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*Full Length Research Paper*

# The effect of foliar application of zinc oxide nanoparticles and *Moringa oleifera* leaf extract on growth, biochemical parameters and in promoting salt stress tolerance in faba bean

**Sherif M. Ragab[1,2]*, Losenge Turoop[1,3], Steven Runo[1,4] and Steven Nyanjom[1,5]**

[1]Department of Molecular Biology and Biotechnology, Pan African University Institute of Science Technology and Innovation, Nairobi, Kenya.
[2]Department of Biochemistry, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
[3]Department of Horticulture and Food Security, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.
[4]Department of Biochemistry, Microbiology and Biotechnology, Kenyatta University, Nairobi, Kenya.
[5]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

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**Salinity is major abiotic stress limiting plant growth worldwide. Plant adaptation to salinity stress involves diverse physiological and metabolic pathways. In this study, we assessed the effects of foliar application of zinc oxide nanoparticles (ZnONPs) and *Moringa* leaf extract (MLE) on salt tolerance in faba beans (cultivar, Sakha 4). Morphological, chemical, and biochemical parameters of plants grown under saline condition (50 and 100 mM NaCl) were assessed 60 days after sowing. Salt stress caused a remarkable reduction in growth traits, photosynthetic pigments, proline, minerals, total phenol, and enzyme activity of the faba bean variety. The results showed that foliar spraying of MLE and ZnONPs on faba bean grown under salt-stressed conditions promoted growth parameters (that is, shoot length, numbers of leaves, relative water content, shoot, roots fresh and dry weights), photosynthetic pigments (that is, chl a, b, total chlorophyll and carotenoids), proline, mineral elements ($Na^+$, $K^+$, $Ca^{+2}$, and $Zn^{+2}$), total phenol and enzyme activity (POX, PPO, APX, and CAT) compared to control plants. Based on these findings, the potential of foliar spraying application of MLE and ZnONPs may help alleviate the negative effect of salinity on growth, photosynthesis efficiency, and biochemical properties of faba bean**.

**Key words:** Faba bean, *Moringa oleifera*, antioxidant enzyme activity, ZnO nanoparticles, salt stress, proline.

## INTRODUCTION

Faba bean (*Vicia faba* L.) is the third most important legume crop grown in more than 60 countries as a cool-season legume (Bohra et al., 2014). It is a high-protein grain legume that provides 20 to 36% of protein to human

*Corresponding author. E-mail: sherifsagher84@gmail.com.

and animal diets (Bulut and Akinci, 2010). Additionally, faba bean is rich in Fe, Mg, Zn, K, and Ca, as well as amino acids, carbohydrates, vitamins, and essential nutraceuticals (Koivunen et al., 2016). However, faba bean production is adversely affected by many abiotic stresses among them salinity and water deficiency. High concentrations of $Na^+$ and $Cl^-$ ions in the soil adversely affect faba bean growth and photosynthesis due to chlorophyll degradation and the damaging effects of both ions on photosystem II (PSII) (Tavakkoli et al., 2010). Additionally, salinity affects seed germination, seedling growth, vegetative growth, reproductive phase, maturity, and grain production (Latef et al., 2021). Overall, salinity limits faba bean productivity in semi-arid regions, causing yield losses of up to 50% (Farooq et al., 2017). *Moringa oleifera* leaf extract (MLE) has been utilized as a natural plant growth enhancer in other crops, boosting plant growth and biomass development while developing tolerance to salt stress (Yasmeen et al., 2013). MLE is high in ascorbates, phenolic compounds, potassium, and calcium, all of which are used as exogenous plant growth stimulants (Waqas et al., 2017).

MLE is a natural plant growth regulator since it includes zeatin, a natural derivative of cytokinin, proteins, vitamins E, phenolics, ascorbic acid, vital amino acids, and different mineral components, making it a possible natural growth booster (El-Hack et al., 2018). MLE foliar spray improved crop production by promoting vigorous plant growth, preserving optimum tissue water status, improving membrane integrity, and increasing antioxidant content (Rehman et al., 2014). Foliar application of $NP_S$ is considered a more convenient and straight forward technique since plants directly absorb unlike the application of chemical fertilizers, reducing soil contamination (Kah et al., 2018). The nanoparticle fertilisers are a technique for boosting the availability of nutrients to plant leaves, hence enhancing the efficiency of plant nutrient absorption and yield (Vishekaii et al., 2019). In agriculture, ZnONPs are employed as fertilizers, growth regulators, pesticides, and herbicides (Khot et al., 2012). The use of ZnONPs has enhanced height, leaf number, fresh and dry weight of leaves, chlorophyll, essential oil, and phosphorus concentration (Vafa et al., 2015). Application of ZnONPs significantly increased *Cyamopsis tetragonoloba* plant biomass, chlorophyll, protein synthesis, rhizospheric microbial population, acid and alkaline phosphatase and phytase activity in the bean rhizosphere (Raliya and Tarafdar, 2013). ZnONPs supplemented with MS medium promoted somatic embryogenesis, shooting, plantlet regeneration, and increased proline synthesis and the activities of superoxide dismutase, catalase, and peroxidase, which improved resistance to biotic stress (Helaly et al., 2014). The green strategy for nanoparticle synthesis is based on the plant supply and the organic molecules present in plants (enzymes, amino acids, proteins, saccharides, vitamins, and organic acids) that may operate as reducing and/or capping agents during metal nanoparticle formation (Zafar et al., 2020). ZnONPs are one of the most widely employed nanoparticles in agriculture because they are linked to secondary metabolite pigment production, protein, and sugar content nutrient translocation, and they scavenge free oxygen radicals generated in stressed plant tissues (Zafar et al., 2016). The natural extract from Moringa oleifera (MO) leaves has been shown to be an efficient reducing/oxidizing, capping, and stabilizing agent in the synthesis of ZnO nanoparticles (Matinise et al., 2017).

The present study, therefore investigated the effect of foliar application of nanoparticle and *Moringa* leaf extract on the growth parameters, biochemical contents, photosynthetic pigment, and antioxidant enzymes in faba bean plants.

## MATERIALS AND METHODS

### Plant and field experiments

Field experiments were carried out in a greenhouse at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Kenya. The experiments were placed from December 2020 to May 2021. Faba bean cultivar seeds (Sakha 4) were obtained from the Agricultural Research Center in Sakha, Kafr El Sheikh, Egypt, and were selected for size and colour consistency. The selected seeds were washed with distilled water sterilized in 1% (v/v) NaClO for 2 min, then rinsed with distilled water and left to dry at room temperature for 2 h. The seeds were then put in a Petri dish on sterilized, moistened filter paper and stacked in darkness for 48 h. Then, the seeds were planted in plastic pots (25 × 40 cm) that contained 91.44% sand, 6.56% silt, and 4.0% clay, and had a pH of 7.5 and EC of 1.3 $dSm^{-1}$. The seed was grown in a plant growth chamber, the temperature was set to 28°C during the day and 20°C at night, and the relative humidity was maintained at 70 to 80%. The experiment was set up in the form of a Randomized Complete Blocks Design (RCBD) with 3 replications. Each pot was irrigated with tap water (control plants) and two salinity levels (50 and 100 mM NaCl).

### Experimental treatments

The salinity levels were obtained by adding appropriate amounts of dry NaCl to water. After that, the pots were irrigated for a day after two days with tap water for 7 days. The treatments were administered 21 days after the seeds were sown. After that, the salt treatments were administered to each pot at 7-day intervals for 90 days. Foliar application of zinc oxide nanoparticles (ZnONPs) 50 mg/L and MLE 50 ml/L was done twice a week from the 30th day after sowing. Tween 20 (0.05%) was added to spray solutions as a wetting agent.

### Preparation of extraction from *M. oleifera* plant leaves

The leaves of *M. oleifera* were gathered from the JKUAT garden. The leaves were taken from the stems and washed with distilled water and left to dry. Phytochemicals from the dried leaves were then extracted with distilled water, according to Pervez et al. (2017). Briefly, the leaves were dried and finely ground to a fine powder. The MLE extraction was done by 100 g of powdered soaking in 1000 mL of distilled water. The mixture was macerated for 48 h at

35°C and later filtered through (Whatman No.1) filter paper and the extraction was stored at -20°C for further uses.

### Synthesis of zinc oxide nanoparticles from leaf extraction

#### *Precipitation method*

ZnONPs were formed from the reaction of zinc nitrate with a variety of other chemicals, as described by Pal et al. (2018). Briefly, 4.75 g zinc nitrate Zn $[NO_3]_2.6H_2O$ was dissolved in 90 mL of $dh_2o$ and stirred for 40 min for complete dissolution. For the synthesis of NPs, a dropwise addition of *M. oleifera* leaf extract solution (10 mL) was made into the zinc nitrate solution with vigorous stirring at 80°C for 3 h. The solution gradually becomes murky yellow in colour. The solution was then centrifuged at 10,000 rpm for 10 min and washed with $dh_2o$ to remove any contaminants or ions that have been absorbed. Finally, the product was dried in a laboratory oven at 70°C for 48 h.

#### *Sample characterizations*

Optical properties of Nanopowder from the amount of Green synthesis of zinc oxide nanoparticles (ZnONPs) were characterized based on UV-Vis Spectrophotometer absorption spectra with the wavelength range of 200 to 600 nm. X-ray diffraction (XRD) patterns of the as-synthesized ZnONPs were determined through the Rigaku D/Max-lllC X-ray diffractometer (Rigaku Int. Corp.Tokyo, Japan). It produced diffractions at a scanning rate of 20 $min^{-1}$ in the 2 to 500 at room temperature with a CuKa radiation set at 40 kV and 20 mA. Fourier transforms infrared (FTIR) spectra were recorded on Jasco FT-IR5300 model spectrophotometer in KBr pellets. High-resolution transmission electron microscopy (SEM) was used to determine the particle size and characteristics of the samples.

### Data collection

Ten randomized plants were selected from each plot at 60 days after sowing (DAS) to determine the plant's development factors, such as height (cm), the number of leaves/plants (shoots, roots, fresh and dry weight), antioxidant enzyme activity, photosynthetic activity, and biochemical content.

### Measurement of plant biomass and growth parameters

Growth measurements for the plants exposed to salt treatments were taken after 30 days of treatment and after 40% of the plants at the highest concentration (100 mM), they are dried. The three replicates taken for each treatment were used to calculate the mean of each measurement. The mean of each measurement was calculated based on the three replicates that were obtained for each treatment. The length of the shoot (SFW), the number of plant leaves, and the size of the root were all measured (RFW). The samples were packaged and kept in an oven at 60°C for 72 h. Following that, the samples were completely dehydrated, and the root dry weight (RDW) and shoot dry weight (SDW) were determined.

#### *Leaf relative water content*

The relative water content (RWC) of the sample was measured and calculated according to Gulen and Eris (2003) using the following formula:

$$RWC = (FW - DW) / (TW - DW) \times 100 \quad (1)$$

where FW = fresh weight, TW = turgid weight, and DW = oven dry weight.

### Analysis of pigment contents

The contents of chlorophyll (Chl a, Chl b and carotenoids) in fresh leaves were measured using spectrophotometry (Khalilzadeh et al., 2016). The fresh leaves were taken from the midst of five major leaves (60 days after sowing). Approximately 1 g of leaf tissue was extracted by grinding in a mortar using 20 ml (80% v/v) acetone and 0.5 g calcium carbonate to equalize the cellular sap acidity. The extract was filtered through a No. 2 filter paper. After complete filtrates were obtained in new test tubes and absorbance was taken at 645, 663, and 470 nm wavelengths using a spectrophotometer. To calculate chlorophyll contents following equations were used.

$$Chl\ a\ (mg\ g-1\ FW) = (12.72 \times OD663 - 2.59 \times OD645) \times V / (1000 \times W) \quad (2)$$

$$Chl\ b\ (mg\ g-1\ FW) = (22.88 \times OD645 - 4.67 \times OD663) \times V / (1000 \times W) \quad (3)$$

$$Total\ Chl\ (mg\ g-1\ FW) = (17.90OD645 + 8.08OD663) \times V / (1000 \times W) \quad (4)$$

$$Carotenoids\ (mg\ g-1\ FW)\ [(1000 \times OD470 - 3.27 \times Chl\ a - 104 \times Chl\ b)/229] \times V/(1000 \times W) \quad (5)$$

where V is the total volume in milliliter (mL) of 80% (v/v) acetone and W is the fresh weight (FW) of the sample (g).

### Proline content

The proline content in leaves following the anthesis stage, which occurred 60 days after planting, was determined with some modifications by Bates et al. (1973). Briefly, approximately 0.5 g sample of fresh leaves were crushed and ground in a mortar with 10 ml of sulfosalicylic acid (3.0%). The homogenate was filtered through Whatman No. 2-filter paper; after that, 2 ml of the extract was mixed with ninhydrin reagent (2 mL) and 2 mL glacial acetic acid then placed in a boiling water bath for 1 h at 100°C until the appearance of red colour. The tubes were then cooled in the ice. Toluene 4 mL was added to the test tube, and the mixture was mixed until the upper coloured layered appeared. Then this layer was separated from the mixture in other test tubes and absorbance was taken at 520 nm. Proline concentration was determined from a standard curve and calculated fresh weight mmol proline (mg. $g^{-1}$

FW).

**Determination of mineral ion contents**

Fresh samples were dried for 48 h at 35°C. A total of 0.3 g of leaves were pulverized into a powder and burnt at 560°C. It was then digested for 1 to 2 h with 10 ml of an acid combination comprising $HNO_3$: $HClO_4$ (2:1 v/v) until the red $NO_2$ emissions stopped. After full digestion, distilled water was used to dilute the colourless digests (2-3 ml) to a volume of 20 ml, which was then filtered using Whatman No. 1 filter paper. The ions $Na^+$, $K^+$, $Zn^{+2}$, and $Ca^{+2}$ were determined by inductively coupled plasma atomic absorption spectrometry using aliquots from this solution (Optima2000DV, Perkin Elmer, USA). The content was determined following the earlier procedure (Stateras and Moustakas, 2018).

**Measurement of phenolic compounds**

The sample extraction method is described with modifications (Neugart et al., 2015). Fresh leaves (20 mg) were immersed in 10 mL of 50% aqueous methanol for 60 min, with sonication below 40°C. The samples were then centrifuged at 14,000× g for 15 min, and the supernatants were collected kept at 4°C for further analysis. Total phenolic content was assayed using the Folin-Ciocalteu colourimetric method. In brief, 0.125 ml of methanolic extract solution was mixed with 0.5 ml of deionized water and 0.125 ml of the Folin-Ciocalteu reagent. Then the mixture was incubated for 1 min before adding 1.25 ml of 7% sodium carbonate ($Na_2CO_3$) solution. Then incubation for 90 min in the dark at room temperature, the absorbance was measured by a spectrophotometer at 760 nm as described by Lim et al. (2014) with some modifications.

**Estimation of the antioxidant enzymes activities**

Fresh leaf samples were used to determine the activities of antioxidant enzymes. Extraction of samples and preparation of supernatants was carried out according to the method (Ahmad et al., 2016). Briefly,1 g leaf samples of faba bean plants were homogenized in an extraction buffer containing potassium phosphate buffer (0.1 M, pH 7.5) and ethylenediaminetetraacetic acid (EDTA, 0.5 mM). Then centrifugation at 12,000 rpm for 10 min at 4°C, the supernatants were collected for the measurements for enzyme assay.

***Catalase (CAT) activities***

Briefly, 3 ml reaction mixture contained 1.5 ml of 50 mM sodium phosphate buffer (pH 7.0), 1 ml of 18 mM $H_2O_2$ and 50 µl enzyme extract. The catalase was estimated by measuring the decrease in the absorbance at 240 nm according to the method of Aebi (1984).

***Ascorbate peroxidase (APX) activities***

In brief, fresh extract assay by 3 ml of enzyme reaction mixture contained 1.5 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.5 ml of 0.5 mM ascorbic acid, 0.1 ml of 0.1 mM EDTA, 100 µl enzyme extract, 0.7 ml of $H_2O$ and 0.1 ml of mM $H_2O_2$. The reaction was initiated by adding 0.1 ml of 3 mM $H_2O_2$. A decrease followed the $H_2O_2$ dependent oxidation of ascorbic acid in the absorbance measured at 290 nm for 3 min at the interval of 30 s (Nakano and Asada, 1981).

***Polyphenol oxidase activity (PPO)***

Leaf tissue (1 g) ground in 10 ml of 100 mM sodium phosphate buffer, pH 6.5. The homogenate was centrifuged for 10 min at 4°C at 12,000 rpm, and the supernatant was employed in the enzyme assays. The reaction mixture contained 1 ml of pyrogallol (10 mM pyrogallol), 1.5 ml of 10 mM phosphate buffer, pH 6.5, and the reaction was initiated by adding 0.5 ml of enzyme extract. The changes in the colour due to the oxidized pyrogallol were read at 420 nm for 1 min at an interval of 15 s. The enzyme activity was expressed as (mg/g FW) (Kar and Mishra, 1976).

***Peroxidase activity (POX)***

About 50 µL of plant extract was added to 1.35 mL 0.1 M potassium phosphate buffer (pH 6.0), 100 µL 45 mM guaiacol, and 500 µL 44 mM hydrogen peroxide. Then, using a UV-Vis spectrophotometer, we recorded kinetic changes in absorbance at 470 nm over a 10-s interval for 30 s at 25°C (MacAdam et al., 1992).

**Statistical analysis**

Plant growth data, biomass, photosynthetic pigment contents (chlorophyll a, b, and carotenoids), mineral contents, total phenol content, and antioxidant enzymes were assessed periodically. The experimental data were computed using two-way analysis of variance (ANOVA) and the treatment effects were ascertained through mean comparison. Means were compared using Tukey's honestly significant difference (HSD) test at P<0.05%.

## RESULTS

### Physical and chemical properties of soil mixture

#### *Soil properties after harvesting*

Irrigation with saline water (50 and 100 mM NaCl) significantly reduced $K^+$, $HCO_3$, and $SO_4$ levels while increasing $Na^+$, $Cl^-$, pH, and EC concentrations in postharvest soil samples to the salt-stressed plants, which utilized distilled water for irrigation. After harvesting, no carbonate was detected in the soil solution. Two levels of salt increased the $Na^+$ concentration in the soil (Table 1).

### Ultraviolet (UV-Vis) analysis of ZnONPs from *M. oleifera* leaf extract

The presence of secondary metabolite in plant (M. oleifera extract) causes zinc ions in the solution to be reduced to zinc oxide. The plant extract not only functions as a reducing agent but also acts as a stabilizing agent. This was validated by doing a UV–visible spectrum investigation in the region of 300 to 600 nm. The spectra revealed a peak at 370 nm, which is specific to ZnO nanoparticles (Figure 1).

### FTIR analysis

The Fourier transform infrared spectroscopy (FTIR) was

**Table 1.** Physical and chemical properties of the soil before and after the field experiments.

| Sample ID (mM) | PH (%) | E.C.mmohs/cm$^3$ | Soil content of cations (meq /L.) | | Soil content of anions (meq/L.) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | K ppm | Na ppm | $HCO_3$ | CL | $CO_3$ | $SO_4$ |
| 0 | 5.7 | 0.255 | TRAC | TRAC | 4.40 | 8.45 | 0.00 | 5.82 |
| 50 | 6.5 | 0.748 | 189 | 284 | 3.30 | 36.75 | 0.00 | 3.35 |
| 100 | 6.8 | 3.46 | 162 | 326 | 1.82 | 45.25 | 0.00 | 1.60 |

Source: Author

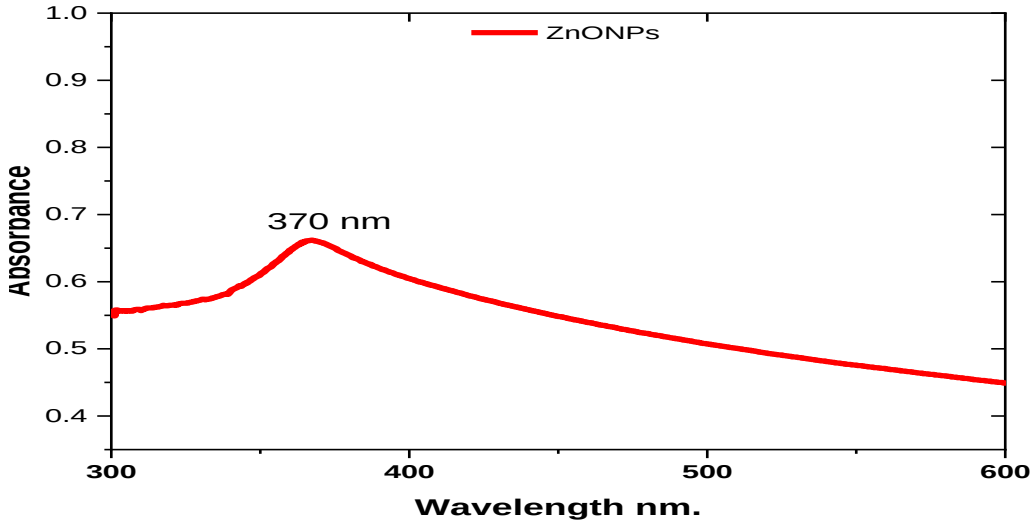


**Figure 1.** UV–Vis absorption spectra of ZnO nanoparticle. (ZnONPs).
Source: Author

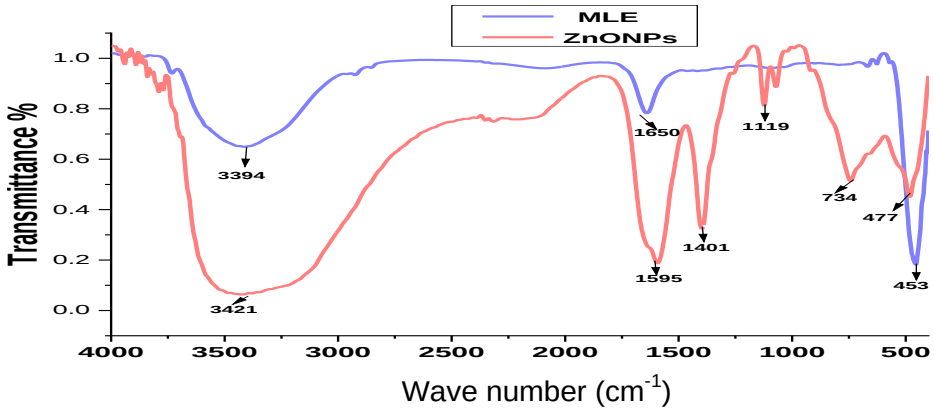


**Figure 2.** FTIR spectra of ZnONPs, and MLE.
Source: Author

utilized to identify the various functional groups included in the produced nanoparticles. The peaks were utilized to identify the functional groups in ZnO nanoparticles, which were then characterized (Figure 2), including *M. oleifera* leaf extract, which revealed an absorption band at 3421,3394 cm$^{-1}$, which is attributable to the asymmetric and symmetric stretching vibrations of the alcohol O-H group, respectively. However, the absorption peak shows

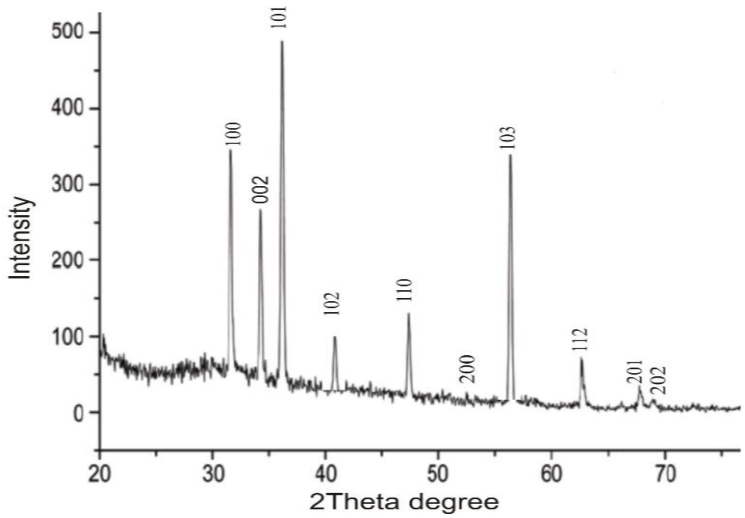


**Figure 3.** XRD patterns of ZnO nanoparticles (ZnONPs).
Source: Author

the C=C bonding of alkene at 1595 $cm^{-1}$. Stretching vibrations at 1401 $cm^{-1}$ revealed the methyl group's C-H stretching. The peak in ZnO represented the amide band of the random coil of protein at about 1401 $cm^{-1}$. The peak indicates the stretching vibrations of ZnO nanoparticles at 734 $cm^{-1}$. A metal-oxygen bond is allocated a range between 400 and 600 $cm^{-1}$. In addition to the absorption bands of the biomolecules utilized as reduction and stabilization (capping agents), the presence of ZnONPs is confirmed by the absorption peak at 477 $cm^{-1}$ owing to Zn-O stretching vibration.

### Analysis of crystal structure (XRD) of ZnONPs from *M. oleifera* leaf extract

The structure and phase purity of the nanoparticles sample produced strong, intense peaks in the spectrum of 2θ values ranging from 25 to 70 that were identified from XRD patterns (Figure 3). The sharp diffraction peaks were observed at 2θ values 26.3, 29.1, 36.33, 39.29, 45.33, 51.52, 53.50, 52.50, 56.23, 64.84, and 67.79°. Diffraction peaks of XRD matched very well with the hexagonal wurtzite structure by comparison with the data from JCPDS card No. 89-1397. All the reflection peaks obtained were corresponding to (100), (002), (101), (102), (110), (103), (200), (112), (201), and (202) diffraction lattice planes, respectively which confirm the hexagonal wurtzite structure for the synthesized nanoparticles.

### Scanning-electron microscope (SEM) analysis of ZnO NPs from *M. oleifera* leaf extract

As shown in Figure 4, scanning electron microscopy (SEM) images were acquired at various magnifications to investigate the form and size of the nanoparticles that were manufactured. The evolution of nanoparticles in their agglomerated condition may be seen in the surface morphology. According to detailed structural parameters, the synthesized products have a spherical and crystalline structure, with diameters in the range of 215 nm. The findings of the SEM analysis indicated that the size and form of the nanoparticles were influenced by the various precursors used in their preparation.

### Effects of MLE and ZnONPs application on growth-related parameters

The growth responses of faba bean under saline conditions were evaluated by measuring growth-related parameters to assess their salt-tolerance capacity. Leaf number, plant height, and total DW of cultivar decreased with the increasing salinity levels (Figure 5). Specifically, when NaCl concentration was increased from 50 to 100 mM, growth-related parameters, such as plant height, leaf number, WC, FW, and DW, were reduced compared to the control plants (Figure 5). Compared with the control, plant height reduced in the 50 and 100 mM NaCl by 84.67 and 76.96%, respectively (Figure 5A). Foliar spray of faba bean plants with MLE and ZnONPs improved plant height (92.71,105.60, 108.90 and 116.83%) at 50 and 100 mM NaCl levels compared to plants sprayed with NaCl alone (Figure 5A). However, the shoot FW (SFW) decreased considerably at 50 and 100 mm NaCl by 87.15 and 73.29% respectively, as compared to control plants (Figure 5B). In contrast, salt levels with treatments (MLE and ZnONPs) had increased SFW compared to salt-stressed plants (101.89, 108.69,

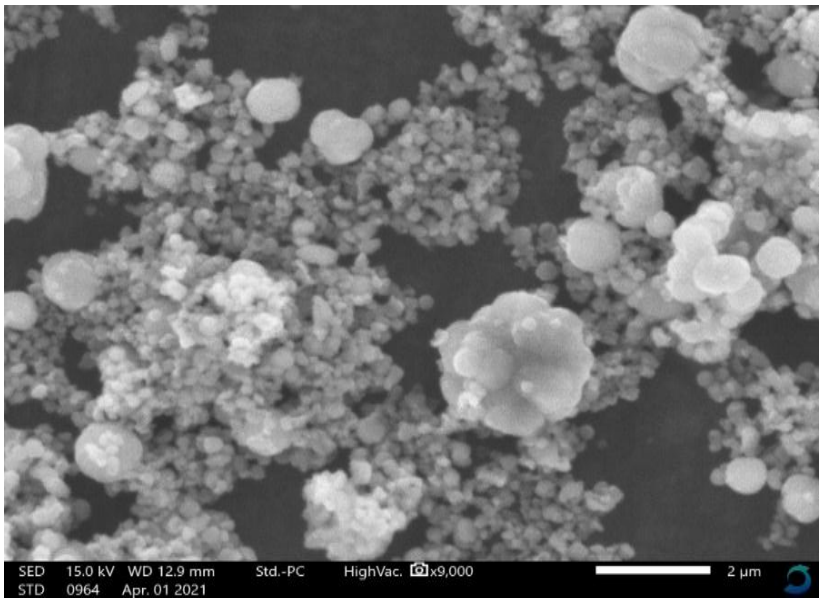


**Figure 4.** SEM images of green synthesized ZnO nanoparticles (ZnONPs).
Source: Author

109.70, and 109.10%), respectively (Figure 5B). The shoot DW(SDW) had a pronounced decrease with alt-stress against control plants by 79.12 and 69.01%, respectively. The treatment with MLE and ZnONPs markedly boosted SDW compared to salt-stressed plants (107.51, 111.84, 117.93, and 117.86%), respectively (Figure 5C). Salt stress reduced the root FW; the reduction was noticed at 50 and 100 mM NaCl than control plants (82.35 and 67.22%, respectively). MLE and ZnONPs application caused a significant increase in root FW in plants grown under salt treatments compared to salt-stressed plants (101.02, 105, 110.20, and 130%), respectively (Figure 5D). Root DW decreased significantly in 50 and 100 mM NaCl treatment compared to non-salt stressed plants by 87.95 and 79.51%, respectively. In contrast, the root DW was increased significantly in all the treatments compared to salt-stressed plants (101.36, 89.39, 108.21, and 124.24%), respectively (Figure 5E). Faba bean stress alone reduced root length by 52 and 60% at 50 and 100 mM NaCl levels, respectively, over the control plants. Foliar spray of faba bean plants with MLE and ZnONPs improved root length (106.65, 99.27, 117.82, and 101.38%), respectively (Figure 5F). Furthermore, a significant decrease in leaf number was observed in 50 and 100 mM NaCl compared to control plants (84.06 and 80.85%, respectively). Foliar spray of MLE and ZnONPs increases the leaf number versus plants exposed to NaCl alone (116.27, 112.40, 131.14, and 134.54%), respectively (Figure 5G). Additionally, the water content (WC%) of faba bean decreased in salt-stressed plants compared to control plants (93.61 and 89.64%, respectively). The application of MLE and ZnONPs increased the water content significantly compared to plants treated with NaCl alone (100.70, 105.36, 112.75, and 121.64%), respectively (Figure 5H).

## Effects of MLE and ZnONPs application on pigment contents and photosynthetic characteristics in faba bean

To evaluate the protective roles of the photosynthetic pigments under NaCl stress, the contents of photosynthetic pigments (Chl a, Chl b, Chl a + b, and carotenoids) in salt-exposed faba bean leaves were determined (Figure 6). Compared with the control sample, there was a significant decrease in Chl a content under different salinity levels (50 and 100 mM NaCl) by 73.52 and 85.29%, respectively. However, plants grown under salt conditions and received MLE and ZnONPs supply restored the concentration of Chl a compared with their salt-stressed plants by 119.37, 113.31, 153.48, and 152.55%, respectively (Figure 6A). On the other hand, it was noticed from the results that the Chl b of faba bean under different salinity levels (50 and 100 mM NaCl) decreased significantly in bean leaves with salinity stress as compared to control plants by 65.45 and 85.45%, respectively. The exogenous application of MLE and ZnONPs increased the concentration of Chl b compared with their salt-stressed plants by (127.77, 102.12, 147.94, and 119.14%), respectively (Figure 6B). However, the results showed that increasing salinity decreased the total chlorophyll of faba bean significantly under different salinity levels (50 and 100 mM NaCl) compared to control plants by 74.11 and 85.88%, respectively. In addition, the

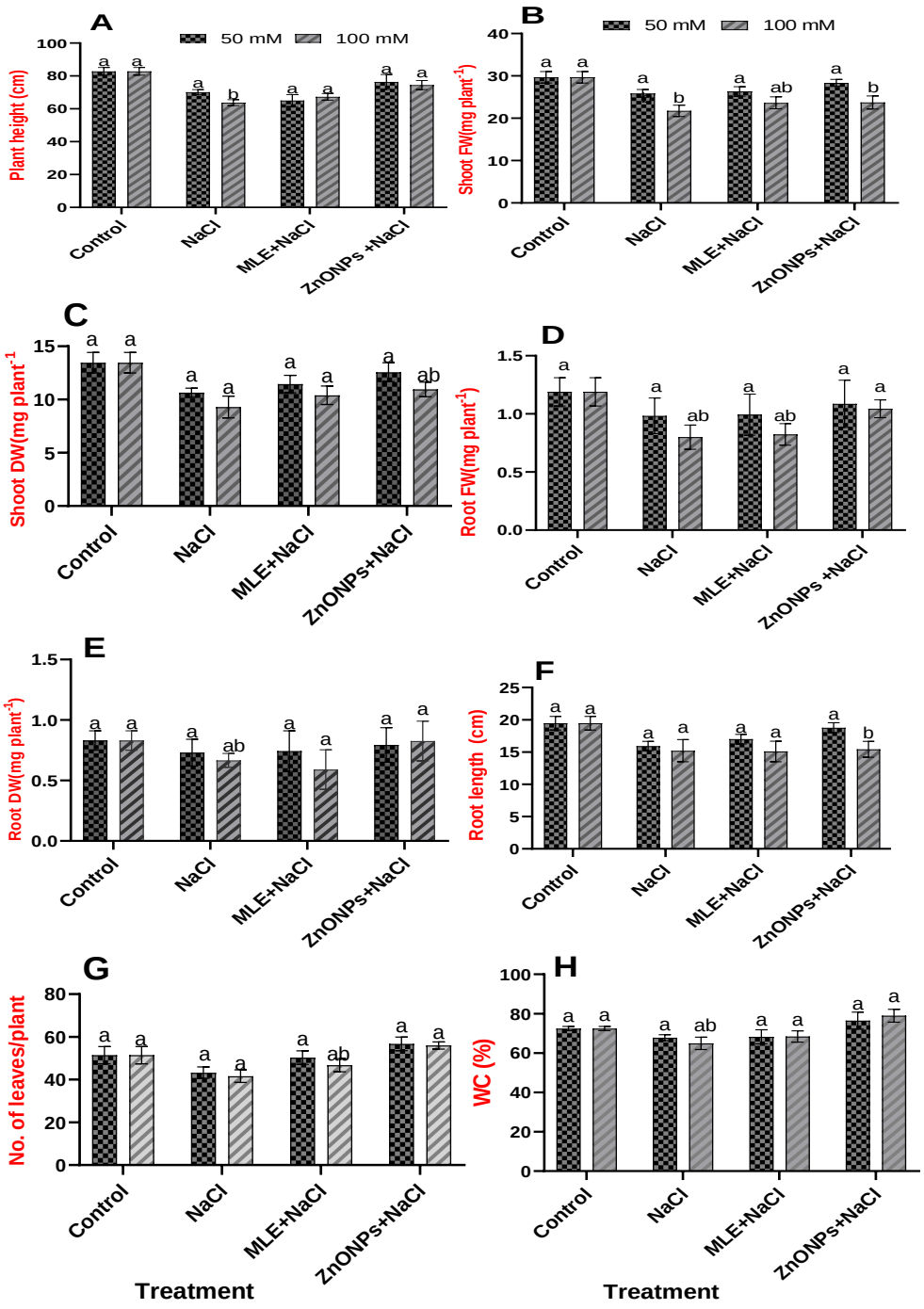


**Figure 5.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract (MLE) treatment on growth characteristics of faba bean, such as (A) plant height; (B) shoot FW; (C) shoot DW; (D) root FW; (E) root DW; (F) root length; (G) No. of leaves/plant; and (H) WC%, from the 60 days of sowing. The values are the mean ± SD of 3 replicates. Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

foliar application of MLE and ZnONPs increased the total chlorophyll concentration compared with their salt-stressed plants by 117.46, 102.73, 152.38, and 128.76%, respectively (Figure 6C). Increasing salinity decreased the carotenoids of faba bean significantly under different salinity levels (50 and 100 mM NaCl) compared to control by 79.31 and 52.29%, respectively. In contrast, spraying of MLE and ZnONPs increased the concentration of

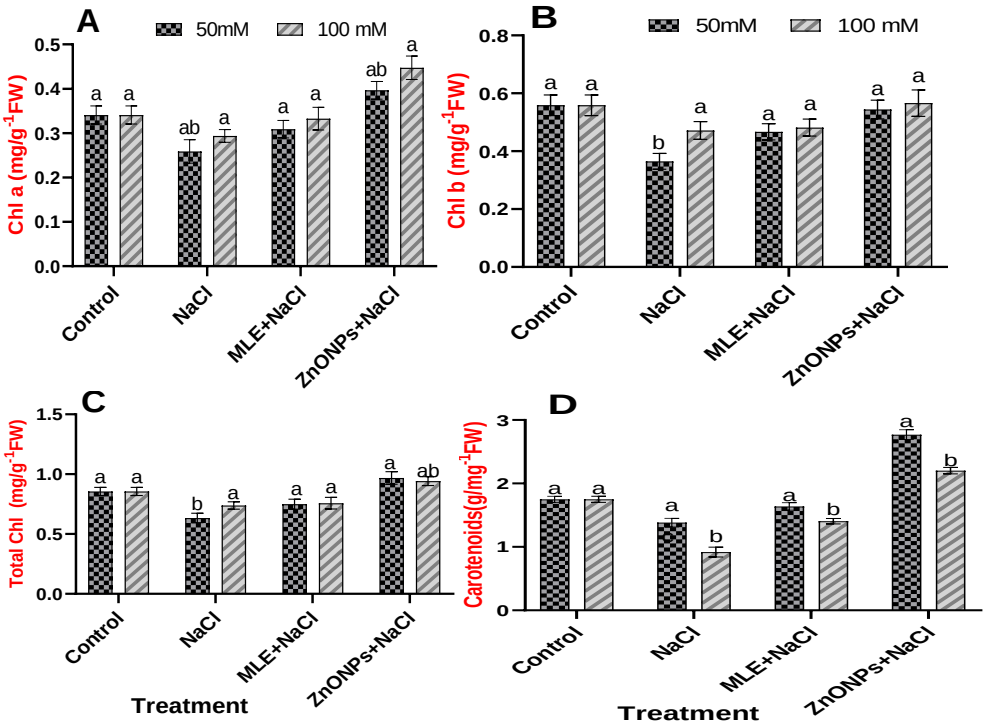


**Figure 6.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract (MLE) treatment on Pigments contents of faba bean. Chl a. (**A**), Chl b (**B**), total Chl (**C**) and Carotenoids (**D**) from the 60 days of sowing. The values are the mean ± SD of 3 replicates. Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

carotenoids compared with their salt-stressed plants by 118.11, 153.84, 200.36, and 241.75%, respectively (Figure 6D).

## Effects of MLE and ZnONPs on proline content of faba bean under salt stress

The proline content of faba bean showed that NaCl treatment significantly increased, whereas 100 mM treatment significantly decreased free proline contents, and MLE and ZnONPs treatment significantly enhanced the proline accumulation (Figure 7). The proline content increased significantly at the salinity level of 100 mM NaCl, then decreased at 50 mM NaCl to control plants by 168 and 136%, respectively. However, the MLE and ZnONPs, when applied to plants, showed a further significant increase in proline accumulation content than the salt-stressed plants (85.29, 73.80, 97.05, and 85.71%), respectively (Figure 7).

## Effects of MLE and ZnONPs application on mineral ion contents in faba under salt stress

Among the mineral ions, $Na^+$ contents of shoot gradually increased with increasing salinity in salinity-treated plants. The $Na^+$ contents of the shoot were also higher than control plants in the combined treatment of MLE + NaCl and ZnONPs + NaCl compared to salt-stressed plants (Figure 8). Compared with control, the plants treated with 50 and 100 mM NaCl had higher $Na^+$ concentrations in their shoots by 321.42 and 307.14%, respectively (Figure 8A). However, the concentration of $Na^+$ in the shoot of plants treated with MLE+50 mM NaCl and ZnONPs+50 mM NaCl was reduced by 57.77 and 80.21%, respectively, when compared with 50 mM NaCl plants (Figure 8A). Moreover, the amount of $Na^+$ in the shoot of plants treated with MLE+100 mM NaCl and ZnONPs+100 mM NaCl was reduced by 76.74 and 78.13%, respectively, compared to the content in 100 mM NaCl plants (Figure 8A). Faba bean plants treated with the salinity levels (50 and 100 mM NaCl) compared to the control sample had a significant increase in leaf $K^+$ content (274.40 and 275.20%), respectively (Figure 8B). Moreover, for the plants treated with the MLE and ZnONPs, the $K^+$ was increased in the shoot by 245.60, 256.80, 237.60, and 257.60%, respectively, against the control plants (Figure 8B). Salinity significantly decreased $Ca^{+2}$ contents in leaves of faba bean plants versus non-stress plants by 82.29 and 80.20%, respectively (Figure 8C). However, foliar application of MLE and ZnONPs

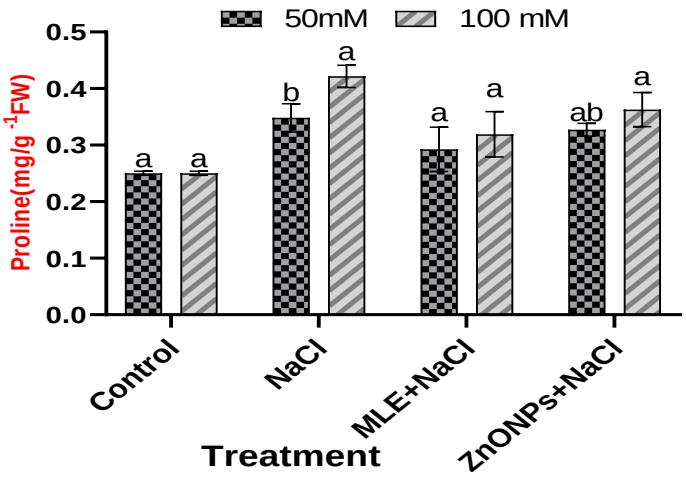


**Figure 7.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract( MLE) treatment on proline of faba bean from the 60 days of sowing. The values are the mean ± SD of 3 replicates. Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

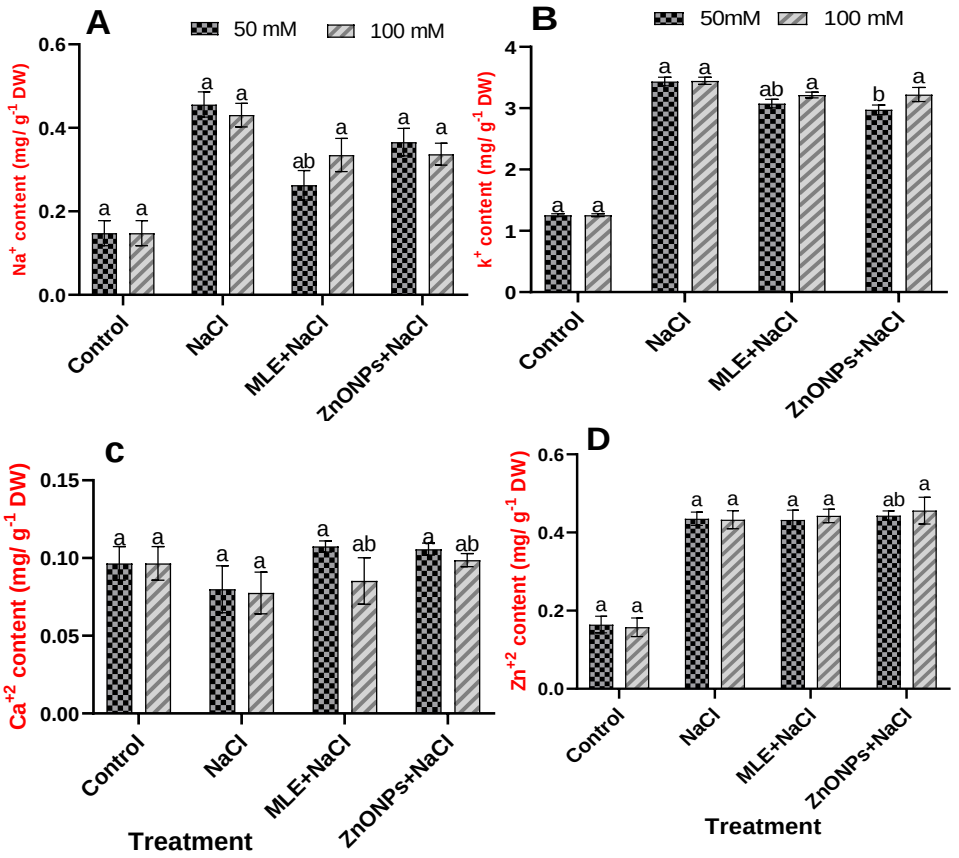


**Figure 8.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract( MLE) treatment on primary mineral ion contents in shoot tissues of faba bean. (A) $Na^{+}$; (B) $K^{+}$; (C) $Ca^{+2}$; and (D) $Zn^{+2}$. The values are the mean ± SD of 3 replicates. Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

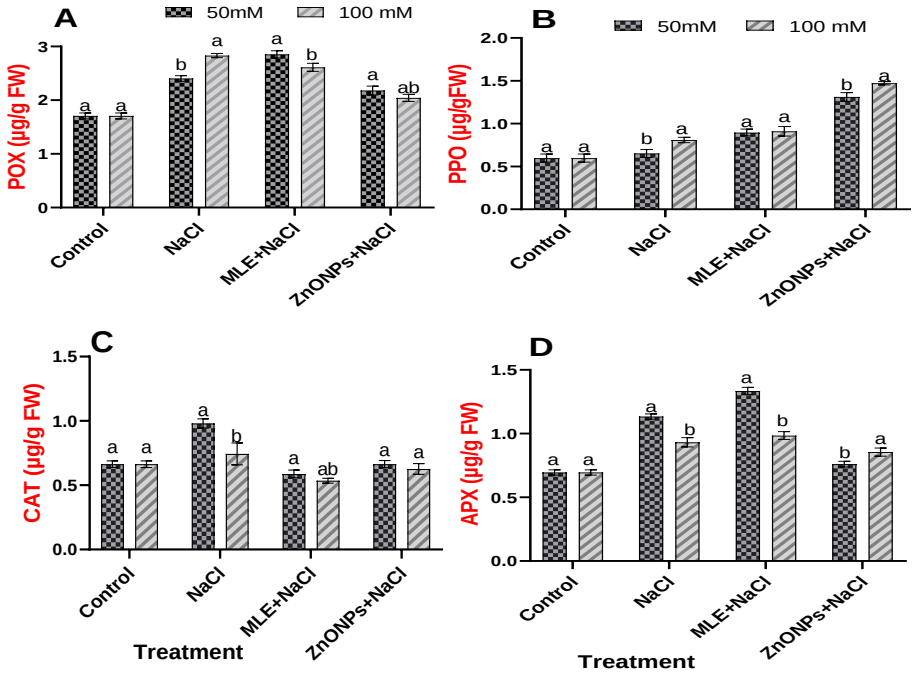


**Figure 9.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract( MLE) treatment on antioxidant enzymes in shoot tissues of faba bean. POX(A), PPO(B), CAT(C), and APX(D). The values are the mean ± SD of 3 replicates. Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

significantly improved the accumulation of $Ca^{+2}$ contents in leaves of faba bean plants compared to only salt-stressed plants by 142.85, 114.28, 132.91, and 127.27%, respectively (Figure 8C). On the other hand, salinity significantly increased the $Zn^{+2}$ content in salt-stressed plants than in the control plants for the shoot of faba bean by 268.75 and 286.66%, respectively (Figure 8D). Moreover, foliar application of MLE and ZnONPs plants showed a further significant increase in the amount of $Zn^{2+}$ in the shoot of faba bean plants versus non-stress plants by 263.41, 293.33, 270.12 and 290.44%, respectively (Figure 8D).

## Effects of MLE and ZnO NPs application on antioxidant enzymes of faba bean under salt stress

The roles of MLE extract and ZnONPs against oxidative stress were assessed by determining the levels of the enzyme activities POX, PPO, CAT, and APX in the leaves of faba bean plants under salinity conditions (Figure 9). In salt-stressed faba bean plants grown under 50 and 100 mM, NaCl levels showed an augmented level of POX, PPO, CAT, and APX activities which increased significantly over the control plants by 141.17, 166.47, 110.16, 135.59, 148.48, 112.12, 163.76, and 134.78%, respectively (Figure 9A to D). Further foliar application of MLE and ZnO NPs significantly increased the activities of antioxidant enzymes such as POX, PPO, CAT, and APX in plants compared to the salt-stressed plants (Figure 9A to D).

## Effects of MLE and ZnONPs application on total phenolic compounds of faba bean under salt stress

Faba bean plants grown in 50 and 100 mM NaCl levels showed a drastic increase in the total phenols content compared to the untreated control sample by 101.57 and 94.62%, respectively (Figure 10). Foliar application of MLE and ZnONPs to NaCl-treated plants slightly increased the total phenols content by 92.77, 102.63, 93.54 and 101.66%, respectively, compared with NaCl-treated plants (Figure 10).

## DISCUSSION

*M. oleifera* leaf extract was used to prepare eco-friendly ZnONPs because the biomolecules in the extract are efficient in stabilizing ZnONPs. The formation of yellow colour in the extracted colour with $ZnNO_3$ solution confirmed the synthesis of ZnONPs (Karnan and Selvakumar, 2016). The shape of ZnONPs was confirmed by XRD analysis (Zak et al., 2011). Scanning electron micrographs (SEM) show several agglomerated

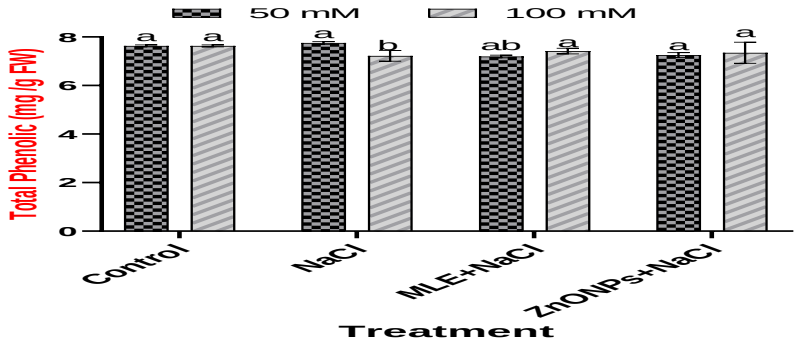


**Figure 10.** Foliar applied of ZnO nanoparticles (ZnONPs) and *M. oleifera* leaf extract (MLE) treatment on total phenols content in shoot tissues of faba bean. The values are the mean ± SD of 3 replicates.Different alphabets represent statistically significant differences between the treatments ($p \leq 0.05$).
Source: Author

particles with uneven shapes in addition to individual particles with hexagonal or elongated structures (Rajendran and Sengodan, 2017). The various functional groups of synthesized nanoparticles were determined using FTIR spectroscopy. Further, proteins, alcohols, aromatic hydrocarbons, aldehydes, ketones, carboxylic acids, and stretching and bending vibrations were related to ZnONPs (Ahmad et al., 2020; Ogunyemi et al., 2019). However, the optical characteristics of the samples were investigated using UV-visible spectroscopy.

Salinity increases the osmotic potential of plants, limiting growth and productivity by imposing toxicity and adverse effects on plant growth. Salt stress affects many aspects of plant metabolism, and, as a result, growth and yields are reduced. Saline soils and saline irrigations are significant production concerns for many crops since saline conditions limit plant growth (Yildirim et al., 2008). The current study shows that salinity adversely affected faba bean growth parameters compared to the control plant. These results agree with Zhu et al. (2004), who detected that treated cucumber plants with 25 50, 100, and 190 mM NaCl reduced several growth parameters, such as fresh and dry weight in the shoot and root. However, compared to non-treated salt stress plants, MLE and ZnONPs applications enhanced the fresh and dry weight of shoot and root, leaf number, plant height, and water content (WC) of faba bean plants. Similar findings were reported by Dhoke et al. (2013), who revealed that ZnONPs treatment increased root biomass and above-ground tissues of *Vigna radiata* seedlings. Additionally, under stress that limits soil water-deficits, plants can absorb water and nutrients due to the provision of ZnONPs, which improves the activity and expression of genes linked to phytohormones (for example, ABA and cytokinin), which promote root growth (Semida et al., 2021; Zhao et al., 2017) found that foliar spraying of ZnONPs on eggplant tissue improves water status in water-stressed plants; it can help preserve cell membrane integrity and increase RWC as metabolically accessible water, indicating plant metabolic activities. In addition, *M. olifera* leaf extract (MLE) significantly enhanced the growth parameters of rocket (*Eruca sativa*) due to its high protein content, which is required for protoplasm synthesis, as well as growth-promoting hormones including auxins and cytokinins (Abdalla, 2013). The high levels of growth-promoting compounds in MLE may be accountable for improving growth traits (Figure 5). This might be attributed to using MLE and ZnONPs, which maintained photosynthesis by improving physiological and biochemical metabolism and enhanced plant growth parameters against salt stress (Figure 5). These positive foliar applications MLE and ZnONPs might be attributed to improved membrane integrity, RWC, and increasing growth parameters (Hafez et al., 2020). This enhancement in growth might be attributed to salinity-induced positive regulation of oxidative and ionic stress (Choudhary et al., 2017).

The chlorophyll content of faba bean leaves reduced as the saline level increased. These findings are in line with those of Stępień and Kłbus (2006), who indicated that chlorophyll content decreased significantly in the leaves of spinach and cucumber plants when NaCl concentration increased. Parida and Das (2005) reported that salt stress considerably decreased the chlorophyll and total carotenoid concentrations in the leaves of many crops. The decrease of these pigments in cells under salt stress is due to sluggish synthesis or quick degradation of the pigments in cells (Ashraf, 2003). This study showed the highest concentrations of photosynthetic pigments (Chl a, b, total Chl, and carotenoids) in faba bean leaves when using an application of MLE and ZnONPs combined with plants under salt stress. Yasmeen et al. (2014) indicated that enhanced chlorophyll concentrations of tomato attributable to MLE application correspond to delayed leaf senescence due to zeatin, ascorbate, and potassium present in MLE. The high

amount of chemicals involved in the chlorophyll structure in moringa leaves, including carotenoids and minerals such as Mg, P, and Ca, may be responsible for the increasing chlorophyll and carotenoid concentrations (El Sohaimy et al., 2015). Further, foliar spraying of MLE and ZnONPs may improve photosynthetic efficiency, increasing metabolites and photosynthesis for faba bean growth. One of the explanations for the decline in photosynthetic activity induced by salt stress might be a decrease in pigment content (Silveira and Carvalho, 2016).

Proline is a critical osmolyte for osmoregulation and the stability of various other macromolecules (Curá et al., 2017). The present study observed that proline levels were significantly higher in salt-stressed plants than in control plants. Plants in the current study had higher proline concentrations, which might be related to treating faba bean plants with MLE and ZnONPs, which relieved salt stress. Our findings are similar to the results obtained in barley (Dbira et al., 2018). Proline has been widely exploited as an effective marker in plant salt tolerance mechanisms due to its critical involvement in plant osmotic adjustment (Uddin et al., 2012). It may be possible that MLE and ZnONPs treatments enhanced proline accumulation by activating proline metabolism enzymes.

Our study indicated that salt stresses caused decreased $Ca^{+2}$ contents (Fig.8C) and a significant increase in other ions concentration ($Na^{+}$, $K^{+}$, and $Zn^{+2}$) compared to non-stressed plants. In this study, the foliar application of MLE and ZnO NPs led to a significant increase in the mineral content ($Na^{+}$, $K^{+}$, $Ca^{+2}$, and $Zn^{+2}$) compared to untreated plants under salt stress. This might be because NPs can permeate tissues and move to other organs through the phloem (Pérez-de-Luque, 2017). The $Na^{+}$ concentration in plant tissues is an essential indicator of salt tolerance since low content indicates minimal ion uptake (Tahir et al., 2006).

Furthermore, the treatments increased K+ concentrations in plant tissues, leading to a $K^{+}/Na^{+}$ ratio regulation in stressed plants compared with the non-stress plants. Rady et al. (2019) demonstrated that under abiotic stress conditions, foliar spraying with MLE induced a decrease in $Na^{+}$ and increased the content of $K^{+}$ and $Ca^{+2}$ in common bean plants compared to untreated plants. In addition, calcium ($Ca^{+2}$) is essential for processes such as maintaining the structural and functional integrity of plant membranes, stabilizing cell wall structures, regulating ion transport and selectivity and controlling ion-exchange behaviour and cell wall enzyme activities (Yildirim et al., 2008). Intracellular $Ca^{+2}$ can influence plant responses to drought and salinity, which has also been attributed to the transmission of drought and salt-stress responses in plants, which play an essential part in
osmoregulation under these environments (Bartels and Sunkar, 2005).

Antioxidant enzymes (e.g., PPO, POX, CAT, and APX.) are induced in faba bean plants as a response to abiotic stress as well as a vital part of the antioxidant defense system to prevent biomarkers of oxidative stress, such as hydroxyl radicals responsible for lipid peroxidation in cell membranes and destructive effects on plant productivity (Abogadallah, 2010). This study showed that the foliar spraying of ZnONPs and MLE enhanced the enzymes activities of faba bean under salt stress. These results agreed with Trivedi et al. (2018), who suggest that with increasing levels of salt stress, polyphenol oxidase activity (PPO) increased in green gram plants. Similarly, Purohit et al. (2020) reported that higher concentrations of salt stress increased the activity of antioxidant enzymes such as catalase (CAT) in groundnut plants. Pal et al. (2004) noted that the antioxidant enzymes such as ascorbate peroxidase (APX) increased in salinity tolerant and sensitive rice cultivars. These findings may be attributable to the high potency active components present in MLE and ZnONPs, which increased enzymatic antioxidants in faba beans, as shown in Figure 9.

Phenolic compounds are the most common secondary metabolite in plants, which have redox characteristics that help plants absorb and scavenge free radicals, quench singlet and triplet oxygen, and decompose peroxides (Osawa, 1994). Consequently, phenolics are potent antioxidants and play an essential role in plant defense against environmental stressors (Elzaawely et al., 2007). The present study showed that the application of MLE and ZnONPs significantly increased total phenolics in faba bean leaves compared to salt stress plants (Figure 9). This results in agreement with Basra et al. (2011) in maize. The result obtained showed that foliar application of ZnONPs and MLE increased total phenolics compared with untreated stress plants.

## Conclusion

In this study, $ZnONP_S$ were synthesized using *Moringa* leaf extract. The UV spectra revealed a peak at 370 nm, which is within the wavelength range of green ZnONPs. XRD peaks confirmed the effectiveness of the crystalline feature and synthesis procedure. The average size of ZnONPs was confirmed by SEM examination and was revealed to be 215 nm. Through FTIR analysis, functional groups of phytoconstituents that act as capping and stabilizing agents were found, endorsed the formation of ZnONPs. Further, the foliar applications of MLE and ZnONPs can reduce the adverse effects of salt stress in faba bean plants by increasing chlorophyll content, photosynthetic activity, growth parameters, biochemical characteristics such as chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, proline, mineral content, antioxidant enzyme activity, and total phenolic. Thus, helping plants to exhibit salt tolerance. Based on these findings, the MLE and ZnONPs treatments could help

reduce the adverse effects of salinity on faba bean growth.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Modulation of ethoxyresorufin-o-deethylase, benzoxyresorufin-o-dealkylase and pentoxyresorufin-o-dealkylase expression by African walnut seeds lipid extract in 3-methylcholanthrene induced breast cancer in Wistar rats

**Esosa Samuel Uhunmwangho*, Onoriode Oyiborhoro and Olafusi Celestina Oluwaseun**

Bioactive Lipids in Cancer and Toxicology Research Laboratory, Department of Biochemistry, Faculty of Basic Medical Sciences, University of Medical Sciences, Ondo, Nigeria.

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**Breast cancer is prevalent among women worldwide. African walnut (*Tetracarpidium conophorum*) seeds extract have been shown to have medicinal properties. This study is to determine the effects of feeding *T. conophorum* seeds lipid extract on 3-methylcholanthrene (MC) induced breast carcinogenesis and the expression of ethoxyresorufin-*O*-deethylase (CYP1A1), benzoxyresorufin-*O*-dealkylase (CYP1B1) and pentoxyresorufin-*O*-dealkylase (CYP2B1). The lipid was extracted using Soxhlet apparatus with *n*-hexane. Forty-five female Wistar rats of 21 days old were used, which were randomly divided into three major groups of 15 animals each. Groups A and B were fed for 12 weeks with diet containing *T. conophorum* seeds lipid extract (10%), and group B animals were administered MC (200 mg/kg body weight) intraperitoneally after 4 weeks of feeding. Group C animals were fed for 12 weeks with diet containing no *T. conophorum* seeds lipid extract and administered MC (200 mg/kg body weight) intraperitoneally after 4 weeks of feeding. Results indicated that CYP1A1, CYP1B1 and CYP2B1 were significantly ($p < 0.05$) reduced in Group B animals liver cells compared to group C with higher expression. Prolonged latency period, reduce tumor weight and size characterized group B animals compared to group C. Histopathology results showed normal morphology of the liver hepatocytes of animals in group B, while necrosis and steatosis were seen in group C. This study therefore showed that *T. conophorum* seeds lipid extract contains bioactive components that may oppose breast carcinogenesis induced by MC.**

**Key words:** 3-Methylcholanthrene, carcinogenesis, *Tetracarpidium conophorum,* fatty acids.

## INTRODUCTION

Walnuts are well known plants in Western Africa especially in Nigeria, and their edible seeds are widely cultivated for their delicacy (Uhunmwangho and Omoregie, 2017a). The tropical African walnut, known as *Tetracarpidium conophorum* belongs to the family Euphorbiaceae (Edem et al., 2009). Adebona et al. (1988) stated that some walnut species are found in the family Olacaceae. The plant African walnut is popularly known as, black walnut or Nigerian walnut (Nwaichi et al., 2017). In Nigeria, among the Yoruba tribe, it is known as *awusa* or *asala*, *ukpa*, or *okeokpokirinya*in Igbo and *gawudibairi* in Hausa, *okhue*or *okwe* among the Bini tribe

of Edo State (Chijoke et al., 2015). The seeds are consumed as snacks (Chijoke et al., 2017). The plant is a woody perennial climber of about 6 to 18 m long (Nwachoko and Jack, 2015). The African walnut is widely grown in the western and eastern parts of Nigeria (Oke, 1995; Nwaichi et al., 2017). All parts of African walnut plant have been used ethnomedically (Janick and Paul, 2008). Nwauzoma and Dappa (2013) reported ethnobotanical uses of African walnut seed extract in the treatment of fibroids, high blood pressure, malaria (Ayoola et al., 2011; Ogunyinka et al., 2015; Uhunmwangho and Omoregie, 2017b). The nut oil contains 48 to 50% dry weight of oil, is golden yellow in colour, with a taste resembling linseed oil (Negi et al., 2011). The oil is highly rich in polyunsaturated fatty acids (Kanu et al., 2015). Dietary fats constitute essential nutrients and are important source of essential fatty acids (FAs) such as α-linolenic, dihomo-dietary-ɣ-linolenic, docosahexaenoic and eicosapentaenoic acids (Uhunmwangho and Omoregie, 2017a). These FAs contribute to the inhibition of diseases involving abnormal and uncontrolled proliferation of cells (Hardman, 2014; Uhunmwangho et al., 2022a). The presence of these essential fatty acids in African walnut seed oil have been demonstrated (Dada and Aguda, 2015; Ayeni and Nuhu, 2018). Cytochromes P450 (CYPs) are enzymes that oxidize substances using iron and are able to metabolize a large variety of xenobiotic substances. CYP enzymes are linked to a wide array of reactions including and O-dealkylation, S-oxidation, epoxidation, and hydroxylation (Lee, 2013). The activity of the typical P450 cytochrome is influenced by a variety of factors, including herbal medications (Sparreboom et al., 2004). The objective of this work is to assess the dietary fats of *T. conophorum* seed oil in the modification of cancer metabolizing enzymes as cancers prevention phytomedicines.

## MATERIALS AND METHODS

### Ethical permission

Ethical permission was taken from UniMed Research Ethics Committee for the use of experimental rats for these studies.

### Reagents/chemicals

All reagents used were of analytical grade.

### Plant (sample collection)

Fresh *T. conophorum* fruits were obtained from farms in Ondo Town, Ondo State, Nigeria and authenticated by a taxonomist of the Botany Department, University of Medical Sciences, Ondo, Nigeria. At each harvest, 40 fruits were collected randomly. The collected fruits were cleaned and the seeds carefully separated from the fruits and dried at 65°C for 4 h in an oven, crushed with a laboratory mortar and pestle and were kept in a well labeled air tight screw-capped bottle at -4°C for extraction.

### Extraction of oil from African walnut

The Soxhlet extraction method was according to AOAC (1996) as reported by Sankeshwari et al. (2018). The seeds of *T. conophorum* were shelled, cut into small pieces, and air dried at room temperature for 2 weeks. The dried seeds of African walnut were ground into powdered form using a blender and further air dried. Fifty grams of African walnut seed powder was packed in a muslin cloth bag and placed in soxhlet apparatus using n-hexane as solvent. At the end of extraction, the thimble was dried in an oven for about 30 min at 100°C to evaporate off the solvent and cool in a desiccator, which was weighed and kept in the refrigerator.

### Feeding the animals with diet containing walnut seed oil

Female Wistar rats (21 day old) were obtained from the University of Medical Science, Ondo, and were housed in metal cages in a well-ventilated room and allowed access to water *ad libitum.* The animals were randomly divided into three major groups of 15 animals each. Group-A animals were fed for 12 weeks with diet containing 10% of *T. conophorum* seeds lipid extract only. Group B animals were fed for 12 weeks with diet containing 10% of *T. conophorum* seeds lipid extract and administered MC (200 mg/kg body weight) intraperitoneally after 4 weeks of feeding. Group C animals were fed for 12 weeks with diet containing no *T. conophorum* seeds lipid extracts and administered MC (200 mg/kg body weight) intraperitoneally after 4 weeks of feeding. The animals were palpated weekly to determine the time of appearance of tumors. Animals from each group were sacrificed after 12 week, mammary glands were exposed and tumors were excised. Tumor incidence, volume and weight were determined and tissues collected for enzymes and biochemical analysis. Portions of liver tissues were preserved in RNA for gene expression studies in formalin (10%) for histopathological studies.

### Preparation of liver microsomes

The preparatory of liver microsomes were as reported by Rani and Kansa (2012).

### Enzyme assays

Microsomal ethoxyresorufin-*O*-de-ethylase, benzoxyresorufin-*O*-dealkylase and pentoxyresorufin-*O*-dealkylase activities were assayed according to the procedure described by Burke et al. (1985) and modified by Teel and Huynh (1998).

### PCR amplification and agarose gel electrophoresis

PCR amplification for the determination of genes whose primers are

*Corresponding author. E-mail: euhunmwangho@unimed.edu.ng.

**Table 1.** List of primers.

| Gene | Accession | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') | Tm (°C) | Amplicon size (bp) |
|---|---|---|---|---|---|
| *Cytochrome P450 1b1* | XM_033762231.1 | TGTACTCGAGCCTGCACATC | GCACCGTCACCGACATCTTC | 61 | 106 |
| *Cytochrome P450 1a1* | NM_012540.3 | ACGTGAGCAAAGAGGCTGAA | TCGTGGTCATAACGTCTGCC | 58 | 145 |
| *Cytochrome P450 2b1* | NM_001134844.1 | CACCACACTCC GCTATGGTT | GCGGTCATCA AGGGTTGGTA | 60 | 118 |
| *Actb* | NM_007393.5 | TATAAAACCCGGCGGCGCA | TCATCCATGGCGAACTGGTG | 56 | 117 |

Source: Uhunmwangho et al. (2022b).

**Table 2.** Effect of feeding *T. conophorum* seeds lipid extract on breast carcinogenesis in MC administered Wistar rats.

| Parameter | Animals fed with *T. conophorum* only (Group A) | Animals fed with *T. conophorum* nut oil + MC (Group B) | Animals fed with MC only (Group C) |
|---|---|---|---|
| Tumor latency period | Symptoms not observed in these animals | 10 weeks | 4weeks |
| Tumor incidence | | 21.8% | 89.4% |
| Tumor weight (g) | | 3.1 ± 1.21 | 9.4 ± 1.35 |
| Tumor volume (mm3) | | 1284 ± 2.21 | 8342 ± 1.51 |

Values are mean ± SEM. *$p$< 0.05.
Source: Uhunmwangho et al. (2022b).

listed in Table 1 was done using the following protocol: PCR amplification was performed in a total of 25 µL volume reaction mixture containing 2 µL cDNA (10 ng), 2 µL primer (100 pmol) 12.5 µL, Ready Mix Taq PCR master mix and 8.5 µL nuclease-free water. Initial denaturation at 95°C for 5 min was followed by 20 cycles of amplification (denaturation at 95°C for 30 s, annealing for 30 s and extension at 72°C for 60 s) and ending with final extension at 72°C for 10 min. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel in Tris-Borate-EDTA buffer (pH 8.4).

### Histopathology

The histopathology study was according to Avwioro (2010).

### Statistical analysis

The values were expressed as mean ± SE. Kruskal-Wallis one-way analysis of variance (ANOVA) was used for the feed intake, tumor weight, tumor volume, and cancer metabolizing enzymes gene expression. A difference with $P$<0.05 was considered statistically significant. All the statistics were carried out in SAS (The SAS System for windows, v8; SAS Institute Inc., Cary, NC, USA).

## RESULTS

A total of 6 rats died within the 6 to 10$^{th}$ week due to carcinogen toxicity, while no mortality was observed in the group pretreated with *T. conophorum* seeds lipid extract and the group that was fed with *T. conophorum* seeds lipid extract only (Table 2). Figure 1 reveals that the growth of animals was similar in rats fed with diet containing *T. conophorum* seeds lipid extract for 12 weeks and the difference in body weight gained was not statistically significant except in animals administered MC. There was a significance increase in the expression of the activities of CYP1B1 and CYP2B1 in the group that received the carcinogen only (Figures 2 and 4). CYP1A1 activity in liver increased in animals in the group administered with carcinogen only and the magnitude of increase doubles activity in the animals in group treated with diet containing *T. conophorum* seeds lipid extract.

## DISCUSSION

Phytotherapy is a potential therapeutic strategy involving the usage of traditional medicinal plants to target various molecular markers altered during cancer, thereby leading to the prevention and treatment of cancer, without inducing side effects (Alugoju et al., 2021). African walnut contain many bioactive components including specific fatty acids, which could be active against cancer (Batirel et al., 2018).*T. conophorum* have been found to have various medicinal properties such as antioxidant, anti-diabetic, anti-inflammatory, anti-proliferative effects, among others (Batirel et al., 2018; Ayeni and Nuhu, 2018). Figure 1 reveals that the growth of animals was similar in rats fed with diet containing *Tetracarpidium conophorum* seeds lipid extract for 12 weeks and the difference in body weight gained was not statistically significant except in animals administered MC. The dietary fat present in *T. conophorum* nut (Uhunmwangho and Omoregie, 2017a), may be responsible for the slight

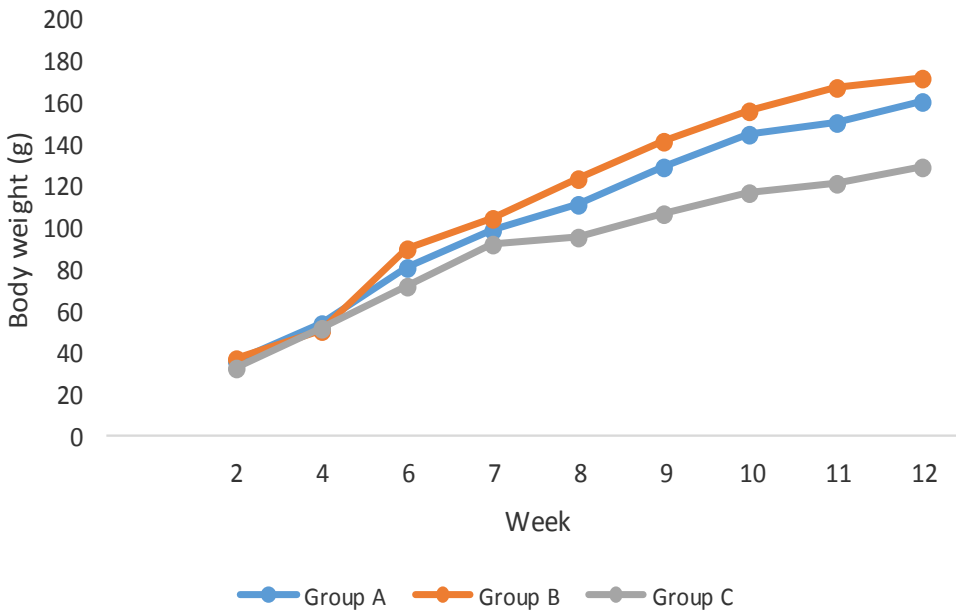


**Figure 1.** Body weight of animals in various groups. Values are mean ± SEM (*p < 0.05). Group A= +VE control (Animals fed with African walnut oil only), Group B =Treated animals with African walnut seed oil + MC, Group C = -VE control (Animals administered MC only).
Source: Uhunmwangho et al. (2022b).

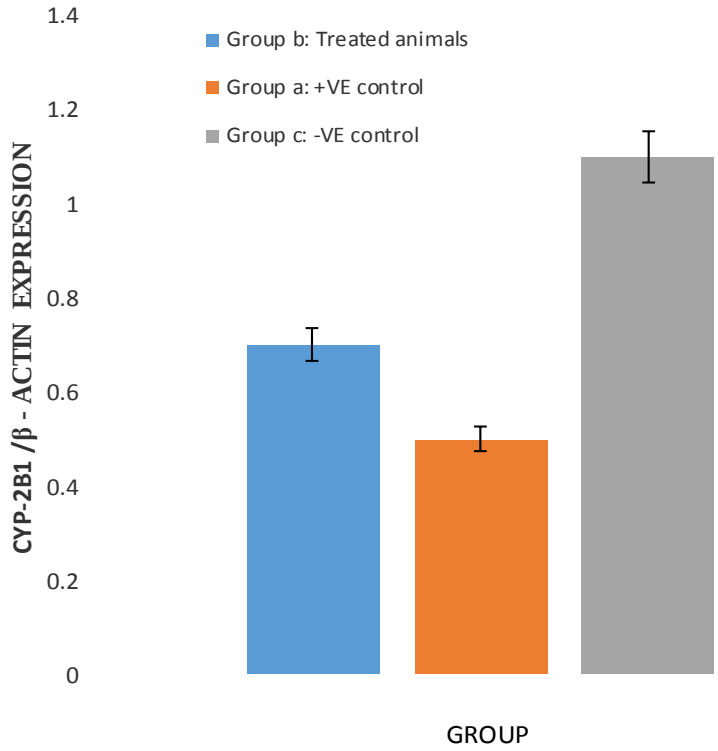


**Figure 2.** Effect of *Tetracarpidium conophorum* seeds oil on the expression of CYP 2B1 in MC-induced carcinogenesis in Wistar rats. Values are mean ± SEM (*p < 0.05). Group A= +VE control (Animals fed with African walnut oil only), Group B =Treated animals with African walnut seed oil + MC, Group C = -VE control (Animals administered MC only).
Source: Uhunmwangho et al. (2022b).

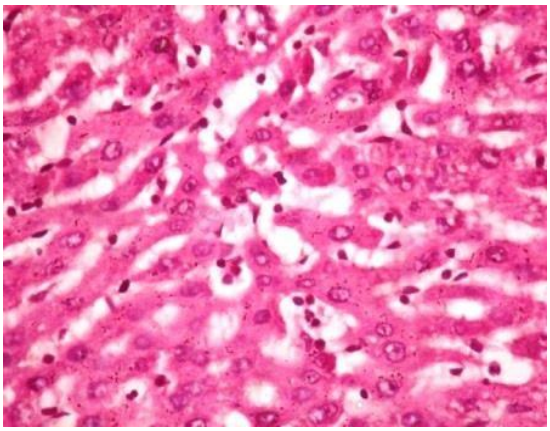

**Plate 1.** Photomicrograph of a liver tissue section of group-A stained by Haematoxylin and Eosin. The photomicrograph shows normal central venules without congestion. The morphology of the hepatocytes appears normal and the sinusoids are mildly infiltrated by inflammatory cells. Histological analysis showing the effects of Walnut seed oil on MC-induced histological changes in the liver of rats. Sections were stained with hematoxylin and eosin ×400.
Source: Uhunmwangho et al. (2022b).

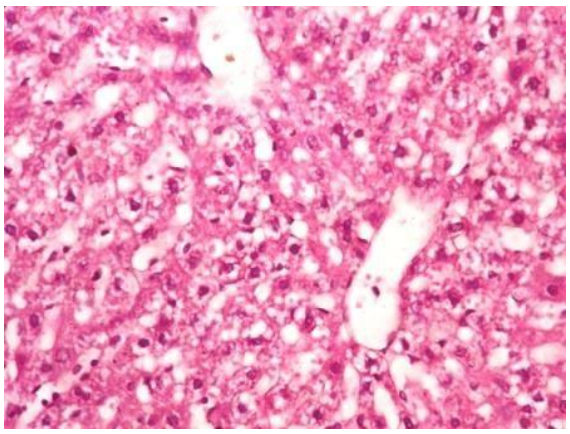

**Plate 2.** Photomicrograph of a liver tissue section of group-B stained by Haematoxylin and Eosin. The photomicrograph shows normal central venules without congestion. The morphology of the hepatocytes shows vacuolation and fat infiltration of their cytoplasma and the sinusoids appear normal, not infiltrated and no pathological lesion seen. Histological analysis showing the effects of Walnut seed oil on MC-induced histological changes in the liver of rats. Sections were stained with hematoxylin and eosin ×400.
Source: Uhunmwangho et al. (2022b).

increase in weight compared to the animals in group not fed with diet containing *Tetracarpidium conophorum* seeds lipid extract. A total of 6 rats died within the 6 to 10$^{th}$ week due to carcinogen toxicity, while no mortality was observed in the group pretreated with *T. conophorum* seeds lipid extract and the group that was fed with *T. conophorum* seeds lipid extract only. Data on incidence, latency period, weight, size and volume of tumors in mammary gland are summarized in Table 1.

The incidence and latency period of tumors on *T. conophorum* seeds lipid extract pretreated animals was 21.8% and 10 weeks respectively, and was significantly ($P$<0.05) lower than animals administered carcinogen only with 89.4% and 4 weeks, respectively. The average size and volume of tumor was generally larger in MC administered group than in *Tetracarpidium conophorum* seeds lipid extract diet treated group. Hence, the feeding of animals which started during the pubescent period of mammary gland development might have resulted in the decreased tumor incidence and progression to malignancy. Histopathology results (Plates 1 to 3) showed normal morphology of the hepatocytes of animals fed with diet containing *T. conophorum* nut oil (groups A and B), while necrosis, steatosis and degeneration of cytoplasm with vacuoles were seen in group that was not fed with diet containing *T. conophorum* seeds lipid extract (group C).

The metabolism of carcinogens involves phase-I and phase-II reactions; in the first reaction, carcinogens are metabolized to reactive molecules by phase-I enzymes, while the active metabolite gets detoxified by several phase-II enzymes. Thus, phase-I and phase-II enzymes reactive reactions would determine the extent of carcinogenesis. The phase-I cytochrome P450 enzymes are membrane bound and their activities are influenced by the fatty acids environment (Ogunyinka et al., 2015; Batirel et al., 2018; Ayeni and Nuhu, 2018). Hence, altering membrane lipid composition by feeding animals on singular source of fat might affect carcinogen metabolism (Talaska et al., 2006). CYP1A1 activity in liver increased in animals in the group administered with carcinogen only and the magnitude of increase doubles activity in the animals in group treated with diet containing *T. conophorum* seeds lipid extract (Figure 3). But the group fed with diet containing the extracted lipid only, the activity of CYP1A1 activity was the lowest. CYP1B1 and CYP2B1 activities in liver remained slightly affected the group treated with diet containing *T. conophorum* seeds lipid extract throughout the period of the experiment compared to group C (group fed with MC only), and there was a significant increase in the expression of the activities of CYP1B1 and CYP2B1 in the group that received the carcinogen only (Figures 2 and 4). *T. conophorum* seeds lipid extract which contains a large amount of saturated and unsaturated fatty acids can modify the degree of unsaturation in lipids and thereby change the physicochemical environment of the microsomal membranes, which may be responsible for the decrease CYP1A1, CYP1B2 and CYP1B1 activities observed in *T. conophorum* seeds lipid extract fed rats. The decrease of these phase I enzymes might have contributed to decreased incidence of mammary tumors

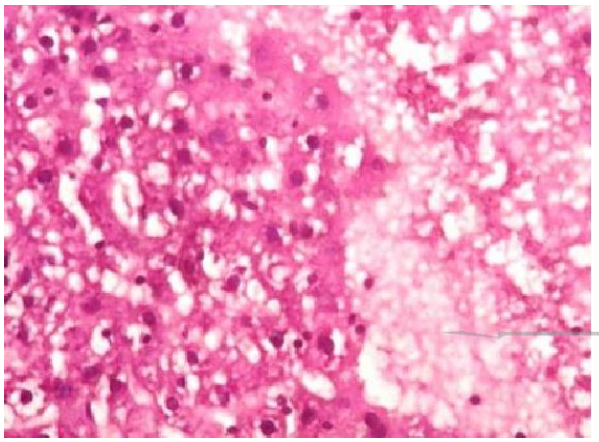

**Plate 3.** Photomicrographofaliver tissue section of group-C stained with Haematoxylin and Eosin. The photomicrograph shows the tissue parenchyma with focal area severe necrosis. The morphology of the hepatocytes shows steatosis and degeneration of cytoplasm with vacuoles.
Source: Uhunmwangho et al. (2022b).

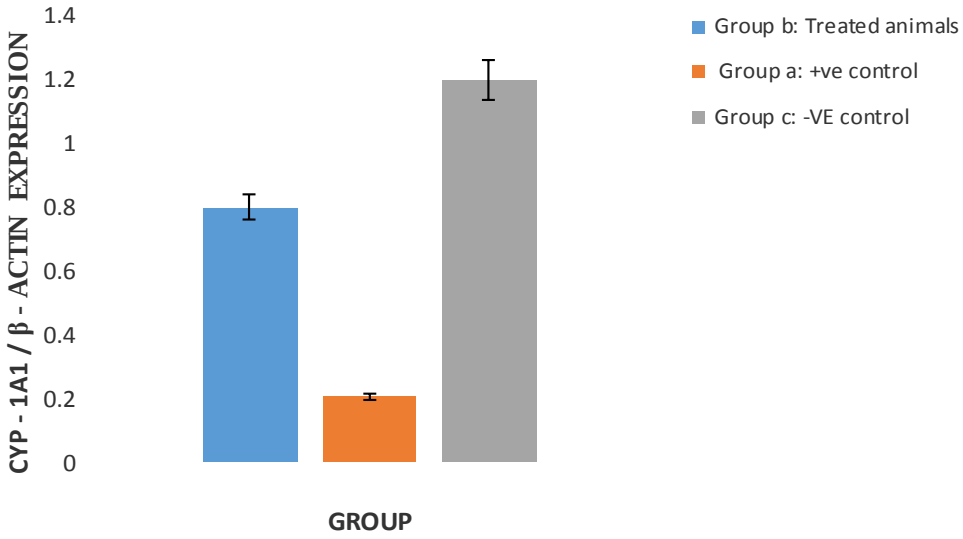


**Figure 3.** Effect of *Tetracarpidium conophorum* seeds oil on the expression of CYP1A1 in MC-induced carcinogenesis in Wister rats. Values are mean ± SEM (*p < 0.05). Group A= +VE control (Animals fed with African walnut oil only), Group B =Treated animals with African walnut seed oil + MC, Group C = -VE control (Animals administered MC only).
Source: Uhunmwangho et al. (2022b).

in *T. conophorum* seeds lipid extract fed rats compared to animals administered with MC only. Conjugated linoleic acid (CLA) and docosahexaenoic acid are well-documented anticarcinogenic agent (Ip et al., 1996).

In an *in vitro* study, CLA (0.5 µM) was reported to inhibit CYP1A1, CYP1A2, and CYP1B1 activities in hamster liver microsome (Teel and Huynh, 1998). *T. conophorum* seeds lipid extract used in this study ontained rich amount of omega-3 fatty acids which include: alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); omega-6 fatty acids which include: linoleic acid, arachidonic acid (AA), gammalinolenic acid (GLA), dihomo-gamma linolenic acid (DGLA); and omega-9 fatty acids which include: oleic acid, eicosenoic acid, erucic and nervonic acid (Uhunmwangho and Omoregie, 2017a). This indicates

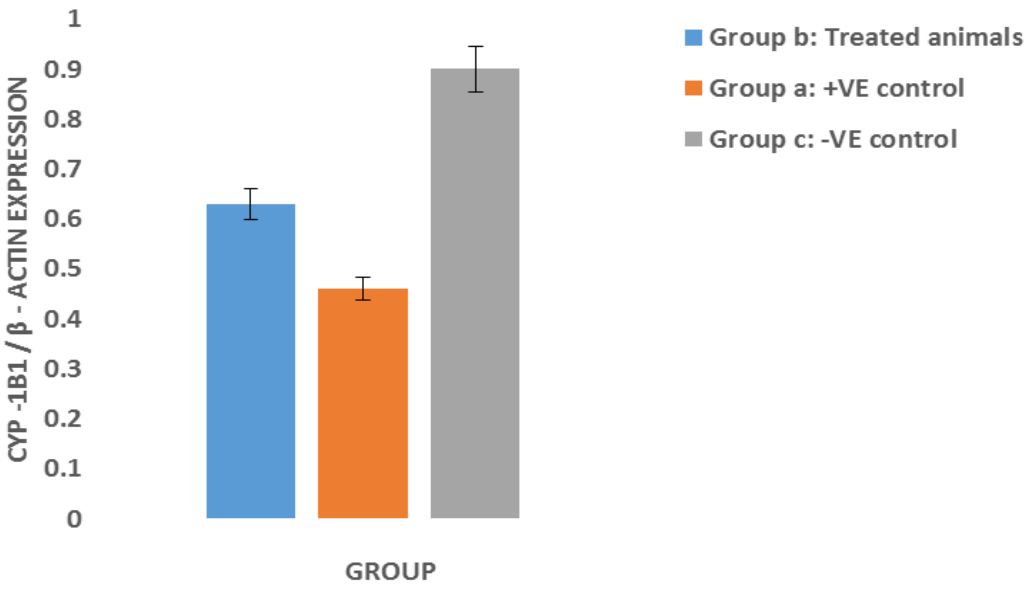


**Figure 4.** Effect of the African walnut seeds oil on the expression of CYP1B1 in MC-Induced carcinogenesis in Wister rats. Values are mean ± SEM (*p < 0.05). Group A= +VE control (Animals fed with African walnut oil only), Group B =Treated animals with African walnut seed oil + MC, Group C = -VE control (Animals administered MC only).
Source: Uhunmwangho et al. (2022b).

the great nutritional and health benefits of *T. conophorum* seed oil and its role in down regulation of the phase-I activities of carcinogen activation, CYP1A1, CYP1B1 and CYP2B1 in liver. These metabolic changes might have contributed to the decrease in 3-methylcholanthrene induced incidence of mammary tumors observed in *Tetracarpidium conophorum* seeds lipid extract fed rats compared to MC untreated animals.

## Conclusion

The result suggests that the cytochrome P450 reactive enzymes could be modulated by biochemical compounds present in the *T. conophorum* nut oil. These biochemical compounds contain anti-cancer properties that may be important in the prevention and treatment of breast cancer.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Assessment of genetic diversity of local rice accessions cultivated in Guinea revealed by SNPs markers, and identification of markers associated with tolerance to iron toxicity

**Mamadou Laho Barry[1,2], Nerbéwendé Sawadogo[1*], Mahamadi Hamed Ouédraogo[1], Tégawende Alphonse Sawadogo[1], Boukaré Kaboré[3], Sawa Camara[2], Mamadou Billo Barry[2], Pauline Bationo-Kando[1] and Mahamadou Sawadogo[1]**

[1]Biosciences, Equipe Genetique et Amélioration des Plantes (EGAP), Laboratoire, Ecole Doctorale Science et Technologies, Université Joseph KI-ZERBO, Burkina Faso.
[2]Institut de Recherche Agronomique de Guinée Conakry (IRAG), Conakry, Guinea.
[3]Institut de l'Environment et de Recherche Agricoles (INERA/CNRST), Farako-Bâ, Burkina Faso.

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**Rice is the staple food in the Republic of Guinea. However, its production is seriously hampered by iron toxicity. The fight against this stress necessarily involves the search for tolerant or resistant varieties. The study aims to determine quantitative trait locus (QTL) associated with tolerance to iron toxicity and to identify genotypes tolerant or resistant to iron toxicity. Thus, 90 local rice accessions collected in two regions of Guinea with high iron toxicity were evaluated using 33,537 single nucleotide polymorphism (SNP) markers. Seventy of the SNP markers expressed a polymorphism rate of 100%. Diversity analysis of the entire collection with these markers revealed a total of 140 alleles, a moderate Nei genetic diversity of 0.426, a fairly high Shannon diversity index of 0.610 and an organization of the accessions into four genetic groups. The study also identified 12 potentially iron-tolerant accessions and five QTLs associated with iron-toxicity tolerance located on chromosomes 1, 2, 3, 11, and 12. The 12 genotypes identified could be exploited in the lowland rice breeding program.**

**Key words:** Oryza, abiotic stress, molecular characterization, variability, molecular markers.

## INTRODUCTION

Rice is the main cereal and staple crop for almost 50% of the world's population (Ahmed et al., 2019; Mahender et al., 2019; Rasheed et al., 2020). It significantly influences food security in most countries (Ara et al., 2017). About 160 million hectares are devoted to rice production worldwide, with an approximate annual production of about 500 million metric tons (Kirby et al., 2017). Indeed, the north-eastern region of India is globally considered one of the hot pockets for rice genetic resources and a potential region with extremely diverse conditions for rice

*Corresponding author. E-mail: nerbewende.sawadogo@ujkz.bf.

cultivation (Myers et al., 2000). But, it is produced in various agroecologies and cropping systems including rain fed upland and lowland, irrigated, and mangrove (Balasubramanian et al., 2007). Thus, worldwide, several studies have focused on the genetic diversity of rice (Chakravarthi et al., 2006; Ogunbayo et al., 2005; Sajib et al., 2012) and its response to biotic and abiotic stresses (Mohammadi-Nejad et al., 2008; Aghaee et al., 2011; Selvaraj et al., 2011; Islam et al., 2012; Onyango et al., 2019).

In the Republic of Guinea, rice is also the staple food of more than 12 million people (ANASA, 2012). Indeed, the per capita consumption of rice is about 100 kg/person/year (SNSA, 2010). Rice is sown on nearly 520,000 ha and represents 65% of the country's cereal production (FAO, 2017). Its annual production has increased considerably in recent decades, from 420,000 tonnes in 1991-1992 to 895 tonnes in 2012 (ANASA, 2012). Despite the significant investments made by the state over the last decade and all potential it has (fertile land, abundant rainfall), rice production in Guinea remains below the expectations of producers and consumers (ANASA, 2012). The country still imports an average of 300,000 tonnes on the international market (CILSS/FAO/MA, 2012) to meet the population's demand for rice. The main causes of low national rice production are high production costs and low yields due to biotic factors including diseases (blast, helminthosporium, yellow mottle, etc.), weeds, and abiotic factors such as iron toxicity. Iron toxicity is one of the most important yield limiting abiotic stresses in flooded lowland rice of humid tropic areas (Becker and Asch, 2005). In Savanna zones of Africa, interflow of ferrous ion occurs from upper slopes (Moorman and Van Breeman, 1978). More than 50% lowland rice is being affected with Fe toxicity in West Africa. This stress is still one of the major constraints of lowland rice cultivation in the Republic of Guinea. The traditional culitvated varieties are poorly adapted to it. Indeed, iron toxicity causes significant yield losses in the West African sub-region, ranging from 12 to 100% depending on the rice cultivar used and the levels of iron toxicity (Masajo et al., 1986; Abifarin et al., 1988, 1989).

Several studies combining conventional and molecular breeding approaches are currently being used by Africa Rice in collaboration with the national research programmes of Guinea, Ghana, Nigeria, and Burkina Faso, Côte d'Ivoire within the STRASA project to develop iron-tolerant rice varieties. Among the promising genetic resources for iron toxicity tolerance, 181 varieties were screened for iron toxicity tolerance in susceptible areas in Nigeria and Burkina Faso and under controlled conditions in station pots. Eighty lines were identified as tolerant on the basis of their yield under stress conditions, their iron toxicity scores and other agronomic traits, and producer choice were tested in the four project countries in Participatory Varietal Selection (PVS) rice gardens. Also, several technological packages such as the development of tolerant or resistant varieties by the Africa Rice Center and the use of silica or fertilisers have been put in place to combat iron toxicity. However, farmers still face enormous difficulties related to the problem of large-scale dissemination of these varieties, their adaptation, the availability of silica, the high cost of fertilisers and the lack of development of plains (Barry, 2020; Barry et al., 2019a). The low success of these previous works in developing iron toxicity tolerant varieties is also related to the complexity of tolerance to this stress in rice.

Local varieties could play an important role in the management of this abiotic rhinestone. Indeed, traditional cultivars are storehouse for a number of desirable alleles. They are the source material for tailoring genotypes for improved agronomic performance, resilience to stresses and enhancement of quality traits (Kapoor et al., 2019). Thus, to improve rice productivity in iron toxicity affected areas, one of the easily accessible and economically profitable avenues for local producers is the use of local iron stress tolerant or resistant cultivars. In addition, previous studies using morphological markers (Barry et al., 2019a; Barry, 2006) revealed the existence of agro-morpho-physiological variability and a positive effect of silica on reducing iron toxicity. However, morphological traits, although they are the primary tools used in breeding programmes, are generally not very informative because of the strong influence of the environment (Anderson et al., 2006).

Unlike morphological and biochemical markers, DNA markers are not influenced by fluctuations in the environment and are independent of the organ analysed and the stage of development of the plant (Tagu and Moussard, 2003). Molecular markers are therefore essential tools in plant breeding, contributing to the acceleration of plant breeding and opening up new perspectives for the breeder. According to Hirschhorn and Daly (2005), the gene-phenotype association reveals missing alleles due to the fact that favourable alleles tend to be rare and difficult to detect. The application of genomic tools has become an essential component in plant breeding programmes and is used to identify novel genes related to agro-nomic or stress tolerance traits. Indeed, studies on rice using single nucleotide polymorphisms (SNPs) have identified loci that can be associated with 14 morphological traits (Xu et al., 2012) such as plant height, grain shape, grain colour, and leaf colour, to name a few. QTLs associated with toxicity tolerance have already been identified in several studies offering prospects for marker-assisted selection (Matthus et al., 2015; Nugraha et al., 2016). Recent prospecting and collection of materials in areas of high iron toxicity has resulted in the collection of 90 traditional rice accessions (Barry et al., 2019b). An evaluation of this local material by integrating tolerant or resistant control varieties would make it possible to select local cultivars that are more adapted and tolerant to iron toxicity. This

study aims (i) at determining the level and structure of genetic diversity of local rice cultivars using SNPs markers, (ii) identifying iron-tolerant or iron-resistant local accessions and (iii) identifying molecular markers associated with iron tolerance and/or resistance.

## MATERIALS AND METHODS

### Plant

The plant material consists of 90 local rice accessions collected inKindia and Macenta prefectures and six introduced improved varieties used as controls of which four (CK 73, ORULUX 6, AZUCENA, NERICA L-19) are considered resistant or tolerant to iron toxicity and two (IR 64, BOUAKE 189) sensitive to iron toxicity.

### Molecular markers

A total of 33,537 SNP markers were used for the analyses and 26,067 SNP markers were identified for this study. These markers were developed specifically for diversity studies of local rice accessions collected in Guinea and the identification of specific markers associated with tolerance to iron toxicity. These markers were generated following partial sequencing of the rice genome in collaboration between AfricaRice and the Kenya-based International Livestock Research Institute. The SNP profiles were identified using bioinformatics processing of the sequencing data.

### DNA extraction

Eight 6 mm diameter leaf discs were punched from 15 days old leaves and collected in 96 deep PCR plates. DNA extraction and sequencing of the plant material was performed by the Integrated Genotyping Support and Service (IGSS) of the Kenya-based International Livestock Research Institute (ILRI) hub (BecA-ILRI hub) using DArTseq™ technology from DArT (Diversity Arrays Technology Pty Ltd) in Australia. DArTseq™ represents a combination of DArT complexity reduction method and next generation sequencing platforms (Kilian et al., 2012; Altshuler et al., 2000). Compared to other similar approaches, DArTseq™ produces a lower marker density (10 thousand and up to 35,000 loci versus> 800,000 loci with a GBS approach) but has significantly higher coverage and results in less missing data (Chen et al., 2016). An additional advantage is that DArTseq™ can directly mark samples as heterozygous/homozygous at each locus with the lower density approach (Chen et al., 2016) and has the ability to produce multiple short, high-quality polymorphic loci using an analytical pipeline custom (Sansaloni et al., 2011; Raman et al., 2014; Al-Beyroutiova et al., 2016) facilitating the optimisation of species-specific projects in the platform.

### Data filtering process and DArTseq SNP call

The DArTseq SNP-derived markers were filtered to eliminate less informative SNPs and genotypes using FlapJack software (Milne et al., 2010), where genotypes with more than 30% missing data, SNP loci with more than 20% missing data and rare SNPs with less than 5% minor allele frequencies (MAF) were pruned. Only 2677 informative DArTseq SNPs (out of 10,219) and 462 genotypes were considered after filtering and quality control of the data. The ICP value of SNPs is high when it is above 0.5 (ICP > 0.5), moderate when it varies between 0.25 and 0.5 (0.25 < ICP < 0.5) and low when it is below 0.25 (ICP < 0.25) (Botstein et al., 1980).

### Construction of linkage maps and QTL detection

For the genotyping data, genotype, environment, and G × E were considered as random factors with a replicate nested in the environment and a block nested in the replicate and environment as a fixed factor. A combined analysis was performed by R using the agricultural package to fit the best linear unbiased prediction (BLUP) model for each genotype at both the Sérédou and Kilissi sites used in GWAS. Marker-trait associations were performed by a compressed linear mixed model implemented in Genome Association and Prediction Integrated Tool (GAPIT).

### Analysis of molecular genetic diversity data

Genetix, FSTAT V2.9.3.2 and Darwin V6.0 software were used to assess the genetic parameters of the whole collection and the diversity according to collection areas (Kindia and Macenta) and species (*Oryza sativa* and *Oryza glaberrima*). GenAlex version 6.501 software was used to evaluate the genetic parameters of the entire collection. These are the average number of alleles per locus (At), the number of effective alleles (Ae), the polymorphism rate (P), the Shannon diversity index (I), the expected heterozygosity (He), the observed heterozygosity (Ho) and the polymorphism information content (PIC). The genetic differentiation index (Fst) was determined with the software FSTAT V2.9.3.2 and the Nei genetic distance between pairs of genetic groups were used to describe the interpopulation diversity. The structuring of the genetic diversity of the collection was carried out by the software DARwin version 6.0. Based on the genetic distance matrix estimated between individuals in the total population by Genetix software, local accessions were identified as potentially tolerant or resistant. Principal component analysis (PCA) was carried out using GenALEx software version 6.501 to identify the associations between the genetic groups obtained and finally to verify in the different groups, the local accessions that are tolerant or resistant to iron toxicity in relation to the resistant controls.

## RESULTS

### Identification of SNPs markers and study of genetic diversity

On the 12 chromosomes, 26,067 SNPs markers (Table 1) were detected with a number varying between 1,413 (Chr 10) and 3,241 (Chr 1). The average marker spacing was 14.3 Kb over the entire rice genome of 372,673.43 Kb. The extreme values ranged from one (1) SNP every 12.25 Kb on chro-mosome 2 to 1 SNP every 16.42 Kb on chromosome 10.

### Selected markers and level of diversity of SNPs markers

The characteristics of selected SNPs markers and values of the genetic diversity parameters are recorded in Table 2. Of the SNP markers identified, only 70 SNPs that expressed a PIC value between 0.3 and 0.5 were retained for the genetic diversity study. These selected SNPs are distributed over all 12 rice chromosomes with a number of SNPs ranging from 02 (chr 9) to 11 (chr 2).

**Table 1.** Distribution of SNP markers on 12 rice chromosomes.

| Chromosome | Physical length (Kb) | Number of SNPs | Average distance/SNP (Kb) |
|---|---|---|---|
| Chr1 | 43,198.16 | 3,241 | 13.33 |
| Chr2 | 35,924.55 | 2,908 | 12.35 |
| Chr3 | 36,396.45 | 2,818 | 12.92 |
| Chr4 | 35,419.56 | 2,376 | 14.91 |
| Chr5 | 29,763.25 | 1,893 | 15.72 |
| Chr6 | 31,209.94 | 2,177 | 14.34 |
| Chr7 | 29,679.40 | 2,033 | 14.60 |
| Chr8 | 28,401.30 | 1,883 | 15.08 |
| Chr9 | 22,947.50 | 1,447 | 15.86 |
| Chr10 | 23,205.33 | 1,413 | 16.42 |
| Chr11 | 29,018.26 | 1,943 | 14.93 |
| Chr12 | 27,509.73 | 1,935 | 14.22 |
| Total | 37,267.43 | 2,6067 | 14.30 |

Kb = Kilo base pairs (1Kb = 1000 base pairs); * = average physical length/number of SNPs.
Source: Authors' analyses

**Table 2.** Genetic diversity parameters of rice collection by SNPs markers.

| N° | SNPs | Chr | P | Q | N | At | Ae | I | PIC | Ho | He | P (95%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3992330\|F\|0-64:A>T-64:A>T | Chr1 | 0.36 | 0.64 | 94 | 2 | 1.86 | 0.65 | 0.46 | - | 0.47 | yes |
| 2 | 3763627\|F\|0-22:G>A-22:G>A | Chr1 | 0.42 | 0.59 | 94 | 2 | 1.94 | 0.68 | 0.49 | 0.01 | 0.49 | yes |
| 3 | 3447628\|F\|0-29:A>T-29:A>T | Chr1 | 0.20 | 0.80 | 94 | 2 | 1.48 | 0.50 | 0.32 | 0.01 | 0.33 | yes |
| 4 | 5398995\|F\|0-31:C>T-31:C>T | Chr1 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | - | 0.37 | yes |
| 5 | 3054375\|F\|0-13:A>G-13:A>G | Chr1 | 0.37 | 0.63 | 94 | 2 | 1.88 | 0.66 | 0.47 | - | 0.47 | yes |
| 6 | 3051369\|F\|0-22:G>C-22:G>C | Chr1 | 0.33 | 0.67 | 94 | 2 | 1.79 | 0.63 | 0.44 | - | 0.45 | yes |
| 7 | 3452391\|F\|0-9:A>G-9:A>G | Chr1 | 0.23 | 0.77 | 94 | 2 | 1.56 | 0.54 | 0.36 | - | 0.36 | yes |
| 8 | 3055204\|F\|0-50:A>G-50:A>G | Chr2 | 0.30 | 0.70 | 94 | 2 | 1.72 | 0.61 | 0.42 | 0.01 | 0.42 | yes |
| 9 | 3445970\|F\|0-35:C>A-35:C>A | Chr2 | 0.28 | 0.72 | 94 | 2 | 1.67 | 0.59 | 0.40 | - | 0.40 | yes |
| 10 | 3054221\|F\|0-11:T>C-11:T>C | Chr2 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | 0.01 | 0.37 | yes |
| 11 | 9754762\|F\|0-15:G>A-15:G>A | Chr2 | 0.22 | 0.78 | 94 | 2 | 1.53 | 0.53 | 0.35 | 0.01 | 0.35 | yes |
| 12 | 100331689\|F\|0-29:A>C-29:A>C | Chr2 | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | 0.01 | 0.38 | yes |
| 13 | 3056152\|F\|0-61:G>A-61:G>A | Chr2 | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | 0.01 | 0.38 | yes |
| 14 | 3755110\|F\|0-8:A>G-8:A>G | Chr2 | 0.37 | 0.63 | 94 | 2 | 1.88 | 0.66 | 0.47 | 0.03 | 0.47 | yes |
| 15 | 3443188\|F\|0-6:G>C-6:G>C | Chr2 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | 0.01 | 0.37 | yes |
| 16 | 3052615\|F\|0-8:A>G-8:A>G | Chr2 | 0.32 | 0.68 | 94 | 2 | 1.77 | 0.63 | 0.44 | -0.00 | 0.44 | yes |
| 17 | 3062322\|F\|0-14:C>A-14:C>A | Chr2 | 0.29 | 0.71 | 94 | 2 | 1.69 | 0.60 | 0.41 | 0.00 | 0.41 | yes |
| 18 | 3048309\|F\|0-57:G>C-57:G>C | Chr2 | 0.44 | 0.56 | 94 | 2 | 1.97 | 0.69 | 0.49 | 0.00 | 0.50 | yes |
| 19 | 3055285\|F\|0-15:G>C-15:G>C | Chr3 | 0.33 | 0.67 | 94 | 2 | 1.79 | 0.63 | 0.44 | 0.00 | 0.45 | yes |
| 20 | 3054138\|F\|0-49:A>G-49:A>G | Chr3 | 0.37 | 0.63 | 94 | 2 | 1.88 | 0.66 | 0.47 | 0.00 | 0.47 | yes |
| 21 | 3048686\|F\|0-16:G>C-16:G>C | Chr3 | 0.43 | 0.57 | 94 | 2 | 1.96 | 0.68 | 0.49 | 0.00 | 0.49 | yes |
| 22 | 6996799\|F\|0-29:A>G-29:A>G | Chr4 | 0.23 | 0.77 | 94 | 2 | 1.56 | 0.54 | 0.36 | 0.00 | 0.36 | yes |
| 23 | 3438153\|F\|0-37:A>G-37:A>G | Chr4 | 0.34 | 0.66 | 94 | 2 | 1.82 | 0.64 | 0.45 | 0.00 | 0.45 | yes |
| 24 | 3058320\|F\|0-20:A>T-20:A>T | Chr5 | 0.21 | 0.79 | 94 | 2 | 1.50 | 0.52 | 0.34 | 0.00 | 0.34 | yes |
| 25 | 3063709\|F\|0-8:T>C-8:T>C | Chr5 | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | 0.01 | 0.38 | yes |
| 26 | 5406581\|F\|0-9:A>G-9:A>G | Chr5 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | 0.00 | 0.37 | yes |
| 27 | 3050023\|F\|0-58:T>A-58:T>A | Chr5 | 0.35 | 0.65 | 94 | 2 | 1.84 | 0.65 | 0.46 | 0.00 | 0.46 | yes |
| 28 | 3057539\|F\|0-6:T>A-6:T>A | Chr6 | 0.33 | 0.67 | 94 | 2 | 1.79 | 0.63 | 0.44 | 0.00 | 0.45 | yes |
| 29 | 100221066\|F\|0-44:A>G-44:A>G | Chr6 | 0.23 | 0.77 | 94 | 2 | 1.56 | 0.54 | 0.36 | 0.00 | 0.36 | yes |
| 30 | 3452699\|F\|0-17:C>G-17:C>G | Chr6 | 0.30 | 0.70 | 94 | 2 | 1.72 | 0.61 | 0.42 | 0.00 | 0.42 | yes |
| 31 | 3442305\|F\|0-37:G>A-37:G>A | Chr6 | 0.33 | 0.67 | 94 | 2 | 1.79 | 0.63 | 0.44 | 0.00 | 0.45 | yes |

**Table 2.** Contd.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 3057879\|F\|0-47:A>G-47:A>G | Chr6 | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.00 | 0.43 | yes |
| 33 | 3056178\|F\|0-64:G>A-64:G>A | Chr6 | 0.35 | 0.65 | 94 | 2 | 1.84 | 0.65 | 0.46 | 0.00 | 0.46 | yes |
| 34 | 3055411\|F\|0-26:C>T-26:C>T | Chr7 | 0.38 | 0.62 | 94 | 2 | 1.90 | 0.67 | 0.47 | 0.00 | 0.48 | yes |
| 35 | 3062799\|F\|0-55:G>A-55:G>A | Chr7 | 0.22 | 0.78 | 94 | 2 | 1.53 | 0.53 | 0.35 | 0.03 | 0.35 | yes |
| 36 | 3752213\|F\|0-32:A>C-32:A>C | Chr7 | 0.40 | 0.60 | 94 | 2 | 1.93 | 0.68 | 0.48 | 0.00 | 0.49 | yes |
| 37 | 3054330\|F\|0-26:C>G-26:C>G | Chr7 | 0.38 | 0.62 | 94 | 2 | 1.90 | 0.67 | 0.47 | 0.00 | 0.48 | yes |
| 38 | 3048748\|F\|0-9:C>T-9:C>T | Chr7 | 0.40 | 0.60 | 94 | 2 | 1.93 | 0.68 | 0.48 | 0.00 | 0.49 | yes |
| 39 | 3054246\|F\|0-44:T>A-44:T>A | Chr8 | 0.33 | 0.67 | 94 | 2 | 1.79 | 0.63 | 0.44 | 0.01 | 0.45 | yes |
| 40 | 3445671\|F\|0-45:A>G-45:A>G | Chr8 | 0.37 | 0.63 | 94 | 2 | 1.88 | 0.66 | 0.47 | 0.00 | 0.47 | yes |
| 41 | 3052888\|F\|0-24:G>C-24:G>C | Chr8 | 0.44 | 0.56 | 94 | 2 | 1.97 | 0.69 | 0.49 | 0.00 | 0.50 | yes |
| 42 | 3049159\|F\|0-28:G>A-28:G>A | Chr8 | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.00 | 0.43 | yes |
| 43 | 3445651\|F\|0-28:G>A-28:G>A | Chr9 | 0.37 | 0.63 | 94 | 2 | 1.88 | 0.66 | 0.47 | 0.00 | 0.47 | yes |
| 44 | 3054235\|F\|0-25:T>C-25:T>C | Chr9 | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.00 | 0.43 | yes |
| 45 | 100385773\|F\|0-35:C>T-35:C>T | Chr10 | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | - | 0.43 | yes |
| 46 | 3049259\|F\|0-67:T>C-67:T>C | Chr10 | 0.45 | 0.55 | 94 | 2 | 1.98 | 0.69 | 0.49 | - | 0.50 | yes |
| 47 | 3050230\|F\|0-41:T>C-41:T>C | Chr10 | 0.39 | 0.61 | 94 | 2 | 1.91 | 0.67 | 0.48 | - | 0.48 | yes |
| 48 | 3054238\|F\|0-50:T>C-50:T>C | Chr11 | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.01 | 0.43 | yes |
| 49** | 5399508\|F\|0-48:C>T-48:C>T | Chr11 | 0.22 | 0.78 | 94 | 2 | 1.53 | 0.53 | 0.35 | 0.06 | 0.35 | yes |
| 50 | 4392105\|F\|0-29:T>C-29:T>C | Chr11 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | - | 0.37 | yes |
| 51 | 3050444\|F\|0-46:T>C-46:T>C | Chr11 | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | - | 0.37 | yes |
| 52 | 100383775\|F\|0-10:G>A-10:G>A | Chr12 | 0.22 | 0.78 | 94 | 2 | 1.53 | 0.53 | 0.35 | - | 0.35 | yes |
| 53 | 3050121\|F\|0-18:G>T-18:G>T | Chr12 | 0.38 | 0.62 | 94 | 2 | 1.90 | 0.67 | 0.47 | - | 0.48 | yes |
| 54 | 12387915\|F\|0-8:A>G-8:A>G | Chr12 | 0.34 | 0.66 | 94 | 2 | 1.82 | 0.64 | 0.45 | - | 0.45 | yes |
| 55 | 3052563\|F\|0-32:C>A-32:C>A | Chr12 | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | - | 0.38 | yes |
| 56 | 3049895\|F\|0-51:A>G-51:A>G | Chr12 | 0.43 | 0.57 | 94 | 2 | 1.96 | 0.68 | 0.49 | - | 0.49 | yes |
| 57 | 100465057\|F\|0-24:C>T-24:C>T | Chr12 | 0.32 | 0.68 | 94 | 2 | 1.77 | 0.63 | 0.44 | - | 0.44 | yes |
| 58 | 3062942\|F\|0-24:C>T-24:C>T | - | 0.23 | 0.77 | 94 | 2 | 1.56 | 0.54 | 0.36 | 0.01 | 0.36 | yes |
| 59 | 3446620\|F\|0-39:C>G-39:C>G | - | 0.39 | 0.61 | 94 | 2 | 1.91 | 0.67 | 0.48 | 0.01 | 0.48 | yes |
| 60 | 100192994\|F\|0-11:G>A-11:G>A | - | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.00 | 0.43 | yes |
| 61 | 100195135\|F\|0-28:G>A-28:G>A | - | 0.22 | 0.78 | 94 | 2 | 1.53 | 0.53 | 0.35 | 0.06 | 0.35 | yes |
| 62 | 3055773\|F\|0-10:G>T-10:G>T | - | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | -0.00 | 0.38 | yes |
| 63 | 3060796\|F\|0-24:G>A-24:G>A | - | 0.32 | 0.68 | 94 | 2 | 1.77 | 0.63 | 0.44 | 0.01 | 0.44 | yes |
| 64 | 4393265\|F\|0-48:T>C-48:T>C | - | 0.35 | 0.65 | 94 | 2 | 1.84 | 0.65 | 0.46 | 0.04 | 0.46 | yes |
| 65 | 12387577\|F\|0-14:C>T-14:C>T | - | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.62 | 0.43 | 0.01 | 0.43 | yes |
| 66 | 100372655\|F\|0-6:C>G-6:C>G | - | 0.43 | 0.57 | 94 | 2 | 1.96 | 0.68 | 0.49 | 0.01 | 0.49 | yes |
| 67 | 4390290\|F\|0-29:C>T-29:C>T | - | 0.25 | 0.76 | 94 | 2 | 1.59 | 0.56 | 0.37 | 0.00 | 0.37 | yes |
| 68 | 100374359\|F\|0-8:G>A-8:G>A | - | 0.26 | 0.75 | 94 | 2 | 1.61 | 0.57 | 0.38 | 0.00 | 0.38 | yes |
| 69 | 3439459\|F\|0-56:G>A-56:G>A | - | 0.30 | 0.70 | 94 | 2 | 1.72 | 0.61 | 0.42 | 0.00 | 0.42 | yes |
| 70 | 100418541\|F\|0-9:A>G-9:A>G | - | 0.28 | 0.72 | 94 | 2 | 1.67 | 0.59 | 0.40 | 0.00 | 0.40 | yes |
| **Average** | | 0.31 | 0.69 | 94 | 2 | 1.74 | 0.61 | 0.42 | 0.01 | 0.43 | 1.00 | |

Chr: Chromosome; At: total number of alleles per locus, Ae: number of effective alleles, He: expected heterozygosity, Ho: observed heterozygosity, PIC: Polymorphism Information Content, I: Shannon diversity index, P: polymorphic loci rate, Fis: fixation index.
Source: Authors' analyses

A total of 140 haplotypes with an average number of two per locus were detected (Figure 1). The genetic diversity of Nei (He) ranged from 0.33 (SNPs 3447628|F|0-29:A>T-29:A>T) to 0.5 (3048309|F|0-57:G>C-57:G>C) with an average of 0.43, respectively. The observed heterozygosity (Ho) was lower and ranged from 0 for SNP 3992330|F|0-64:A>T-64:A>T to 0.064 for SNP 5399508|F|0-48:C>T-48:C>T with a mean of 0.006. As for the Shannon diversity index (I), it expressed higher values ranging from 0.52 for the SNP marker 3058320|F|0-20:A>T-20:A>T to 0.69 for the markers 3049259|F|0-67: T>C-67:T>C. Polymorphism information content (PIC) ranged from 0.32 for the SNP marker 3447628|F|0-29:A>T-29:A>T to 0.49 for 3049259|F|0-67:T>C-67:T>C with a mean of 0.42. The fixation index or intrapopulation genetic differentiation (Fis), which is a

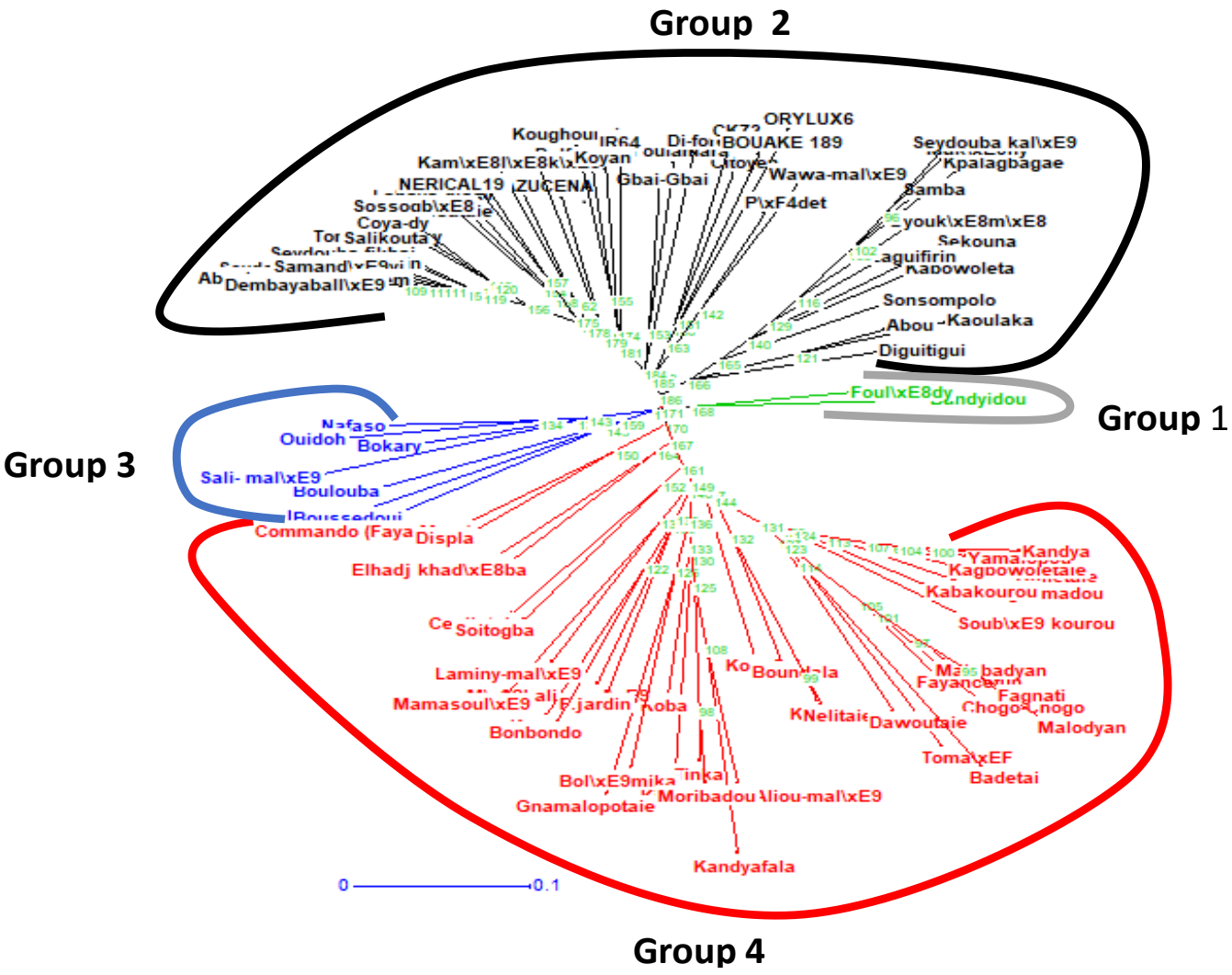


**Figure 1.** Radial representation of the dendrogram constructed from the dissimilarity matrix obtained for all 90 local accessions and the 6 rice controls with the Neighbour-Joining method.
Black for tolerant, red for highly susceptible, blue for less susceptible, and green for susceptible. The numbers from 1 to 94 represent the individuals studied.
Source: Authors' analyses

measure of the difference between the population of individuals found in the observed heterozygous state (Ho) and the expected heterozygous (He) showed a relatively high positive value (0.98).

## Structuring the genetic diversity of rice in Guinea

### *Comparison of accessions according to collection area*

The values of the diversity parameters of accessions in the Kindia area are higher than those in the Macenta area (Table 3). Indeed, the Kindia area revealed an effective number of alleles of 1.78; an expected heterozygosity of 0.44; a Shannon index of 0.62% and a polymorphism information content of 0.60. However, in Macenta, an effective number of alleles of 1.68; an expected heterozygosity of 0.40; a Shannon index of 0.58, a polymorphism rate of 100% and a low polymorphism information content of 0.30 were recorded. The polymorphism rate of the SNPs loci is 100% for both areas. The Nei genetic distance and the estimated genetic differentiation index between the accessions of the two zones revealed significant differences between the local accessions of the zones (Table 4).

### *Comparison of accessions by species (O. sativa and O. glaberrima)*

The values of the diversity parameters of the *O. sativa*

**Table 3.** Genetic diversity parameters of rice according to collection area.

| Location | N | At | Ae | I | PIC | He | P (%) |
|---|---|---|---|---|---|---|---|
| Kindia | 43 | 2 | 1.780 | 0.623 | 0.602 | 0.443 | 100 |
| Macenta | 51 | 2 | 1.680 | 0.581 | 0.007 | 0.402 | 100 |
| Mean | 47 | 2 | 1.730 | 0.602 | 0.305 | 0.423 | 100 |

**N**: Number of individuals, **At**: total number of alleles per locus, **Ae**: number of effective alleles, **He**: expected heterozygosity, **PIC**: Polymorphism Information Content, **I**: Shannon diversity index, **P**: polymorphic loci rate.
Source: Authors' computaions

**Table 4.** Inter-area genetic differentiation of local rice accessions.

| Climatic zone | Minimum distance from Nei | | Differentiation index Fst | |
|---|---|---|---|---|
| | Kindia | Macenta | Kindia | Macenta |
| Kindia | 0 | | 0 | |
| Macenta | 0.019* | 0 | 0.05* | 0 |

**Table 5.** Distribution of genetic diversity of rice accessions by species.

| Rice species | N | At | Ae | I | He | Pic | P(%) |
|---|---|---|---|---|---|---|---|
| *O. glaberrima* | 17.000 | 2.000 | 1.660 | 0.567 | 0.408 | 0.383 | 100.00 |
| *O. Sativa* | 77.000 | 2.000 | 1.749 | 0.614 | 0.430 | 0.424 | 100.00 |
| Average | 47.000 | 2.000 | 1.705 | 0.591 | 0.419 | 0.403 | 100.00 |

At: Total number of alleles, Ae: number of effective alleles, He: expected heterozygosity, PIC: Polymorphism Information Content, I: Shannon diversity index, P: polymorphic loci rate, N: number of individuals.
Source: Authors' computaions

**Table 6.** Genetic differentiation of local rice accessions according to species.

| Rice species | Nei' Minimum distance | | Fst Differentiation index | |
|---|---|---|---|---|
| | *O. glaberrima* | *O. sativa* | *O. glaberrima* | *O. sativa* |
| *O. glaberrima* | 0 | - | 0 | - |
| *O. sativa* | 0.019 | 0 | $0.45^{ns}$ | 0 |

Source: Authors' computations

species accessions are higher than those of the *O. glaberrima* species (Table 5). Indeed, *Oryza sativa* species revealed an effective number of alleles of 1.75; an expected heterozygosity of 0.43; a Shannon index of 0.61; a polymorphism rate of 100% and a polymorphism information content of 0.42. For *O. glaberrima*, the effective number of alleles was 1.66; expected heterozygosity, 0.41; Shannon index, 0.57; polymorphism information content, 0.38 and polymorphism rate of SNPs loci 100%. The genetic differentiation index revealed a significant difference between the two species (*O. sativa* and *O. glaberrima*) on tolerance and/or resistance to iron toxicity (Table 6). The genetic distance matrix between the species indicates a difference of 0.45 between *O. sativa* and *O. glaberrima* species. These relatively low values indicate that the species are genetically close in tolerance and/or resistance to iron toxicity.

## Organisation of genetic diversity by markers and description of genetic groups

The estimated distances between individuals in the total population ranged from 0 to 0.56. Examination of the dendrogram of the total population (Figure 1) allows us to distinguish four distinct genetic groups (1, 2, 3, 4) at the

**Table 7.** Characteristics of genetic groups.

| Group | N | At | Ae | I | He | PIC | P (%) |
|---|---|---|---|---|---|---|---|
| 1 | 2.000 | 0.861 | 1.389 | 0.270 | 0.389 | 0.200 | 38.889 |
| 2 | 45.000 | 2.000 | 1.506 | 0.489 | 0.325 | 0.320 | 100.000 |
| 3 | 7.000 | 1.792 | 1.610 | 0.512 | 0.406 | 0.358 | 88.889 |
| 4 | 40.000 | 1.972 | 1.757 | 0.598 | 0.426 | 0.427 | 97.222 |
| Total | 23.500 | 1.656 | 1.566 | 0.467 | 0.386 | 0.326 | 81.250 |

At: Total number of alleles, Ae: number of effective alleles, He: expected heterozygosity, PIC: Polymorphism Information Content, I: Shannon diversity index, P: polymorphic loci rate, N: number of individuals.
Source: authors

**Table 8.** Genetic differentiation between genetic groups of rice accessions.

| Genetic group | Nei' minimum distance | | | Fst differentiation index | | |
|---|---|---|---|---|---|---|
| | Group 1 | Group 2 | Group 3 | Group 1 | Group 2 | Group 3 |
| Group 1 | 0 | | | 0 | | |
| Group 2 | 0.181 | 0 | | 0.083* | 0 | |
| Group 3 | 0.177 | 0.137 | 0 | 0.725* | 0.008** | 0 |
| Group 4 | 0.251 | 0.186 | 0.235 | 0.033* | 0.008** | 0.008** |

Source: authors

threshold of the 5% confidence interval independent of the collection area and consisting, respectively of 2 accessions for group 1, 47 accessions for group 2, 7 accessions for group 3 and 40 accessions for group 4. Estimation of the genetic diversity parameters of the four identified genetic groups showed that a variation in the number of effective alleles from 1.389 (group 1) to 1.506 (group 2) and in the expected heterozygosity 0.325 for group 2 to 0.426 for group 4. The percentage of polymorphism was generally high except for group 1 which expressed the lowest rate of 38.889%. Finally, the Shannon diversity index and polymorphism information content ranged from 0.270 to 0.489 for groups 1 and 2 and from 0.512 to 0.598 for groups 3 and 4, respectively. In general, group 2 showed the highest values of diversity parameters, while groups 1 and 3 expressed the lowest values (Table 7).

### *Comparison of genetic groups*

The average value of the intergroup differentiation index (Fst) is 0.14. Most of the total genetic variability (86%) is explained by intrapopulation variation. The genetic distances of 0.181 between genetic groups 1 and 2 and 0.177 between groups 2 and 3 (Table 8). These are relatively low. This would indicate that the accessions have a high genetic proximity. The Principal Coordinates Analysis (PCA) indicates that axis 1 (Coord.1) explains 57.57% of the total genetic variation, axis 2 (Coord.2) explains 29.18% and confirms that groups 2 and 3 are genetically close and axis 3 which explains 13.24% of the variability indicates that groups 1 and 4 are the most distant. These two groups would therefore be genetically more distant (Figure 2).

### Identification of alleles associated with tolerance to iron toxicity

Of the 12 rice chromosomes, five chromosomes carry Quantitative Trait Locus (QTL) associated with tolerance to iron toxicity. These potentially influential QTLs are located on chromosomes 1, 2, 3, 11, and 12 (Table 9). The most significant SNP (3438008 | F |0-40: T> C-40: T>C) associated with tolerance is located on chromosome 2 at 25224.27 kb with a P-value of 0.000136 and followed by SNP 3049240 | F |0-25:C>T-25: C>T, which is located on chromosome 1 at 27586.15 kb (Figure 3).

## DISCUSSION

Analysis of the genetic diversity of local Guinean rice accessions shows higher variability in *O. sativa* than in *O. glaberrima. O. sativa* is the most cultivated species with higher yields than *O. glaberrima. O. glaberrima* has completely disappeared in some regions such as East Africa (Bezançon, 1995). In contrast, the study revealed that it still occurs in Guinea to a considerable degree in the two study regions which are the rice granaries of

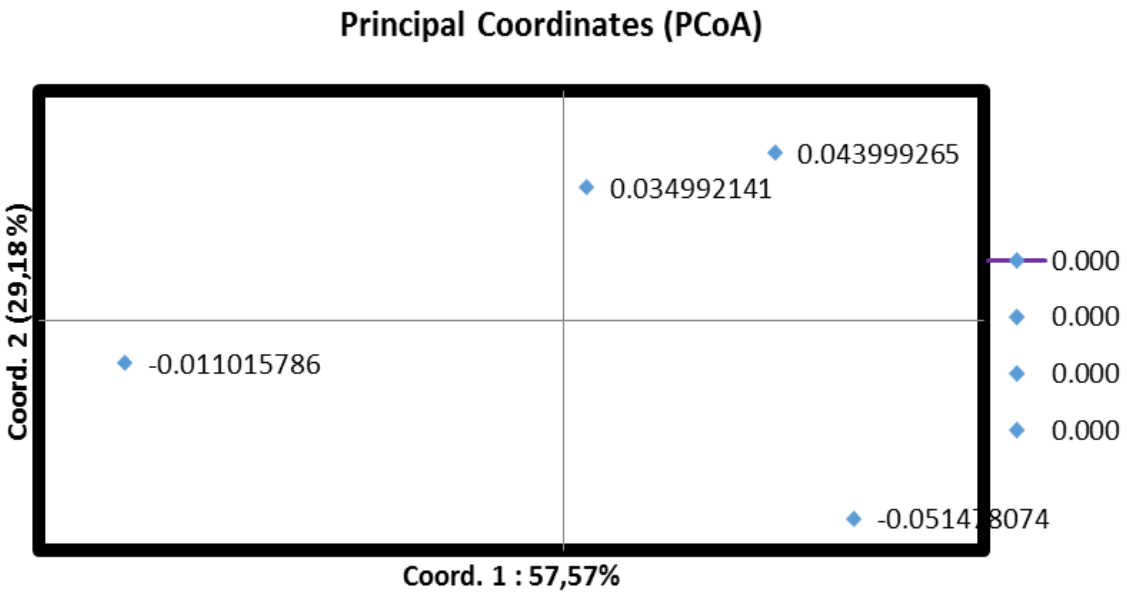


**Figure 2.** PCA representation of four genetic groups in the ½ plane.
Source: authors

**Table 9.** QTLs were identified with significant association with iron toxicity tolerance.

| SNPs | Chromosome | Position (kb) | P.value | Maf |
|---|---|---|---|---|
| 3049240\|F\|0-25:C>T-25:C>T | 1 | 27586.15 | 0.000629 | 0.253846 |
| 3438008\|F\|0-40:T>C-40:T>C | 2 | 25224.27 | 0.000136 | 0.071429 |
| 3052218\|F\|0-29:G>A-29:G>A | 3 | 14704.16 | 0.000739 | 0.184783 |
| 3453008\|F\|0-22:T>G-22:T>G | 11 | 4764.300 | 0.000891 | 0.240000 |
| 3057943\|F\|0-14:C>G-14:C>G | 12 | 18040.38 | 0.000830 | 0.120370 |

Source: authors

Guinea. These results have already been reported by several authors (Second, 1985; Ghesquiere and Miezan, 1982; Barry et al., 2007) on a larger sample. A comparison of the average genetic diversity at common loci of the Guinea accessions with samples used elsewhere for other studies was made. These accessions are drawn from the collection of local rice varieties in Guinea and are assessed for iron tolerance through their yield. Yield is a parameter controlled by several genes corresponding to yield components. Thus, the use of resistant or tolerant controls and SNPs markers allowed the identification of iron-tolerant and iron-resistant rice accessions. Molecular characterisation of these local rice accessions (*O. glaberriama* and *O. sativa*) in Guinea revealed multiple traits in local accessions at the different study sites that define the yield increase and the tolerance of certain accessions to iron toxicity.

Furthermore, the markers used helped to identify the alleles of interest of these local accessions evaluated in Kindia and Macenta. This was further confirmed by the distribution of the accessions through the phylogenetic tree. Analysis of the dendrogram of the 96 local accessions shows a clustering of accessions by similarity in terms of Nei genetic distance. The 12 local rice accessions that were selected in both study sites all fall into the same group as the resistant control varieties. Groups 2 and 3 are the closest genetically. Group 4, on the other hand, is significantly further away from the other three groups 1, 2 and 3 with distances of 0.251, 0.186 and 0.235, respectively. The selected local accessions are predisposed to a more significant yield performance than other accessions that are in other groups to which they are similar. This could be explained by the additive effect of the different traits accumulated by the latter. However, the nature of these effects needs to be verified by agronomic data such as yield. In the same sense, Xiao et al. (1986) state that genetic distances obtained by RAPD or SSR markers can be useful in predicting the yield potential and heterosis of intraspecific species. However, the effect of the accumulation of several traits in a line is not clearly defined. The 70 validated SNP markers revealed diversity within the local rice accessions

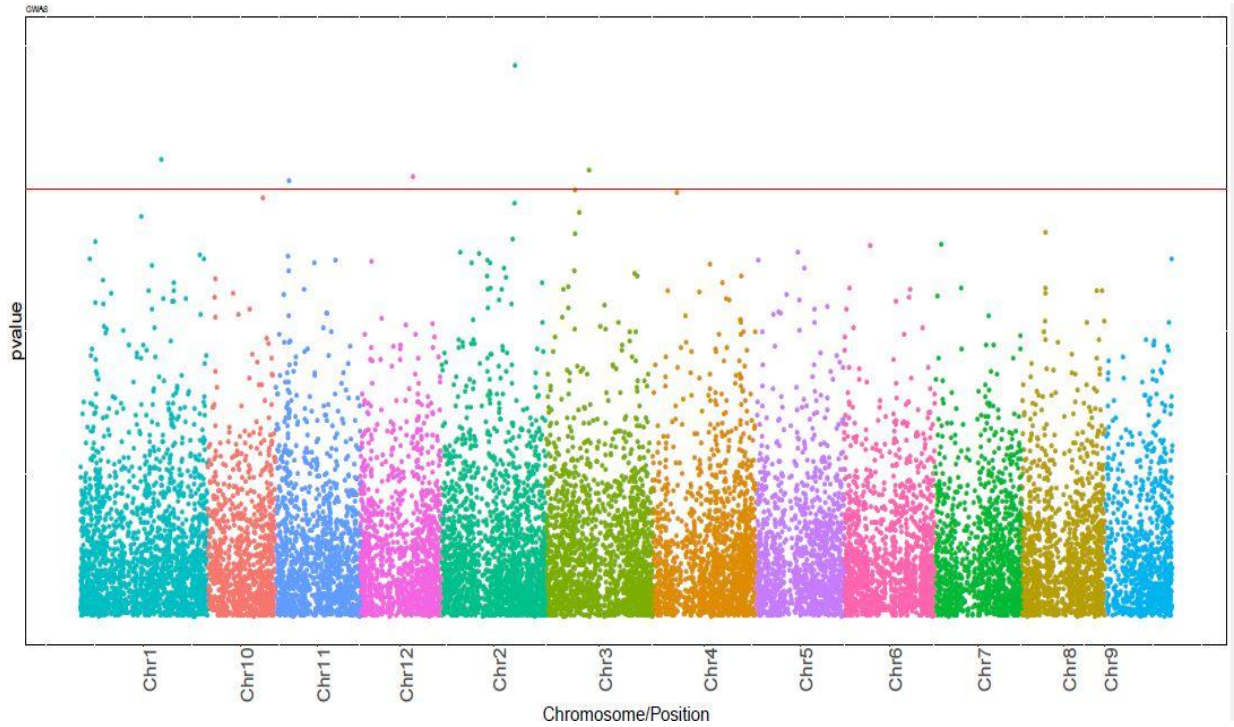


**Figure 3.** p-value of identified markers per chromosome for tolerance to iron toxicity.
Source: authors

studied with a polymorphism rate of 100% at the 5% threshold. Previous works (Maravilla et al., 2017; Adeniji et al., 2012) reported higher or lower rates of 97 and 65.12%, respectively. The positive difference between the expected (0.44) and observed (0.064) heterozygosity and the higher positive value of the fixation index (Fis) (0.98) indicate a deficit of heterozygotes with an inbreeding coefficient of 2%, which indicates that the accessions collected in Kindia and Macenta prefectures are poor in heterozygous individuals. This high level of inbreeding could be explained by the preferentially self-pollinating mode of reproduction of rice.

According to Jordana et al. (2003), inbreeding modifies genotypic frequencies and the consequence is a loss of genetic variability over generations. The expected heterozygosity of 0.44 with an effective number of alleles ranging from 1.66 to 1.78 per locus and the genetic distances between accessions (0 to 0.56) reflect a relatively high genetic diversity of the collection of local rice accessions established in Kindia and Macenta prefectures. These differences can be explained by the genotypic diversity, the sample size, and the origin of the plant material. Indeed, the allelic richness of a population is known to depend on the sample size, since the chances of discovering a new allele increase each time a new individual is observed (Foulley and Ollivier, 2006). Similarly, according to (M'Hamed et al., 2008), the number of alleles per locus is affected by several factors such as genotype primer sequences and minor variations in amplification protocols. Moreover, according to Zhu et al. (2012), the diversity revealed in a collection is all lower when the markers used are developed on another species. Indeed, the SNPs markers used in this study were developed on local rice accessions and are therefore specific to these species (*O. sativa* and *O. glaberrima*). The influence of the climatic zone factor on the level and organisation of genetic diversity is very low. This low genetic differentiation between local accessions of the two prefectures is considered due to a large and continuous exchange of plant material between producers through weekly markets, population migration on the one hand, and the self-pollination rate (98%) of rice on the other. Similar results were reported by Barry (2006) in Guinea Maritime and revealed the existence of exchanges of planting material in different villages between families and acquaintances.

The analysis of the genetic diversity of *O. sativa* and *O. glaberrima* accessions using tested and validated SNPs markers has shown their effectiveness in revealing polymorphisms within rice. These SNPs markers tested and validated in this study are potential markers for the study of the genetic diversity of rice at the national, subregional and even international levels with a view to preserving and enhancing these species (*O. sativa* and *O. glaberrima*) which are well exploited throughout the world for a breeding programme and comparison of entities within collections. It also revealed the existence of a distribution of accessions into genetic groups based

on tolerance and/or resistance to iron toxicity. Genetic structuring is much more determined by the collection area factor and by the species factor (*O. glaberrima* and *O. sativa*). Finally, five QTLs associated with tolerance to iron toxicity have been also identified on chromosomes 1, 2, 3, 11 and 12. Furthermore, Nugraha et al. (2016) identified TBGI380435 located on 14.45 Mbp of chromosome 9 as associated to leaf bronzing and relative shoot weight characters. A major QTL for salt tolerance named Saltol was mapped on chromosome 1 (Mohammadi-Nejad et al., 2008).

## Conclusion

The study highlighted significant genetic diversity within local rice accessions grown in the Republic of Guinea. The diversity is organised in four genetic groups and allowed the detection of 12 accessions potentially tolerant to iron toxicity. Five markers linked to iron toxicity were also identified.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# The role of N-terminal module of PhyB in modulating root and hypocotyl growth length in Arabidopsis

**Njimona Ibrahim[1,2*] and Baluška František[3]**

[1]Institute of Medical Research and Medicinal Plants Studies (IMPM), Ministry of Scientific Research and Innovation, P. O. Box 6133, Yaounde, Cameroon.
[2]Bamenda University of Science and Technology (BUST), Bamenda, Cameroon.
[3]Institute of Cellular and Molecular Botanics (IZMB), University of Bonn, Bonn, Germany.

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**Phytochrome belongs to red/far-red light family of photoreceptors. It exists in two spectral forms named red light absorbing form (Pr), said to be the inactive form and far-red light absorbing form (Pfr) which is the active form. This photoreceptor is structurally divided into two modules- the amino acid (N-) terminal photosensory module and caboxylic acid (C-) terminal His kinase-like catalytic output module. Five different types exist in Arabidopsis (PhyA-E). Roots and hypocotyls elongation in Arabidopsis is regulated by photoreceptors one class of which is phytochrome. The role of phytochrome B (PhyB) in red light responses has been established through studies using PhyB mutant and truncated versions. N-terminal module of PhyB containing 651 amino acids was shown to be biologically active in regulating photomorphogenesis. Meanwhile, the C-terminal module was long assumed to be involved in downstream signal transduction. Recently, this module was suggested to play a role in integrating red and blue light signaling to circadian clock. Here, the study shows that the C-terminal module of PhyB is needed for root growth and strongly modulates the root to hypocotyl ratio at 22°C. At an elevated temperature (34°C), this ratio was altered suggesting a role of this module in temperature signaling during plant growth.**

**Key words:** Phytochrome, red/far-red, Roots, hypocotyls.

## INTRODUCTION

In all living organisms, responses to stimuli are mediated by signal transduction pathways governed by their physiological performance under given established conditions. In both prokaryotes and eukaryotes, one of the key strategies from which originates intracellular signal-transduction is protein phosphorylation. Using ATP as the phosphate donor, protein kinases catalyze auto-phosphorylation (phosphorylation of themselves) or trans-phosphorylation (phosphorylation of other protein substrates) at particular amino acid residues. Until now,

*Corresponding author. E-mail: njimona@yahoo.com.

protein phosphorylation has been identified on His, Ser, Asp, Tyr, Thr, Lys, Arg and Cys residues (Matthews, 1995; Khoury et al., 2011). As regards this substrate specificity, protein kinases have been grouped into three main categories, viz: Tyr kinases, Ser-Thr kinases, and His kinases. In eukaryotic systems, the Tyr and Ser-Thr kinases are dominant. In bacteria, His kinases are dominant and are involved in diverse responses like thermosensing, nutrient sensing, photoreceptor, chemotaxis and osmosensing. Here, while His kinases perceive the signal, signal transduction occurs by means of phosphotransfer to another class of signal transducer proteins known as the response regulators (RRs). Signal transduction of this type is called, “Two Component System” (TCS), since it comprises two kinds of conserved proteins, namely: His kinases and their corresponding RRs. Two types of this mode of phosphotransfer are now well described: the first type is found mainly in bacteria and involves transfer of the phosphoryl group directly from the His kinase to the response regulator and the second type that is more complex and involves a hybrid His kinase where the phosphotransfer occurs from the conserved His to the conserved Asp residue, existing within the same protein. Here, there is mediation of the phosphotransfer to the response regulator by an additional protein, the His-containing phosphotransfer protein, which on its own has a conserved His phosphorylation site. Two component systems encoded by many genes have been identified and properly described in both Prokaryotes and Eukaryotes including plants (Mijakovic et al., 2016; Chang and Stewart, 1998). This kinase activity which is strongly modulated by temperature could possibly be one of the mechanisms which plants use to adjust their internal temperature amidst constant fluctuations of environmental temperature (Josse et al., 2008).

Plants are sisal organisms which have no other option than to optimize their growth in the area in which they find themselves. Plants show both developmental plasticity and an adaptive ability for growth development and modification in response to the constant changing of environmental signals (Franklin and Quail, 2010). Among important environmental signals are light, gravity and temperature, with plant growth development, survival and productivity found to be greatly affected by light and temperature. Major photoreceptor families saddled with signaling and light perception in plants consist of red (R)/far-red (FR) reversible phytochromes, blue (B)/ultraviolet-A (UV-A) responsive cryptochromes, phototropins, and ultraviolet-B (UV-B) absorbing photoreceptors (Yang et al., 2009; Galva˜o and Fankhauser, 2015; Li and Mathews, 2016; Somers and Quail, 1995; Goosey et al., 1997). The integrated signalling governed by these photoreceptors lead to regulation of numerous light- and temperature-dependent growth and developmental responses, comprising skotomorphogenesis, photomorphogenesis and thermomorphogenesis, chloroplast differentiation and development, leaf development, and other processes occurring throughout the life cycle, like flowering and senescence. Light has distinct effects on different tissues during the process of photomorphogenesis such as inhibition in hypocotyl growth, but promotes growth and development in cotyledons and emerging leaves, as well as in roots. Such antagonistic responses in distinct tissues could be maintained by means of distinct pools of photoreceptors regulating growth promotion in roots or cotyledons, as well as distinct photoreceptors inhibiting elongation of hypocotyl (Tóth et al., 2001).

In photomorphogenesis, phytochromes and cryptochromes are the main players which accumulate at different patterns and levels in different tissues owing to developmental signals (Somers and Quail, 1995; Goosey et al., 1997; Tóth et al., 2001; Sharrock and Clack, 2002). Nevertheless, significant overlap is also exhibited by these photoreceptors in their expression patterns, which only partly supports a role for spatially distinct photoreceptors in the control of antagonistic growth responses that are light-dependent in different tissues (Tóth et al., 2001). Therefore, the distinct impacts of light on growth promotion in some tissues alongside inhibiting expansion in others is possible due to distinct signalling cascades downstream of the activated photoreceptors in distinct tissues.

Arabidopsis phytochrome (Phy) photoreceptor family consists of two types: type 1(PhyA) and type 2 (PhyB-E) which are further classified into five members (PhyA-E). The type11 to which PhyB belongs is the most abundant and light stable. These Phys function as molecular light switches. In the dark, Phys appear in their inactive red light-absorbing (Pr) form. After capturing a photon by the covalently bound linear tetrapyrrole chromophore, they are converted to the active far-red light-absorbing conformer (Pfr). Pfr initiates downstream signaling events in the cytosol or in the nucleus. The active Pfr form is converted to Pr by far-red light (Rockwell et al., 2006) or by a thermally driven process named dark reversion in the absence of light (Mancinelli, 1994). The Pfr conformers of Phys are transported into the nuclei, where they form characteristic nuclear bodies (Kircher et al., 2002). Interestingly, Pr and Pfr forms of phytochromes have overlapping absorption spectra which permit them to monitor F/FR ratio of sunlight. Phytochrome B (PhyB) is the major red/far-red light-absorbing phytochrome receptor in light-grown plants. The characteristic domain structure of PhyB and analysis of PhyB mutants displaying altered light sensing or signaling capabilities suggested that the N-terminal domain is required for light absorption, whereas downstream signaling cascades are activated by the C-terminal His kinase like module (Park et al., 2000). However, it has been shown that N-terminal fragments of PhyB containing 651 amino acids of the

photoreceptor fused to bacterial GUS protein (providing dimerization motifs) and nuclear localization signals (NLS) were biologically active in regulating photomorphogenesis (Matsushita et al., 2003; Oka et al., 2004). These reports have also demonstrated that the 651-amino acid, N-terminal fragment retained the function of PhyB in controlling flowering. This could possibly imply that the His kinase-like subdomain, which is located within the C-terminal module of PhyB, is dispensable for downstream signaling mediating photomorphogenesis and flowering. Nonetheless, other reports (Palágyi et al., 2010) provide proof that the carboxyl-terminal module is required to mediate circadian entrainment in white light, indicating a role for this module in integrating red and blue light signaling to the clock (Njimona and Lamparter, 2011). It has previously been suggested that, the C-terminal of bacterial phytochrome Agp1 may have a thermosensing function. Also, the spectral properties of cynobacterial phytochromes Cph1 was shown to be modulated by temperature (Njimona et al., 2014). Here, the research shows that PhyB derivatives containing 651 amino acid residues of the N-terminal module is functional in mediating inhibition of root growth and that this inhibition is altered at an elevated temperature (34°C) in Arabidopsis. This research further provides evidence that the root to hypocotyl ratio is reduced by a factor of 2 in the 651-NLS compared to the wild type, hence the carboxyl-terminal module may be required to mediate root length elongation in the nucleus at this temperature, suggesting a role for this module in integrating light and temperature signaling to root elongation. This further supports the fact that this part of the phytochrome molecule is involved in downstream signal transduction.

## MATERIALS AND METHODS

### Plant materials and growth conditions

The WT *Arabidopsis thaliana* used in this study is Columbia-0 (Col-0). The studies conducted in Germany in the city of Karlsruhe and Bonn showed that both results were the same hence no influence on the environment. The mutants of it B651-NLS, B651-NES, phyB-9 and BFL lines were described previously (Palágyi et al., 2010). All seeds were surface-sterilized with 6% (v/v) bleach solution for about 10 min, rinsed several times with sterile water and then sown on half-strength Murashige and Skoog (1/2 MS) medium containing 0.8% (w/v) agar. Thereafter, the seeds were stratified for 2 days at 4°C in darkness to synchronize germination; the plates were transferred to a growth chamber with continuous white light (about 100 µmol $m^{-2}$ $s^{-1}$) and maintained at 23°C for 5 days. Germinated seeds were then incubated at different temperatures as given in the text for 72 h on vertical Petri dishes. All treatments were done in white light. Thereafter images were taken, hypocotyl and root lengths were measured using Image J. A total of 50 plants each in 5 separate repeats were considered for each mutant's line and wild type. The root and the corresponding hypocotyls of each of the plants were measured. The mean length of the root and hypocotyls were calculated. Error bars are the standard error.

## RESULTS AND DISCUSSION

The hypocotyls growth of seedlings is inhibited by light, mediated by phytochromes and cryptochromes (Poppe et al., 1998). The N-terminal photosensory module of PhyB alone, consisting of 651 amino acids, was reported by many groups to be biologically active in regulating photomorphogenesis. All of these studies were done at ambient temperature. To examine whether the C-terminal His-kinase catalytic output module plays a role in root and hypocotyls elongation at different temperatures under continuous white light, we grow Arabidopsis wild type and four PhyB mutants: B651-NLS carrying a nuclei localization signal at the C-terminal, B651-NES with nuclei exclusion signal at the C-terminal, PhyB deficient mutant (PhyB-9) and PhyB complementation line PhyB-OX (all lines were previously described (Palágyi et al., 2010). The data shows that no significant difference was found between hypocotyls of the lines grown at 23°C except for phyB-9 mutant line with ca 0.53 cm taller (Figure 1A). Interestingly, the wild type and 651-NLS have hypocotyls to root ratio of 1:3 and all other mutant lines showing less (Figure 1B). On a warm day (34°C), the C-terminal His kinase like catalytic output module suppresses both root and hypocotyls elongation (Figure 2A). At this temperature, the hypocotyls to root ratio was found to be 1:5 for the wild type and PhyB-OX line, surprisingly the mutants PhyB-9 and 651NES line has factor of ca 2 less. This strongly shows that the C-terminal module of PhyB modulates hypocotyls to root ratio under these conditions.

In nature, root systems of most terrestrial plants are shielded from light exposure by growing in a dark soil environment. Although only shoots are normally exposed to light in nature, it has been reported that from about 14 different types of photoreceptors expressed in Arabidopsis, most of them are also found in the root where they participate in root growth control (Kiss et al., 2003; Briggs and Lin, 2012). There are also reports that light can only penetrate a few millimeter into the soil due to its high absorbance (Woolley and Stoller, 1978). Nevertheless, human action on the ground and natural disaster such as route construction, farming, sudden changes in temperature, earthquake, heavy rain, wind etc. often happen which allows light to penetrate deeper and come into contact with some roots. Decades ago, transparent Petri dishes were introduced and are currently in use as a simple system for cultivating plants. This type of cultivation allows for easy access to the root system for imaging and analysis, but in the standard set-up it does not prevent light interference with roots. Main functions of roots include the acquisition of water and nutrients from the soil. Since nutrient availability may be

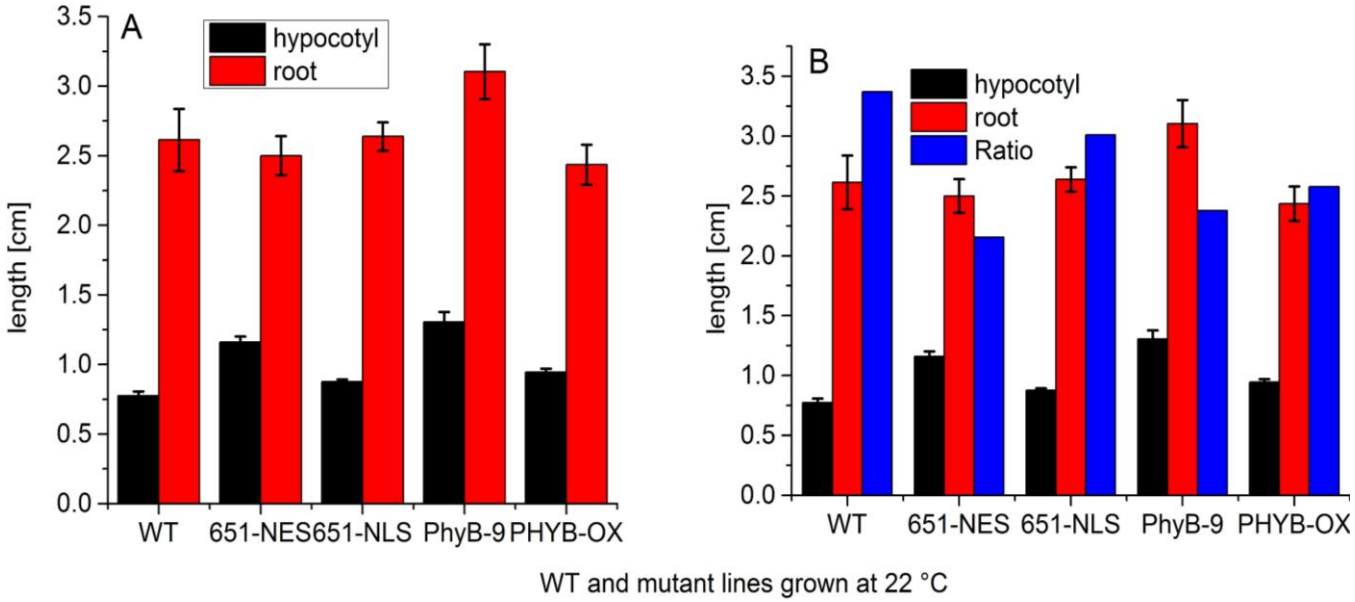


**Figure 1.** Hypocotyls and root lengths of Arabidopsis wild type and mutants grown at 22°C under continuous white light. Mean values ± standard error.

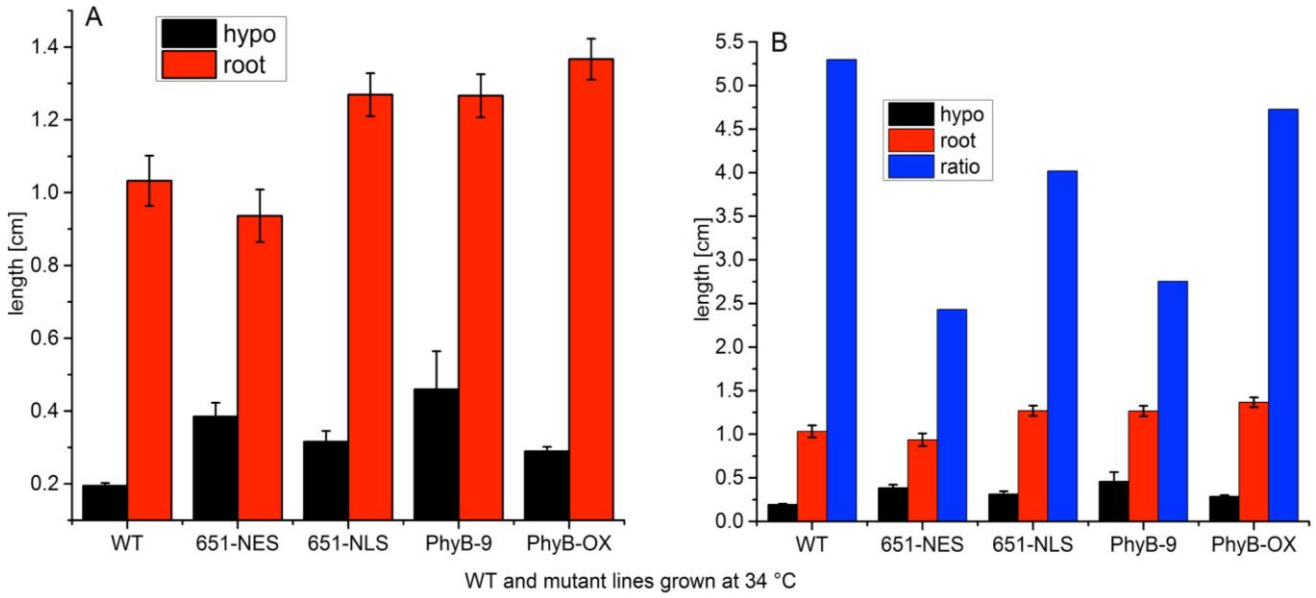


**Figure 2.** Hypocotyls and root lengths of Arabidopsis wild type and mutants grown at 34°C under continuous white light. Mean values ± standard error.

limited, plants form root hairs that increase the total surface of primary and lateral roots, as well as enhance nutrient acquisition (Gilroy and Jones, 2000). In our cultivation system, the whole plant was exposed to white light (ca 100 µmol $m^{-2}$ $s^{-1}$). We cannot exclude the fact that this type of cultivation could alter the ratio of root to hypocotyl length compared to when grown in its natural environment. What is really striking is the difference in root to hypocotyl length ratio between the wild type and the various mutants. The work from Koini et al. (2009) was amongst the first to demonstrate that plant acclimation to high temperature requires PhyB interacting

protein PIF4, suggesting a pivotal role of PhyB in temperature sensing; in line with this, we suggest that this could possibly be due to different strength of interactions at the C-terminal of PhyB with PIF4 or/and other phytochromes interacting factors.

## Conclusion

This study shows that PhyB act as both photo-sensing and thermal-sensing molecular in Arabidopsis.

## Recommendation

Further experiments are however needed to pool-down PIF4 together with C-terminal at this temperature.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# The effect of substrates on the growth, yield, nutritional and phytochemical components of *Pleurotus ostreatus* supplemented with four medicinal plants

**Sirri Vera Nsoh, Walter Ndam Tacham, Mercy Veyeh Ngwang and Tonjock Rosemary Kinge***

Department of Biological Sciences, Faculty of Science, The University of Bamenda, P. O. Box 39, Bambili, Northwest Region, Cameroon.

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**The aim of this study was to investigate the effect of substrates on the growth, yield, nutritional, and medicinal value of *Pleurotus ostreatus* supplemented with four medicinal plants. A completely randomized block design was laid out with 4 treatments replicated 4 times with and without medicinal plants. T1 (sawdust), T2 (sawdust + corncobs), T3 (palm cones), and T4 (elephant stalks) were the treatments used. *Croton macrastarchus, Harungana madagascariensis, Tithonia diversifolia,* and *Rauwolfia vomitoria* were the medicinal plants used. Nutritional and phytochemical analysis was carried out. Sawdust + corn cobs indicated the highest effect on growth as it had the highest mean height (19.5 ± 3.3 cm), diameter (29.0 ± 4.3 cm) and mean weight of individual fruiting bodies (175.8 ± 84.3 cm). Biological efficiency was highest in palm cones (77.1%), second by sawdust + corn cobs (61.1%), saw dust (53.0%) and elephant stalk (6.3%). The protein content was highest in sawdust + corn cobs (12.4 g), lipid concentration highest in sawdust only (1.51 g), total carbohydrate highest in palm cones (82.98 g), and total ash highest in sawdust only (7.32 g) per 100 g. The supplementation of sawdust + corn cobs with *R. vomitoria* had the highest phytochemical components.**

**Key words:** Medicinal properties, mushroom cultivation, growth and yield, nutritional content, *Pleurotus ostreatus*, medicinal plants substrates.

## INTRODUCTION

Mushrooms are fleshy saprophytic fungi, and it can be found growing on damp rotten log of wood trunk of trees, decaying organic matter and in damp soil rich in organic substances. Edible mushrooms are highly nutritious and can be compared with eggs, milk and meat (Mata et al., 2005). *Pleurotus ostreatus* (*Basidiomycota*), is of the Pleurotaceae family. *Pleurotus* originated from China; however, nowadays it is distributed all over the world, except for the north-west Pacific because of the arctic climate (Wojewoda, 2003). Cultivation methods were developed in Germany during World War I and then successfully applied on a large scale. This was the result of the search for new food sources, due to the problem of hunger in Germany. In Poland, *P. ostreatus* is a common species (Wojewoda, 2003). *P. ostreatus* (white-rot fungus), also known as oyster mushroom, is

*Corresponding author. E-mail: rosemary32us@yahoo.com. Tel: (+237) 674625339.

commercially important in the world mushroom market.

Oyster mushroom is an edible mushroom having an excellent flavour and taste. *P. ostreatus* has received increased attention for applications in bio-bleaching and the catalysis of difficult chemical conversions in the paper industry, textile dye decolorization, and detoxification of environmental pollutants (Park et al., 2014). Oyster mushrooms are prized for their exclusive flavour and deliciousness. They are rich in proteins, contain less fat, less carbohydrates, salt and rich in fibres and have high vitamin B12 and folic acid which are uncommon in vegetables. High availability of lysine and tryptophan and other amino acids usually absent in cereals make them ideal for food for patients suffering from hypertension, diabetes and obesity (Carel et al., 2013). Mushrooms require carbon, nitrogen and inorganic compounds as their nutritional sources and the main nutrients are carbon sources such as cellulose, hemicellulose and lignin. The use of various wastes is recommended for the growth of *P. ostreatus* (Vendrusco et al., 2008)

The waste obtained from fruit processing industry is extremely diverse due to the use of wide variety of fruits and vegetables, the broad range of processes and the multiplicity of the product. Full utilization of horticultural produce is a requirement and a demand that needs to be met by countries wishing to implement low-waste technology in their agribusiness. Food processing industries generate high amount of waste and direct disposal of such residues poses a serious threat to the environment and represents an important loss of biomass (Vendrusco et al., 2008). Apple pomace is an important fruit industrial waste that remain unutilized and creating an environmental pollution. Therefore, it can be utilized along with the wheat straw as a substrate for *P. ostreatus* production. Several million tons of Apple pomace is generated because of its high carbohydrate content which is used for microbial processes for single cell protein, enzymes, ethanol, low alcoholic drinks and pigments (Bhushan et al., 2008). Therefore, this horticultural waste can be utilized and replaced with wheat straw for mushroom cultivation under mushroom house.

Extracts from wild species of *Pleurotus* have been reported to be used in treating some ailments (Osemwegie et al., 2007). Wild species are collected for consumption because they are a good source of carbohydrates, digestible proteins, fibres and vitamins (Barros et al., 2008). Structurally, polysaccharides and proteins comprise the main components of dry matter of *Pleurotus*, while lipid content is low. Chitin, glycogen, mannitol and trehalose are typical carbohydrate constituents. *Pleurotus* occupy the third position in the production of edible mushrooms, behind the species of the genus *Agaricus* and *Lentinula* (Cardoso et al., 2013). *Pleurotus* spp. are found in tropical and subtropical rainforests around the world, and can be artificially cultivated (Bonatti, 2004) due to their ability to colonize and degrade a wide variety of substrates containing cellulose, hemicellulose and lignin, using them in their own development (Pokhrel et al., 2013). Furthermore, these species have a quick mycelium growth, fruiting and a low cost of culture, being slightly affected by diseases, and requiring minimal monitoring of the cultivation environment due to an easy adaptation and maintenance (Pokhrel et al., 2013). Therefore, due to nutritional and functional characteristics, *Pleurotus* spp. is considered increasingly popular in a commercial point of view.

In general, the production of mushrooms may be divided into several stages: composting and filling, sterilization, inoculation, incubation, fruiting, and harvesting (Loss, 2009). Contrary to others, *Pleurotus* genus does not require a composting substrate (Fan et al., 2000), due to the presence of a powerful enzyme complex (with cellulases, hemicelulases, ligninases, peroxidases, laccases, proteases, among other enzymes) (Hyde et al., 2019). *Pleurotus* spp. only requires crushed materials to acquire the desired texture for a good mycelial colonization. The production of *Pleurotus* spp. has been tested using different substrates *e.g.*, cotton waste textile (Chang et al., 1981), rice straw (Mehta et al., 1990) by products of corn (Loss, 2009), bark of coffee (Dias et al., 2003), wheat straw (Ramos et al., 2011) and sugarcane (Cardoso et al., 2013). The adaptation of this genus to new wastes represents one of the main methods for bioconversion of agro-industrial waste into edible products with high nutritional value (Cohen et al., 2002). The recycle of different materials is one of the most important contributions of fungi in nature (Sanchez et al., 2002).

*P. ostreatus* is often attacked by various fungal, bacterial, viral pathogens and this leads to a great loss of yield. So, these pathogens must be controlled to save crop production (Dawoud et al., 2005). Hot water extract of *Curcuma aromatic* inhibited the mycelia growth of the causative agent of root rot of cotton and wilt of tomatoes (Raja and Kurcheve, 1999). The incorporation of medicinal plant products with *P. ostreatus* is for the phytochemical control of pathogenic microbes during mushroom cultivation. Some medicinal plants used during mushroom cultivation include Eucalyptus, Neem cake, Citrus lemon and Lemon grass. The fine powder of these medicinal plant's substances is mixed with mushroom substrates for yield improvement of various strains of oyster mushrooms (Nazir et al., 2010). The aim of this research was to investigate the effect of substrates on the growth, yield, nutritional and medicinal value of *P. ostreatus* when supplemented with four medicinal plants.

## MATERIALS AND METHODS

### Description of cultivation site

This research was conducted at the Mushroom Programme Training and Research Center (MUPTAREC) farmhouse at Ntarinkon Mankon-Bamenda. The farmhouse is situated in Mezam division in the Northwest Region of Cameroon. Bamenda also

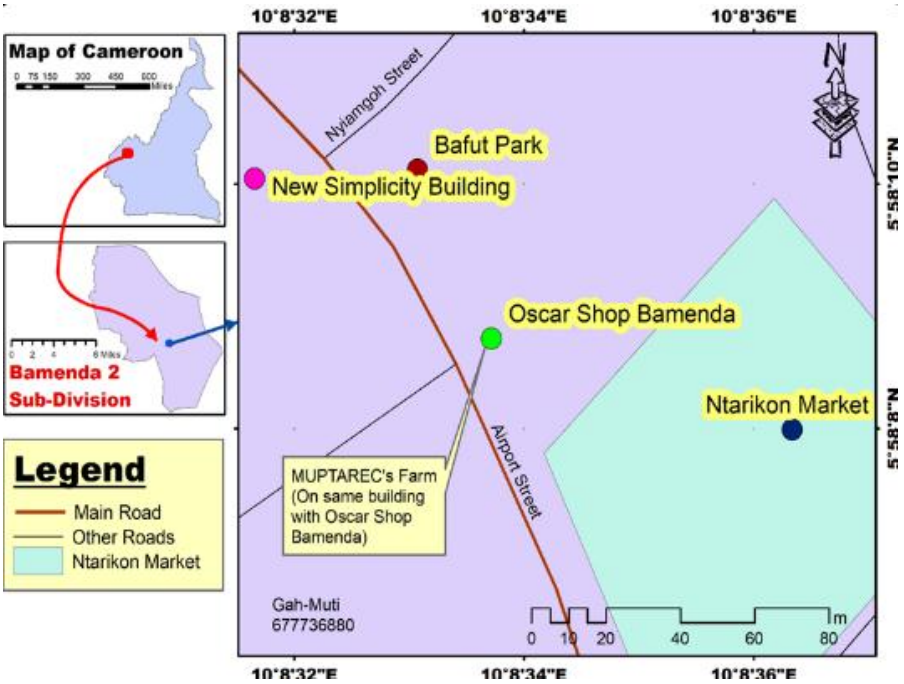


**Figure 1.** Map of Ntarinkon Mankon-Bamenda indicating MUPTAREC'S Farmhouse. Source: Authors

known as Abakwa town and Mankon town is a city in the Northwest Region and has a population of about 2 million inhabitants located at 366 km Northwest of the Cameroonian capital Yaounde. Bamenda is known for its cool climate. Mankon has coordinates 5°56[1]N 10E and 5.933N 10.167°E (Figure 1). The area has a tropical savanna climate, bordering on a tropical monsoon climate with long wet season and short dry season. The soil type in Mankon is sandy loam (Alakeh et al., 2020).

### Sterilization procedure

All apparatus, equipment, metallic instruments, glass wares and culture media were sterilized locally in a drum. The culture room was cleaned by washing with detergents followed by 70% ethyl alcohol.

### Mother spawn production

An exotic strain of *P. ostreatus* was obtained from Belgium and multiplied using Potato Dextrose Agar. 100 kg bag of sawdust, 25 kg bag of rice bran, 50 kg bag of rice husk, and 500 g of slake lime (Calcium Carbonate) were mixed using clean spades and 30 L of water added to obtain 65% moisture content. Mixing was done properly to obtain a homogenous mixture. This mixture was put in bottles and closed with bottle lids that have been perforated at the center of the lid using a nail and cotton fitted on the perforated area to permit the supply of small amount of oxygen to the mycelium after planting. These bottles were sterilized in a sterilization drum for 4 h. Cooling was allowed to take place for an hour followed by inoculation of the bottles in an inoculator for 90 min. Planting of substrate in bottles took place with seeds obtain from mother spawn that was already prepared three weeks before. The bottles were then placed in an already prepared spawn room for colonization for 3 weeks after which they were suitable for planting substrates.

### Substrate preparation

Sawdust, sawdust mixed with corn cobs, palm cones and elephant grass were mixed with equal quantities (1:1) of rice bran and $CaCO_3$. Water was added to the mixture and mixed to homogeneity. The substrates used; sawdust, sawdust mixed with corn cobs, palm cones and elephant grass, were filled in plastic bags. Four (4) replications were made from each substrate, a kilogram in each bag. Tags were made on the bags for clear identification and differentiation. The substrate bags were tied and sterilized in a 25 ml metallic drum for 4 h and allowed in the drums for continuous gradual sterilization overnight. Bags were removed the next day and allowed to cool then spawning was done.

### Substrates, composition and replications

The cultivation of *P. ostreatus* on different substrates was done using the procedure of Anagho (2008). This experiment was laid out in a completely randomized design with 4 treatments replicated 4 times. The treatments were supplemented with and without the 4 medicinal plants and each had 4 replications. Table 1 shows the various substrates (treatments) and composition used in the cultivation of *P. ostreatus* with the various replications.

### Substrate preparation with medicinal plants

Substrates were mixed as stated earlier and equal amount of the different medicinal plants; 35 g each were added to the substrates

**Table 1.** Substrates and composition used in the cultivation of *P. ostreatus* and replications.

| Treatment | Substrates and composition | Replications |
|---|---|---|
| Treatment 1 | Saw dust+ rice bran | 4 |
| | Saw dust + rice bran +M1 | 4 |
| | Saw dust + rice bran +M2 | 4 |
| | Saw dust + rice bran +M3 | 4 |
| | Saw dust +rice bran + M4 | 4 |
| Treatment 2 | Saw dust + corn cobs + rice bran | 4 |
| | Saw dust +corn cobs rice bran +M1 | 4 |
| | Saw dust +corn cobs rice bran +M2 | 4 |
| | Saw dust +corn cobs rice bran +M3 | 4 |
| | Saw dust +corn cobs rice bran + M4 | 4 |
| Treatment 3 | Palm cones+ rice bran | 4 |
| | Palm cones + rice bran +M1 | 4 |
| | Palm cones + rice bran +M2 | 4 |
| | Palm cones + rice bran +M3 | 4 |
| | Palm cones +rice bran + M4 | 4 |
| Treatment 4 | Straw + rice bran | 4 |
| | Straw + rice bran +M1 | 4 |
| | Straw + rice bran +M2 | 4 |
| | Straw + rice bran +M3 | 4 |
| | Straw +rice bran + M4 | 4 |

M1= *Croton macrastarchus;* M2=*Harungana madagascariensis;* M3=*Tithonia diversifolia;* M4=*Rauwolfia vomitoria.*
Source: Authors

and mixed thoroughly for homogeneity. The four (4) medicinal plants used were *Croton macrastarchus*, *Harungana madasgariensis*, *Tithonia diversifolia* and *Rauwolfia vomitoria.* These medicinal plants were collected, chopped into pieces, dried in a bakery oven and ground into powder in a grinding mill. 20 replications were made from the substrates with different medicinal plants (4 replications per medicinal plant). 1 kg of compost was filled in each substrate bag. Tags were made on each substrate bag for clear identification. Bags were tied and sterilized in a 250 ml drum together with the control experiment for 4 h. Bags were removed the next day, and allowed to cool.

**Spawning**

The bags were removed from the drum and carefully placed in a spawning room. Cooling was allowed to take place for an hour before spawning. Washing and sterilization was done and spawning was carried out by use of a spoon. Hands and spoons were sterilized using medical alcohol. Planting spoons was used to prevent contamination and to mix spawn in the bags without damaging it. Bags were then untied and bottles of “sub mother” spawn weighing 760 g were used to plant substrates. 50 seeds were used to plant 100 substrates, that is planted at the ratio of 1 bottle of seed for two bags of substrate (1:2). The planted bags were properly tied to avoid the entering of microorganisms and to enhance proper colonization of the mycelium network. The substrates were then moved to the mushroom cultivation house with dark room with shelves where it was placed for colonization. After 21 days when the mycelium was fully colonized, the windows were open for ventilation to enhance and provide necessary conditions for fruiting. The temperature and moisture content of the room was maintained for healthy fruiting to take place. Watering of substrate to improve on the moisture content was done only after the first fruiting.

**Morphological data collection**

The trial was carried out according to the complete randomized block design with factorial arrangement using four replications for each treatment. The 100% saw dust substrate (treatment 1) was selected as the control. The pertinent data on growth and yield parameters such as time to spawn running, to pinhead formation, to first harvest; height of pileus, diameter of pileus, total yield, biological efficiency was collected during the experimental period according to Kinge et al. (2016).

**Nutritional analysis**

A laboratory analysis was done according to Association of Official Analytical Chemists (AOAC, 1984) in the National Polytechnic Bambui Nutritional Laboratory Bamenda to compare composition of nutrient in *P. ostreatus* cultivated on five substrates. The data was

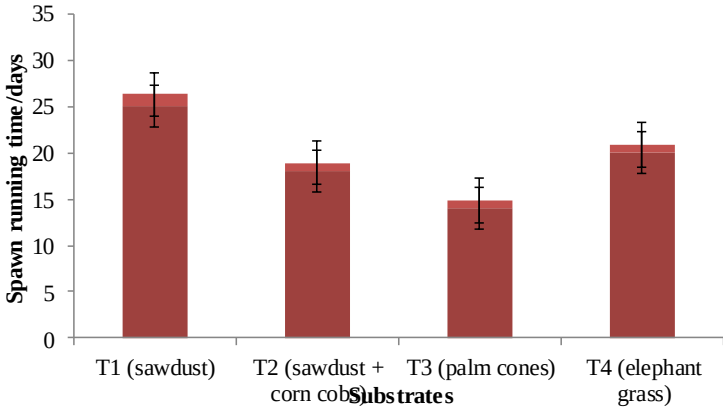


**Figure 2.** Days to spawn run on different substrates.
Source: Authors

recorded on crude protein, crude fat, crude fiber ash, organic matter, and dry matter according to Raghuramulu et al. (2003).

#### Phytochemical analysis

The phytochemical analysis was done using standard procedures (AOAC, 1984). The phytochemical test was analysed after extraction in 1:1 methanol and methylene chloride. The concentrated residues were used to detect the secondary fungi metabolites such as alkaloids, flavonoids, steroids, saponins, phenolics and tannins using standard methods with some modifications (MacNee, 2005).

#### Data analysis

The data for growth and yield parameters were analyzed by descriptive analysis using M.S Excel version 2010 where the results on growth and yield were compared using bar charts. Also, statistical evaluations on the growth and yield parameters were done by one way ANOVA in SPSS version 20 and the comparisons of the means realized by the Turkey HSD multiple comparison at $p<0.05$.

## RESULTS

### Spawn running time

Spawn running time tested on the 4 different substrates took 14 to 25 days. Palm cones took 14.4 ± 0.8 days; corn cobs+ saw dust 18.4 ± 0.9 days, elephant straw 20.4 ± 0.8 days and saw dust 24.2 ± 1.3 days. Figure 2 and Table 2 show spawn running time measured by counting number of days of the different substrates.

### Time required for primordial initiation (pinhead formation)

Two replications from treatment 1 and one replication from treatment 2 were destroyed by green mould fungus. The time required for the formation of pinhead was least in treatment 3 (21.8 ± 2.1 days), treatment 2 (26.9 ± 2.2 days), treatment 4 (31.0 ± 2.8 days) and treatment 1(46.3 ± 1.7 days). Figure 3 and Table 2 show time required for pinhead formation measured by counting number of days of the different substrates.

### Days from pinhead formation to harvesting

The number of days it took from fruiting to harvesting was equal in treatment 1, 2 and 3 (6.7 ± 0.7 days) and highest in treatment 4 (11.7 ± 0.7 days). Figure 4 and Table 2 show the time required for pinhead formation to harvesting measured by counting number of days of the different substrates.

### Height of fruiting bodies

The mean height of individual fruiting bodies per treatment was measured in centimeters using a tailor's tape beginning from where the stalk gets attached to the substrate to the pileus. The highest mean height was recorded in treatment 2 (19.5 ± 3.3 cm), followed by treatment 1 (15.7 ± 2.6 cm), treatment 3 (14.3 ± 1.3 cm) and treatment 4 (10.1 ± 1.3 cm). Figure 5 shows the mean height of fruiting bodies of the different substrates.

### Diameter of the pileus of individual fruiting bodies

The mean diameter of individual fruiting bodies measured (which is the distance round the pileus) using a tailor's tape in cm was recorded. The highest mean diameter was recorded in treatment 2 (29.0 ± 4.3 cm), treatment 1 (24.7 ± 3.5 cm), treatment 3 (23.5 ± 2.3 cm) and

**Table 2.** Comparison of growth and yield parameters based on treatment levels.

| Parameter | Means ± SD of parameters based on treatment levels | | | | | Significant difference (ANOVA) | | |
|---|---|---|---|---|---|---|---|---|
| | T 1 (control) | T2 | T3 | T 4 | Total | df | F-value | *p* - value |
| Spawning running time (Days) | 24.2±1.3 | 18.4±0.9 | 14.4±0.8 | 20.4±0.8 | 19.4±3.7 | 3 | 163.141 | *0.000* |
| Primordial initiation (Days) | 46.3±1.7 | 26.9±2.2 | 21.8±2.1 | 31.0±2.8 | 31.5±9.5 | 3 | 222.286 | *0.000* |
| Pinhead formation to harvesting (days) | 6.8±0.4 | 6.7±0.5 | 6.7±0.7 | 11.7±0.7 | 7.9±2.2 | 3 | 186.630 | *0.000* |
| Height of fruiting bodies (cm) | 15.7±2.6 | 19.5±3.3 | 14.3±1.3 | 10.1±1.3 | 14.9±4.0 | 3 | 29.182 | *0.000* |
| Diameter of pileus fruiting bodies (cm) | 24.7±3.5 | 29.0±4.3 | 23.5±2.3 | 11.3±1.2 | 22.1±7.3 | 3 | 61.282 | *0.000* |
| Number of fruiting bodies in a cluster | 5.1±0.9 | 4.7±1.4 | 4.5±1.2 | 2.2±0.6 | 4.1±1.5 | 3 | 14.971 | *0.000* |
| Weight of individual fruiting bodies (g) | 114.1±55.0 | 175.8±84.3 | 70.9±20.6 | 11.5±3.8 | 93.0±78.4 | 3 | 18.195 | *0.000* |
| Biological yield (g) | 2225.0±0.0 | 2645.0±0.0 | 1721.0±0.0 | 110.0±0.0 | 1675.3±1109.8 | 3 | ND | ND |
| Biological efficiency (%) | 53.0±5.8 | 61.1±17.7 | 77.1±7.1 | 6.3±3.1 | 49.4±28.4 | 3 | 91.339 | *0.000* |

Source: Authors

treatment 4 (11.3 ± 1.2 cm). Figure 6 and Table 2 show the mean diameter of the pileus of individual fruiting bodies measured in cm of the different substrates.

**Number of individual fruiting bodies in a cluster**

The mean number of individual fruiting bodies per treatment was recorded by counting. This number was almost equal in treatment 1, 2 and 3 (4.5 ± 1.2) and least in treatment 4 (2.2 ± 0.6). Figure 7 and Table 2 show the mean number of individual fruiting bodies of the different substrates.

**Weight of individual fruiting bodies**

The mean fresh weight of individual fruiting bodies was measured in grams and recorded. The highest mean weight of individual fruiting bodies was found in treatment 2 (175.8 ± 84.3 g), second in treatment 1 (114.1 ± 55.0 g), third in treatment 3 (70.9 ± 20.6 g) and least in treatment 4 (11.5 ± 3.8 g). Figure 8 and Table 2 show the mean weight of individual fruiting bodies of the different substrates.

**Biological yield**

This is the total fresh weight of fruiting bodies on the various treatments measured in grams using an electronic scale. Biological yield was highest in treatment 2 (2645 g), second in treatment 1 (2525 g), third in treatment 3 (1721 g) and least in treatment 4 (110 g). Figure 9 and Table 2 show the biological yield of the different substrates.

**Biological efficiency**

The biological efficiency as calculated from the total fresh weights of mushrooms divided by the dry weight of substrates x 100. The highest biological efficiency (77.5%) was obtained in treatment 3, treatment 2 (61.2%), treatment 1 (53.1%) and the lowest in treatment 4(6.3%). Figure 10 and Table 2 show the biological efficiencies of the different substrates.

**Nutritional analysis**

Table 3 shows the composition of various nutrients found in *P. ostreatus* on different substrates. Protein content was highest in sawdust + corn cobs (12.4 g), lipid concentration highest in sawdust only (1.51 g), total carbohydrate highest in palm cones (82.98 g), fibers highest in sawdust + corn cobs (19.78 g) and total ash highest in sawdust only (7.32 g) per 100 g.

**Phytochemical analysis**

Table 4 shows the various phytochemical compounds and their concentrations found in *P. ostreatus* on different treatments. According to our finding, steroids were highly concentrated in most of the treatments (those with and without medicinal

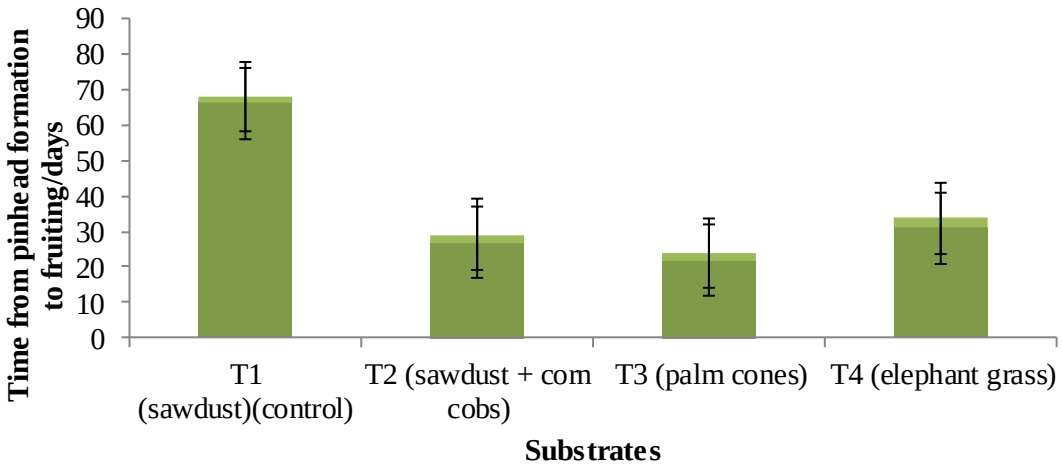


**Figure 3.** Days of pinhead formation on substrates.
Source: Authors

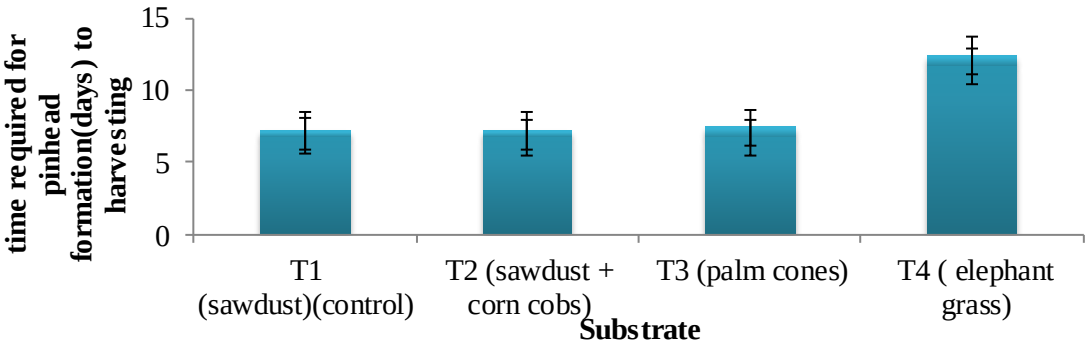


**Figure 4.** Time from pinhead formation to harvesting on substrates.
Source: Authors

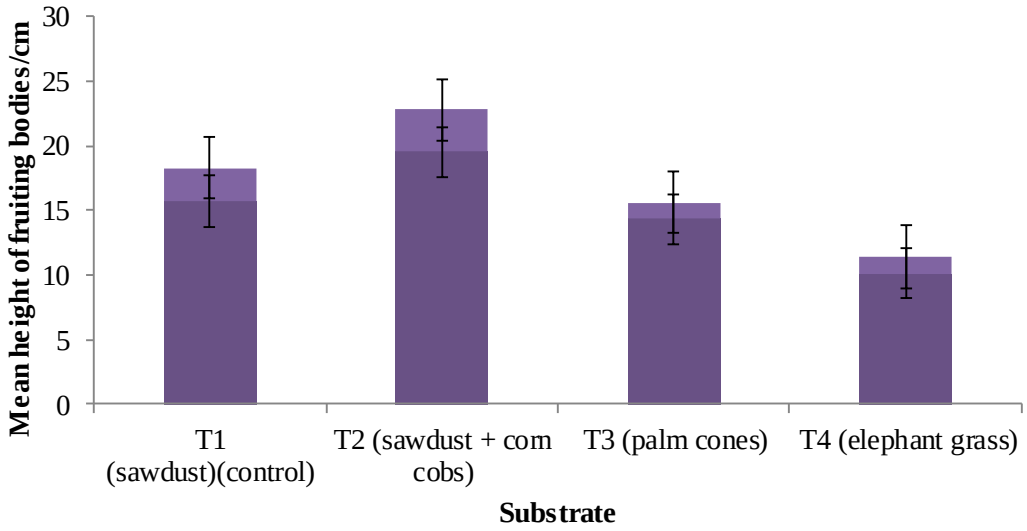


**Figure 5.** Mean height of individual fruiting bodies on substrates.
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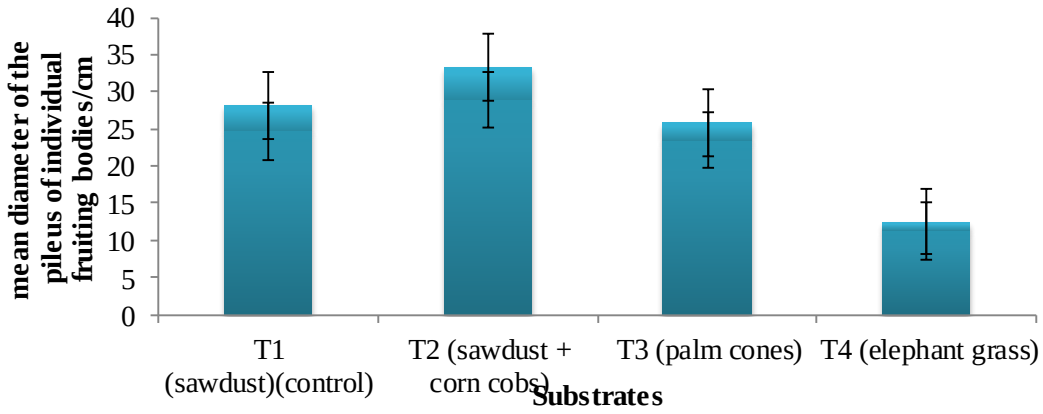


**Figure 6.** Mean diameter of the pileus of fruiting bodies on substrate.
Source: Authors

plants). Supplementation of SCB (Sawdust + corncobs) with *R. vomitoria* presented the highest concentrations of steroids, flavonoids and saponins (+++) and presence of alkaloids (+). SCB (sawdust + corncobs without supplementation) presented the lowest concentration of bioactive components with steroids highly concentrated (+++), saponins just present (+) and other components absent. Alkaloids were present in some of the treatments supplemented with medicinal plants (DBH, DBT, DBR, SCR, PBC, PBH and PBR).

## DISCUSSION

### Effects of different substrates on the growth and yield of *P. ostreatus*

Mycelia growth was faster in palm cones (T3) (14.4 ± 0.8 days) and slowest in sawdust (24.2 ± 1.3 days). Formation of pinhead appeared earlier in palm cones (21.8 ± 2.1 days) after the period of incubation and last in sawdust (46.3 ± 1.7 days). Pin head formation is affected by temperature and humidity; below 17°C pinhead formation is delayed. Composition of growth medium also affects pinhead formation time (Pathmashini et al., 2008); shortest time of primordial initiation in corncobs and palm cones has been reported by Ajonina and Tata (2012). Pinhead formation was prevented in some replications as a result of fungal infection; two replications of the treatment 1 and 1 replication of treatment 2 (sawdust and corn cobs + sawdust). This prevented the expected mycelium growth. Earnshaw et al. (2012) and Oseni et al. (2012) have reported similar infections and their detrimental effects on mycelium.

Days from pinhead formation to harvesting was significantly similar in the treatments 1, 2, 3 (corn cobs and palm cones) when compared with the control (sawdust) (6.8 ± 0.4 days) and higher in treatment 4 (elephant grass) (11.7 ± 0.7 days). The time from the pinhead formation to the first harvest for *P. ostreatus* was around 6±1 days, agreeing with those of Iqbal et al. (2005) who conducted similar research and who reported 46±3 days for this stage of development. Height of fruiting bodies differed on the different substrates. T2 (corn cobs + sawdust) presented the highest height (19.5 ± 3.3 cm) while the least height was recorded in T4 (elephant grass) when compared with sawdust (control). This result is in line with the findings of Rambey et al. (2018) who reported that sawdust can be mixed with corncobs to enhance productivity. Diameter of pileus of the fruiting bodies was higher in corncobs + sawdust (29.0 ± 4.3 cm) and lowest in elephant grass (11.3 ± 1.2 cm) when compared with sawdust (control) in *P. ostreatus*. Pileus diameter on corn cobs + saw dust was statistically similar. The outcome is in line with the findings of Ajonina and Tatah (2012) who reported highest diameter of pileus in corncobs and palm cones during their experiment. Mean number of effective fruiting bodies in the various treatments were significantly similar in treatment 1, 2 and 3 (4.7 ± 1.4) (corncobs + sawdust and palm cones) and lower in treatment 4 (elephant grass) when compared with sawdust. This outcome is similar to the findings of Ajonina and Tatah (2012) who reported highest number of fruiting bodies in sawdust and corn cobs. These results might be due to the presence of glucose, fructose and trehalose in the substrate as reported by Kitamoto et al. (1995). The highest weight of individual fruiting bodies of *P. ostreatus* was recorded in corncobs + sawdust (175.8 ± 84.3 g) and lowest in elephant grass (11.5 ± 3.8 g) when compared with sawdust. This result is similar to the findings of Rambey et al. (2018) who confirmed best yield of *P. ostreatus* when cultivated on corncobs mixed with sawdust.

Biological yield varied significantly because of different substrate compositions. The highest biological yield (2645 g) was obtained in corncobs + sawdust and the lowest biological yield (110 g) was obtained in elephant grass. Biological yield in corncobs+ sawdust was

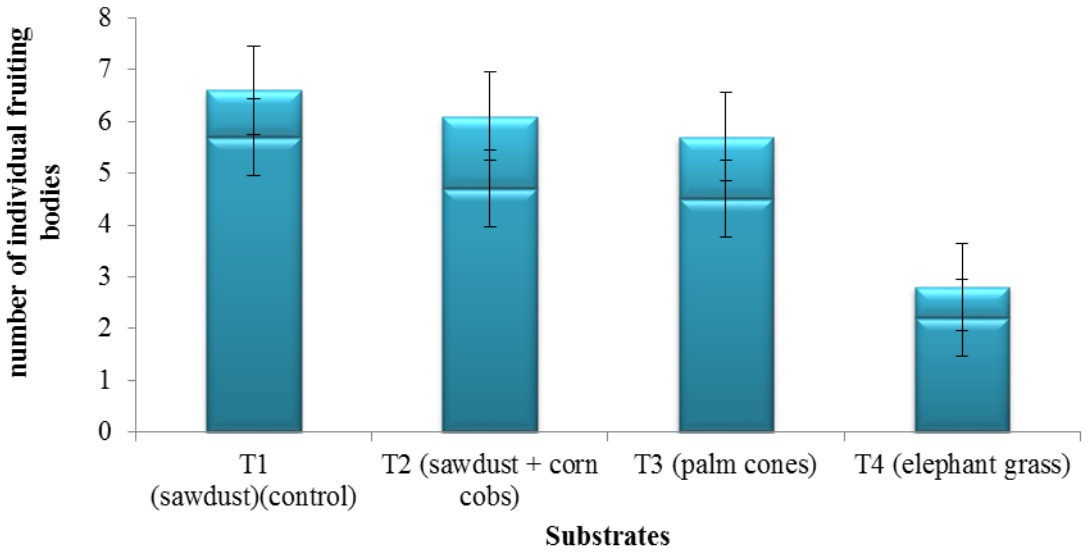


**Figure 7.** Mean number of fruiting bodies on substrate
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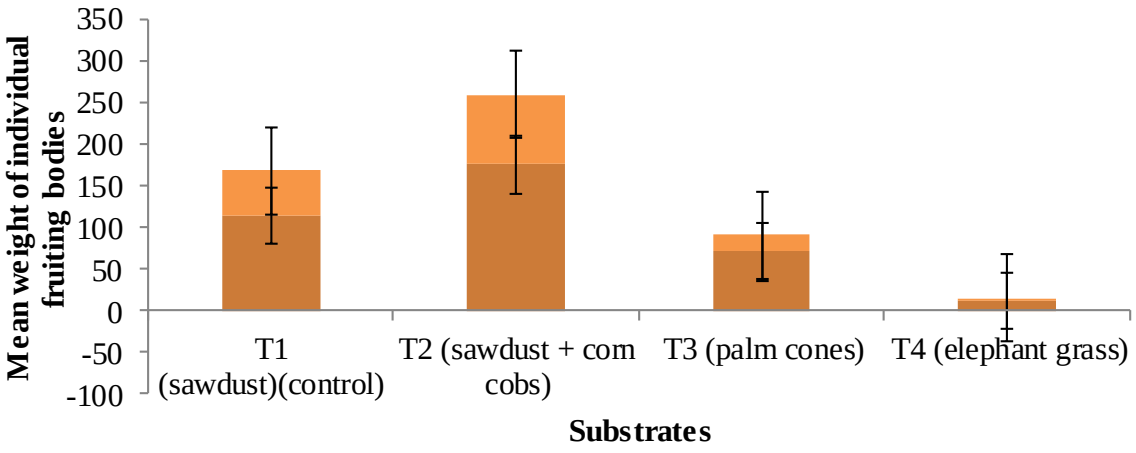


**Figure 8.** Mean weight of individual fruiting bodies on substrate.
Source: Authors

Significantly similar with biological yield on sawdust (control). Biological yield value of 2645 g obtained on the sawdust + corn cobs substrate was higher than the biological yield value of 213.5 g reported by Hawrez (2018) on wheat straw. Biological efficiency is the most important parameter in mushroom growing. Highest biological efficiency (77.1 ± 7.1%) was recorded in palm cones, (61.1 ± 17.7%) in corncobs + sawdust, (53.0 ± 5.8%) in sawdust (control), and lowest in elephant grass (6.3 ± 3.1%). The highest biological efficiency value of (77.1%) obtained on the palm corn substrate was higher than the biological efficiency (66.41 %) reported by Hawrez (2018) on wheat straw substrate.

**Nutritional analysis**

Nutritional composition of mushroom is affected by many factors among which the composition of the substrate is of major importance. This can be supported by the results in the findings where *Pleurotus florida* and *P. ostreatus* grown on sawdust gave a significant nutritional value than that cultivated on corn cobs (Shah et al., 2004). During our trial we discovered that protein content was highest in sawdust + corn cobs substrate (12.48 g/100 g) and least in palm cones (8.22 g/100 g). It is well known that the protein content of mushrooms varies with the type of substrate as a result of the differences in nutrient

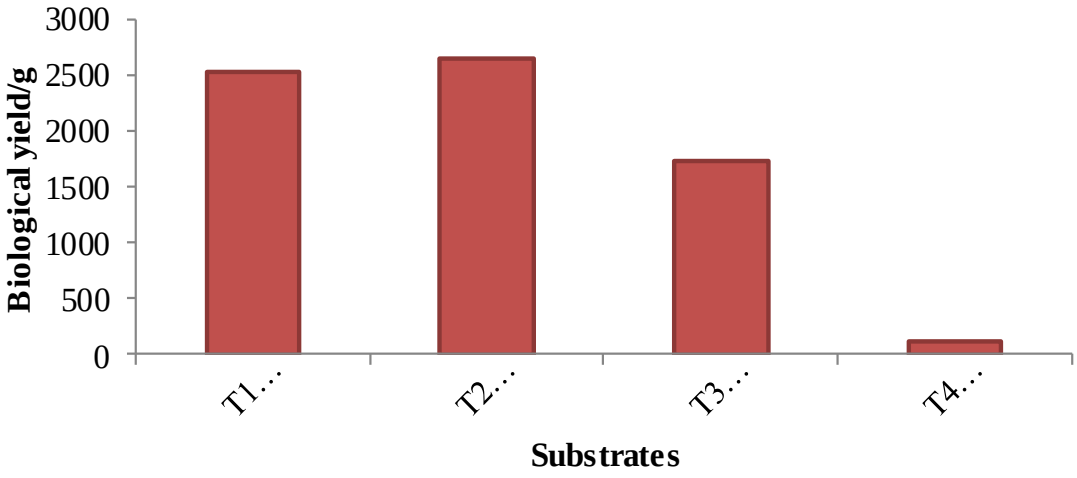


**Figure 9.** Biological yield of fruiting bodies on substrate.
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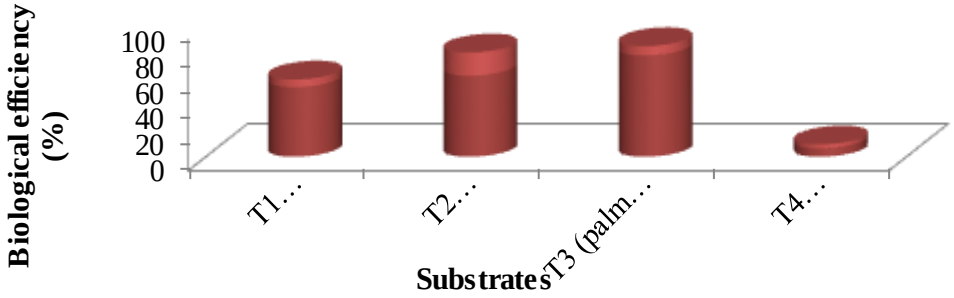


**Figure 10.** Biological efficiency of *P. ostreatus* on different types of substrates.
Source: Authors

supply (Gupta et al., 2013). This result is similar to that of Jin et al. (2018) who reported a protein value closer to our finding but contradicts the findings of Kinge et al. (2016) and Hawrez (2018) who reported of protein value higher than that of our findings.

Lipid content ranged from 1.43 to 1.51 g/100 g. Lipid content of oyster mushroom was affected by different substrates. Lipid content was maximum in sawdust (1.51 g/100 g) which was the control and minimum in palm cones (1.43 g/100 g). This result is similar to the findings of Barros et al. (2015) who reported lipid content range from 1.16-1.67 g/100 g for *P. ostreatus* but contradicts the report of Kinge et al. (2016) who reported a higher value of lipid content to that of our findings. Total carbohydrate ranged from 78.89 to 82.98 g/100 g. Total carbohydrates content varies depending on the type of substrates used. Total carbohydrate content was highest in palm cones (82.98 g) and minimum in sawdust (78.89g). The results of our finding were higher than the reported 61.9% for Croatian wild variety of *P. osreatus* studied by Beluhan and Ranogajec (2011), but less than the reported values of 73.2 to 78.1% in *P. ostreatus* studied by Fernandes et al. (2015). Crude fibre content ranged from 17.07 to 19.78 g/100 g. Crude fibre content varies depending on the type of substrate used. Fibre content was maximum in sawdust + corncobs (19.78 g) and minimum in the control which is saw dust only (17.07 g). This result is similar to the findings of Kinge et al. (2016) who reported a crude fibre content value maximum in corncobs (16.69 g) and minimum in sawdust (5.08 g) but contradicted by the findings of Teke et al. (2021) who reported a crude fibre value of 30.69 g obtained from the analysis of eight most preferred wild mushroom species from the Kilium-Ijim mountain forest in Cameroon. Total ash content varied depending on the type of substrate material used. Ash content ranged from 6.85 to 7.32 g/100 g. Total ash content was recorded as maximum in sawdust (7.32 g) which is the control and minimum in sawdust + corncobs (6.85 g). This result is similar to that of Hawrez (2018) who reported the ash

**Table 3.** Nutrient composition of various mushroom samples.

| Sample (g/100 g) | T1 | T2 | T3 |
|---|---|---|---|
| Protein | 12.09±0.20 | 12.48±0.50 | 8.22±0.27 |
| Lipids | 1.51±0.17 | 1.45±0.11 | 1.43±0.21 |
| Total carbohydrates | 78.89±1.25 | 79.88±0.00 | 82.98±1.78 |
| Fibers | 17.07±0.51 | 19.78±0.50 | 18.75±0.61 |
| Ash | 7.32±.21 | 6.85±0.32 | 7.10±0.25 |

Source: Authors

content value in the range of 6.38 to 7.48 g/100 g.

## Phytochemical analysis

Lindequist et al. (2005) stated that the nutritional and chemical compositions of mushroom are responsible for their medicinal values. The phytochemical analysis of *P. ostreatus* when supplemented with 4 medicinal plants revealed the presence of the following bioactive components; steroids, flavonoids, alkaloid, phenolic, tannins and saponins present in different concentrations in the different substrates and replications. Flavonoids, phenolic, tannins and saponins were present in *P. ostreatus* while alkaloid was absent (Kinge et al., 2016). During our experiment, we observed an increase in the concentration of bioactive components in *P. ostreatus*. The concentration and type of bioactive component varied depending on the substrate material used and the type of medicinal plant supplement. This is similar to the findings reported by Lindequist et al. (2005) who stated that the nutritional and chemical values of mushrooms are responsible for their medicinal values and equally, nutritional value of mushroom is affected by the type of substrate material used (Kinge et al., 2016).

The increase in the phytochemical component and thus the medicinal component of *P. ostreatus* is as a result of the supplementation with medicinal plants as medicinal plants contain phytochemical components which give them their medicinal properties. This result is in line with the findings of some researchers who reported an increase in the yield of oyster mushroom as a result of phytochemical components. This has been supported by an increase in the concentration of substances present in Neem cake which contain a mixture of tetranor triterpenoids (Govindachari et al., 1998), flavonoid, nimbosterol, liminoids, quercentinm, tannic acid, and substances found in citrus lemon, that is, volatile oil (Zeringue and Bhatnagar, 1994), limonene, alpha-pinene, beta-pinene, citral, coumarins, and bioflavonoids (Sammbamurty and Subrahmanyan, 2000). According to our findings, the concentrations of flavonoids, phenolic, tannins and saponins was highest in sawdust+ corn cobs + *R. vomitoria*. Alkaloids which are reported to be absent in oyster mushroom (Kinge et al., 2016) were present in some of the replications with medicinal plants in our trial. Alkaloids were present in replications: PBH, DBT, DBR, SCR, PBC, PBH, and PBR. Therefore, the supplementation of substrate with medicinal plants does not only improve on the growth and yield of the oyster mushroom according to Nazir et al. (2010) but also improves on the medicinal value.

## Conclusion

Cultivation of oyster mushroom (*P. ostreatus*) on corn cobs substrate mixture with sawdust has a significant positive effect on the growth and yield of *P. ostreatus*. The nutritional content of oyster mushroom depends on the type of substrate material used. Protein and fibres content was highest in sawdust + corn cobs substrate making it the best substrate under investigation. Phytochemical components were present in oyster mushroom supplemented with medicinal plants more than in the control. Best result was presented by supplementation of sawdust + corn cobs with *R. vomitoria*. *R. vomitoria* is an anti-cancer plant. Oyster mushroom substrate can be supplemented with this plant during cultivation and the mushroom giving to cancer patients as a treatment. Therefore, mushroom growers are encouraged to supplement their oyster mushroom substrates with medicinal plants to improve on its medicinal value.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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**Table 4.** Phytochemical screening of *P. ostreatus* supplemented with and without medicinal plants.

| Sample | Phytochemical constituents | | | | | |
|---|---|---|---|---|---|---|
| | **Steroid** | **Flavonoid** | **Alkaloid** | **Phenolic** | **Tannins** | **Saponins** |
| DB | +++ | + | - | - | - | + |
| DBC | +++ | - | - | - | - | + |
| DBH | +++ | - | + | - | + | - |
| DBT | +++ | - | + | - | + | - |
| DBR | +++ | - | + | - | - | - |
| SCB | +++ | - | - | - | - | + |
| SCC | +++ | + | - | - | - | + |
| SCH | +++ | ++ | - | + | + | ++ |
| SCT | +++ | + | - | ++ | + | ++ |
| SCR | +++ | +++ | + | - | - | +++ |
| PB | +++ | + | + | - | + | + |
| PBC | +++ | + | + | - | - | - |
| PBH | +++ | + | + | - | - | + |
| PBT | +++ | - | - | - | + | + |
| PBR | +++ | + | + | - | - | - |

(+) Present, (-) absent, (++) moderate concentration, (+++) abundant concentration. DB= Sawdust + rice bran; DBC= Sawdust + rice bran+ *Croton macrastarchus;* DBH= Sawdust + rice bran+ *Harungana madagascariences;* DBT =Sawdust + rice bran+ *Tithonia diversifolia;* DBR= Sawdust + rice bran+ *Rauwolfia vomitoria;* SCB=Sawdust + corncobs + rice bran; SCC= Sawdust + corncobs + rice bran + *Croton macrastarchus;* SCH= Sawdust + corncobs + rice bran + *Harungan madagascariences;* SCT= Sawdust + corncobs + rice bran + *Tithonia diversifolia;* SCR= Sawdust + corncobs + rice bran + *Rauwolfia vomitoria;* PB= Palm cones + rice bran; PBC= Palm cones + rice bran + *Croton macratarchus;* PBH= Palm cones + rice bran + *Harungana madagascariences;* PBT= Palm cones + rice bran + *Tithonia diversifolia;* PBR= Palm cones + rice bran + *Rauwolfia vomitoria.*
Source: Authors

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