *F. falciforme* CBS 475.67 (NR_164424). Another 6 isolates (25%) shared at least 99.57% nucleotide sequence identity to *F. foetens* (NR_159865) from the Netherlands. Three isolates (FOX003_ITS1, FOX005_Nyanga and FOX016_Marondera) shared 100% nucleotide sequence identity to *F. fujikuroi* (NR_111889), a rice-infecting isolate. Isolates FOX009_Msasa and FOX012_Msasa were at least 99.3% related to *F. chlamydosporum* var. *chlamydosporum* from Honduras (NR_172283). Isolate FOX006_Nyanga was most closely related (99%) to *Fusarium perseae* from Italy while isolate FOX011_Kutsaga was most closely related (99.3%) to *Fusarium nygamai* (NR_130698) (Table 2).

## Phylogenetic analysis

Phylogenetically, the sequences of the *Fusarium* isolates were grouped into four distinct clusters, A-D. Cluster A was made of *F. fujikuroi* and *F. nygamai* isolates while Cluster D had F. *falciforme*, and *F. perseae*. *F. chlamydosporum* was in cluster B while *Fusarium foetens* was in cluster C (Figure 4).

## DISCUSSION

Pathogen characterization is necessary to help understand the biology of a pathogen prior to the

**Table 2.** Identity of the *Fusarium* isolates recovered infecting potato and tobacco in Zimbabwe.

| Isolate ID | Host plant | Accession number | Isolate identity |
|---|---|---|---|
| FOX001_ITS1 | Potato | OP445592 | *F. foetens* |
| FOX002_Burma | Tobacco | OP458568 | *F. foetens* |
| FOX003_ITS1 | Tobacco | OP473984 | *F. fujikuroi* |
| FOX004_Banket | Potato | OP474046 | *F. foetens* |
| FOX005_Nyanga | Potato | OP474047 | *F. fujikuroi* |
| FOX006_Nyanga | Potato | OP474048 | *F. perseae* |
| FOX007_Nyanga | Potato | OP474049 | *F. foetens* |
| FOX008_Kutsaga | Potato | OP474050 | *F. foetens* |
| FOX009_Msasa | Potato | OP474051 | *F. chlamydosporum* |
| FOX010_Mhangura | Potato | OP474052 | *F. foetens* |
| FOX011_Kutsaga | Tobacco | OP474054 | *F. nygamai* |
| FOX012_Msasa | Potato | OP474053 | *F. chlamydosporum* |
| FOX013_Burma 2 | Tobacco | OQ423103 | *F. falciforme* |
| FOX014_Headlands | Tobacco | OQ423104 | *F. falciforme* |
| FOX015_Rusape | Tobacco | OQ423105 | *F. falciforme* |
| FOX016_Marondera | Tobacco | OQ423106 | *F. fujikuroi* |
| FOX017_Trelawney | Tobacco | OQ423107 | *F. falciforme* |
| FOX018_Mhangura | Tobacco | OQ423108 | *F. falciforme* |
| FOX019_Chinhoi | Tobacco | OQ423109 | *F. longifundum* |
| FOX020_Mvurwi | Tobacco | OQ423110 | *F. falciforme* |
| FOX021_Shamva | Tobacco | OQ423111 | *F. falciforme* |
| FOX022_Mazowe | Tobacco | OQ423112 | *F. falciforme* |
| FOX023_Centenary | Tobacco | OQ423113 | *F. falciforme* |
| FOX024_Guruve | Tobacco | OQ423114 | *F. falciforme* |

implementation of any disease management strategies. This study was undertaken to characterize *Fusarium* spp. that are associated with wilts and rots of potatoes and tobacco in Zimbabwe. Seven *Fusarium* spp. were reported, with *F. falciforme* dominant in tobacco, while *F. foetens* and *F. fujikuroi* were detected in both potatoes and tobacco. To the best of our knowledge, this is the first time that these seven *Fusarium* spp. have been reported infecting tobacco and potato crops in Zimbabwe. Prior to this study, only *F. oxysporum* f. sp. *nicotianae* and *F. solani* had been reported infecting tobacco while *Fusarium culmorum, F. oxysporum, Fusarium vasinfectum*, and *F. solani* were reported infecting potato in Zimbabwe (Masuka et al., 1998). This study increased our knowledge of the range of fusaria that infect potatoes and tobacco in Zimbabwe. In addition, it provides protocols that can either be adapted or adopted for use in *Fusarium* characterization. In this study, the ITS region which has been identified as the standard marker for fungal identification (Iwen et al., 2002; Badotti et al., 2017), was utilized to identify the Zimbabwean *Fusarium* isolates.

*F. falciforme* has been reported infecting many crops in different parts of the world. It is an emerging pathogen that has been reported to cause wilt of chrysanthemum in Vietnman (Thao et al., 2021), root rot of chickpea, cucurbits, tobacco, tomato, and soybean (Vega-Gutierrez et al., 2019; Qiu et al., 2020, 2022; Velarde Felix et al., 2021; Li et al., 2022; Xu et al., 2022), and postharvest fruit rot in watermelon (Balasubramaniam et al., 2023). *F. falciforme* is a trans-kingdom fungal pathogen that has also been reported infecting humans (Edupuganti et al., 2011; Ma et al., 2013). Given that *F. falciforme* is a generalist pathogen, there is high likelihood that it could spread and infect other crops in Zimbabwe and potentially cause serious yield losses.

*F. foetens* was first reported infecting begonia in the Netherlands (Schroers et al., 2004), but has also been reported infecting tea (*Camellia sinsensis*) and rooibos (*Aspalathus linearis*) in South Africa (Lamprecht and Tewoldemedhin, 2017). *F fujikuroi,* a primary pathogen of cereals such as rice, barley, maize, millet and sugarcane (Hsuan et al., 2011), also infects soybean (Qiu et al., 2020). Both *F. foetens* and *F. fujikuroi* are mycotoxins producers (González-Jartín et al., 2019; Zakaria, 2023). The infection of potato and tobacco by these fungi poses potential health hazards to consumers.

Phylogenetic analysis revealed that the *Fusarium* spp. reported in this study belong to four species complexes: *F. fujikuroi* species complex (FFSC), *F. chlamydosporum* species complex (FCSC), *F. oxysporum* species complex (FOSC), and *Fusarium solani* species complex (FSSC).

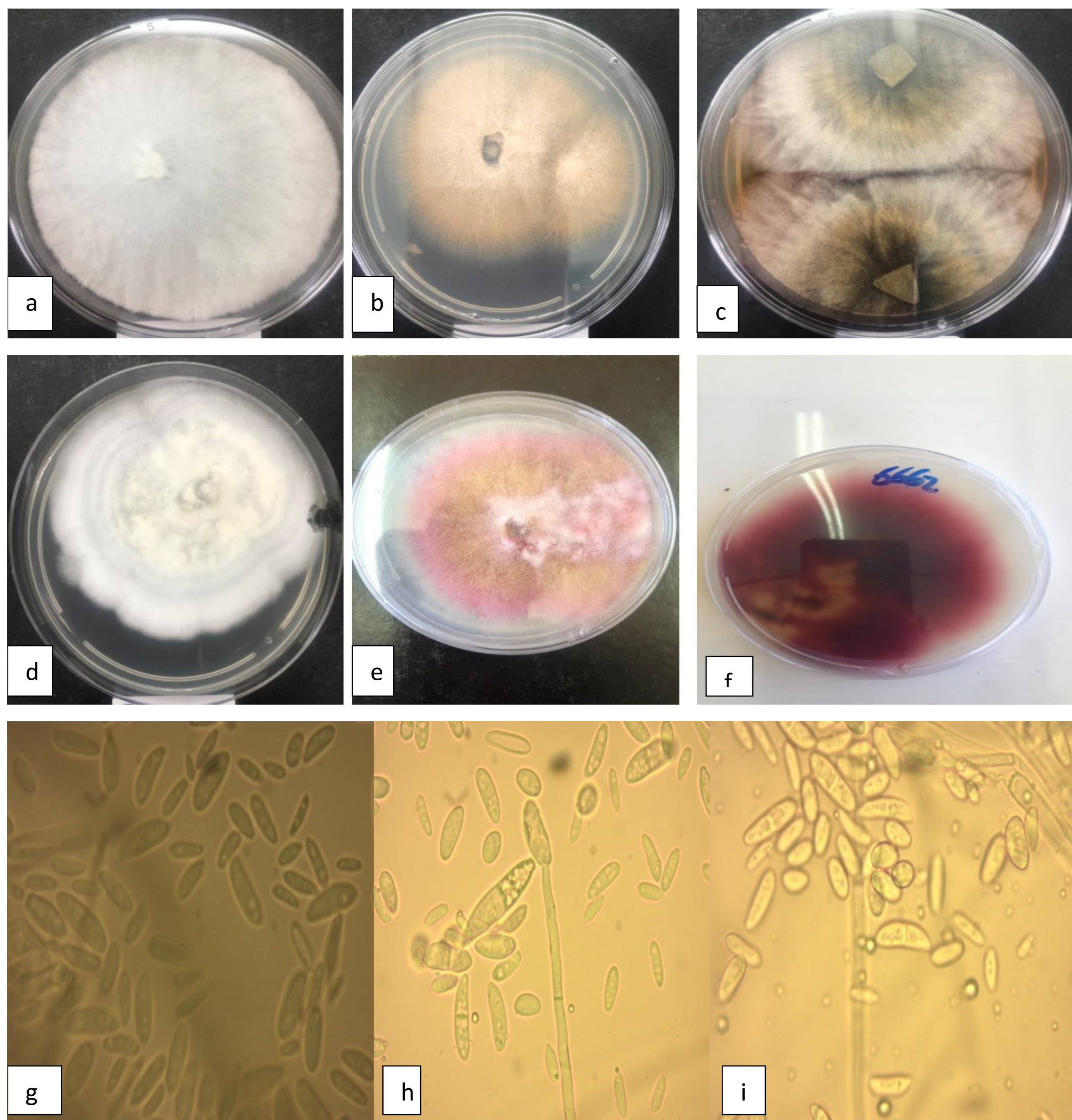


**Figure 1.** Morphological and cultural characteristics used in the characterization of 24 *Fusarium* isolates obtained from potato and tobacco plants. The isolates produced different coloured colonies (a-f) on PDA. There were also different growth patterns as exemplified by concentric rings (d). The *Fusarium* spp. also produced different macroconidia (g-i).

The FFSC, one of the biggest complexes with 65 accepted species (Yilmaz et al., 2021), was represented by *F. fujikuroi* and *F. nygamai.* The FCSC was represented by *F. chlamydosporum*. This species complex is one of the smallest till date, with 9 accepted species (Lombard et al., 2019). Both *F. foetens* and *Fusarium lognifundum* are members of the FOSC (Navale et al., 2023) while *F. falciforme* and *F. perseae* belong to the FSSC (Gleason et al., 2020).

The detection of these fusaria on potato and tobacco could be evidence of an expanding host range for the pathogens in the country. It could also be evidence of improved diagnostics as DNA sequencing was used for the first time ever in studying *Fusarium* wilt and rot pathogens of tobacco and potato in Zimbabwe. Molecular assays, though expensive, offer quick and unambiguous identification of pathogens (Luchi et al., 2020). This is critical if effective disease management strategies are to be developed and implemented.

Some of these newly-reported pathogens were probably introduced into Zimbabwe through plant material imports. In the past two decades, Zimbabwe has been

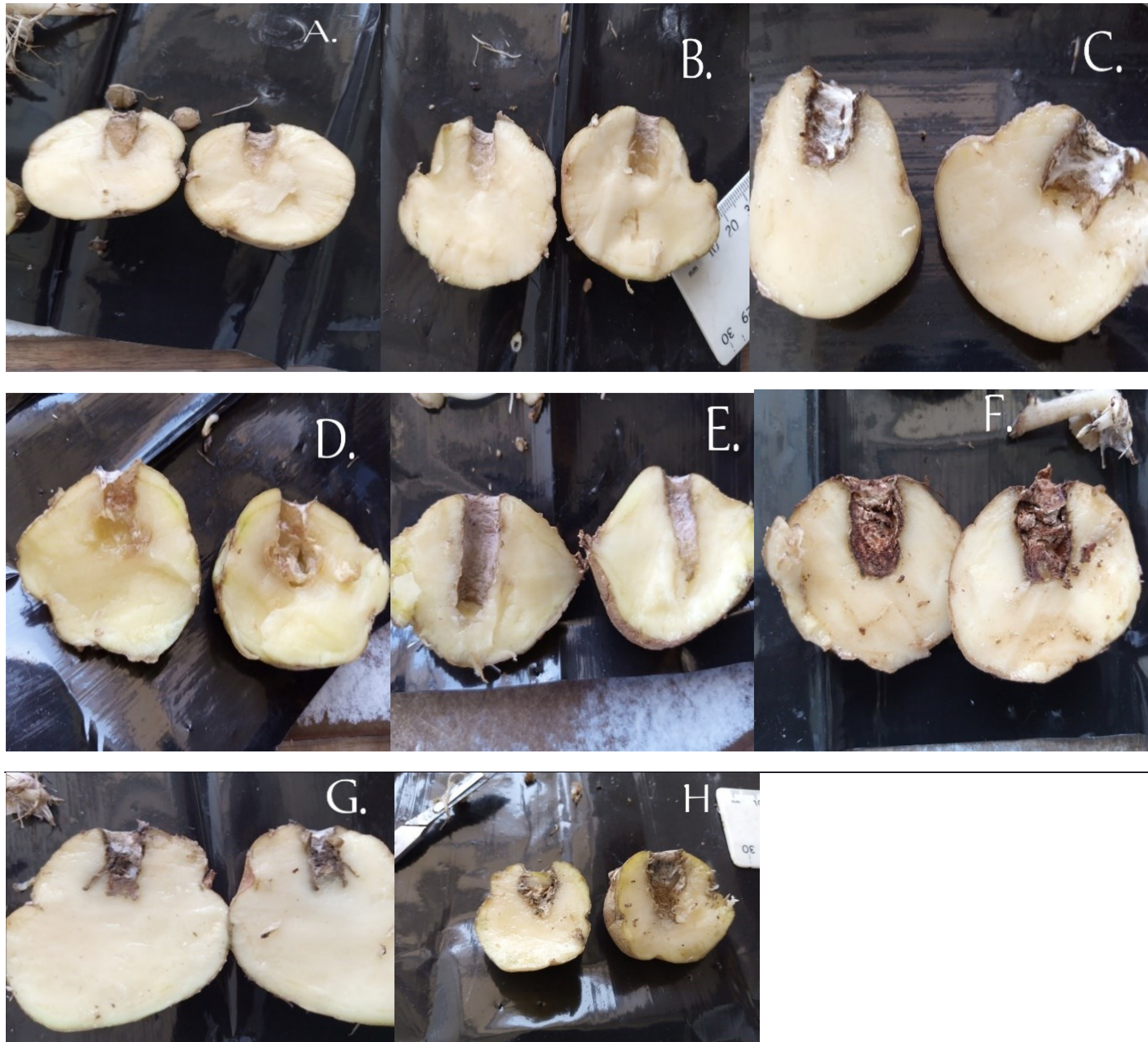


**Figure 2.** Response of potato tubers when inoculated with different *Fusarium* isolates. Potato tubers were inoculated with PDA plugs containing mycelium of A (control- sterile PDA plug), B (*F. nygamai*), C (*F. foetens*), D (*F. perseae*), E (*F. fujikuroi*), F (*F. chlamydosporum*), and G (*F. falciforme*).

importing potatoes from neigbouring countries, most notably South Africa, to meet local demand. A pathogen like *F. foetens* which has been reported on tea and rooibos in South Africa (Lamprecht and Tewoldenedhin, 2017) could have jumped hosts to infect potatoes which were then imported into Zimbabwe. Inadequate screening of imported plant materials at the country's ports of entry and smuggling of agricultural commodities could have aided pathogen introduction into Zimbabwe.

The pathogenicity tests confirmed the capacity of the isolates to cause disease in potatoes. However, the isolates did not show significant differences in pathogenicity in the *in vitro* tests. The severity of *Fusarium* diseases is conditioned by various factors, including the climate conditions, cultural practices and host plant physiology (Trabelsi et al., 2017). Different host plants represent different selective environments for fungal pathogens and could result in functional trade-offs that would limit the general fitness of the pathogen in the host (Sacristan and Garcia-Arenal, 2008).

This study showed that *Fusarium* has wide diversity in Zimbabwe. This corroborates findings by other researchers on the diversity of *Fusarium* in the world (Singha et al., 2016). Numerous partial ITS sequences are currently present in databases and are regularly used to identify different species of fungi. This study added 24

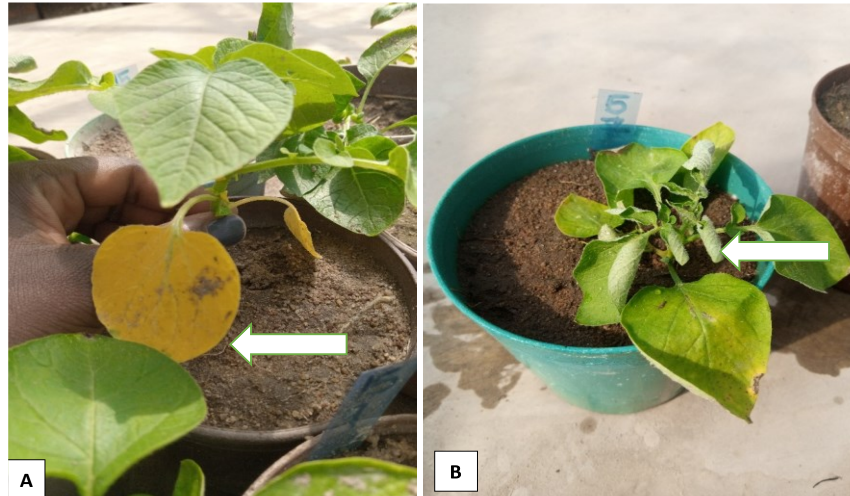


**Figure 3.** Potato plants showing symptoms of hemiplegic yellowing (A), leaf curling and wilting of the lower leaves (B). These were the common symptoms in all the plants that were inoculated with the *Fusarium* isolates in this study.

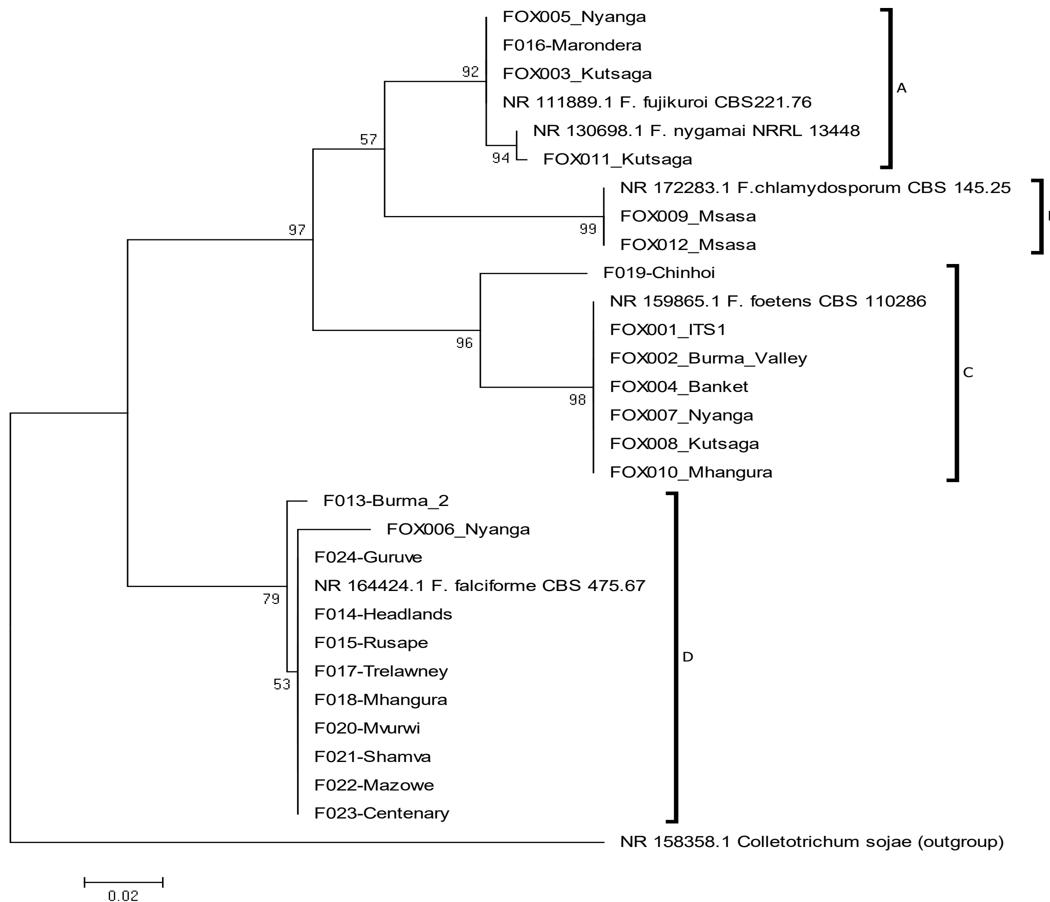


**Figure 4.** Phylogenetic analysis of *Fusarium* isolates obtained from Zimbabwe. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The numbers shown next to branches indicate the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. The analysis was performed with 30 sequences. Evolutionary analyses were conducted in MEGA 6.

partial ITS sequences to these databases for use in future studies to understand the diversity of *Fusarium* spp.

## Conclusion

This study identified *F. chlamydospoum, F. falciforme, F. foetens, F. fujikuroi, F. longifundum, F. nygamai*, and *F. persea* infecting tobacco and potato in Zimbabwe. *F. falciforme* was reported predominantly infecting tobacco while *F. foetens* and *F. fujikuroi* infected both crops. Knowing the range and diversity of these pathogens is an important step in developing effective disease management strategies against diseases infecting both tobacco and potatoes.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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