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African Journal of Biotechnology

*Full Length Research Paper*

# Establishment of an efficient regeneration protocol of six different varieties of wheat (*Triticum aestivum* L.)

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**An efficient callogenesis and regeneration protocol was optimized for six different wheat *(Triticum aestivum* L.) varieties including Chakwal-50, Galaxy-01, NARC-09, Pakistan-13, Millat-11, and Borlog-14. For callogenesis, mature seeds of each variety were used as explant, surface sterilization, and culturing were carried out on MS medium enhanced with dissimilar levels (0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D). For regeneration, six different treatments with different combinations of IAA (1.5, 3, and 4.5 mg/L) and Kinetin (1, 2, 3, and 4 mg/L) were applied on each variety. Highest frequency of callogenesis (41.66%) was attained on MS medium in addition with 2.5, 5.5 and 3.5 mg/L 2,4-D in Millet-11, followed by Chakwal-50 (33.33%) and Borlog-14 (29.16%), respectively. Maximum regeneration (41.66%) was observed in Millat-11 on medium enhanced with 1.5 mg/L IAA and 2 mg/L Kinetin. Among all, Millet-11 appeared most responsive genotype towards callogenesis and regeneration, while NARC-09 (16.66%) was least responsive. This study will open new avenues for biofortification of Mellet-11 wheat variety and improvement against biotic and abiotic stress.**

**Key words:** Wheat, tissue culture, callogenesis, regeneration, Millet-11, 2,4-D Kinetin.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is mostly growing cereal crop around the world. With the increment of biotic and abiotic stresses, annual outcome of wheat is less than its potential (Rashid et al., 2012). Wheat is providing energy

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and nutrients to more than two billion people and have essential role in food security as it facilitate with 20% of world calories (Bhatta et al., 2017). Conventional breeding faces two major limitations, that is, restricted gene pool and extended duration. Simultaneously, wheat genome size is ~17000 Mb, which is challenging to improve genetically. As compared to traditional breeding, robust techniques of biotechnology such as CRISPR/ Cas9 and other most advanced technology to modify DNA with addition or deletion of nucleotides are more precise and encompass all challenges (El-Sappah et al., 2021), additionally, inter- and intra-species transmission of stress associated traits is also possible with a shortest duration (Singh et al., 2021). To employ aforementioned techniques, a robust and highly efficient regeneration protocol is mandatory (Yu et al., 2008).

Dedifferentiation of cells into tissues and altering gene expression pattern within a cell are basic need for regeneration of plants in controlled environment (Bull and Michelmore, 2022). *In-vitro* regeneration of plants happen by three different pathways including repairing of tissues, clonal embryogenesis, and *de novo* organogenesis (Long et al., 2022). First pathway of tissue repairing involves the process of regeneration of tissues after roots, shoots and tips of leaf get wound or cut, and this mostly used in those techniques that utilized non-reproductive plant parts (Xu and Huang, 2014). In cell, tissue and organ cultivation techniques, plants are mostly regrown through non-reproductive cells as well as through *de novo* organogenesis (Hill and Schaller, 2013). *De novo* organogenesis can occur through two ways including direct and indirect *de novo* organogenesis. Direct *de novo* organogenesis takes place naturally while indirect *de novo* organogenesis takes place *in-vitro*. At the time of *de novo* indirect organogenesis, cells of explants pass through dedifferentiation and different plant growth regulators including auxin and cytokinin activate cell division (Sugimoto and Meyerowitz, 2013). Numerous explants of wheat namely seed, immature as well as mature embryo, endosperm, root tips and shoot apical meristem can be employed for *in-vitro* formation of callus and tissues regeneration (Kumar et al., 2017; Mahmood and Razzaq, 2017). Among all, young and immature embryos are best choice (Kumar et al., 2017). For example, immature zygotic embryos of barley (*Hordeum vulgare* L.) displayed significantly high callogenesis and regeneration (Abbas et al., 2023). In order to develop heat stress resistant maize verities (El-Sappah et al., 2022), immature embryos of maize (*Zea mays*) were employed to induce callogenesis and regeneration, which resulted in significant regeneration rate (Wei et al., 2022). Nonetheless, pre-mature embryos are not accessible consistently throughout the year. Contrastingly, mature seeds are available around the year, therefore, considered best choice (Rahman et al., 2008). In this study, we employed mature seeds in callogenesis and regeneration to investigate their potential.

Murashige and Skoog (MS) media containing specific concentration of hormones for accelerating growth such as 2,4-D, IAA, and Kinetin is promising media for plant callogenesis and regeneration. MS supplemented with 200 µL/L 1-naphthaleneacetic acid (NAA) and 500 µL/L IBA resulted in significant regeneration of *Populus trichocarpa* (Nisqually-1) (Abbas et al., 2020). Similarly, other researchers propagated *P. trichocarpa* on MS medium in order to perform GUS staining and identified expression of *PtrCslD5* gene in roots (Peng et al., 2019). Pre-mature embryos of barley (*H. vulgare* L.) grow very well at 2.5 mg/L concentration of 2,4-D on MS medium and displayed excessive callogenesis, while MS medium enhanced with 1 mg/L Kinetin came out in significant regeneration, and supplementation with 1 mg/L IAA resulted in maximum rooting (Abbas et al., 2023). Callus initiation medium enhanced with MS media and dicamba (2 mg/L) showed maximum efficiency of callus induction of 98.22 and 97.33%, respectively, and shoot development media with addition of zeatin at concentration of 5 mg/L 6-benzylaminopurine (BAP) concentration of 5 mg/L 2,4-D concentration of 0.25 mg/L NAA concentration of 0.25 mg/L, and copper sulfate ($CuSO_4$) concentration of 20 mg/L showed maximum shoot induction in wheat (Phogat et al., 2023). Here, we streamlined MS media enhanced with various concentrations of growth promoting hormones.

Various channels of tissue culture were laid out to upgrade callus formation efficiency in wheat which are extensively including genotypic, culture medium, explant dependent and over expression of callus regenerative gene (Roesler et al., 2018; Chaimae et al., 2021; Yu et al., 2023). For all cultivars, new plants, or species using more than one hormone, tissue culture and growth procedures should be investigated through careful design and testing experiments. Therefore, this study aims to find the most responsive genotypes and most important individuals of wheat having high callus formation and regrowth efficiency, and additionally accelerate the propagation of wheat genotypes that exhibit superior characteristics for genetic advancement based on limited resources and time. The point of our research was to decide reasonable protocol and the selection of most efficient wheat genotype for tissue culture.

## MATERIALS AND METHODS

### Experimental sites and explant selection

In order to establish tissue culture and regeneration in wheat, we selected common, approved and high yield wheat verities being extensively cultivated by farmers. We collected mature seeds of wheat in March 2023, packed in clean and microbe free plastic bags, labeled and stored at normal temperature for further use in experiment. Notably, no specific permission was required to collect samples for experimental purpose. Mature seeds of genotypically different six wheat varieties namely Chakwal-50, Galaxy-01, NARC-09, Pakistan-13, Millet-11 and Borlog-14 were employed for callogenesis and regeneration. Laboratory conditions were adjusted as follows: 25±2°C temperature, 60-80% humidity, and white light

**Table 1.** MS medium enhanced with dissimilar concentrations of IAA and Kinetin for regeneration, while 2,4-D for callogenesis.

| S/N | Treatment | IAA + Kinetin (mg/L) | 2,4 -D (mg/L) |
|---|---|---|---|
| 1 | H1 | 1.5 + 1 | 0.5 |
| 2 | H2 | 3 + 1 | 1.5 |
| 3 | H3 | 4.5 + 1 | 2.5 |
| 4 | H4 | 1.5 + 2 | 3.5 |
| 5 | H5 | 1.5 + 3 | 4.5 |
| 6 | H6 | 1.5 + 4 | 5.5 |

intensity was 7000 lux. Prior to kick-off experimental work, laminar airflow cabinet was sterilized with UV lamp for 20 min.

#### Surface sterilization and inoculation of seeds

Mature seeds of all six wheat varieties were picked out as an explant material and disinfected three times with 50% Clorox for 20 min with constant agitation in a laminar airflow hood. Washing of seeds were performed thrice for 10 min with $ddH_2O$ to remove Clorox and placed at sterilized filter paper to become dry. Disinfected seeds were then shifted on an autoclaved Whatmann filter papers to absorb excessive water. After that, disinfected seeds were cultured on MS media supplemented with regeneration and callus initiation media with the help of sanitized forceps. All experimental procedure was performed inside 6-inch vicinity of spirit lamp.

#### Regeneration and callogenesis

In order to initiate regeneration, surface sterilized dry seeds of wheat were cultured on MS media supplemented with different concentrations of IAA and Kinetin (Table 1). Each variety of wheat was tested at six different combinations of hormones in order to observe their regeneration potential, and response to various combinations of hormones, on the base of different genetic makeup. Each treatment was performed thrice, data was collected, and average was calculated to avoid any error. Inoculated seeds were incubated in growth room at temperature at 25 ± 1°C, 60-80% humidity, and 7000 lux intense white light.

Germination initiated after 4 days of culturing and day-by-day germination data was collected and saved on excel sheet. For the purpose of callus production, inoculation of surface sterilized seeds was performed on MS media enhanced with various levels of 2,4-D (Table 1), and cultures were placed in dark condition for prompt callus induction. Notably, after 2 weeks of incubation in dark condition significant callus formation was observed in almost all wheat verities. All reading were obtained in next 3 to 4 weeks, then separation of calli from seeds was performed with the help of sterilized forceps under sterilized conditions in order to identify best wheat variety with maximum potential of callogenesis.

#### Statistical analysis

Collected data were statistically analyzed by using slide writer software and ANOVA to check out the differences between treatments and within the treatments, in concern of percentage of callus formation and tissues regeneration. To get optimum result, 20 explants of every variety used for each treatment in three replications. The mean was processed from each treatment.

## RESULTS

### Study aim

The aim of this study was to foster a strategy for compelling crop improvement via genetic modification. For this purpose, an established and reproducible callus induction and regeneration protocol was optimized using mature seeds of six genetically different wheat genotypes. Although, fully mature seeds of wheat are available around the year, but we collected seeds of six different verities of wheat in March 2023, labelled name of each wheat variety, and stored in sterile bags in order to avoid exposure to microbes such as fungal spores. Notably, it is very hard to induce callus formation in mature seeds as compared to immature embryo, cotyledons or leaves, but mature seeds are available around the year. This is why we employed mature seeds for callogenesis and regeneration. This optimized callus induction and regeneration protocol will be further employed in development of biofortified wheat verities with biotic and abiotic stress resistance. For the purpose to check the potency of six wheat cultivars for callus development and regeneration, six unique combinations of hormones (IAA, Kinetin and 2,4-D) for six treatments were applied.

### Regeneration

Diverse concentrations and combinations of IAA (1.5, 3, and 4.5 mg/L) and Kinetin (1, 2, 3, and 4 mg/L) were considered to advance the regeneration combination (Table 1). To obtain high regeneration activity, various amount of Kinetin was mixed in MS media enhanced with multiple levels of IAA. The outcomes showed significant contrast among wheat varieties against growth regulators (IAA and Kinetin) of various levels.

Regeneration of tissues is genotypes dependent; therefore, behavior of different wheat genotypes was different at various concentrations of growth regulators.

**Figure 1.** The maximum regeneration obtained (41.66%) on regeneration medium having 1.5 mg/L IAA and 2 mg/L Kinetin by the var. Millet-11.

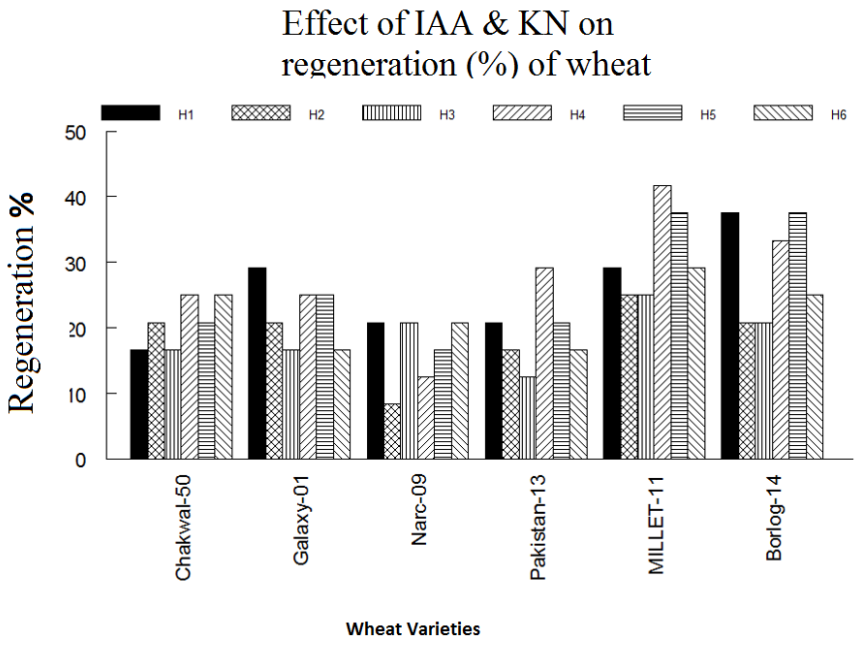


**Figure 2.** Effect of IAA & Kinetin (KN) on regeneration of wheat.

Millet-11 showed the highest regeneration potency on IAA concentration of 1.5 mg/L and on Kinetin concentration of 5 mg/L on tissue regeneration media (Figure 1). Behavior and regeneration ability of each genotype altered with alteration of IAA and Kinetin levels (Figure 2). However, maximum average regeneration among varieties at all combination of growth regulators was examined in Millet-11 (31.24%), next to 29.16% in Borlog-14, and 27.7% mean maximum regeneration was acquired among all varieties at combination of IAA and Kinetin 1.5 + 2 mg/L, respectively. Wheat cultivar NARC-09 was considered a poor genotype in terms of regeneration as it produced minimum regeneration percentage of 16.6% declared as least active genotype to

**Table 2.** Effect of IAA and KN on regeneration (%) of wheat.

| Variety | H1 | H2 | H3 | H4 | H5 | H6 | Mean |
|---|---|---|---|---|---|---|---|
| IAA | 1.5 mg/L | 3 mg/L | 4.5 mg/L | 1.5 mg/L | 1.5 mg/L | 1.5 mg/L | |
| Kn | 1 mg/L | 1 mg/L | 1 mg/L | 2 mg/L | 3 mg/L | 4 mg/L | |
| Chakwal-50 | 16.66 | 20.83 | 16.66 | 25 | 20.83 | 25 | 20.83 |
| Galaxy-01 | 29.16 | 20.83 | 16.66 | 25 | 25 | 16.66 | 22.22 |
| NARC-09 | 20.83 | 8.33 | 20.83 | 12.5 | 16.66 | 20.83 | 16.66 |
| Pakistan-13 | 20.83 | 16.66 | 12.5 | 29.16 | 20.83 | 16.66 | 19.44 |
| Millet-11 | 29.16 | 25 | 25 | 41.66 | 37.5 | 29.16 | 31.24 |
| Borlog-14 | 37.5 | 20.83 | 20.83 | 33.33 | 37.5 | 25 | 29.16 |
| Mean | 25.69 | 18.74 | 18.74 | 27.77 | 26.38 | 22.22 | |

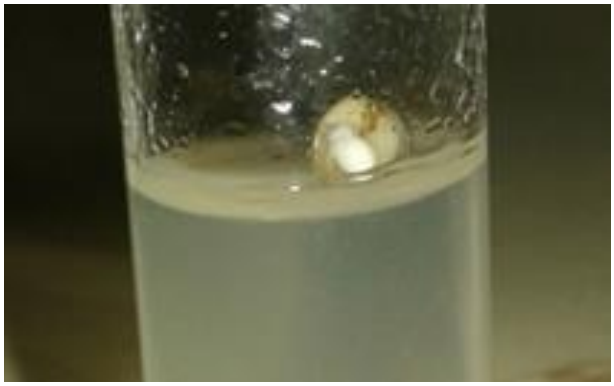

**Figure 3.** Initiation of callus started as a white spongy tissue.

among all wheat cultivars for the purpose of tissue culturing (Table 2). However, outcomes of this research presented Millet-11 cultivars as best wheat cultivars for use of tissue culture on the base of giving maximum percentage of regeneration and Borlog-14 was considered as second-best genotypes having potency of regeneration.

## Callus induction

Callus formation is the essential step in different processes involved in applied and analytical tissue culture. The callus formation was divided into two different calli. Calli that were dense in look, nodules like shape, creamy-whitish to light greenish in color and embryo-like structures were named as “embryogenic calli” (Figure 4a). Calli that showed color of dirty-whit and soft-watery texture were named as “non-embryogenic calli” (Figure 4b). For callus induction, hormones play important role and, in our research, we found that only 2,4-D was prime for callus development and it was elaborated that 2,4-D is the mostly utilized growth regulators for the purpose of callus formation and its maintenance (Aadel et al., 2016; Eshagi et al., 2021; Phogat et al., 2023).

For the purpose of callus development, various concentrations of hormone 2,4-D (0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mg/L) were evaluated. In order to find out excellent wheat genotype in terms of callus induction, six wheat genotypes were treated with six different levels of 2,4-D. Callus formation examined as a white spongy tissue on upper side of seed during the periods of 10 to 12 days and it was also genotype and medium dependent (Figure 3). Almost same types of results were disclosed by Mehmood et al. (2013). Culturing was carried out in 2,4-D hormones for up to three weeks and after that data was calculated.”

The data indicated that behavior of each variety was significantly different under unchangeable cultural environment and medium concentration because of their potency to develop callus. However, callus induction media ought to be well standardized to study the potency of a genotype to make maximum callus on the base of its genetic makeup. Results showed that in the light of different levels of 2,4-D, Millet-11 (41.66%) recovered maximum number of calli percentage at 2,4-D concentration of 2.5 mg/L next to 33.33% by variety Chakwal-50 and 29.16% by variety Borlog-14 (Figure 5).

Other than this, variety Chakwal-50 gave the best

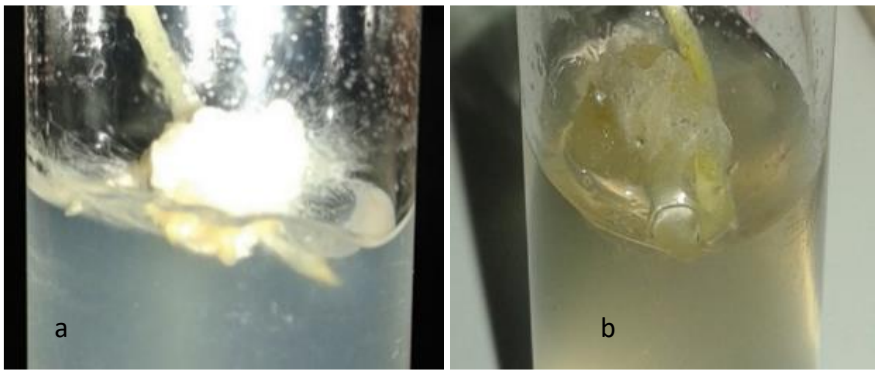


**Figure 4.** Different types of callus induction in wheat: (a) embryogenic callus; (b) non-embryogenic caluss.

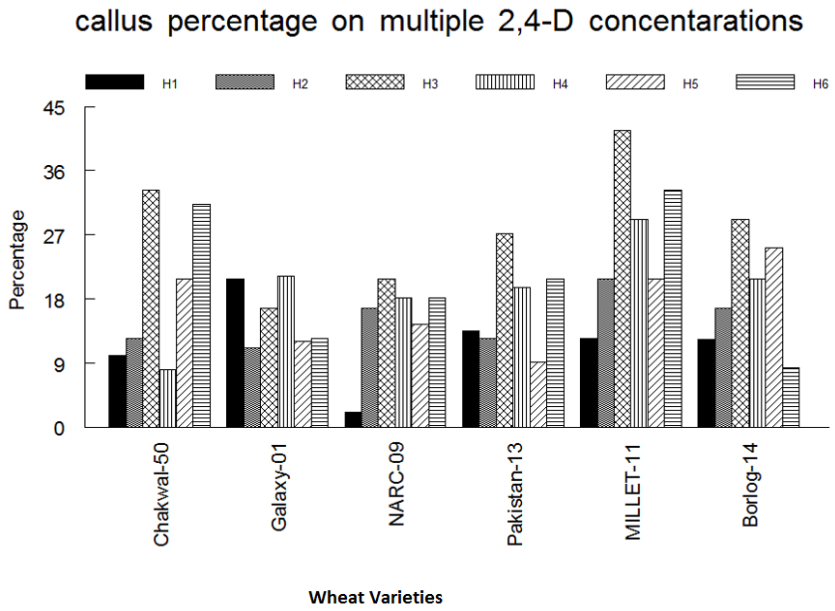


**Figure 5.** Callus formation percentage of various wheat varieties in response to multiple 2,4-D concentrations.

outcomes by producing maximum callus weight (0.26 g) at MS media holding 3.5 mg/L 2,4-D followed by Borlog-14 (0.16 g) and Millet-11 (0.14 g) (Figure 6). In general examination of multiple varieties at every giving level of 2,4-D media disclosed that Millet-11 (26.39%) is a genotype exhibited superior characteristics for callus development and 2.5 mg/L concentration of 2,4-D was viewed as best concentration for production of maximum callus (Table 3), and 3.5 mg/L 2,4-D was viewed best for wheat crop to produce highest weight of callus (Table 4).

## DISCUSSION

Tissue culture is one of the latest techniques that involves exposure of plant tissue to a specific type of nutrients, different kinds of hormones and artificial light under *in-vitro* circumstances to develop many plants, all of them are clones of explants, over a very small interval of time. Plant tissue culture is a crucial technique for making disease-free, stress resistant planting material and in short time production of uniform plants in developing countries. In botany, callus cells are that kinds of cells that heal plant wound after injury. Callus formation is stimulated from any type of explants materials after being sterilized and *in-vitro* planting on culture medium contains different hormones and growth regulators. Plants growth controlling hormones such as IAA, Kinetin, and 2,4-D are added in plating medium for the purpose to commence callus production or somatic embryogenesis.

In the present research, six wheat genotypes were utilized to observed *in-vitro* effect of different hormones

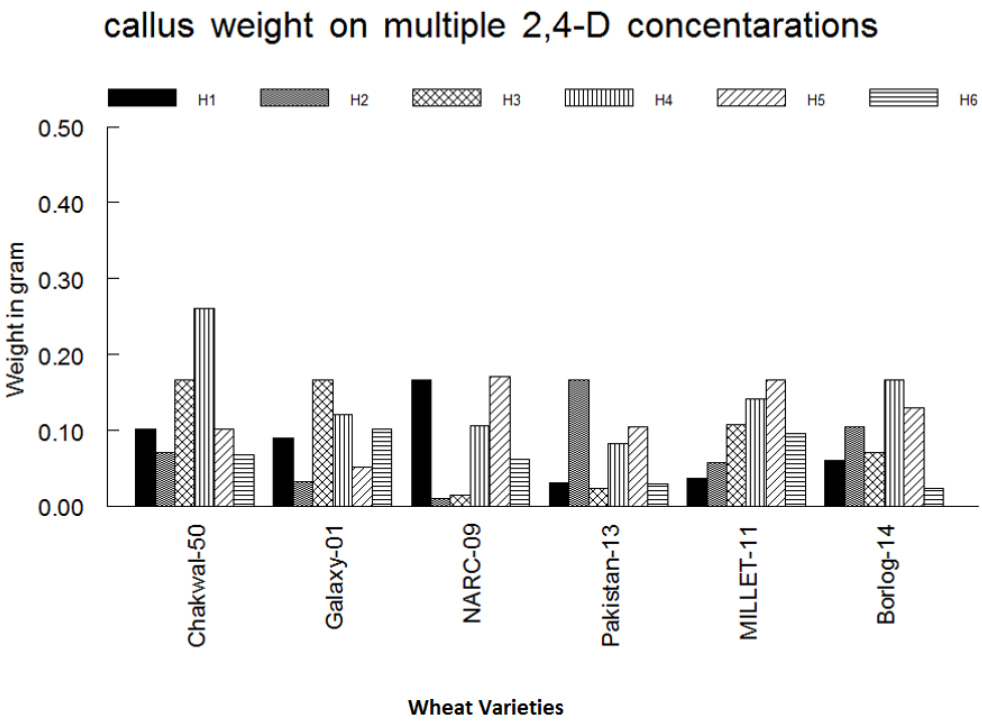


**Figure 6.** Callus weight of various wheat varieties in response to multiple 2,4-D concentrations

**Table 3.** Callus percentage (%) on multiple 2,4-D concentrations.

| Variety | H1 | H2 | H3 | H4 | H5 | H6 | Mean |
|---|---|---|---|---|---|---|---|
| 2, 4-D (mg/L) | 0.5 | 1.5 | 2.5 | 3.5 | 4.5 | 5.5 | (g) |
| CH-50 | 10.12 | 12.50 | 33.33 | 8.01 | 20.83 | 31.22 | 19.34 |
| GA-01 | 20.83 | 11.08 | 16.66 | 21.20 | 12.05 | 12.50 | 15.72 |
| NARC-09 | 2.08 | 16.66 | 20.83 | 18.16 | 14.50 | 18.16 | 15.07 |
| Pak-13 | 13.50 | 12.50 | 27.16 | 19.66 | 9.08 | 20.83 | 17.12 |
| Millet-11 | 12.50 | 20.83 | 41.66 | 29.17 | 20.83 | 33.33 | 26.39 |
| Borlog-14 | 12.38 | 16.66 | 29.16 | 20.83 | 25.16 | 8.33 | 18.75 |
| Mean (%) | 11.9 | 15.04 | 28.13 | 19.5 | 17.08 | 0.07 | |

by applying different growth regulators. The role of IAA alone for plant regeneration was not effective but combined effect of IAA and BAP together was vital in case of whole plant regeneration. These findings are consistent with Malik et al. (2004), they elaborated the combined effect of IAA and BAP on whole plant regeneration is necessary as compared to apply these hormones separately on wheat genotypes and found regeneration of plant step-up 84 and 52% in wheat cultivars Inqilab-91 and Pavon-76, respectively.

Pattern of callus development and tissue regeneration was varied in all studied genotypes by altering the IAA and Kinetin concentration. However, ability and efficiency of Millet-11 for callus development and plant regeneration was highest in comparison to other genotypes, maybe because of high expression of callus formation and plant regeneration genes at genetics level. Highest amount of callus production was obtained at 2.5 mg/L 2,4-D concentration (Figure 5) while highest callus weight was generated at 3.5 mg/L 2,4-D concentration in wheat (Figure 6). High weight of callus is necessary to establish a good protocol for gene transformation and fast development of plantlets through tissue culture. By the way, the output of our current experiments for callus formation and plant regeneration are consistent with other researchers who also applied different combinations of hormones to establish a tissue culture protocol in wheat. For example, Malik et al. (2021) obtained the most

**Table 4.** Callus weight (g) on multiple 2,4-D concentration.

| | H1 | H2 | H3 | H4 | H5 | H6 | Mean |
|---|---|---|---|---|---|---|---|
| 2, 4-D | 0.5 mg/l | 1.5 mg/l | 2.5 mg/l | 3.5 mg/L | 4.5 mg/l | 5.5 mg/l | g |
| CH-50 | 0.10 | 0.07 | 0.16 | 0.26 | 0.10 | 0.06 | 0.12 |
| GA-01 | 0.09 | 0.03 | 0.16 | 0.12 | 0.05 | 0.10 | 0.09 |
| NARC-09 | 0.16 | 0.01 | 0.01 | 0.10 | 0.17 | 0.06 | 0.08 |
| Pak-13 | 0.03 | 0.16 | 0.02 | 0.08 | 0.10 | 0.03 | 0.07 |
| Millet-11 | 0.03 | 0.05 | 0.10 | 0.14 | 0.16 | 0.09 | 0.10 |
| Borlog-14 | 0.06 | 0.10 | 0.07 | 0.16 | 0.13 | 0.02 | 0.09 |
| Mean (g) | 0.08 | 0.07 | 0.09 | 0.14 | 0.12 | 0.06 | |

noteworthy regeneration in Pakistani wheat varieties AS-2002 and Wafaq-2001 which yielded maximum embryogenic calli at concentration of 3.0 mg/L 2,4-D and 3.5 mg/L 2,4-D containing induction medium, respectively, and Mahmood et al. (2012) described perfect regeneration of plant tissues (41.19%) in wheat cultivars namely GA-2002 on culture media enhanced with 1.0 mg/L concentration of Kinetin. Shah (2023) tested 10 different Pakistani wheat genotypes with changeable concentrations of 2,4-D, IAA and Kinetin and observed maximum regeneration by variety Atta Habib at 0.1 to 0.4 mg/L (IAA- Kinetin) while maximum response of callus induction was 21 to 94%.

Numerous researchers have normalized optimal levels of 2,4-D for genetically dissimilar varieties of wheat. Iqbal et al. (2016) recovered utmost callus on MS media together with 4 and 6 mg/L 2,4-D. Similarly, Naz et al. (2021) recorded the highest proliferation and callus induction at concentration of 2 mg/L 2,4-D, in wheat, while highest plants regeneration was observed at concentration of IAA 6 mg/L and 6 mg/L Kinetin. Afzal et al. (2010) mentioned the highest callus formation at 3 mg/L of 2,4-D. Miroshnichenko et al. (2017) observed 10 shoots per explant in wheat by applying 3 mg/L Dicamba concentrations, 50 mg/L concertation of Daminozide, and with TZD concentration of 0.25 mg/L. Callus formation activity of wheat varieties to 2,4-D explained that genetic makeup of each variety have different demands of 2,4-D for maximal callus formation. Different cultivars of wheat have different behavior toward various amounts of plant growth hormones. Callogenesis and regeneration of varieties and variation among them were found to be genotype-dependent (Kagami et al., 2016; Ahmad et al., 2021).

## Conclusion

Optimize concentration of the plant growth controlling hormones is crucial for maximal plantlets regrowth for most researches involving tissue development techniques. Among six researched wheat cultivars, high potential for tissue regeneration and callus development was observed by the variety Millet-11. The results also suggest that regeneration potency of a genotype can speed up to a particular level by combining 1.5 mg/L concentration of IAA and 2 mg/L Kinetin concentration. Similarly, 2,4-D is a necessary plant growth hormones and appropriate amount is crucial for different plant species for callus formation and in wheat, 3.5 mg/L 2,4-D concertation was described best for high weight callus induction, so these results might be useful in future for gene transformation for the improvement of agronomic traits. Maximum potential for tissue culture of Millet-11 suggests the opportunity to use it for wheat improvement programs. These findings provide precise knowledge for plant breeders to develop new potential varieties of wheat having strong biotic and abiotic stress resistance in order to get maximum yield to feed growing population.

## Author Contributions

Conceptualization, BC, MT, and F; designed the experiments, BC, and MT; performed the experiments BC, MT, SZ, F, HL, JL, HMA, and CMG; analyzed the data, BC, MT, MA, and AJR; wrote the manuscript, AJR, MT, BC, and MA. All authors reviewed the manuscript.

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## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Multi-resistance to carbapenems by the production of Imipenemase (IMP)-types carbapenemases in Gram-negative bacilli in Burkina Faso

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**The growing prevalence of Imipenemase (IMP) metallo-beta-lactamases (MBL) producing strains of Gram-negative bacilli (GNB) pathogens is a real concern for clinicians in the light of therapeutic impasses driven. However, resistance genes encoding these enzymes are hardly documented in Burkina Faso. This study aims to show carbapenem-resistance mediated by the production of IMP-type carbapenemase in GNB clinical strains collected in Ouagadougou, Burkina Faso. Strains resistance profile to imipenem, meropenem, ertapenem, doripenem and aztreonam was determined by the disk diffusion method. Classical polymerase chain reaction (PCR) was carried out to detect β-lactamase (*bla*) gene of IMP in resistant strains. Out of 158 GNB collected, 91 (57.6%) were resistant to at least one of the carbapenems and/or to aztreonam. The highest prevalence of resistant strains was observed in *Escherichia coli* (45.1%; n=41) and *Klebsiella pneumoniae* (26.5% n=24). Among 32 resistant strains *bla*$_{IMP}$ gene positive (35.2%), *Escherichia coli* was the predominant species carrying resistance gene (18.7%, n=17/91). The findings strengthen the scarce existing scientific data on antimicrobial resistance mediated by metallo-beta-lactamases (MBL) in Burkina Faso.**

**Key words:** Carbapenem resistance, carbapenemase, blaIMP, Gram-negative bacilli, Burkina Faso.

## INTRODUCTION

Antibiotic therapy aims to kill pathogen bacteria. Unfortunately, these bacteria to protect themselves develop resistance to antibiotics used to treat them. Bacteria resistant to several types of antibiotics are sometime referred to as multi-resistant. Increased and inappropriate use of antimicrobial drugs promotes the emergence of antimicrobial resistant bacterial strains and related infections (Masoud et al., 2021; Kim et al., 2023). Some of those bacteria have become resistant to several antibiotics, including carbapenems and third-generation cephalosporins (Palacios-Baena et al., 2021; Arumugham et al., 2022). The emergence and spread of multidrug-resistant Gram-negative bacilli (MDR) has become a major public health concern worldwide (Dembele et al., 2020). The spread of MDR Gram-negative bacilli is increasingly reported in both hospital and community

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settings worldwide (Manenzhe et al., 2015). In the last decade, an alarming increase in the prevalence of carbapenemase-producing Gram-negative bacilli of serious nosocomial infections has been shown worldwide (Bourafa et al., 2018; Haji et al., 2021). Indeed, carbapenems were considered as last line drugs for controlling multidrug-resistant Gram-negative pathogens including extended-spectrum beta lactamases (ESBL)-producing Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumanii* (Haji et al., 2021; Ibrahim et al., 2021; Dikoumba et al., 2023). However, over-prescribing of carbapenems has led to emergence and global dissemination of carbapenem-aresistant organisms, including carbapenem-resistant Enterobacteriaceae, carbapenem-resistant *P. aeruginosa* and carbapenem-resistant *A. baumanii* (Aruhomukama et al., 2019; Haji et al., 2021) which are on World Health Organization (WHO) list of critical priority 1 pathogens of urgent need for new antimicrobial development (Tacconelli et al., 2018). Carbapenemase enzymes are the most common mechanism by which resistance to carbapenems occurs in these GNB (Shaker et al., 2018; Aruhomukama et al., 2019; Dziri et al., 2020; Haji et al., 2021). Several types of carbapenemases have been described. However, KPC (*K. pneumonia* carbapenemase, class A carbapenemase), class B metallo-beta-lactamases (New Dehli metallo-beta-lactamase-NDM, Verona Integron-encoded Metallo-beta-lactamase-VIM, Imipenemase-IMP) and OXA-48 and its variants (class D carbapenemase) are the most predominant carbapenemases in carbapenem-resistant GNB (Halat and Moubareck, 2020; Owusu et al., 2023). Imipenemases (IMPs), Verona Integron-encoded Metallo-β-lactamases (VIMs) and New Delhi Metallo-β-lactamase (NDMs) are the three MBLs types with an increasing challenge for clinical care and were identified in many human pathogens including Enterobacteriaceae and *P. aeruginosa* (Yang et al., 2023). The genes encoding these MBLs are genetically located within a variety of integrons, where they have been incorporated as gene cassettes (Halat and Moubareck, 2020), and these integrons associated with plasmids or transposons facilitated their spread by horizontal transfert between bacteria (Diene and Rolain, 2014). IMPs enzymes, subject of this study, are able to hydrolyse nearly all beta-lactams, specifically the carbapenems (Li et al., 2023). Many IMPs variants are involved in carbapenem resistance. The first genetically transferable IMP-1 was identified in *P. aeruginosa* and *Serratia marcescens* in Japan in 1990 (Watanabe et al., 1991). Currently, at least 91 IMP variants have been discovered worldwide (Li et al., 2023) including IMP-68 in Japan (Kubota et al., 2019) and IMP-89, IMP-91 and IMP-96 recently identified in Chinese clinical isolates (Li et al., 2023). Thus, South East Asian region remains the largest reservoir of IMP-type MBL (Halat and Moubareck, 2020; Li et al., 2023). However, these MBL have been reported in various other countries: Italy and Portugal (Cornaglia et al., 1999; Riccio et al., 2000), USA (Limbago et al., 2011), Australia (McCarthy et al., 2017) and Lebanon (Halat et al., 2017). In Africa, IMP enzymes had been in Egypt (Masoud et al., 2021; Benmahmod et al., 2019; Abbas et al., 2019), Morocco (Barguigua et al., 2013), Tunisia (Chouchani et al., 2011; Dziri et al., 2020), Tanzania (Mushi et al., 2014) and Sudan (Adam and Elhag, 2018). In Burkina Faso, most of the studies were much focused on ESBL-producing Enterobacteriaceae which occurs resistant to beta-lactam (Metuor Dabire et al., 2013; Metuor et al., 2019a; Metuor Dabire et al., 2019b; Tiemtore et al., 2022) at the expense of carbapenemases, mainly MBL despite the threat they represent for heath care. Nevertheless, previous studies (Sanou et al., 2020; Dembele et al., 2021; Kabore et al., 2022, 2023) have reported resistance to carbapenems in GNB by the production of carbapenemases. Although, $bla_{IMP-2}$ gene was detected in *E. coli* clinical isolate from rural settings by Dembele et al. (2021); no other study, to the researchers knowledge, have reported IMP-type carbapenemase gene in urban settings, especially in hospital settings. Thus, this study aimed to report carbapenems multi-resistance mediated by IMP-type carbapenemase producing in GNB from urban hospitals in Burkina Faso.

## METHODS

### Sample and bacterial isolates identification

This study was conducted in Ouagadougou, Burkina Faso. This was a cross-sectional study with retrospective data collection of 158 bacterial strains which were collected from September 2018 to October 2018 and from September 2022 to August 2022 in Bacteriology Department of the Laboratories of University Hospital Center of Tengandogo (CHU-T) and of Saint Camille Hospital of Ouagadougou (HOSCO) in Burkina Faso. These bacterial samples were GNB strains resistant to at least one third generation cephalosporin and/or aztreonam, including 130 isolates from HOSCO and 28 others from CHU-T. They were isolated in the following specimens: Urine, stool, pus, blood cultures, vaginal and vulvar swabs and peritoneal fluids collected from outpatients and hospitalized patients. API 20E gallery tests (BioMerieux S.A., Marcy Etoile, French) were used for bacterial species identification.

### Antibiotic susceptibility testing

Disk diffusion method on Mueller-Hinton (MH) agar (Liofilchem, Italia) was used to perform the antibiotic susceptibility test of the strains, while following the recommendations the Antibiogram Committee of the French Microbiology Society/European Committee for Antimicrobial Susceptibility Testing (CA-SFM/EUCAST) versus 2022 (EUCAST/CASFM, 2022). The following antibiotics, purchased from Liofilchem (Italia) were used to carry out carbapenem-resistant strains: Imipenem IPM (10 µg), Meropenem MRP (10 µg), Ertapenem ETP (10 µg), Doripenem DOR (10 µg) and Aztreonam ATM (30 µg). Susceptible strains were categorized «Sensitive, S» and strains with intermediate susceptibility or resistant were classified «Resistant, R», using critical limits of inhibition diameters according to CA-SFM vs 2022 guidelines (EUCAST/CASFM, 2022).

**Table 1.** Primers used for *bla*$_{IMP}$ gene detection.

| Researched gene | Primers | Sequence (5'- 3') | Size (pb) | Reference |
|---|---|---|---|---|
| *bla*$_{IMP}$ | IMP-F | CATGGTTTGGTGGTTCTTGT | 488 | Huang et al. (2012) |
| | IMP-R | ATAATTTGGCGGACTTTGGC | | |

**Molecular detection of gene encoding IMP-type carbapenemase**

***Extraction of bacterial DNA***

Bacterial DNA was extracted from isolated colonies obtained from previously strains stored in Luria Bertani storage medium, which were awakened by culturing on MH agar for 18 to 24 h at 37°C. Bacterial DNA extraction was done by the boiling method (Dashti et al., 2009) with few modifications. A total of 2 to 3 identical colonies were suspended in 200 µL of distilled sterile water in 1.5 mL labeled Eppendorf tube. The resulting suspension was boiled at 100°C for 15min in water bath (MEMMERT, Rost fret) to release bacterial genetic material and then centrifuged (NF 048 centrifuge) at 12000 rpm for 10 min to remove genetic material from others debris. The supernatant containing DNA was transferred to a new Eppendorf tube. DNA extract was assayed for quantity and purity, using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and then stored at -20°C for further usage.

**Molecular identification of gene encoding IMP carbapenemase**

All isolates resistant to at least one carbapenem and/or aztreonam were screened by conventional PCR (Polymerase Chain Reaction), using specific oligonucleotide primers (Table 1) to gene encoding beta-lactamase of *bla*$_{IMP}$ family. Reaction mixture of 20 µL was composed of 4 µL Firepol® Master Mix 5X, 0.5 µL of each primer (Forward and Reverse), 14 µL of PCR water and 1 µL of DNA extract. Amplification reactions were performed using GeneAmp System PCR 9700 Thermal Cycler (Applied Biosystems, California, USA) according to PCR program shown in Table 2.

After amplification, 6 to 8 µL of each PCR product were separated by 1.5% agarose gel electrophoresis at 100 volts for 35 min in TAE 1X buffer mixed to ethidium bromide (1 µg/mL) in migration tank. A molecular weight marker, DNA ladder (Solis Biodyne, Estonia) 100 pb was used as a reference. Amplified DNA bands were visualized using a UV transilluminator (E-Box Vilber).

## RESULTS

### Bacterial strains and antibiotic susceptibility testing

In this study, 57.6% (91/158) GNB strains resistant to at least one carbapenem and/or aztreonam were detected (Figure 1). Among the 91 resistant strains, 11 bacterial species were identified and the majority of strains 81.3% (n=74) were from Saint Camille Hospital of Ouagadougou (Table 3). *E. coli* 45.1% (n=41) and *K. pneumoniae* 26.5% (n=24) were the predominant resistant species followed by *P. aeruginosa* 9.9% (n=9), *P. mirabilis* 4.4% (n=4) and *S. marcescens* 4.4% (n=4) (Table 3). Most resistant strains were from cytobacteriological examinations of urines (50.5%, n=46), stools (33%, n=30) and pus (13.2%, n=12) and from hospitalized patients (60.4%, n=55), mostly female (51.6%, n=47) were children and adults from 0 to 4 years and 25 to 64 years, respectively.

The distribution of bacterial species according to antibiotic resistance patterns are shown in Table 4. The resistance rates observed were: 94.5% (n=86) for aztreonam, 44.0% (n=40) for ertapenem and 22.0% (n=20) for each imipenem, meropenem and doripenem. For ertapenem, the carbapenem with the highest resistance, *E. coli* strains recorded the highest level of resistance (20.9%, n=19/91) followed by *K. pneumoniae* (11%, n=10/91) and *P. aeruginosa* (6.6%, n=6/91). On the other hand, the susceptibility of the isolates to antibiotics revealed that almost all strains were highly resistant to aztreonam.

### Molecular detection of gene encoding IMP carbapenemase

Molecular characterization of gene encoding IMP-type carbapenemase performed by conventional PCR, using specific primers (Table 1) revealed that, out of 91 resistant isolates, 32 strains (35.2%) harbored *bla*$_{IMP}$ gene as showing DNA bands appeared approximatively at 488 pb (Figure 2).

More than half of the bacterial strains carrying bla$_{IMP}$ gene were *E. coli* (18.7%, n=17/91) as indicated in Table 5 showing resistance gene distribution according to resistant bacterial species. Also, most of resistant isolates positive to carbapenemase-IMP encoding gene were recovered from urines samples (46.9%, n=15/32) collected from patients predominantly hospitalized (71.9%, n=23/32) and female (50%, n= 16/32). As regard correlation between carbapenem resistance profile and bla$_{IMP}$ gene detection, ertapenem non-susceptible strains exhibited the highest prevalence of *IMP* gene (68.8%, n=22/32), compared to gene detection rates in isolates resistant to other carbapenems: 25.0% (n=8/32) for doripenem and 31.3% (n=10/32) each for imipenem and meropenem.

## DISCUSSION

Carbapenems over-prescribing has led to emergence and global increased dissemination of carbapenem-resistant bacteria which reduced the effectiveness of these antibiotics in the treatment of related infections

**Table 2.** PCR program for *bla*$_{IMP}$ gene detection.

| Amplification step | Temperature/duration |
|---|---|
| Initial denaturation | 96°C / 5 min |
| Denaturation | 96°C / 30 s |
| Hybridization | 54°C / 30 s |
| Elongation | 72°C / 30 s |
| Final elongation | 72°C / 7 min |
| Number of cycles | 30 |

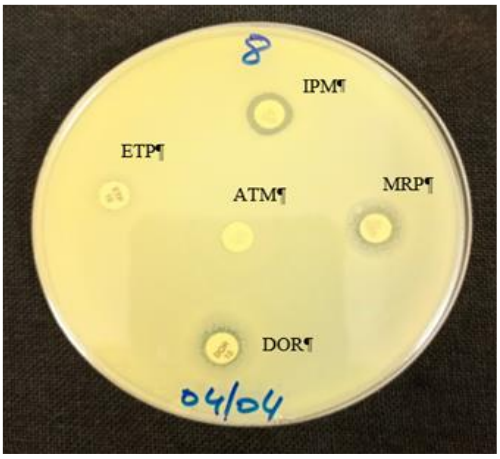


**Figure 1.** Petri dishes showing resistance of strain to antibiotics tested.

**Table 3.** Frequencies of bacterial resistant strains according to area sampling.

| Bacterial species | Area sampling | | Total (n/%) |
|---|---|---|---|
| | HOSCO (n/%) | CHUT (n/%) | |
| *Escherichia coli* | 31 (34.1) | 10 (11.0) | 41 (45.1) |
| *Klebsiella pneumoniae* | 21 (23.1) | 3 (3.3) | 24 (26.4) |
| *Pseudomonas aeruginosa* | 9 (9.9) | 0 | 9 (9.9) |
| *Proteus mirabilis* | 4 (4.4) | 0 | 4 (4.4) |
| *Enterobacter cloacae* | 2 (2.2) | 1 (1.1) | 3 (3.3) |
| *Enterobacter aerogenes* | 1 (1.1) | 0 | 1 (1,1) |
| *Citrobacter freundii* | 2 (2.2) | 0 | 2 (2.2) |
| *Serratia marcescens* | 3 (3.3) | 1(1.1) | 4 (4.4) |
| *Serratia odorifera* | 1(1.1) | 0 | 1(1.1) |
| *Klebsiella oxytoca* | 0 | 1(1.1) | 1(1.1) |
| *Salmonella arizonae* | 0 | 1(1.1) | 1(1.1) |
| Total | 74 (81.3) | 17 (18.7) | 91 (100) |

n = number of resistant strains of each bacterial species; % = proportion of resistant strains of each bacterial species, HOSCO = Saint Camille Hospital Center in Ouagadougou; CHU-T = Tengandogo University Hospital Center.

(Aruhomukama et al., 2019; Haji et al., 2021; Das, 2023). An alarming increase in the prevalence of carbapenemase-producing Gram-negative bacilli of serious nosocomial infections has been reported worldwide (Bourafa et al., 2018; Haji et al., 2021).

This current study was undertaken to show

**Table 4.** Distribution of bacterial species according to antibiotic resistance patterns.

| Bacterial species | IPM$^R$ (n'/%) | MRP$^R$ (n'/%) | ETP$^R$ (n'/%) | DOR$^R$ (n'/%) | ATM$^R$ (n'/%) |
|---|---|---|---|---|---|
| *E. coli (n=41)* | 7 (7.7) | 7 (7.7) | 19 (20.9) | 8 (8.8) | 38 (41.8) |
| *K. pneumoniae (n=24)* | 4 (4.4) | 7 (7.7) | 10 (11.0) | 5 (5.5) | 23 (25.3) |
| *P. aeruginosa (n=9)* | 7 (7.7) | 2 (2.2) | 6 (6.6) | 4 (4.4) | 9 (9.9) |
| *P. mirabilis (n=4)* | 0 | 0 | 1 (1.1) | 0 | 4 (4.4) |
| *E. cloacae (n=3)* | 1 (1.1) | 1 (1.1) | 1 (1.1) | 0 | 2 (2.2) |
| *E. aerogenes (n=1)* | 0 | 0 | 0 | 0 | 1 (1.1) |
| *C. freundii (n=2)* | 0 | 0 | 0 | 0 | 2 (2.2) |
| *S. marcescens (n=4)* | 0 | 1 (1.1) | 1 (1.1) | 1 (1.1) | 4 (4.4) |
| *S. odorifera (n=1)* | 0 | 0 | 0 | 0 | 1 (1.1) |
| *K. oxytoca (n=1)* | 1 (1.1) | 1 (1.1) | 1 (1.1) | 1 (1.1) | 1 (1.1) |
| *S. arizonae (n=1)* | 0 | 1 (1.1) | 1 (1.1) | 1 (1.1) | 1 (1.1) |
| Total | 20 (22.0) | 20 (22.0) | 40 (44.0) | 20 (22.0) | 86 (94.5) |

R = Resistant; n= strain number of each bacterial species; n' = number of resistant strains of each bacterial species to antibiotic overall of the total of strains; %= percent correlated to the total number of resistant strains bacterial; IPM= Imipenem, MRP= Meropenem, ERT= Ertapenem, DOR = Doripenem, ATM = Aztreonam.

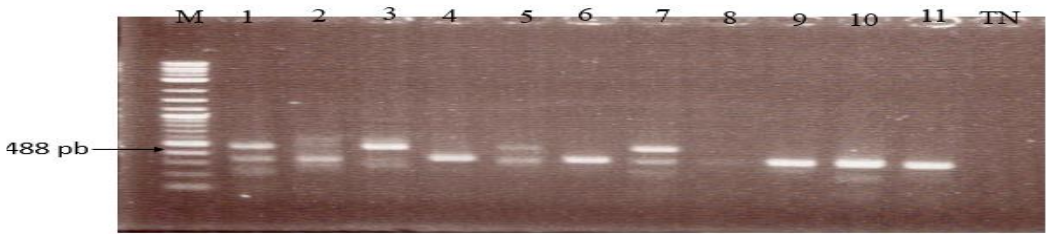


**Figure 2**. Agarose gel electrophoresis of bla$_{IMP}$ gene amplicons (488 bp). M= molecular weight marker, DNA ladder 100 pb (Solis Biodyne, Estonia); TN = negative control; Lanes 1-11 correspond to strains with positive IMP carbapenemase gene.

carbapenem resistance in GNB by production of IMP-type carbapenemase from urban hospitals centers in Ouagadougou, Burkina Faso. Overall 158 GNB strains collected in HOSCO and in CHU-T, 57.6% (n=91) of them were carbapenem-resistant including *E. coli* (45.1%, n=41) and *K. pneumoniae* (26.5%, n=24) which were the predominant resistant species followed by *P. aeruginosa* (9.9%, n=9). In Ghana, Owusu et al. (2023) reported these similar findings, where *E. coli* and *K. pneumoniae* were the predominant GNB species at 46% (n=83) and 17% (n=30), respectively. Furthermore, studies conducted in other countries have reported both same species with the most carbapenem-resistant strains (Haji et al., 2021; Armin et al., 2023). The results are in agreement with the study in Burkina Faso by Kabore et al. (2022) who reported that GNB is mainly more resistant to antibiotics including carbapenems were *E. coli* and *K. pneumoniae*, but at higher rate (82.69%) and lower rate (9.62%) respectively. In contrast, Balkhair et al. (2023) found a lowest resistance rate to carbapenems for *E. coli* (2.9%) and a higher carbapenem level resistance at 46.4 and 29.9% respectively for *K. pneumoniae* and *P. aeruginosa*. One the other hand, this study carbapenem-resistant strains prevalence (57.6%, n=91/158) was higher than those (30.9, 37 and 27.7%) observed respectively by Haji et al. (2021), Armin et al. (2023) and Balkhair et al. (2023). Indeed, any strain exhibiting reduced sensitivity to at least one of carbapenems and/or aztreonam was categorized resistant and therefore was suspicious of carbapenemase production. This fact and sample size would explain the prevalence of carbapenem-resistant strains. The majority of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates were originated from urine (50.5%) collected mainly from female patients (51.6%). Also, Haji et al. (2021) observed urine samples were the dominant type (62%) from which these species were isolated.

Regarding antibiotic resistance profile, the strains showed slightly high resistance level to carbapenems (Table 4). Thus, imipenem with 22.0% as resistance rate, is currently one the most effective carbapenem as reported by Masoud et al. (2021). However, Haji et al. (2021) have recorded slightly higher resistance rates for imipenem (41%) and meropenem (40%) and a slightly low resistance level for ertapenem (36%). In this study,

**Table 5.** Distribution of *IMP* gene according to resistant bacterial species.

| Bacterial species | *IMP* gene '+' (n/%) | *IMP* gene '-' (n/%) | Total |
|---|---|---|---|
| *E. coli* | 17 (18.7) | 24 (26.4) | 41 (45.1) |
| *K. pneumonia* | 6(6.6) | 18 (19.8) | 24 (26.4) |
| *P. aeruginosa* | 3 (3.3) | 6 (6.6) | 9 (9.9) |
| *P. mirabilis* | 1 (1.1) | 3 (3.3) | 4 (4.4) |
| *E. cloacae* | 1 (1.1) | 2 (2.2) | 3 (3.3) |
| *E. aerogenes* | 1 (1.1) | 0 | 1 (1.1) |
| *C. freundii* | 0 | 2 (2.2) | 2 (2.2) |
| *S. marcescens* | 2 (2.2) | 2 (2.2) | 4 (4.4) |
| *S. odorifera* | 0 | 1 (1.1) | 1 (1.1) |
| *K. oxytoca* | 1 (1.1) | 0 | 1 (1.1) |
| *S. arizonae* | 0 | 1 (1.1) | 1 (1.1) |
| Total | 32 (35.2) | 59 (64.8) | 91 (100) |

n = number of resistant strains of each bacterial species harboring or not resistance gene; '+'indicating gene detected; '-'= indicating gene not detected; %= percent correlated to the total number resistant strains bacterial.

carbapenem resistance profile was carried out by an automated method (Hu et al., 2019) which improve resistance detection. Whereas, in this study phenotypic detection of carbapenem-resistant strains has been performed using manual disk diffusion method (EUCAST/CASFM, 2022), by which it is difficult to demonstrate carbapenem resistance (Kabore et al., 2023).

All bacterial isolates resistant to carbapenem and/or to aztreonam therefore suspicious of carbapenemase production were screening by molecular approaches to detect *IMP* gene. In this current study, *bla*$_{IMP}$ gene was found in 35.2% (n=32/91) of carbapenem-resistant strains, while in 64.8% (n=59/91) other strains none resistance *IMP* gene was found (Table 5). A previous study of Dembele et al. (2021) in Burkina Faso has demonstrated the presence of *bla*$_{IMP-2}$ gene in Imipenem-resistant *E. coli* strains isolated from children with diarrhea in rural settings. Whereas, in this study, carbapenemase-IMP encoding gene was identified not only in *E. coli*, but also in other enterobacteria (Table 5) and then in *P. aeruginosa* recovered from children or adult patients in urban health centers in Ouagadougou, Burkina Faso.

*IMP* gene was recorded at a slightly high prevalence (35.2%, n=32/91) and more than half of gene positive strains (18.7%, n=17) were *Escherichia coli*. These findings are comparable to those reported in Iraq, where detection rate of *bla*$_{IMP}$ gene was 43% (n=23/53) in clinical GNB strains, and this gene most prevalent in *Escherichia coli* (50%) (Haji et al., 2021). Our carbapenemase-*IMP* gene frequency (35.2%, n=32/91) was highest than those recorded in previous studies in Sudan (26.4%) (Adam et Elhag, 2018), in Egypt (11.8%) (Abbas et al., 2019) and in Iran (13%) (Armin et al., 2023). This increasing detection rate of *bla*$_{IMP}$ comparing to frequencies of these previous studies would suggested a currently worrying spread of carbapenem-resistant GNB producing IMP-type carbapenemase (Li et al., 2023). In addition, to the best of our knowledge, our study is one of the first in our country to record detection of *bla*$_{IMP}$ gene in *Pseudomonas aeruginosa* carbapenem resistant clinical strains with a prevalence of 3.3% (Table 5) in health centers of Ouagadougou. Until then, Kabore et al., in their study, had been limited to the phenotypic detection of MBL in Imipenem-resistant *Pseudomonas aeruginosa* clinical strains . This detection rate of *bla*$_{IMP}$ gene in *Pseudomonas aeruginosa* is certainly underestimated given the relatively small sample size of our study. Thus, our study firstly reporting the circulation of *Pseudomonas aeruginosa* producing IMP-type carbapenemases in health centers of Ouagadougou, Burkina Faso.

In contrast, out of 64.8% (n=59/91) resistant strains, none strain has harbored *bla*$_{IMP}$ gene encoding IMP-type carbapenemase although some of them (12.1%, n=11/91) were resistant to all carbapenems and aztreonam tested. This fact could be explained by the existence of other resistance mechanisms in our isolates, such as the production of ESBLs or other carbapenemases and defects in cell wall permeability. Indeed, in a previous study conducted in 2022 by Tiemtore et al. (2022) in Burkina Faso, *bla*$_{GES}$ gene encoding Guiana Extended-Spectrum (GES) carbapenemase has been detected in Imipenem-resistant enterobacteriaceae strains included in this study. Thus, eventual presence of GES-2 variant, exhibiting an increased hydrolysis of carbapenems (Poirel et al., 2001), in our bacterial strains, might justified carbapenem resistance of *IMP* gene-negative isolates. Interestingly, the encoding-carbapenemase genes, such as *bla*$_{NDM}$, *bla*$_{VIM}$ and *bla* $_{KPC}$ were found in GNB clinical isolates as previously reported in other studies in this country (Dembele et al., 2021; Kabore et al., 2023; Ouattara et

al., 2023; Bambara et al., 2023). These carbapenem resistance genes not researched in this study could also explained the finding. Further, non-enzymatic resistance mechanisms to carbapenems such as defecting in wall cell permeability through modification or loss of porins (Zango et al., 2019; Eichenberger and Thaden, 2019; Çekin et al., 2021; Onishi et al., 2023) and over-expression of efflux pumps (Lee et al., 2021; Onishi et al., 2023) would explained the result.

Finally, *IMP* gene was detected in 9.9% (n=9/91) strains which were susceptible to all carbapenems except aztreonam. This presence of MBL gene encoding IMP enzyme in GNB isolates sensitive to carbapenems finding in this study was reported in previous studies in other countries with various frequencies including NDM and VIM (Anoar et al., 2014; Adam and Elhag, 2018). These results corroborate hypothesis that, ordinarily, MBL genes presence among carbapenems sensitive strains indicate that there might be hidden MBL genes not detected by phenotypic tests, leading to the silent spread of these genes in the hospitals and the community (Adam and Elhag, 2018).

## Conclusion

Carbapenem-resistant Gram-negative bacilli are a growing threat to public health worldwide. The study aimed to determine carbapenem multi-resistance mediated by IMP carbapenemase production in GNB at Saint Camille Hospital of Ouagadougou (HOSCO) and University Hospital Center of Tengandogo (CHU-T) in Ouagadougou, Burkina Faso. The GNB strains exhibited a high resistance level to antibiotics tested with a prevalence of 57.6%. This study revealed a slightly high prevalence of *bla*$_{IMP}$, gene encoding IMP-type metallo-beta-lactamase in carbapenem-resistant strains. These results confirm the presence of GNB resistant to antibiotics producing IMP-type carbapenemases at HOSCO and CHU-T in Ouagadougou, Burkina Faso. The findings of this study strengthen the scarce existing scientific data on antimicrobial resistance mediated by metallo-beta-lactamases in Burkina Faso. The study recommended an urgent implementation of an antibiotic resistance surveillance system combining clinical aspects of infections related to bacteria producing MBL and other carbapenemases, in order to prevent, monitor and control the spread of antimicrobial resistance gene in our country. Further studies on sequence analysis of *IMP* gene amplicons are also needed to know different IMP variant profile in carbapenem-resistant GNB in Burkina Faso.

## Ethics approval

The institutional ethic committee of CERBA/LABIOGENE reviewed and approved the study protocol.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## AUTHORS' CONTRIBUTIONS

Damis Yves Patrik Bouniounou, Amana Metuor Dabire and Yasmine Rahimatou Wend-Kouni Tiemtore and Serge Sougue designed the study, analyzed and interpreted data; Damis Yves Patrik Bouniounou performed the experiments and drafting the manuscript; Yasmine Rahimatou Wend-Kouni Tiemtore and Pegdwende Rose Bonkoungou collected samples and carried out the laboratory investigations under following the recommendations of Amana Metuor Dabire; Jacques Simpore was general supervisor of the study. All authors were involved in critically reviewing the manuscript and have read and approved the final published version of the manuscript.

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# The effect of clarification methods on quality attributes of sugarcane-watermelon wine fermented by palm wine yeast (*Saccharomyces cerevisiae*)

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**Clarification of wine is aimed at improving the quality of the product by removing haze. In this study, the effect of two clarification methods namely membrane filtration technique and the use of Keiselguhr diatomaceous earth powder on the physico-chemical and sensory attributes of wine produced using sugarcane (*Saccharum officinarum* L) and watermelon (*Citrullus vulgaris* L) juice blended in the ratio 1:1 (v/v) and fermented by *Saccharomyces cerevisiae* isolated from palm wine was determined. Sugarcane-watermelon wine not clarified was the control. Physicochemical analysis of the wine at 0 h indicates the following: sugar (10.73 °Brix), specific gravity (1.043 kg/m$^3$), pH (3.9), alcohol content (5.9%), titratable acidity (0.720 g/l), turbidity (94.32 NTU) and colour intensity (0.892 nm). During maturation of wine, the sugar content, specific gravity, pH, alcohol content, titratable acidity, turbidity and colour intensity of the samples clarified by membrane filtration/diatomaceous earth powder at 72 and 336 h were 6.5/9.9 and 7.2/10.8 °Brix, 1.026/1.040 and 1.029/1.043 kg/m$^3$, 3.7/3.8 and 3.0/3.13, 3.57/5.48 and 3.7/5.7%, 0.375/0.405 and 0.517/0.628 g/l, 29/32.6 and 15/20 NTU, and 0.649/0.873 and 0.642/0.628 nm, respectively. The cumulative sensory scores of wine clarified using Keiselguhr diatomaceous earth powder were slightly higher than the wine clarified by membrane filtration. Taking other parameters into consideration, the clarification of sugarcane-watermelon wine using membrane filtration is relatively better than Keiselguhr diatomaceous earth powder.**

**Key words:** Alcoholic beverages, fruits and vegetables, fruit wine, fermentation.

## INTRODUCTION

Wine is a popular beverage that contains alcohol. People of different social status drink wine to their delight (Ogbeide and Ele, 2015). Wine is produced by fermenting fruit juice preferably grape juice (Biri et al., 2015; Zainab et al., 2018). A single fruit or combination of different fruits depending on individual's choice is used for wine production (Saranraj et al., 2017; Velić et al., 2018). Production and consumption of wine is part of the history of man (Wurz, 2019). The type of fruit/vegetable and yeast strain(s) selected for wine production influences its sensory characteristics (Okemini and Dilim, 2017; Pino et al., 2019). The alcohol content of wine is within the range

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5-13%. Wine, also called fruit wine is an undistilled alcoholic beverage. A situation whereby grape is not used to produce wine, it is a convention to add the name of the fruit eventually used when the product is being referred to (Swami et al., 2014; Ire et al., 2020; Kantiyok et al., 2021).

Tropical fruit wine are prepared using fruits grown in subtropical and tropical regions of the world (Chakraborty et al., 2014). Watermelon red, cashew, banana, guava, pawpaw, orange, pineapple, mango and watermelon wines of acceptable quality were prepared by Djouldedarman et al. (2010), Awe and Olayinka (2011), Idise and Odum (2011), Nikhanj and Kocher (2015), Umeh et al. (2015), Patharkar et al. (2017), Qi et al. (2017), Ogodo et al. (2018), and Zainab et al. (2018), respectively. According to Ire et al. (2020), any kind of fruit is suitable for the production of wine. Vegetables such as fluted pumpkin (*Telfairia occidentalis*) leaves and cucumber (*Cucumis sativus* L.) have also been used to produce wine of acceptable quality (Ebana et al., 2019). Production of wine is possible using tigernut and sugarcane juices obtained from *Cyperus esculentus* and *Saccharum officinarum* L., respectively (Okemini and Dilim, 2017; Shah et al*.,* 2020).

Clarification is an important stage during wine production which takes place after fermentation. Acceptance or rejection of wine by consumers is influenced by sensorial assessment of the product based on sight, taste, and smell. The essence of wine clarification by filtration and/or fining is to remove cloudiness/turbidity of wine during production (Muñoz-Castells et al., 2022). After clarification of wine is completed, the final product becomes bright and clear. In order to remove dead or unreacted yeast, bacteria and grape debris during production of wine, it has to be filtered whereas the addition of fining agents is capable of removing soluble substances such as proteins, colouring phenols and polymerized tannins associated with a cloudy wine (Awe, 2018). Fining agents are placed in different groups referred as earths, animal proteins, plant proteins, wood charcoal, synthetic polymers and silicon dioxide based on their general nature (Kemp et al., 2022). A wide range of fining agents obtained from animal protein, vegetable protein and inorganic compounds is used in the wine industry with varying effects on the product (Chagas et al., 2012).

For more than 2,000 years, the medicinal use of wine has been established (Wurz, 2019). It is moderate for adults to consume 1 to 2 glasses of wine per day. The health benefits associated with wine consumption include longevity, increased cognitive performance and insulin sensitivity, reduced risk of stroke, prevention of cancer and cardiovascular diseases. A combination of food and wine helps digestion to take place. Intake of wine is healthy for the skin. Wine could also play a role to prevent blindness (Fehér et al., 2007; Wurz, 2019, Ire et al., 2020). However, too much consumption of wine could damage cellular processes responsible for bone tissue formation and the long-term effect is fractures occurring at high frequency (Oladipo et al., 2014).

Sugarcane (*S. officinarum* L.) is a tall perennial grass that grows abundantly in the tropics and warm temperate regions. It is cultivated mainly because it is the major raw material for production of sugar (Aina et al., 2015; Wada et al., 2017). The country of origin of sugarcane is yet to be substantiated. Available information suggests that *S. officinarum* and *Saccharum robustum* originated from New Guinea; *Saccharum barberi* from India; and *Saccharum sinense* from China (Brumbley et al., 2008). Sugarcane is a snack to many people who enjoy chewing the stem and swallow its juice (Aina et al., 2015; Williams et al., 2016). Sugarcane juice is one of the popular products of sugarcane (Singh et al., 2015; Pino et al., 2019). It is a good source of carbohydrates, minerals and amino acids (Williams et al., 2016; Arif et al., 2019). Sugarcane juice possess anticancer and antioxidant properties which confer health benefits to consumers. Regular consumption of sugarcane juice fights chronic diseases associated with old age (Hameed et al., 2016). Patients experiencing jaundice are advised to drink it. Sugarcane juice is associated with anti-inflammatory, diuretic, analgesic and hepatoprotective effects in humans. It also functions as a laxative, aphrodisiac, cooling, antiseptic, demulscent and tonic (Singh et al., 2015). Abundant nutrients mainly sugar, yeasts and bacteria especially *Leuconostoc* species present in sugarcane juice are responsible for easy spoilage of the juice (Bag et al., 2022).

Watermelon (*Citrullus vulgaris* L.) is a xerophytic tropical and subtropical fruit that grows abundantly in most African countries and South East Asia (Yusufu et al., 2018; Dube et al., 2020). North-East Africa is believed to be the origin of watermelon, reported more than 5,000 years ago (Mezue and Aghimien, 2016). According to Reetu and Tomar (2017), watermelon originated from Kalahari Desert. In Nigeria, watermelon is consumed as nectars, juice and fruit cocktails after the fruit has been fermented and blended (Kantiyok et al., 2021). The method of preparation will determine whether watermelon is going to be consumed as snack or appetizer (Ogodo et al., 2015; Zainab et al., 2018). During drought seasons, watermelon serves as a source of drinking water for people living in parts of Nigeria and Sudan (Dube et al., 2020). Watermelon is a good source of carotenoids such as β-carotene, lycopene, phytoene, phytofluene, neurospnene and lutein. It also contain carbohydrates, protein, fats, dietary fiber, vitamin A, $B_1$, $B_6$ and C as well as minerals such as potassium, iron, manganese and magnesium (Reetu and Tomar, 2017). Lycopne present in watermelon is associated with health benefits. Watermelon helps the body to fight arteriosclerosis, diabetes, hypertension, cancer, diabetes, arthritis, macular degeneration and some coronary heart diseases (Zainab et al., 2018; Asante et al., 2020).

Palm wine is the sap obtained from trees that grow abundantly in the tropical region belonging to the family Palmae (Agwuna et al., 2019). In Southern Nigeria, palm wine tapped from *Elaeis guineensis*, *Raphia hookeris* and *Raphia vinifera* is a milky alcoholic beverage consumed by the people (Ogodo et al., 2015). It is a potential source of yeast strains for producing industrially fermented products such as wine, bread, etc (Olowonibi, 2017; Zainab et al., 2018). Spontaneous fermentation of palm wine which contains sugars, amino acids, proteins, and vitamins create a favourable environment for yeast and bacteria to increase in large numbers (Onwumah et al., 2019; Kantiyok et al*.,* 2021).

Wine of acceptable quality was produced by Soibam et al. (2016) using a blend of sugarcane (*S. officinarum* L.) and watermelon juice (*C. vulgaris* L.). The researchers used *Saccharomyces cerevisiae* strain isolated from palm juice to ferment sugarcane-watermelon juice. However, the researchers did not evaluate the effect of using different fining agents on the quality of wine produced. The acceptability of wine by consumers could be affected if the product is not clarified using an appropriate clarifying agent/method (Verlić et al., 2019). Therefore, this study is aimed at evaluating the effect of membrane filtration and the use of Kieselguhr diatomaceous earth powder as clarification methods on the physico-chemical and sensory quality of sugarcane-watermelon wine fermented by palm wine yeast.

## MATERIALS AND METHODS

Fresh and mature sugarcane and watermelon were purchased from Choba market along East- West Road, Obio-Akpor Local Government Area, Rivers State, Nigeria. About 1.5 L of fresh palm wine was obtained from palm wine tappers in Ozuoba also in Obio-Akpor Local Government Area, Rivers State using a sterile plastic jerry can that has a cork. The watermelon, sugarcane and palm wine were transported to Microbiology Laboratory, University of Port Harcourt, using clean big shopping bags within 2 h for laboratory analyses.

### Extraction of watermelon juice

About 5 kg of watermelon was washed thoroughly using distilled water. A clean stainless steel knife sterilized with 70% ethanol was used to peel the watermelon. The fleshy part of watermelon which is reddish was deseeded and chopped into small pieces and transferred into sterilized electric blender (Sonic food processor 2203) for crushing. Thereafter, the slurry was filtered using a clean muslin cloth to obtain 2 L of watermelon juice.

### Extraction of sugarcane juice

About 5 kg of sugarcane was washed thoroughly with distilled water and peeled using a clean sterilized stainless knife. Thereafter, the knife was used to chop the sugarcane into smaller pieces before transferring them quantitatively into electric blender (sonic food processor 2203) for crushing. The slurry containing insoluble sugarcane fibre was filtered using a clean muslin cloth to obtain 2 L of sugarcane juice.

### Preparation of ‘must’

The ‘must’ was prepared using a mixture of sugarcane juice (2 L) and watermelon juice (2 L) which represent a ratio of 1:1 v/v. A total volume of 4 L of ‘must’ was achieved. The bowl containing the ‘must’ was carefully covered after 14.2 mL of 3.14% sodium metabisulphite ($Na_2S_2O_5$) was added to sterilize ‘must’ (Berry, 2000).

### Preparation of yeast starter culture

Ten fold serial dilution of the palm wine was aseptically carried out using a sterile peptone water. A sterile pipette was used for each transfer from one dilution to the next. With the aid of inoculating loop sterilized in a Bunsen flame, a loopful of palm wine from dilution $10^{-4}$ was streaked on potato dextrose agar (PDA). The plate was covered and incubated at a temperature of 27°C for 72 h. The Petri-dishes were examined for growth of microorganisms.

### Identification of the isolate

A speck of the fungal colony was mounted on a glass slide and viewed under the microscope for cellular characteristics. Standard morphological and physiological tests as described by Nwachukwu et al. (2006) and Ogbulie et al. (2007) for yeast identification were employed. The tests include morphology, surface characteristics, presence of pseudomycellium, ascospore formation, vegetative reproduction, growth in 10% NaCl + 50% yeast extract. The isolates were subcultured on potato dextrose broth incorporated with streptomycin to inhibit bacterial growth. The inoculated plates were incubated for 24 h and stored at 28°C.

### Fermentation process

#### *Aerobic phase of fermentation*

Standard inoculum (*S. cerevisiae*) using MacFarland Standard which is equivalent to $1.5 \times 10^8$ CFU/mL was prepared and added into 1 mL. equivalent of sugarcane-watermelon juice. The fermentation broth was covered using a sterile cotton wool. Fermentation was allowed to proceed for 2 days at 30±2°C.

#### *Anaerobic phase of fermentation*

The fermenting vessel was made air tight by covering it with a lid and sealing the edge with paper tape to prevent contamination from undesirable microorganisms, ensure adequate nutrition, and growth of desirable yeasts as well as prevent excessive heat and oxidation. The fermentation process lasted for 14 days (Okoro, 2007). A constant alcohol content of 5.9% was reported.

### Clarification of wine

#### *Membrane filtration technique*

The method described by Rosária et al. (2022) with some modification was adopted. Exactly 1 L of unfiltered wine sample was passed through membrane filtration apparatus. A special

porous membrane designed to trap microorganisms and sediments larger than 0.45 µm in size was used.

***Application of Kieselguhr diatomaceous earth powder***

A commercially available diatomaceous earth powder was dissolved in a 10 mL sterile water to form a cake layer of 800 g/m$^2$ on the surface of a filter paper. The wine was poured onto the pre-coated filter paper and allowed to flow into a collecting flask. Thereafter, 7 g/100 mL of the powder was re-added to the wine as a bodyfeed to trap the sediments and haze that may have been left unfiltered during the initial passage through the pre-coated filter paper (Devolli et al., 2017).

***Aging***

The wine was allowed to age in order to improve the flavour, palatability, appearance, clarity, and colour.

***Wine pasteurization***

The wine was pasteurized at 121°C for 15 min to eliminate the need for $SO_2$ addition at the time of bottling.

**Bottling**

The bottles were properly washed, dried and autoclaved at 121°C at 15 psi for 15 min. Aseptically, the bottles were filled with wine very close to a Bunsen burner with flame and screw capped. The bottled wine were refrigerated at 4°C for further maturation.

**Physico-chemical analysis of the wine**

***Determination of pH***

The pH of wine was monitored at 0, 72, and 336 h during clarification using pH meter (model PHS- 25C Precision pH/mV). About 10 mL of wine sample was poured inside a sterile beaker. Standard pH buffer 7.0 and 4.0 was used to standardize the pH meter. Afterwards, the glass electrode was immersed into the sample. The result was recorded after 2 min when the pH reading on the display was stable (Kantiyok et al., 2021).

***Determination of specific gravity***

The specific gravity (g/mL) was measured using the Triple Scale Hydrometer (Model HY110) for beer and wine. The value was taken from calibration on the stem. The specific gravity of the wine was determined at 0, 72, and 336 h.

***Determination of total alcohol content***

The total alcohol content of the wine was determined at 0, 72, and 336 h using the method described by Kantiyok et al. (2021). Exactly 100 mL of the sample was transferred to a graduated cylinder marked 100 mL l as the highest volume. The measuring cylinder and its content was kept inside a refrigerator for 15 min. When the temperature of the wine dropped to 15°C, alcohol meter was gently placed on the wine and allowed to float. The reading on the alcohol meter was noted and expressed as % alcohol (v/v). Using the following formula, purified alcohol content of the wine was calculated.

$$\text{Purified alcohol (L)} = \frac{\text{Volume of alcohol (L)} \times \text{Alcohol percentage}}{100}$$

***Determination of titratable acidity***

The wine sample was agitated to remove excess gas present (degassing). One millilitre (1 mL) phenolphthalein indicator was added to 200 mL of water to adjust the pH. To neutralize the water, 0.1 N NaOH was continuously added until a faint pink colour indicative of the end point was observed. Five millilitres of the degassed sample was pipetted into a 250 mL conical flask after which 100 mL of boiling water was poured inside the flask. The mixture was swirled to release $CO_2$. Titration of 0.1 N NaOH against the mixture inside the flask was carried out until a pale pink colour was observed (end point) and persisted for 30 s. Calculation of titratable acid was done using the following formula. The test was performed on the wine samples at 0, 72, and 336 h (Kantiyok et al., 2021).

$$\% \text{ Tartaric acid} = \frac{(\text{ml alkali}) \times (\text{normality of alkali}) \times 7.5}{\textit{Weight of sample (mls of sample)}}$$

***Determination of total sugar content***

Total sugars of the wine samples were determined using the method described by Dubois et al. (2022). One millilitre of the sample was pipetted into a test tube followed by 1 mL of 5% phenol. From a burette containing concentrated $H_2SO_4$, a total volume of 5 mL of the acid was added to the mixture inside the test tube. The concentrated sulphuric acid was added rapidly. To achieve good mixing, the stream of the acid was directed against the liquid surface rather than the side of the test tube. The mixture was shaken and allowed to stand for 10 min. A second shaking of the mixture inside the test tube was done before it was placed in a water bath for 20 min at 25 to 30°C. The optical density of the solution was read using a spectrophotometer at 490 nm. Preparation of the blank was carried out by substituting 1 mL of distilled water for the sample. A standard curve of glucose was used to estimate the total sugar content of the sample expressed as °Brix. The test was performed on the wine samples at 0, 72, and 336 h.

***Determination of turbidity***

The clarity of the wine was measured using a benchtop turbidity (2100P Turbidometer) nephelometery meter which measured the suspended particulates in the wine sample. The unit of measurement is nepholmetric turbidity units, usually abbreviated and referred to as NTUs. The principle of the test involves shinning infrared light on the wine sample and the light scattered by particles in the wine being measured. The wavelength of the scattered light was measured in nanometer and converted to NTUs. The turbidity of sample was determined at 0, 72, and 336 h.

***Determination of colour intensity***

The colour of the sample is characterized as absorptivity at 420 nm by a Helios-α spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). According to Sudraud method described by Giosanu and Vijan (2013), colour intensity (I) is the sum of

**Plate 1.** Sugarcane juice.

**Plate 2.** Watermelon juice.

absorbances measured at 420 and 520 nm using the UV-1800 Spectrophotometer. The colour intensity (I.C.) was determined at 0, 72, and 336 h. Calculation of colour intensity involves a simple formula presented:

$c = a + b$

where 'c' is the colour intensity; 'a' is the absorbance at 420 nm; and 'b' is the absorbance at 520 nm.

**Sensory evaluation**

The method described by Okafor et al. (2014) with slight modifications was adopted. Sensory evaluation of the pasteurized wine samples was carried out by 10 semi-trained panelist who were undergraduate students in University of Port Harcourt. Each of the panelist was tasked to independently assign a sensory score for

**Plate 3.** Sugarcane-watermelon wine.

aroma, colour, clarity, appearance, and overall acceptability to each wine sample using a 9 point Hedonic scale (9 represents liked extremely and 1 is disliked extremely). The samples were presented to the panelist using a transparent glass cup coded with alphabets. Potable water was also provided for each panelist to rinse his or her mouth before evaluating the next sample.

**Statistical analysis**

Data obtained from the sensory report was analyzed using Microsoft excel and SPSS statistical software. Data source was compared using one way analysis of variance (ANOVA) and significant differences were accepted at $P<0.05$.

## RESULTS

Yeast isolated from palm wine was moist, dull white, and smooth. Microscopic characteristics showed that the isolate was oval to round; large elipsidal budding yeast-like. *S. cerevisiae* count in sugarcane-watermelon wine slightly increased from 8.18 to 8.21 $\log_{10}$ CFU/ mL within 72 h, but declined to 7.15 $\log_{10}$ CFU/ mL at 336 h. Freshly prepared sugarcane and watermelon juice is depicted in Plates 1 and 2, respectively. Plate 3 shows a labeled sugarcane-watermelon wine ready for consumption.

Figure 1 shows the pH of clarified wine samples and the control during maturation. At 72 h, the pH of sugarcane-watermelon wine slightly reduced from initial value of 3.9 at 0 h to 3.8 and 3.7 for the product clarified using Kieselguhr diatomaceous earth powder and membrane filtration, respectively. At 336 h, the pH of clarified sugarcane-watermelon wine samples further reduced to 3.2. The control maintained the pH of 3.2 throughout the period of maturation.

Figure 2 shows the specific gravity of clarified wine samples and the control undergoing maturation. At 0 h, the specific gravity (SG) of sugarcane-watermelon wine

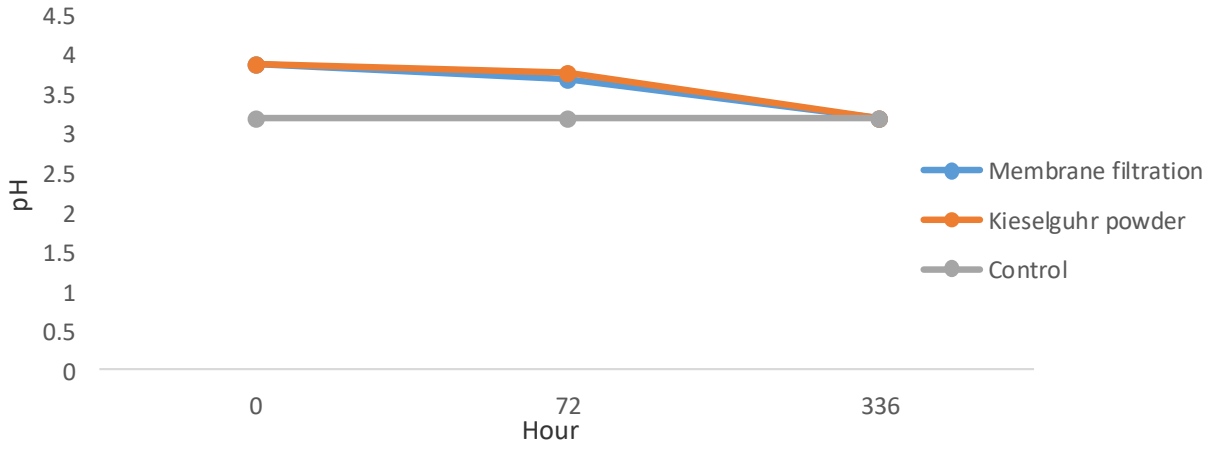


**Figure 1.** pH of clarified wine samples and the control undergoing maturation.

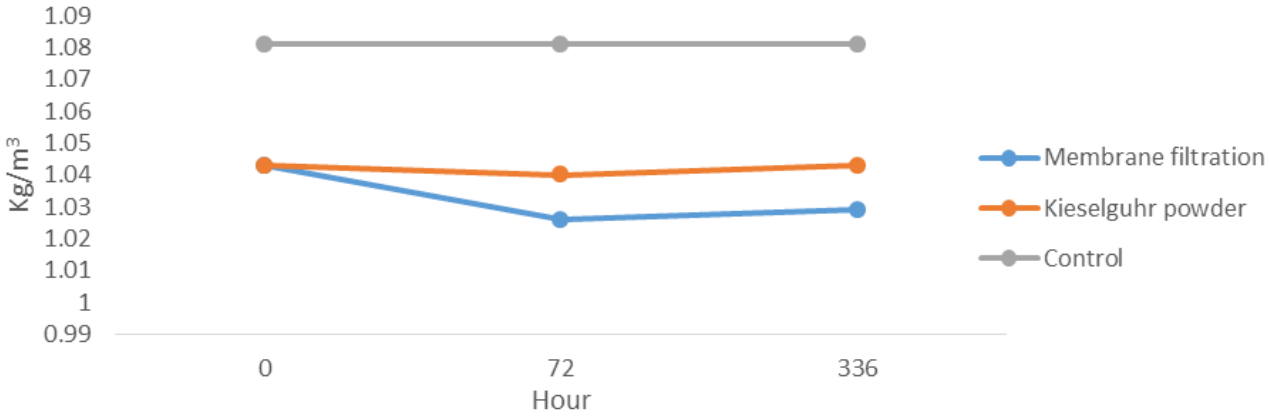


**Figure 2.** Specific gravity of clarified wine samples and the control undergoing maturation.

subjected to clarification and the control was 1.043 and 1.081 kg/m$^3$, respectively. During maturation, there was a slight reduction in SG of the wine samples with the exception of the control.

Figure 3 shows the sugar content of clarified wine samples and the control undergoing maturation. At 0 h, the sugar content of the clarified wines and the control was 10.73 and 19.52°, respectively. While the sugar content of the clarified wine samples reduced to 9.9 °Brix (Kieselguhr powder) and 6.5 °Brix (membrane filtration) at 72 h, the control remains unchanged. At 336 h, the sugar content of the samples slightly increased to 10.8 and 7.2 °Brix for wine clarified using Kieselguhr powder and membrane filtration, respectively.

Figure 4 shows the alcohol content of clarified wine samples and the control undergoing fermentation. There was a reduction in alcohol content of wine clarified using Kieselguhr powder and membrane filtration from 5.9% in both samples at 0 h to 5.48 and 3.47% at 72 h, respectively. At 336 h, there was a slight increase in alcohol content of wine clarified using Kieselguhr powder (5.7%) and membrane filtration (3.7%) whereas the control remain unchanged.

Depicted in Figure 5 is the titratable acidity (TA) of clarified wine samples and the control undergoing maturation. The result shows that TA of clarified wines at 0 h had the same value (0.72 g/l) which reduced to 0.375 (membrane filtration) and 0.405 g/l (Kieselguhr powder) at 72 h. At 336 h, the TA of the clarified wine increased to 0.517 and 0.628 g/l for wine samples clarified using membrane filtration and Kieselguhr powder. However, the specific gravity of the control was 0.9 g/l.

Figure 6 shows the colour intensity of clarified wine samples and the control undergoing maturation. At 0 h, the colour intensity of the clarified wine samples was the same (0.892), but the values reduced to 0.649 and 0.873 in wine samples clarified with membrane filtration and Kieselguhr powder, respectively. Further reduction in

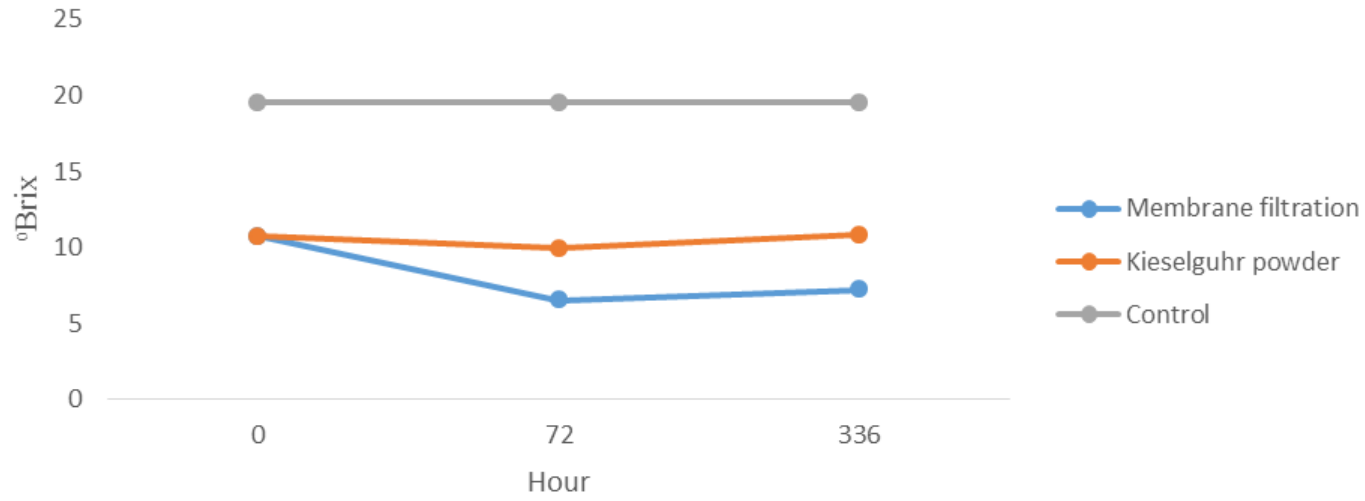


**Figure 3.** Sugar content of clarified wine samples and the control undergoing maturation.

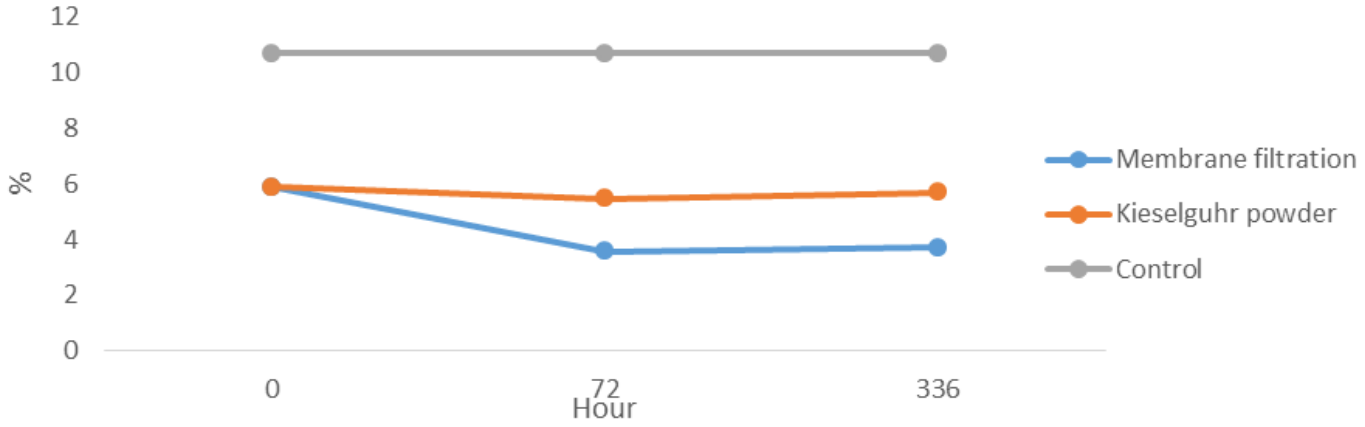


**Figure 4.** Alcohol content of clarified wine samples and the control undergoing maturation.

colour intensity to 0.628 was reported in both clarified wine samples. However, the values for the control was 0.33 throughout the period of maturation.

Figure 7 shows the turbidity of clarified wine samples and the control undergoing maturation. The result shows that turbidity of the control was constant (0.5 NTU) during the period of maturation whereas the samples clarified using membrane filtration and Keiselguhr diatomaceous earth powder which was 94.32 NTU at 0 h steadily reduced to 15 and 20 NTU at 336 h, respectively.

Table 1 shows the average score for each sensory attribute of clarified sugarcane-watermelon wine and the control evaluated by the panelist. The sensory report shows that the control was the most preferred wine followed by the wine clarified using membrane filtration and the least was wine clarified using Keiselguhr diatomaceous earth powder. There was significant difference ($p<0.05$) among the clarified wine samples including the control with regards to each of the sensory attribute.

## DISCUSSION

The result obtained from this study shows that yeast isolated from palm wine was *S. cerevisiae*. It is in agreement with earlier studies which involved the use of *S. cerevisiae* isolated from fresh palm wine to ferment fruit juice into wine (Okoro, 2007; Ogodo et al., 2015; Okeke et al., 2015; Hafsat et al., 2015; Nwinyi and Hassan, 2021). A slight increase in population of *S. cerevisiae* during fermentation of watermelon-sugarcane 'must' is an indication that metabolizable nutrients in the medium were low. Secondly, the physico-chemical properties of the medium might not provide optimum growth conditions for *S. cerevisiae.*

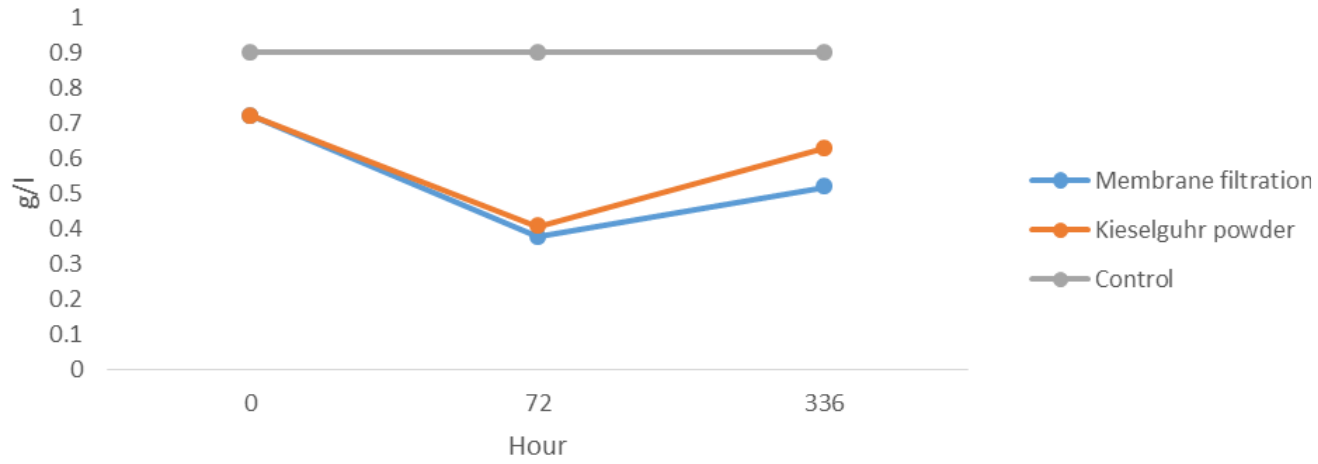


**Figure 5.** Titratable acidity of clarified wine samples and the control undergoing maturation.

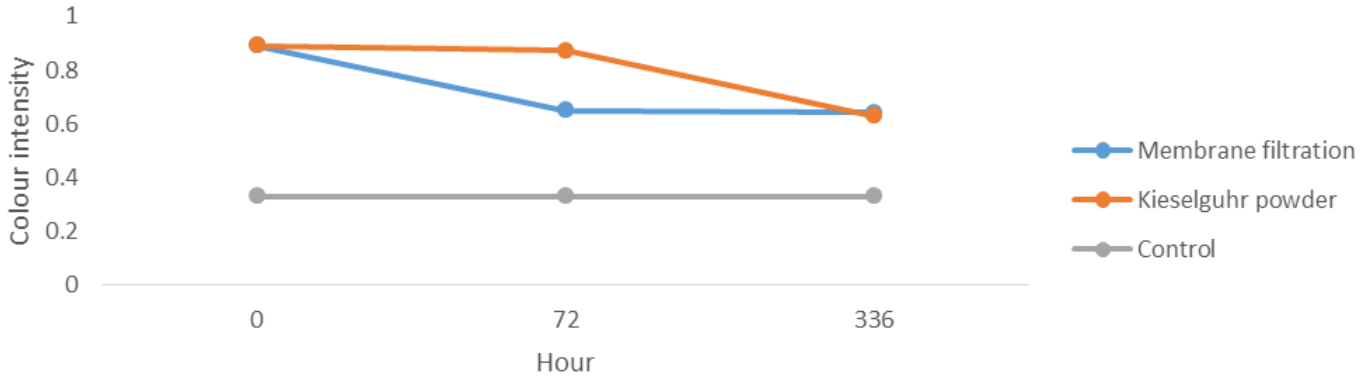


**Figure 6.** Colour intensity of clarified wine samples and the control undergoing maturation.

The reduction in pH of the clarified wine samples during maturation is in agreement with a related study carried out by Zainab et al. (2018) and Soibam et al. (2016). It could be attributed to formation of acetic acid by acetic acid bacteria in the clarified wine samples. The possible release of $CO_2$ which forms a weak acid in the wines is a contributory factor to reduction in pH reported in this study (Ire et al., 2020). At 0 h, the pH of the clarified wines was 3.9. Since the pH of the clarified wine samples was lower than 3.5 at the end of maturation, it is an indication that the product was high in acid content. The pH of wine samples clarified using Keiselguhr diatomaceous earth powder/membrane filtration at 72 and 336 h was 3.8/3.7 and 3.13/3.0, respectively. During the maturation period, the pH of the control was constant (3.2). Aging, clarifying or fining of wine is influenced by pH. Wine that has a pH below 3.5 is suitable for most fining and clearing agents of wine (Saranraj et al., 2017).

Initial reduction of titratable acidity of the wine samples clarified using Keiselguhr diatomaceous earth powder and membrane filtration between 0 and 72 h is in agreement with a related study by Okafor et al. (2014). Increase in titratable acidity of clarified wines between 72 and 336 h is a sign of maturation. The titratable acidity of the clarified wines and the control was within the range 0.5 - 1.0% recommended for good quality wines. According to Okafor et al. (2014), the titratable acidity of table wines is within the range 0.6 - 0.9%. So, the wine samples clarified using Keiselguhr diatomaceous earth powder and membrane filtration and the control are table wines. According to Ire et al. (2020), titratable acidity of wine influences its sensory attributes. Low pH and high titratable acidity are conditions that give competitive advantage to fermentative yeasts in their natural environment. Spoilage microorganisms present in wines are inhibited by low pH, but the condition encourage the growth of desirable organisms such as fermentative yeasts.

At 0 and 72 h, the specific gravity of sugarcane-watermelon wine clarified using membrane filtration/

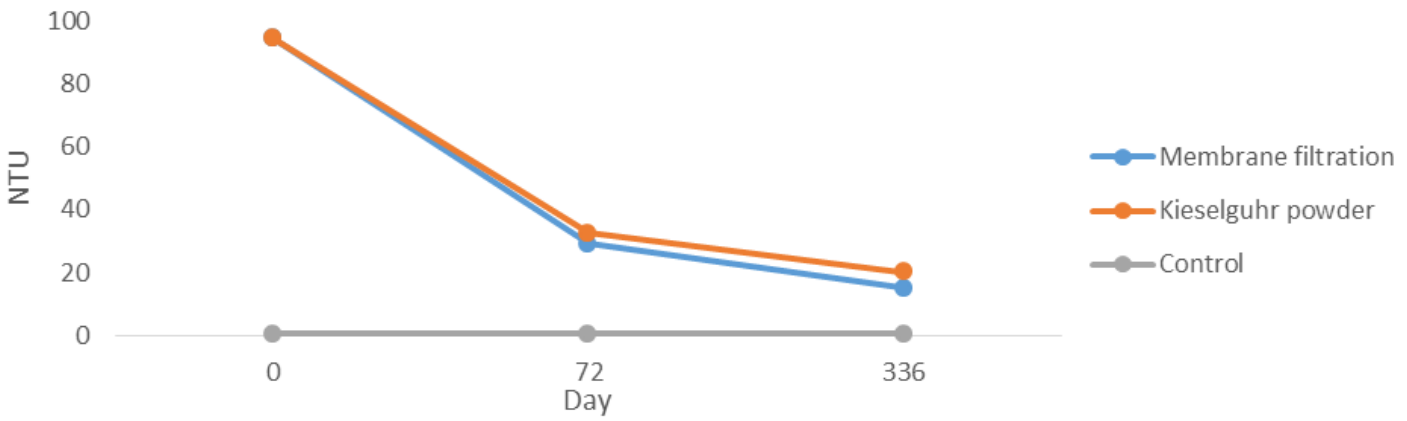


**Figure 7.** Turbidity of clarified wine samples and the control undergoing maturation.

**Table 1.** Average score of sensory attributes (Hedonic scale 1-9) of clarified sugarcane-watermelon wine and the control.

| Attribute | Membrane filtration | Kieselguhr diatomaceous earth powder | Control |
|---|---|---|---|
| Clarity | 7.2±0.63$^{b}$ | 6.1±1.20$^{a}$ | 8.3±0.67$^{c}$ |
| Aroma | 5.4±0.97$^{a}$ | 7.2±1.14$^{b}$ | 8.2±0.79$^{c}$ |
| Appearance | 7.1±1.10$^{ab}$ | 6.4±1.17$^{a}$ | 8.0±0.94$^{b}$ |
| Colour | 5.4±0.97$^{a}$ | 7.3±0.95$^{b}$ | 9.0±0.00$^{c}$ |
| Overall acceptibility | 7.3±0.95$^{b}$ | 6.3±0.95$^{a}$ | 9.0±0.00$^{c}$ |

Values show means of sensory scores of ten panelists ±SD. Values with different superscript across the row are significantly different (P<0.05). Hedonic scale: 9- like extremely; 8- like very much; 7- like moderately; 6- like slightly; 5- neither liked nor disliked; 4- disliked slightly; 3- disliked moderately; 2- disliked very much; 1- disliked extremely.

Keiselguhr diatomaceous earth powder was 1.043/1.026 and 1.043/1.040 kg/m$^3$, respectively. The reduction in specific gravity of the wines agrees with the findings by Okeke et al. (2015). In a related study, Zainab et al. (2018) reported that specific gravity of watermelon 'must' and watermelon wine was 1.075 and 1.020, respectively. This could be attributed to the activities of *S. cerevisiae* in the wine. At 336 h, the specific gravity of sugarcane-watermelon wine clarified using membrane filtration and Keiselguhr diatomaceous earth powder was 1.029 and 1.043 kg/m$^3$, respectively.

During maturation of wine, the reduction in sugar content of sugarcane-watermelon wine clarified using membrane filtration (10.73 to 6.3 °Brix) and Keiselguhr diatomaceous earth powder (10.73 to 9.9 °Brix) is in agreement with the report by Hafsat et al. (2015). This could be as a result of yeast utilizing sugar present in the medium for production of alcohol and other by-products of fermentation. This study shows that wines clarified by membrane filtration (7.2 °Brix) had a lower sugar content compared with wines clarified using Keiselguhr diatomaceous earth powder (10.8 °Brix). According to Nilar (2020), alcoholic beverage regarded as sweet wine contains residual sugar after fermentation within the range of 1 to 14%. During fermentation, sugar is a very important substrate for the production of ethanol, lactic acid and $CO_2$ (Saranraj et al., 2017). According to Okemini and Dilim (2017), insufficient quantity of sugar in fermenting 'must' is a challenge encountered during production of non-grape wine. In order to overcome the challenge, sugar is usually added to 'must' in the course of producing wine.

Findings from this study show that alcohol content of clarified sugarcane-watermelon wine reduced during maturation between 0 and 72 h. At 0 h, the alcohol content of the clarified wine was 5.9%. The alcohol content of wine clarified using membrane filtration and Keiselguhr diatomaceous earth powder at 72 h reduced to 3.57 and 5.48%, respectively. At 336 h, it was observed that alcohol content of wine clarified using membrane filtration (3.7%) was lower than wine clarified using Keiselguhr diatomaceous earth powder (5.7%). Throughout the period of maturation, the alcohol content of the control was constant (10.71%). According to Schmidtke et al. (2012), wine transported across a semi-permeable membrane or membrane is aimed at reducing its ethanol content. Since the alcohol content of clarified sugarcane-watermelon wine and the control was below the standard (7 - 24% v/v alcohol) stipulated in the Federal Alcohol Administration Act of the United States of

America (USA), the final product could be regarded as a dealcoholized wine. A slight increase in alcohol content during maturation of the clarified wines could be attributed to the release of various by-products of fermentation which include ethanol and alcohols. It suggests that fermentation was not completed. An increase in alcohol content during wine maturation agrees with the findings by Hasfat et al. (2015) and Soibam et al. (2016).

Findings from this study shows that colour intensity of sugarcane-watermelon wine clarified using membrane filtration and Keiselguhr diatomaceous earth powder steadily reduced during maturation. At 0 h, the colour intensity of clarified wines was 0.892 nm. The result for the wine clarified using membrane filtration/Keiselguhr diatomaceous earth powder at 72 and 336 h was 0.649/0.873 and 0.642/0.628 nm, respectively. The result is in agreement with the findings by Babincev et al. (2016) from a related study. On average, the colour intensity of sugarcane-watermelon wine clarified using Keiselguhr diatomaceous earth powder had a higher colour intensity than sugarcane-watermelon wine clarified by membrane filtration. Although the colour intensity of the control (0.330 nm) was constant during maturation, it was quite lower than sugarcane-watermelon wine clarified using membrane filtration and Keiselguhr diatomaceous earth powder.

During maturation of sugarcane-watermelon wine, the turbidity of unclarified wine was relatively stable (0.5 NTU) whereas the samples clarified using Keiselguhr diatomaceous earth powder and membrane filtration is within the range 15 - 94.32 NTU. According to Awe (2018), aging of wine without applying clarifying agent is a better approach to clarify wine. The turbidty of clarified wines at Day 0 was higher than the control sample. This result could be as a result of fining agent slurry added to the wine. The result is in agreement with the findings by Awe (2018). Among the two clarification methods sugarcane-watermelon wine samples were subjected to, findings from this study show that membrane filtration was more effective in reducing wine turbidty than the use of Keiselguhr diatomaceous earth powder. According to Awe (2018), filtration is not as effective as the use of fining agents which can only remove dead yeast cells and fruit fragments present in wine. The use of fining agent such as bentonite, kieselsol, casein, kaolin, albumin, gelatin and silicon dioxide is capable of removing soluble substances present in wine which include proteins, polymerized tannins, and colouring phenols.

The sensory report indicates that appearance, clarity and overall acceptability of sugarcane-watermelon wine samples clarified by membrane filtration were assigned a higher sensory scores than wine samples clarified using Keiselguhr diatomaceous earth powder. With regards to aroma and colour, wine samples clarified using Keiselguhr diatomaceous earth powder were assigned a higher sensory scores than sugarcane-watermelon wine samples clarified by membrane filtration. In a related study, Soibam et al. (2016) reported that wine produced using watermelon-sugarcane juice blended at 1:1 (v/v) had a good sensory rating with regards to colour, flavour and overall acceptability. It is worthy to note that all the sensory attributes of the control (sugarcane-watermelon wine without clarification) were assigned higher sensory scores than sugarcane-watermelon wine samples clarified using Keiselguhr diatomaceous earth powder or membrane filtration.

## Conclusion

Winemakers who intend to use non-grape fruit(s) such as watermelon and other juices e.g. sugarcane juice to produce an acceptable wine in commercial quantity should evaluate the effect of clarification agent/method on the physicochemical and sensorial quality of the product. The acceptability of wine by consumers is influenced by the choice of fermentation substrate, yeast strain, among other factors. This study has proven that Keiselguhr diatomaceous earth powder or membrane filtration as clarification method/agent had some effect on the quality of sugarcane-watermelon wine fermented by *S. cerevisiae* isolated from palm wine.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Distribution of cassava mosaic begomoviruses in the North-Western provinces of Democratic Republic of Congo

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**This study was carried out for the first time in the North-Western Democratic Republic of Congo (DR Congo) as part of the Viral Epidemiology in West and Central Africa (WAVE) program. Its dual aim was to identify and map the viruses responsible for African cassava mosaic in this part of the country. Cassava leaf samples were collected during a geo-referenced survey conducted from 1st February to 31st March, 2022 in three provinces: Mongala, Nord Ubangi, and Sud Ubangi. Molecular diagnostics were carried out to identify the viral strains associated with Cassava Mosaic Disease (CMD). The results showed the presence of African Cassava Mosaic Virus (ACMV) and East African Cassava Mosaic Cameroon Virus (EACMCV) in the study area. EACMCV was present in all provinces, while ACMV was only reported in the province of Nord-Ubangi. This study recommends good agricultural practices and participatory surveillance as a strategy for managing CMD in the Democratic Republic of Congo.**

**Key words:** Epidemiology, cassava, viruses, East African Cassava Mosaic Cameroon (EACMCV), African Cassava Mosaic Virus (ACMV).

## INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important food crops in sub-Saharan Africa. It is a source of income and a key element of food security for poor farmers in the eastern and central Great Lakes region (Maruthi et al., 2004; Legg et al., 2017). In the Democratic Republic of Congo (DR Congo), cassava covers the daily

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**Figure 1.** Cassava leaf showing symptoms of CMD.

calorific needs of over 70% of the population (Monde et al., 2013; Akinpelu et al., 2012). Its consumption amounts to 250 kg/person/year, making the country one of the world's leading cassava consumers (FAO, 2018). DR Congo is ranked the second producer of cassava in Africa after Nigeria and fifth in the world, with production estimated at over 45 million tonnes (FAO, 2022).

Despite its ability to withstand extreme growing conditions: low mineral supply (Hillocks et al., 2002), drought, and climatic disturbances (Rey and Vanderschuren, 2017; Jarvis et al., 2012; El-Sharkawy, 2006; De Tafur et al., 1997; Cock et al., 1985), cassava is subject to strong biotic pressure (Legg et al., 2011). Diseases and pests affect its growth and development and consequently reduce its yield in several production zones in Africa (Bakelana et al., 2019; Legg et al., 2011). Cassava mosaic disease (CMD) is one of the major constraints of cassava production in the 21st century (Winter et al., 2010; Alicai et al., 2007). The disease is spread by infected cuttings and by whitefly vectors (*Bemisia tabaci*) (Chi et al., 2020; Njoroge et al., 2017; Maruthi et al., 2005). CMD is widespread in several countries in East, Central, and West Africa and towards Southern Africa (Chikoti et al., 2015; Tresh and Cooter, 2005; Sseruwagi et al., 2004).

CMD is caused by a Cassava Mosaic Begomoviruses (CMBs) complex composed of eleven species of bipartite Begomoviruses, nine of them have been reported in Africa (Crespo-Bellido et al., 2021; Maruthi et al., 2004). The distribution of these begomoviruses associated with CMD symptoms often varies from one country to another and/or from one region to another within the same country (Monde et al., 2010; Patil and Fauquet, 2009; Ndunguru et al., 2005; Ariyo et al., 2005; Were et al., 2004).

Several epidemiological studies carried out in the Democratic Republic of Congo (DR Congo) reported the presence of CMD in the majority of cassava-growing areas. These studies have focused on incidence and severity, identification and/or characterization of the viruses responsible, as well as assessment of the impact of the disease on certain cassava varieties/accessions, and finally, screening for resistance in different agroecosystems in the country (Biola et al., 2022; Bisimwa et al., 2019, 2015, 2012; Monde et al., 2012, 2010; Tata-Hangy et al., 2007). Despite these various studies carried out in DR Congo, the distribution of African cassava mosaic viruses remains less documented in several provinces. The present study aims to identify and map the distribution of CMD-associated viruses in North-Western DR Congo.

## MATERIALS AND METHODS

### Cassava leaves sampling

A total of two hundred and seventy-six CMD-symptomatic cassava leaves were collected from cassava fields aged between 3 and 9 months (Figure 1). Fields were prospected at intervals of 5 to 10 km in the three north-western provinces of the Democratic Republic of Congo where any CMD-associated virus survey had been conducted (Figure 3). The leaves were air-dried in herbarium beds and then taken to the WAVE Molecular Diagnostic Laboratory at INERA M'vuazi for analysis. The coordinates of each sample were recorded using GPS Garmin 62s.

### DNA extraction

Total DNA extraction was performed using the modified CTAB method (Lodhi et al., 1994). 0.5 g of each sample was ground in 100 ml extraction buffer containing 2 g Cethyl Triethyl Ammonium Bromide (2%), 4.09 g NaCl (1.4 M), 200 µl B-mercaptol-Ethanol (0.2 M), 0.37 mg EDTA $2H_2O$ (20 mM), 0.6 g Tris-HCl (100 mM), and 100 ml water. The DNA obtained was kept cold (-20°C) before being used for biomolecular analysis.

**Table 1.** Primers used to identify viruses associated with CMD.

| No. | Primers used | Primer sequences | Target region | Size (bp) | References |
|---|---|---|---|---|---|
| 1 | JSP 001/JSP002 | 5 'ATGTCGAAGCGACCAGGAGAT 3'<br>5' TGTTTATTAATTGCCAATACT 3' | ACMV DNA-A (CP) | 783 | Pita et al. (2001) |
| 2 | ACMVB F/R | 5' TCGGGAGTGATACATGCGAAGC 3'<br>5' GGCTACACCAGCTACCTGAAGCT 3' | ACMV DNA-B (BV1/BC1) | 628 | Matic et al. (2012) |
| 3 | JSP001/JSP003 | 5 ' ATGTCGAAGCGACCAGGAGAT 3'<br>5' CCTTTATTAATTTGTCACTGC 3' | EACMV DNA-A (CP) | 780 | Pita et al. (2001) |
| 4 | CMBRepR F/EACMVRe R | 5' CRTCAATGACGTTGTACCA 3'<br>5' GGTTTGCAGAGAACTACATC 3' | EACMV DNA-A (AC1) | 650 | Alabi et al. (2008) |
| 5 | VNF031 F/R | 5' GGATACAGGATAGGGTTCCCAC 3'<br>5' GACGAGGACAAGAATTCCAAT 3' | EACMV-CM DNA-A (AC2/AC3) | 560 | Fondong et al. (2000) |
| 6 | ACMV21 F/R | 5' CRTCAATGACGTTGTACCA 3'<br>5' GGTTTGCAGAGAACTACATC 3' | DNA-A (AC3-AC2-AC1) of ACMV, EACMV, EACMCV, EACMKV, EACMMV, EACMZV, and SACMV | 552 | Matic et al. (2012) |

**Polymerase chain reaction (PCR) amplification**

PCR amplification was carried out according to the harmonized protocol of the Central and West African Virus Epidemiology Program. The PCR Mix was prepared to a final volume of 25 µl containing 5 µl of extracted DNA, previously diluted 1:50, 11.375 µl of distilled water, 0.5 µl of dNTP, 1 µl of $MgCl_2$, and 5 µl of colourless green Buffer and 1 µl of each of the specific primer pairs listed (Table 1). The PCR program was performed at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 1 min, hybridization at 54°C for 1 min, elongation at 72°C for 1 min, and final elongation at 72°C for 10 min.

**Analysis of PCR products by agarose gel electrophoresis**

Ten microliters of amplicons (PCR products) from each sample were migrated in electrophoresis under 90 V at 58 mA for 60 min in 1.2% agarose gel: 2.4 g agarose in 200 ml TAE (1x). Bands were visualized on the UV transilluminator at 254 nm. A 1 Kb DNA molecular weight marker was used to determine amplicon size (Figure 2).

## RESULTS

Two viral strains are associated with CMD in North-Western DR Congo. PCR detected 2 viral strains of CMD in the cassava leaf samples diagnosed (Figure 2).

CMD is widespread in north-western DR Congo. Two viral strains were identified in the three provinces as mentioned in Table 2. These viral strains are ACMV and EACMCV (Figure 2). The EACMCV strain was identified in all three provinces surveyed, while ACMV was only found in the province of Nord-Ubangi. The proximity of the province of Nord-Ubangi to the Central African Republic could justify the presence of the ACMV strain in this part of the national territory. Indeed, in his study on the epidemiology of African CMD in the Central African Republic in 2022, Zinga et al. (2012) identified two viral strains. The distribution of EACMCV in the three provinces surveyed is explained by the evolution of CMD from east to west DR Congo. This strain is different from what was characterized in the majority of the various agroecological zones of the DR Congo (Yangambi and South Kivu). The low rate of virus identification is due to the primers used in this study and suggests that they are not specific to the CMD virus in DR Congo that is widely infected by the Uganda variant (Monde et

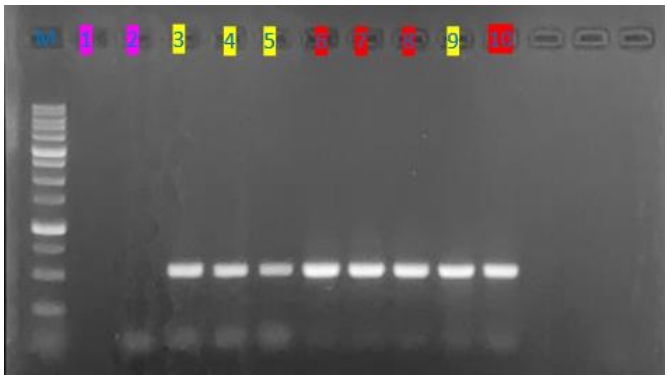


**Figure 2.** Gel electrophoresis of ACMV (628 pb: 3, 4, 5 & 9); EACMCV (650 pb: 6, 7, 8 & 10), and Negative control (1 & 2).

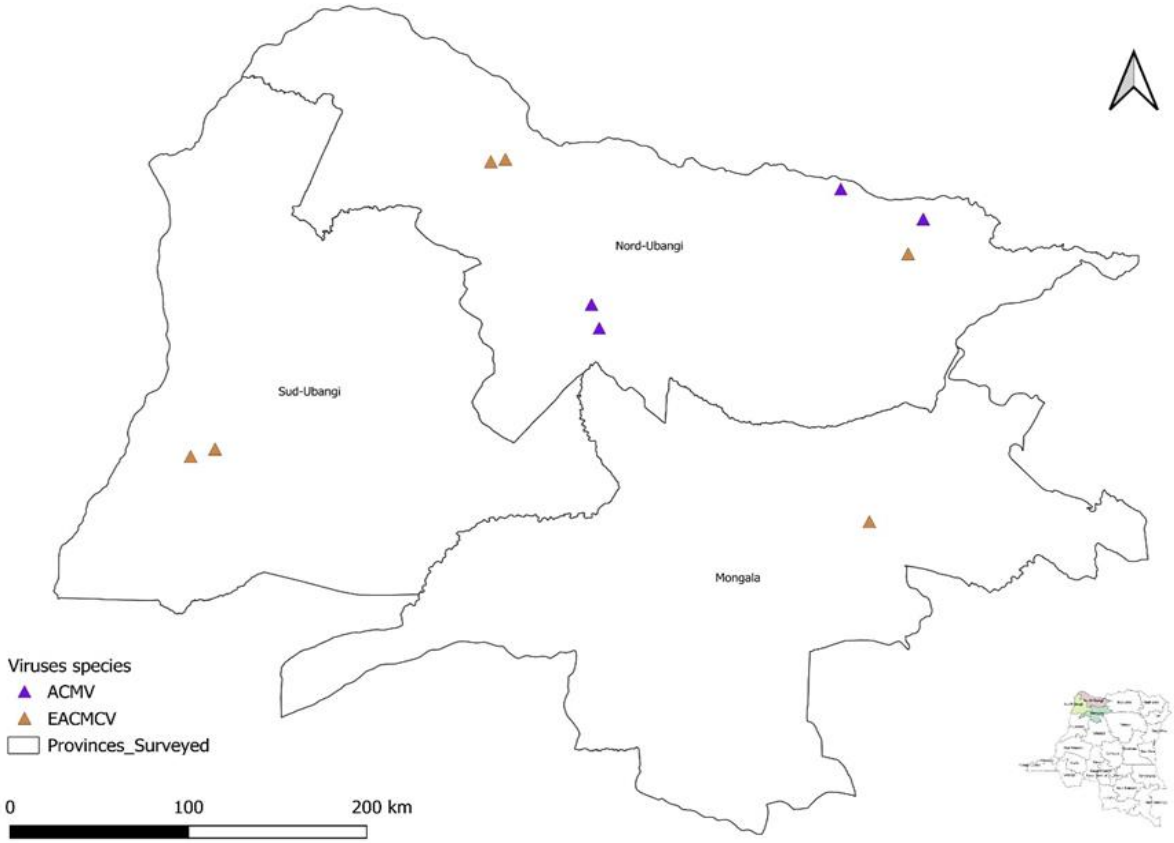


**Figure 3.** Distribution of cassava mosaic begomoviruses isolates in three surveyed provinces.

al., 2010; Bisimwa et al., 2012).

## Distribution of CMD-associated viruses in the Mongala, Nord-Ubangi, and Sud-Ubangi provinces

The CMD-associated virus distribution identified in the three north-western provinces of DR Congo was mapped using geospatial coordinates (latitude, longitude, and altitude) recorded for collected samples using QGIS software version 3.14.15 (Figure 3). It showed the two strains were differently distributed in the three provinces. ACMV has been identified in the province of Nord-Ubangi, while EACMCV has been identified in all three

**Table 2.** Two viral strains associated with CMD in North-Western DRC.

| Province | Total samples | Positive samples (%) | Negative samples (%) | Virus identified |
|---|---|---|---|---|
| Mongala | 38 | 1 (2.63) | 37 (97.37) | EACMCV |
| Nord-Ubangi | 122 | 7 (5.74) | 115 (94.26) | EACMCV (3), ACMV (4) |
| Sud-Ubangi | 105 | 2 (1.91) | 103 (98.09) | EACMCV |

provinces.

## DISCUSSION

### Two viral strains are responsible for CMD in north-western DR Congo

CMD is caused by eleven cassava mosaic geminiviruses (CMGs), nine of which are present in Africa, singly or in combination (Legg and Fauquet, 2004; Ndunguru et al., 2005; Bull et al., 2006; Patil and Fauquet, 2009; Harimalala et al., 2012; Tiendrébéogo et al., 2012; Zinga et al., 2016). The results of molecular analyses revealed the presence of two viral strains in the three north-western provinces of DR Congo: African Cassava Mosaic Virus (ACMV) and East African Cassava Mosaic Cameroon Virus (EACMCV). These two viral strains are the most widespread in most African countries (Legg et al., 2011). EACMCV is present in all provinces, while ACMV has only been identified in the province of Nord-Ubangi. This province shares borders with the Central African Republic and has been hosting Central African Republic (CAR) refugees for several years. This situation suggests the possibility of the introduction of propagation materials from the CAR, where ACMV has been identified and characterized as a single infection or mixed infection on cassava (Zinga et al., 2012). In contrast, ACMV is thought to come from other eastern provinces of DR Congo neighbouring Uganda and move westwards before reaching the CAR (Tocko-Marabena et al., 2017). Viral diseases of cassava, including CMD transmission, spread from one country to another or from one region to another (De Brun et al., 2012). CMD has evolved from East Africa through Central Africa to West Africa (Legg et al., 2011) and is mostly spread through the infected cuttings that farmers take from their own or neighbouring fields (Armel et al., 2023). In DR Congo, CMD is present in all cassava-growing regions across the country. The EACMV-Ug and ACMV strains prevalent in north-western DR Congo are thought to have originated in areas previously infected by this strain, namely the agroecosystems of mountainous South Kivu (Bisimwa et al., 2012) and the Yangambi region (Monde et al., 2010), where this viral strain was identified and characterized on cassava and other host plants (*Pueraria javanica* and *Centrosoma pubescent*). EACMCV founded in DRC suggests the expansion of the Cameroon strain in Central and West African countries from Cameroon where it has been characterised (Alabi et al., 2008b). EACMCV were also found in Nigeria on Cassava and alternate host (Alabi et al., 2008a), Burkina Faso (Soro et al., 2021) and in Côte d'Ivoire (Amoakon et al., 2023).

### Conclusion

This study aimed to identify and map the CMD viruses in the provinces of Mongala, Nord-Ubangi and Sud-Ubangi in North-Western DR Congo. To achieve this, cassava leaves in fields on plants showing characteristic symptoms of CMD were sampled in three North-Western provinces of DR Congo. These cassava samples were sent to WAVE INERA-M'vuazi for molecular diagnosis. The laboratory results revealed the presence of two strains that are associated with CMD: ACMV and EACMCV. These two strains are distributed across the three provinces in different pathways. The results of the present study suggest the use of various primers to identify cassava viruses that cause the CMD in DR Congo and recommend participatory surveillance as a control strategy against African CMD in DR Congo in general and in the northwest of the country in particular.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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