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# Waterlogging tolerance, phenolic and flavonoid contents, and genetic diversity among Moringa oleifera self-pollinated lines

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ABSTRACT

*Moringa oleifera* Lam. (drumstick) is a valuable vegetable with considerable health benefits. The cultivation of *M. oleifera* is limited to well-drained soil as they are poorly tolerant to waterlogged conditions. In this study, self-pollinated lines, derived from a parental drumstick exhibiting high tolerance to waterlogging in Hue city, Vietnam, were studied. Seventy six lines were assayed for their morphological traits and waterlogging tolerance. Furthermore, seven RAPD primers and three SRAP primer pairs were employed to explore the genetic diversity among the *M. oleifera* parental and 76 self-pollinated lines. A high level of genetic diversity was observed within the germplasm. The dendrogram divided the germplasm genetically into five main groups. Finally, their phenolic and flavonoid contents were determined to identify lines with the highest concentration of bioactive compounds. These findings can aid future breeding programs to create elite cultivars of *Moringa oleifera*.



## 1. Introduction

*Moringa oleifera* Lam. (commonly known as drumstick) is a multipurpose tree species, nutritional rich and is distributed throughout South India, Southeast Asia, South America and Africa (Dhakad et al., 2019; Singh et al., 2020; George et al., 2021; Alavilli et al., 2022). Drumstick leaves and pods are used as a vegetable for human consumption and serve as ingredients for animal feeds. Additionally, *M. oleifera* parts are also rich in minerals, protein, vitamins, phenolic and flavonoid compounds (Singh et al., 2020; Hassan et al., 2021). Furthermore, hydrogels prepared with *M. oleifera* seed extract help to promote wound healing (Ali et al., 2022). The market size of moringa products was projected to reach 15 billion US dollars in 2028 (Fortune Business Insights, 2022), calling for the expansion of cultivation areas. Currently, the requirement for well-drained soil makes it unsuitable for drumstick to be cultivated in areas with frequent rainfall and floods (Dania et al., 2014).

Since *M. oleifera* is a cross-pollinated species and is also naturalised in many areas, they exhibit variations in morphologies, yields and phytochemical contents (Lakshmidevamma et al., 2021; Leone et al., 2015). Morphological diversity was also observed among drumstick landraces in Myanmar (Chan et al., 2018) and Ghana (Amoatey et al., 2012). Similarly, differences in leaf sizes, stem colours, tree shapes and heights were observed among the drumstick accessions from the South-Southeast of Mexico (Hernández *et al.*, 2021) and India (Kurian et al., 2021). Gandji and co-workers (2019) also observed diversity in morphological traits of *M. oleifera* with changing climate and cultivation practice. Thus, these traits are influenced not only by genetic factors but also by environmental factors (Drisya et al., 2021; Ruiz-Hernández et al., 2022).

Using molecular markers to assess the genetic diversity of a germplasm is essential for conservation, selection and breeding programs. Previous works have employed Random Amplified Polymorphic DNA (RAPD) markers to explore the genetic diversity of cultivated or wild accessions of M. oleifera (Mgendi et al., 2010; Popoola et al., 2014; Yusuf et al., 2011; Silva et al., 2012; Drisya et al., 2022; Saini et al., 2013; Kleden et al., 2017; Rufai et al., 2013). Furthermore, Truong et al. (2018) observed genetic diversity not only among accessions collected from different countries (Thailand, USA, Philippines, Taiwan and Vietnam), but also among individuals derived from the same accession, suggesting that the varieties have been mixed in the process of breeding through cross pollination.

As *M. oleifera* is poorly tolerant to waterlogged conditions, it is critical to develop cultivars with high tolerance to waterlogged conditions, to expand drumstick cultivation areas. This has not been successfully addressed in the *M. oleifera* field of research. A potential approach to solve this problem is to obtain self-pollinated offspring from waterlogged tolerant drumstick plants and to keep selecting for waterlogging tolerant trait. Pure breeds can be obtained, which can

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then be outcrossed to create elite lines of *M. oleifera* that are tolerant to waterlogged conditions. In this work, seedlings derived from a waterlogged tolerant *M. oleifera* parental tree was characterised for their morphological traits and tolerance to waterlogged conditions. Furthermore, their genetic diversity and phytochemical compounds were evaluated in attempts to identify suitable lines of *M. oleifera* for future breeding programs.

## 2. Materials and methods

### 2.1. Plant materials

A hundred self-pollinated seeds were randomly harvested from a single parental plant of accession VI048718, kindly provided by AVRDC - The World Vegetable Center (Truong et al., 2017). The parental plant was planted in 2015 and survived a historical flood in 2020 while all other accessions cultivated in the same area did not. The seeds were a result of self-pollination in 2020 and were matured in 2021. The seeds were sowed in pots containing a 1:1:1 mixture of sand, garden soil and commercial organic fertilizer. Drumstick seedlings were generated as described in AVRDC International Cooperators' Guide: Suggested Cultural Practices for Moringa (Palada and Chang, 2003). The germination rate was 82% and the survival rate was 93%. The seedlings (76 self-pollinated lines, SPLs) were placed in a net house for eight weeks before being transplanted to plastic pots (36 × 29 × 29 cm) containing 25 kg of alluvial soil, 20 g of N:P:K (30:30:30) and 150 g of Super Organic 3–2–2. Soil properties (Table 1) were measured as described in Ruíz-Valdiviezo and co-workers (2010).

### 2.2. Waterlogging tolerance assay

After transplanting for forty days, the waterlogging tolerance of the SPLs was assayed as described by Abud-Archila et al. (2018). Each pot was watered with 10 L of water everyday for twenty days. Growth parameters including leaf number, plant height (cm), stem circumference (cm), biomass yield (g), stem fresh yield (g), leaf fresh yield (g), leaf dry yield (g) and leaf dry matter (%) were measured. Colours were determined using the Methuen Handbook of Colours (Kornerup and Wanscher, 1978).

### 2.3. Genetic diversity analysis

*DNA extraction*

Genomic DNAs of the parental plant and 76 SPLs were extracted from fresh leaves following the CTAB (cetyl-trimethyl ammonium bromide) procedure of Doyle and Doyle (1986). In particular, 0.5 g of leaves was washed and ground with a mortar and pestle in 500 $\mu$L of CTAB extraction buffer (100 mM Tris.HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB). The mixture was transferred to 1.5 mL tubes and incubated at 65 °C for 30 min. Afterwards, an equal volume of chloroform:isoamyl alcohol (24:1) mixture was added and the mixture was shaken at 500 rpm for 30 mins. The tubes were centrifuged at 17,000 *x g* for 10 mins at 4 °C and the aqueous phases (upper phases) were transferred to clean 1.5 mL tubes. Isopropanol (2/3 vol) was added, inverted to mix and the mixture was incubated in −20 °C for 30 mins to precipitate DNA. Genomic DNA was harvested by centrifugation (17,000 *x g* for 10 mins at 4 °C) and the pellets were washed with 500 $\mu$L of 70% ethanol (17,000 *x g* for 10 mins at 4 °C). DNA pellets were air-dried for 10 mins on the bench to remove ethanol residues before being dissolved in 100 $\mu$L TE buffer (pH 7.5). DNA quality was examined by gel electrophoresis (1% agarose in 0.5 x TBE buffer). Genomic DNAs were either used directly or subjected to further purification using spin columns (DNeasy Plant mini kit, QIAGEN, Germany).

*RAPD PCR*

A total of 200 UBC RAPD primers (Bioneer, Korea) were used to pre-screen the parental plant (*P*) and three SPLs (33, 48 and 71). The primer pairs yielding polymorphism were then confirmed using five SPLs (33, 48, 71, 19 and 27) and *P*. The polymorphic UBC RAPD primers were used to genotype 76 SPLs. PCR reactions were carried out as described previously (Truong et al., 2013). Briefly, 15-$\mu$L PCR reactions contained 1x MyTaq DNA polymerase mix (Bioline-Meridian, UK), 0.67 $\mu$M of primers and 100 ng of genomic DNA. The thermocycling program included 94 °C for 3 min, 40 cycles of amplification (94 °C for 1 min, 37 °C for 1 min and 72 °C for 2 min), followed by a final extension at 72 °C for 7 min. The PCR products were resolved by gel electrophoresis (2% agarose gel in 0.5 × TBE buffer) for 4 h at 120 V, stained with SYBR Green I (Invitrogen, USA) and visualized under UV illumination.

*Sequence-related amplified polymorphism (SRAP) PCR*

Sequence-related amplified polymorphism was examined using fifteen primer combinations (three forward and five reverse primers) (Ridwan *et al.*, 2020). The PCR reactions were performed as above, in which thermocycling program included an initial denaturation at 94 °C for 5 min, 40 cycles of amplification (94 °C for 1 min, 50 °C for 45 s and 72 °C for 2 min) and a final extension at 72 °C for 5 min. The PCR products were resolved by gel electrophoresis (2% agarose gel in 0.5 x TBE buffer), stained with SYBR Green I and visualized under UV illumination.

*DNA gel analysis*

Clear and undistorted DNA bands were scored as "1″, and absent (or faint) bands were scored as "0″. The size of each band was estimated based on the molecular weight markers. This logical matrix data were used to determine the genetic diversity using POPGENE version 1.32 (Yeh et al., 1999). The phylogenetic tree was constructed using the UPGMA algorithm in NTSYSpc (version 2.1), in which the distance matrix was established based on simple matching similarity coefficient (Sokal and Michener, 1958).

**Table 1**
Characteristics of the soil used growing 76 *M. oleifera* self-pollinated lines.

| Soil property | Values |
|---|---|
| Soil density (g/cm$^3$) | 1.07 |
| Absolute density (g/cm$^3$) | 2.50 |
| Porosity (%) | 52.04 |
| $pH_{KCl}$ | 5.70 |
| Total N (%) | 0.18 |
| Available N (mg/100 g) | 3.13 |
| Total P (%) | 0.42 |
| Available $P_2O_5$ (mg/100 g) | 45.40 |
| Total K (%) | 0.81 |
| Available $K_2O$ (mg/100 g) | 35.24 |
| OC (%) | 3.20 |
| Cu (mg/kg) | 25.14 |
| Pb (mg/kg) | 0.22 |
| Zn (mg/kg) | 112.0 |

### 2.4. Total phenolic content assay

The total phenolic content of *M. oleifera* leaves was determined using the Folin–Ciocalteu assay as previously described (Siddhuraju and Becker, 2003) with modifications. Briefly, leaves were dried in an oven at 50 °C for 48 h and then were ground with a mortar and pestle. Next, 50 mg of ground powder were extracted with 1 mL of 70% aqueous ethanol in 2-mL tubes and shaken (500 rpm) at 30 °C for 24 h. Then, the tubes were centrifuged at 13,000 rpm for 5 min. The ethanol extract was diluted in 70% ethanol (20 $\mu$L of extract in 980 $\mu$L of 70% ethanol) and 0.2 mL of the diluted extract was added to 1.2 mL of MilliQ water in 2-mL tubes. Folin–Ciocalteu's phenol reagent (0.1 mL) was added to the mixture, mixed and incubated for 5 min. Next, 0.3 mL of 20% $Na_2CO_3$ solution was added, followed by 0.2 mL of MilliQ water. After a 45-min incubation at room

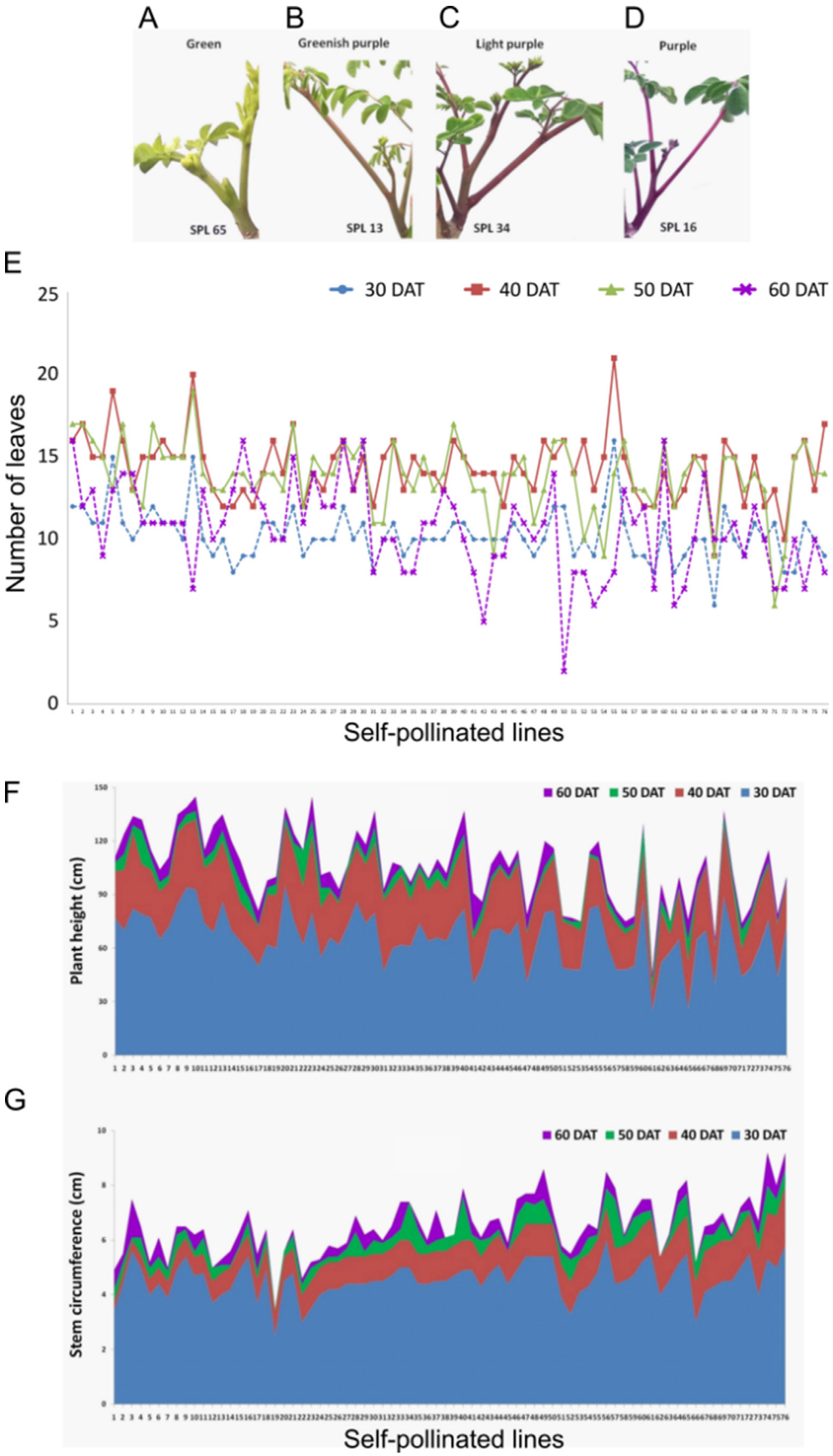


**Fig. 1.** Waterlogging tolerance of 76 *M. oleifera* self-pollinated lines (SPLs) at 40 days after transplanting. (A-D) Colour variation observed in young shoots of *M. oleifera* self-pollinated lines. (E-G) Growth parameters observed in *M. oleifera* SPLs following waterlogging treatment. (E) Number of leaves, (F) plant height and (G) stem circumference prior to waterlogging treatments (30 DAT and 40 DAT), 10 days (50 DAT) or 20 days (60 DAT) into the waterlogging treatment (60 DAT). DAT: days after transplanting.

temperature, the absorbance was measured at 758 nm (Hitachi U-2910, Japan). Standards of gallic acid were prepared in 70% ethanol (20, 40, 60, 80, 100 and 120 mg/L). Total phenolic content of *M. oleifera* leaves was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight. Results represent averages of three technical repeats.

### 2.5. Total flavonoid content assay

The ethanol extract was prepared as above, and a ten-fold dilution was carried out in 70% ethanol. The total flavonoid content was determined as described by Siddhuraju and Becker (2003). In 2-mL tubes, 0.12 mL of diluted ethanol extract, 1.36 mL of 30% methanol, 0.06 mL of $NaNO_2$ (0.5 M) and 0.06 mL of $AlCl_3{\cdot}6H_2O$ (0.3 M) were mixed. After 5 min, 40 $\mu$L of NaOH (1 M) was added to the mixture. The absorbance was measured at 506 nm (Hitachi U-2910, Japan). The standard curve was constructed using rutin standard solutions (100, 200, 300, 400 and 500 mg/L). The total flavonoid contents were expressed as milligrams of rutin equivalents per gram of dry weight. Results represent averages of three technical repeats.

## 3. Results

### 3.1. Morphology and waterlogging tolerance

At 40 days post transplantation, morphological variations were observed amongst 76 SPLs. As an example, young shoot color varied from green, greenish purple, light purple to purple (Fig. 1A-D). Leaf number ranged from nine leaves (SPL 65) to 21 leaves (SPL 55) (Fig. 1E, red line). Plant heights varied between 36 cm (SPL 61) and 132 cm (SPL 10) (Fig. 1F, red upper edge). Stem circumferences varied between 3.4 cm (SPL 61) and 8.0 cm (SPL 23) (Fig. 1G, red upper edge). Furthermore, the number of leaves, plant height and stem circumference of self-pollinated line population were distributed normally (Fig. S1), thus these traits were likely to be regulated by multiple genes.

Waterlogging treatment was carried out for 20 days, during which the number of leaves, plant heights and stem circumferences were monitored. Ten days into the waterlogging treatment, *M. oleifera* leaves from most SPLs turned yellow (Fig. 2). Leaf dropping was observed in most SPLs at the end of the 20-day waterlogging treatment (Fig. 1E and Fig. 2C). Overall, leaf gain was observed in only three SPLs following the waterlogging treatment: 7, 18 and 65. Furthermore, the rates of plant height and stem circumference increase reduced during the waterlogging treatment (Fig. 1F-G). Taken together, these observations demonstrated poor tolerance of SPLs towards waterlogged conditions.

Following the 20-day waterlogging treatment, the drumstick biomasses were harvested by cutting at position of 55 cm from the soil surface. Variations in biomass yield, stem fresh yield, leaf fresh yield and leaf dry yield were observed among 76 SPLs (Fig. 3). The highest biomass yield and stem fresh yield were obtained in SPL 23 (220.3 g and 213.4 g, respectively), followed by SPL 1 (168.1 g and 138.3 g, respectively). The highest leaf fresh yield and leaf dry yield were found in SPL 24 (42.3 g and 11.1 g, respectively), followed by SPL 12 (41.5 g and 9.8 g, respectively). SPL 61 had the lowest biomass yield, stem fresh yield, leaf fresh yield and leaf dry yield (0.9 g, 0.8 g, 0.1 g and 0.02 g, respectively). Although the highest biomass yield and stem fresh yield were recorded in SPL 23, its leaf fresh yield was low (6.95 g), thus, the ratio of leaf fresh yield and biomass yield was only 3.15%. The highest leaf fresh yield and leaf dry yield were recorded in SPL 24, and the highest ratio of leaf fresh yield/biomass (34%).


**Fig. 2.** Waterlogging treatment on *Moringa oleifera* self-pollinated lines. (A) Before, (B) 10 days into the waterlogging treatment or (C) at the end of the 20-day waterlogging treatment. (D) Differences in waterlogging tolerance ability amongst *M. oleifera* self-pollinated lines 10 days into the waterlogging treatment (at 50 days after transplanting).

### 3.2. Genetic polymorphism

Polymorphism was screened on the parental plant and three randomly selected SPLs 33, 48 and 71, using a total of 200 UBC RAPD primers and 15 SRAP primer pairs. Of these, 17 UBC RAPD primers and eight SRAP primer pairs were found to yield polymorphism (Fig. 4A).

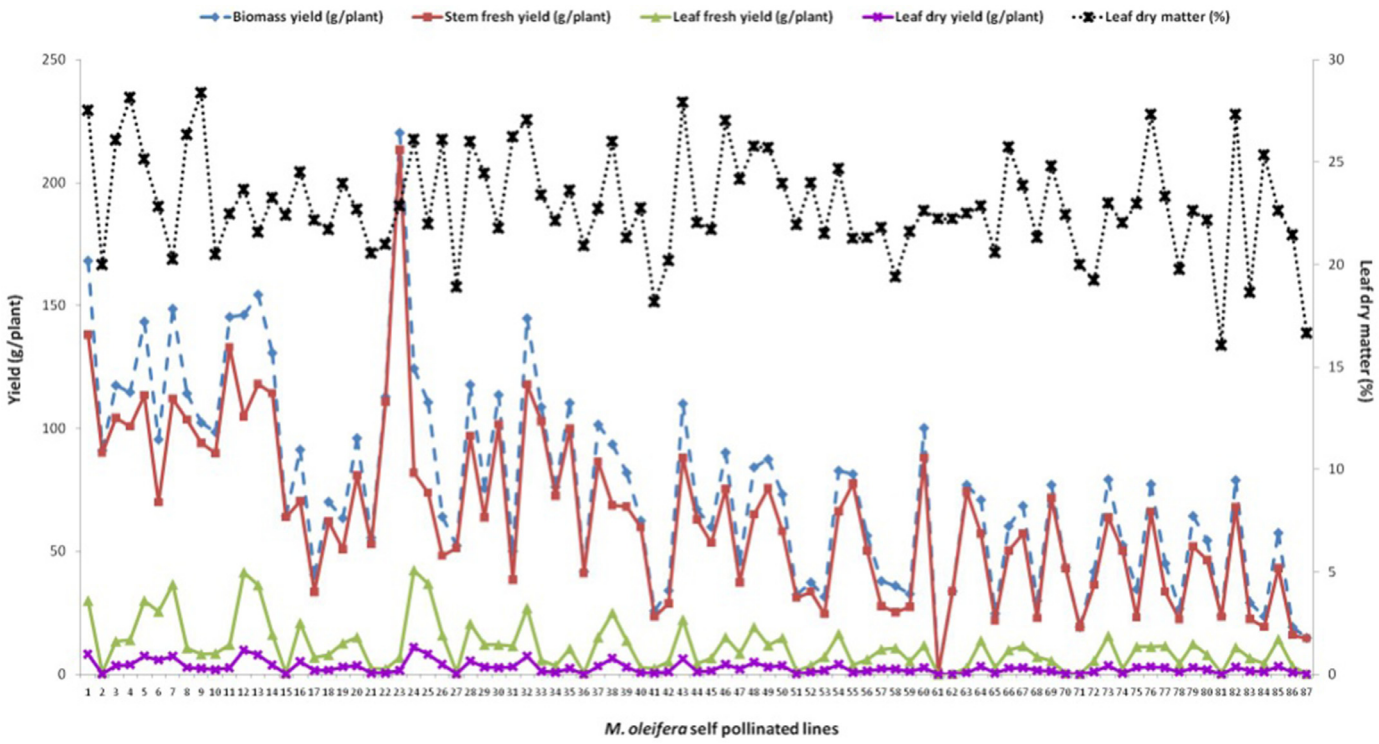


**Fig. 3.** Biomass yield, stem fresh yield, leaf fresh yield, leaf dry yield and leaf dry matter of *Moringa oleifera* self-pollinated lines following the waterlogging treatment.

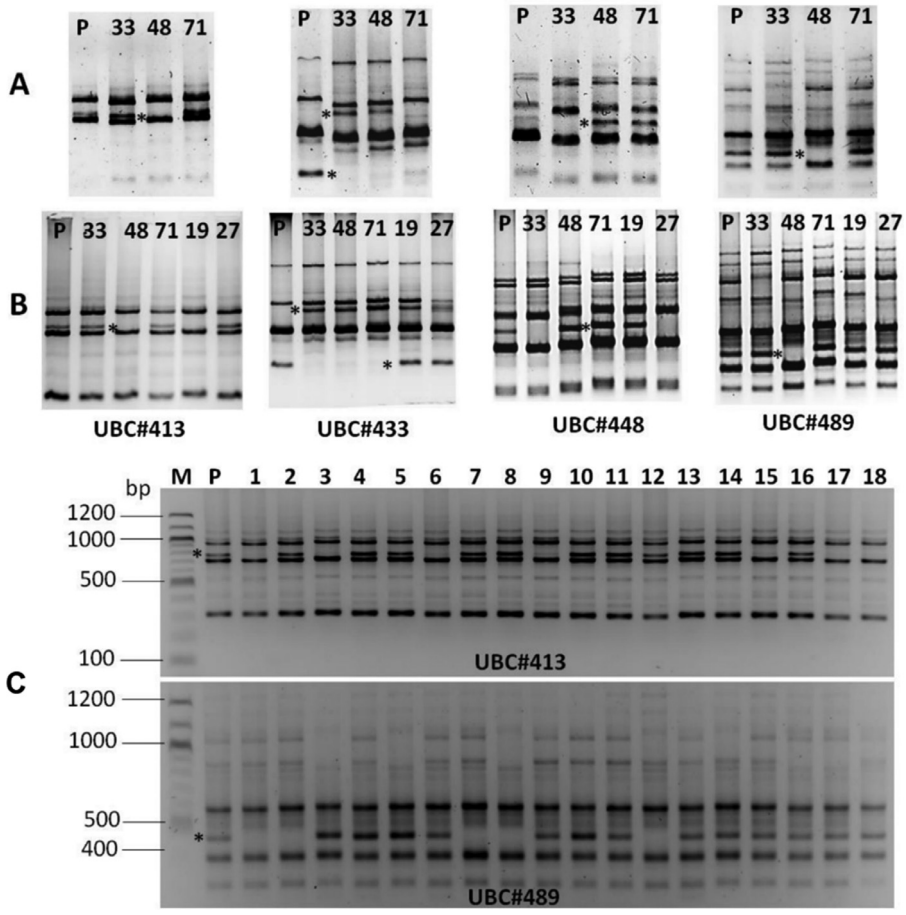


**Fig. 4.** Polymorphism within the *M. oleifera* parental (*P*) and self-pollinated lines shown by RAPD markers. (A) Three SPLs (33, 48 and 71) were randomly selected to screen for suitable primers in a collection of 200 UBC RAPD primers and 15 SRAP primer pairs. (B) The screen was expanded to include SPLs 19 and 27 to identify seven UBC RAPD primers and three SRAP primer pairs for polymorphic analyses. (C) PCR products obtained with RAPD UBC#413 and UBC#489 primers and DNA from the *M. oleifera* parental plant (*P*) and 18 self-pollinated lines. Products were resolved on 2% agarose gel. *M*, 100-bp molecular weight markers; asterisk denotes polymorphic bands.

**Table 2**
Sequence of primers used for characterizing polymorphism in 76 *M. oleifera* self-pollinated lines.

| No. | Oligo name | Sequence (5′–3′) |
|---|---|---|
| 1 | UBC#350 | TGACGCGCTC |
| 2 | UBC#368 | ACTTGTGCGG |
| 3 | UBC#413 | GAGGCGGCGA |
| 4 | UBC#433 | TCACGTGCCT |
| 5 | UBC#437 | AGTCCGCTGC |
| 6 | UBC#448 | GTTGTGCCTG |
| 7 | UBC#489 | CGCACGCACA |
| 8 | me_1F | TGAGTCCAAACCCGATA |
| | em_4R | GACTGCGTACGAATTTGA |
| 9 | me_2F | TGAGTCCAAACCGGAGC |
| | em_1R | GACTGCGTACGAATTAAT |
| 10 | me_2F | TGAGTCCAAACCGGAGC |
| | em_4R | GACTGCGTACGAATTTGA |

When the screen was expanded to include SPLs 19 and 27, only seven UBC RAPD primers and three SRAP primer pairs yielded clear and stable polymorphic fragments (Fig. 4B, Table 2). These primers were then used to genotype the 76 *M. oleifera* self-pollinated lines and the parental plant (Fig. 4C).

### 3.2.1. PCR result with rapd primers and srap primer

The polymorphic analyses obtained from PCR reactions using seven RADP primers and three SRAP primer pairs were displayed in Tables 3 and 4. A total of 92 bands were observed, with 25 bands being polymorphic (27%). The band sizes ranged from 300 to 1800 base pairs. Most primer pairs yielded low polymorphic band ratios, except UBC#350 and the pair me_1F/em_4R, both of which gave rise to a polymorphic rate of 50%. The pair me_2F/em_4R yielded the most polymorphic bands (6 bands, Table 4). In contrast, primer UBC#433 yielded the lowest rate of polymorphic band (10%). One

**Table 3**
Number of PCR bands observed when genomic DNA of *M. oleifera* parental and self-pollinated lines were amplified using ten different primers/primer pairs.

| No. | Line | UBC# | | | | | | | SRAP primer pair | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 350 | 368 | 413 | 433 | 437 | 448 | 489 | me_1F/em_4R | me_2F/em_1R | me_2F/em_4R | |
| **1** | *P* | 4 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 78 |
| **2** | SPL 1 | 4 | 7 | 8 | 10 | 9 | 7 | 10 | 4 | 7 | 10 | 76 |
| **3** | SPL 2 | 5 | 7 | 9 | 9 | 9 | 6 | 10 | 4 | 7 | 10 | 76 |
| **4** | SPL 3 | 5 | 7 | 8 | 10 | 9 | 6 | 9 | 4 | 7 | 11 | 76 |
| **5** | SPL 4 | 5 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 79 |
| **6** | SPL 5 | 8 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 11 | 81 |
| **7** | SPL 6 | 4 | 7 | 8 | 9 | 9 | 7 | 11 | 4 | 7 | 10 | 76 |
| **8** | SPL 7 | 6 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 80 |
| **9** | SPL 8 | 6 | 7 | 9 | 10 | 9 | 6 | 8 | 4 | 7 | 11 | 77 |
| **10** | SPL 9 | 4 | 7 | 8 | 10 | 9 | 6 | 11 | 4 | 6 | 11 | 76 |
| **11** | SPL 10 | 5 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 80 |
| **12** | SPL 11 | 5 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 80 |
| **13** | SPL 12 | 4 | 7 | 9 | 10 | 9 | 6 | 9 | 4 | 7 | 11 | 76 |
| **14** | SPL 13 | 6 | 7 | 9 | 9 | 9 | 7 | 11 | 4 | 7 | 10 | 79 |
| **15** | SPL 14 | 4 | 7 | 9 | 9 | 9 | 6 | 10 | 4 | 7 | 10 | 75 |
| **16** | SPL 15 | 4 | 7 | 8 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 78 |
| **17** | SPL 16 | 4 | 7 | 9 | 9 | 9 | 7 | 10 | 4 | 7 | 10 | 76 |
| **18** | SPL 17 | 4 | 7 | 8 | 10 | 9 | 7 | 10 | 4 | 7 | 10 | 76 |
| **19** | SPL 18 | 4 | 7 | 8 | 10 | 9 | 6 | 11 | 4 | 7 | 10 | 76 |
| **20** | SPL 19 | 4 | 7 | 8 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 77 |
| **21** | SPL 20 | 6 | 7 | 8 | 10 | 9 | 7 | 9 | 4 | 7 | 10 | 77 |
| **22** | SPL 21 | 4 | 7 | 9 | 10 | 9 | 6 | 9 | 4 | 7 | 10 | 75 |
| **23** | SPL 22 | 4 | 7 | 8 | 10 | 9 | 6 | 10 | 4 | 7 | 10 | 75 |
| **24** | SPL 23 | 4 | 7 | 9 | 9 | 9 | 7 | 10 | 4 | 7 | 11 | 77 |
| **25** | SPL 24 | 4 | 7 | 8 | 9 | 9 | 7 | 11 | 4 | 7 | 10 | 76 |
| **26** | SPL 25 | 5 | 7 | 9 | 10 | 9 | 6 | 11 | 4 | 7 | 11 | 79 |
| **27** | SPL 26 | 4 | 7 | 9 | 9 | 9 | 7 | 11 | 4 | 7 | 11 | 78 |
| **28** | SPL 27 | 4 | 7 | 9 | 10 | 9 | 6 | 10 | 4 | 7 | 11 | 77 |
| **29** | SPL 28 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 78 |
| **30** | SPL 29 | 4 | 7 | 8 | 10 | 9 | 6 | 11 | 4 | 7 | 10 | 76 |
| **31** | SPL 30 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 10 | 77 |
| **32** | SPL 31 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 78 |
| **33** | SPL 32 | 4 | 7 | 9 | 9 | 9 | 7 | 9 | 4 | 7 | 11 | 76 |
| **34** | SPL 33 | 4 | 7 | 9 | 9 | 9 | 6 | 10 | 4 | 7 | 11 | 76 |
| **35** | SPL 34 | 7 | 7 | 8 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 81 |
| **36** | SPL 35 | 4 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 10 | 76 |
| **37** | SPL 36 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 10 | 77 |
| **38** | SPL 37 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 78 |
| **39** | SPL 38 | 4 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 78 |
| **40** | SPL 39 | 6 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 11 | 79 |
| **41** | SPL 40 | 6 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 81 |
| **42** | SPL 41 | 4 | 7 | 9 | 9 | 9 | 7 | 10 | 4 | 7 | 11 | 77 |
| **43** | SPL 42 | 4 | 7 | 9 | 10 | 10 | 7 | 11 | 4 | 7 | 12 | 81 |
| **44** | SPL 43 | 8 | 7 | 9 | 9 | 9 | 6 | 10 | 4 | 7 | 11 | 80 |
| **45** | SPL 44 | 4 | 7 | 8 | 10 | 9 | 6 | 11 | 4 | 7 | 10 | 76 |
| **46** | SPL 45 | 4 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 79 |
| **47** | SPL 46 | 4 | 7 | 8 | 9 | 9 | 6 | 10 | 4 | 7 | 11 | 75 |
| **48** | SPL 47 | 4 | 7 | 8 | 9 | 9 | 7 | 11 | 4 | 7 | 11 | 77 |

(*continued*)

**Table 3** (*Continued*)

| No. | Line | UBC# | | | | | | | SRAP primer pair | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 350 | 368 | 413 | 433 | 437 | 448 | 489 | me_1F/ em_4R | me_2F/ em_1R | me_2F/ em_4R | |
| **49** | SPL 48 | 4 | 8 | 8 | 10 | 9 | 7 | 9 | 4 | 7 | 11 | 77 |
| **50** | SPL 49 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 12 | 79 |
| **51** | SPL 50 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 78 |
| **52** | SPL 51 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 10 | 77 |
| **53** | SPL 52 | 4 | 7 | 8 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 77 |
| **54** | SPL 53 | 4 | 7 | 8 | 10 | 10 | 7 | 11 | 4 | 7 | 11 | 79 |
| **55** | SPL 54 | 4 | 7 | 9 | 9 | 9 | 7 | 9 | 4 | 7 | 11 | 76 |
| **56** | SPL 55 | 4 | 7 | 9 | 9 | 9 | 6 | 11 | 4 | 7 | 11 | 77 |
| **57** | SPL 56 | 4 | 7 | 9 | 9 | 9 | 7 | 11 | 4 | 7 | 11 | 78 |
| **58** | SPL 57 | 4 | 7 | 9 | 10 | 10 | 7 | 11 | 4 | 7 | 11 | 80 |
| **59** | SPL 58 | 4 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 11 | 77 |
| **60** | SPL 59 | 4 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 12 | 80 |
| **61** | SPL 60 | 4 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 10 | 76 |
| **62** | SPL 61 | 4 | 7 | 8 | 10 | 9 | 7 | 11 | 4 | 7 | 11 | 78 |
| **63** | SPL 62 | 4 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 78 |
| **64** | SPL 63 | 5 | 7 | 9 | 10 | 9 | 6 | 11 | 4 | 7 | 10 | 78 |
| **65** | SPL 64 | 4 | 7 | 9 | 9 | 9 | 7 | 11 | 4 | 7 | 11 | 78 |
| **66** | SPL 65 | 5 | 7 | 9 | 10 | 9 | 6 | 10 | 4 | 7 | 10 | 77 |
| **67** | SPL 66 | 5 | 7 | 9 | 10 | 9 | 7 | 9 | 4 | 7 | 12 | 79 |
| **68** | SPL 67 | 4 | 7 | 8 | 10 | 9 | 6 | 10 | 4 | 7 | 12 | 77 |
| **69** | SPL 68 | 5 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 79 |
| **70** | SPL 69 | 5 | 7 | 9 | 10 | 10 | 7 | 10 | 4 | 7 | 10 | 79 |
| **71** | SPL 70 | 4 | 7 | 9 | 10 | 9 | 6 | 9 | 4 | 7 | 11 | 76 |
| **72** | SPL 71 | 7 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 12 | 83 |
| **73** | SPL 72 | 5 | 7 | 9 | 10 | 9 | 7 | 10 | 4 | 7 | 11 | 79 |
| **74** | SPL 73 | 4 | 7 | 9 | 10 | 10 | 7 | 9 | 4 | 7 | 10 | 77 |
| **75** | SPL 74 | 4 | 7 | 9 | 10 | 9 | 6 | 8 | 4 | 7 | 11 | 75 |
| **76** | SPL 75 | 4 | 7 | 9 | 10 | 9 | 6 | 11 | 4 | 7 | 11 | 78 |
| **77** | SPL 76 | 4 | 7 | 9 | 10 | 9 | 7 | 11 | 4 | 7 | 10 | 78 |
| **Total** | | 346 | 540 | 672 | 752 | 698 | 516 | 784 | 308 | 538 | 824 | 5978 |

**Table 4**
Polymorphic analysis of the *M. oleifera* self-pollinated lines based on PCR products obtained with ten primers/ primer pairs.

| Primer/ Primer pair | Number of bands | Number of polymorphic bands | Percentage of polymorphic bands (%) | Size (bp) |
|---|---|---|---|---|
| **UBC#350** | 8 | 4 | 50.0 | 570 - 1517 |
| **UBC#368** | 8 | 1 | 12.5 | 450 - 1550 |
| **UBC#413** | 9 | 1 | 11.1 | 300 - 1150 |
| **UBC#433** | 10 | 1 | 10.0 | 300 - 1517 |
| **UBC#437** | 10 | 2 | 20.0 | 450 - 1300 |
| **UBC#448** | 7 | 1 | 14.3 | 400 - 1250 |
| **UBC#489** | 11 | 3 | 27.3 | 300 - 1500 |
| **me_1F and em_4R** | 6 | 3 | 50.0 | 320 - 1800 |
| **me_2F and em_1R** | 9 | 3 | 33.3 | 450 - 1800 |
| **me_2F and em_4R** | 14 | 6 | 42.9 | 300 - 1700 |
| **Total** | 92 | 25 | 27.2 | 300 - 1800 |

characteristic band (450 bp), which appeared only in the PCR products of SPL 48 and not in others, was observed when primer UBC#368 was used. Across SPLs, the combined number of amplification bands from ten primers/primer pairs ranged from 75 to 83, with SPL 71 yielding the highest number of amplification bands (Table 3).

### 3.2.2. Genetic diversity analysis

POPGENE (version 1.32) was employed to determine the genetic diversity indices. The number of expected alleles, the number of effective alleles, Nei's gene diversity ($h$) and Shannon's information index ($I$) were found to be 1.2609, 1.1358, 0.0791 and 0.1200 respectively (Table 5). These figures indicated that the self-pollinated lines were quite diverse genetically. Genetically, the parental and 76 self-pollinated lines were separated into five major groups: group I included SPL 5 and SPL 43, having a similarity coefficient of 0.80 (Fig. 5). Group II consisted of SPL 3 and SPL 13 whereas group III involved SPL 12 and SPL48. Next, group IV included 14 SPLs (7, 8, 23, 25, 34, 39, 67, 68, 69, 70, 72, 73, 74 and 75) whereas the rest, which

**Table 5**
Genetic diversity indices of *Moringa oleifera* self-pollinated lines.

| Indices | Number of expected alleles | Number of effective alleles | Nei's gene diversity ($h$) | Shannon's information index ($I$) |
|---|---|---|---|---|
| | 1.2609 | 1.1358 | 0.0791 | 0.1200 |
| **Standard error** | 0.4415 | 0.2951 | 0.1590 | 0.2301 |

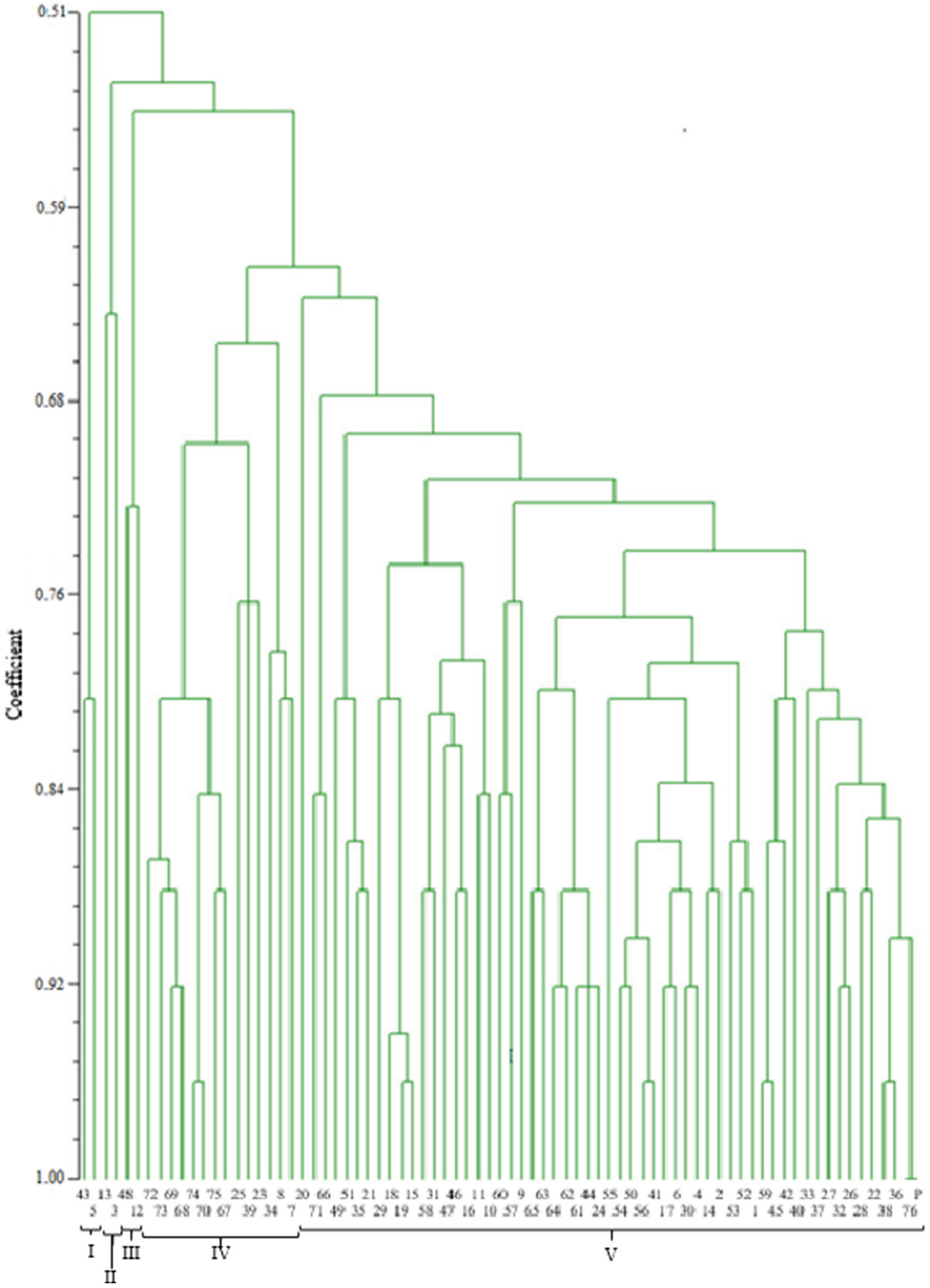


**Fig. 5.** Dendrogram showing the genetic relationship between the *Moringa oleifera* parental (*P*) and 76 self-pollinated lines (SPLs).

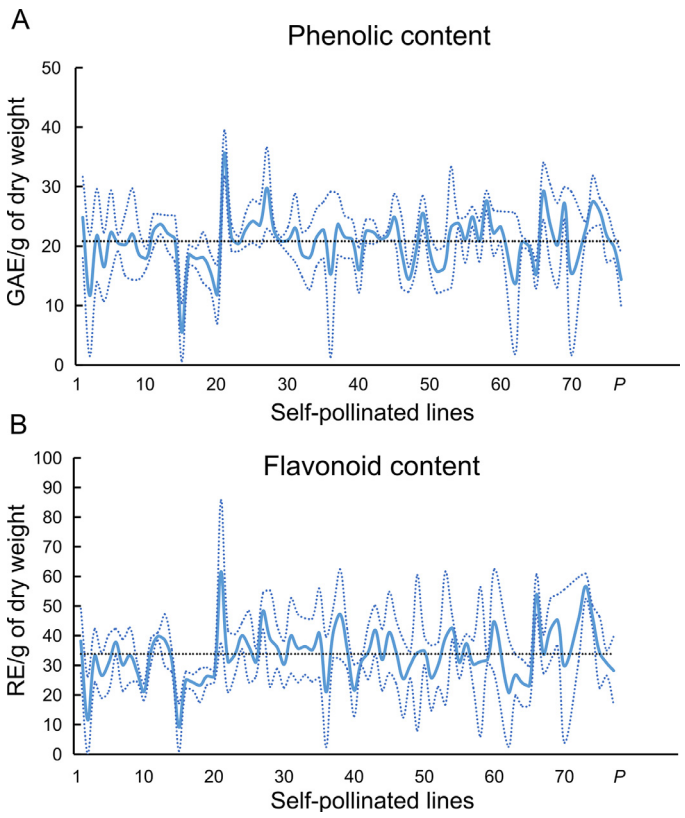


**Fig. 6.** Total phenolic and flavonoid contents measured in *M. oleifera* parental (*P*) and 76 self-pollinated lines. (A) Total phenolic content was determined as mg of gallic acid equivalents per gram of dry weight (GAE/g of dry weight). (B) Total flavonoid content was determined as mg of rutin equivalents per gram of dry weight (RE/g of dry weight). Solid lines and dashed blue lines represent the mean and standard deviations (three repeats) respectively. Dashed black lines represent averaged values across the parental and 76 self-pollinated lines.

included the parental and 56 SPLs, belonged to the largest group - group V. SPL 76 and *P* were genetically close. The lowest similarity was observed between SPL 43 and SPL 48 (Table S1).

### 3.3. Phenolic and flavonoid contents

The total phenolic and flavonoid contents were measured in the *Moringa oleifera* parental and self-pollinated lines (Fig. 6). The variations in phenolic contents mirrored those of flavonoid contents (compared Fig. 6A and 6B), which is consistent with the fact that flavonoids are a group of chemicals in the phenolic family. Across the self-pollinated lines, SPL 21 had the highest phenolic and flavonoid contents (35.6 mg of GAE/g of dry weight and 61.6 mg of RE/g of dry weight respectively). The SPLs with the second and third highest phenolic contents were SPL 27 and SPL 66 (29.7 and 29.2 mg of GAE/g of dry weight respectively) (Fig. 6A). On the other hand, SPL 15, SPL 2 and SPL 20 had the lowest, second and third lowest phenolic contents (5.5 mg, 11.7 mg and 12.0 mg of GAE/g of dry weight respectively). The phenolic content of the parent was 14.4 mg of GAE/g of dry weight, below the averaged value of 75 SPLs (20.8 mg of GAE/g of dry weight). The SPL with the highest phenolic content (SPL 21) had more than six-fold higher phenolic content than that of the lowest (SPL 15).

The SPLs with the second and third highest flavonoid contents were SPL 73 and SPL 66 (56.7 and 53.9 mg of RE/g of dry weight respectively) (Fig. 6B). On the other hand, SPL 15, SPL 2 and SPL 62 had the lowest, second and third lowest flavonoid contents (9.1 mg, 11.6 mg and 20.9 mg of RE/g of dry weight respectively). The flavonoid content of the parent was 28.2 mg of RE/g of dry weight, below the averaged value of 75 SPLs (33.8 mg of RE/g of dry weight). The SPL with the highest flavonoid content (SPL 21) had almost seven-fold higher flavonoid content than that in SPL 15, which contained the lowest amount of flavonoids.

## 4. Discussion

*Moringa oleifera* leaves are consumed as a vegetable in parts of Asia, although its nutritional and health benefits have not been fully realised. Due to their poor tolerance for waterlogging, it is useful to develop cultivars that are more tolerant to waterlogged conditions to expand cultivation areas. In this study, 76 self-pollinated lines derived from a waterlogging tolerant *M. oleifera* tree were characterised. They exhibited a range of morphologies, yields and tolerance to waterlogged conditions. Following a 20-day waterlogging treatment, leaf gain was only observed in three SPLs: 7, 18 and 65, indicating high levels of tolerance to waterlogged conditions by these lines. However, the phenolic and flavonoid contents in these SPLs were only around the averaged values.

On the other hand, SPL 21 had the highest phenolic and flavonoid contents among the 76 SPLs and doubled those from the parental tree. The averaged phenolic content reported in this work is similar to those obtained in *M. oleifera* from Madagascar (24 mg GAE/g of dry weight; Rodríguez-Pérez et al., 2015), South Africa (15–32 mg GAE/g of dry weight; Chitiyo et al., 2021) and Indonesia (25–30 mg GAE/g of dry weight; Sulastri et al., 2018) but somewhat lower than those reported by Siddhuraju and Becker (2003) (89–123 mg GAE/g of dry weight). Similarly, the averaged flavonoid content reported here is also similar to those measured by Chitiyo and co-workers (2021) but lower than values obtained by Siddhuraju and Becker (2003) (58–140 mg RE/g of dry weight). The variations in phenolic and flavonoid contents among SPLs were large, with the richest line (SPL 21) containing six- and seven-fold higher phenolic and flavonoid contents respectively than the poorest line (SPL 15). The variation is consistent with the differences in morphologies, waterlogging tolerance and genetic diversity; all pointed towards allelic segregation in the self-pollinated lines.

The genetic diversity within *M. oleifera* germplasms is well-known; previous studies using SRAP markers and RADP markers have shown a polymorphism percentages to be in the range of 48% to 90% (Ridwan et al., 2021; Saini et al., 2013; Drisya *et al*., 2022). In terms of the number of expected alleles, the number of effective alleles, Nei's gene diversity and Shannon's diversity index, figures observed in this study (Table 5) are somewhat lower than those reported by Drisya and co-workers (2022), but comparable to those reported by Rufai and co-workers (2013). However, previously reported germplasms were collected from various geographical locations, and therefore the polymorphic ratios were higher than that observed in this study (27%), reflecting a higher genetic diversity.

## 5. Conclusion

Most investigations on *M. oleifera* cultivation have been focussed on traits such as yields (Zheng et al., 2016), seed oil content and resistance to pests (Leone et al., 2016). This work presents a new direction where *M. oleifera* was selected for waterlogging tolerance and high phenolic and flavonoid contents. The waterlogged tolerant lines were found to be SPLs 7, 18 and 65. However, these lines only contained averaged amounts of phenolic and flavonoid compounds. The lines with the highest phenolic contents were SPLs 21, 27 and 66 and the lines with the highest flavonoid contents were SPLs 21, 73 and 66. Future work will focus on creating pure breeds from accessions with high waterlogging tolerance (SPLs 7, 18 and 65), and high phenolic and flavonoid contents (SPLs 21, 27, 66 and 73), before outcrossing can be carried out to create elite *M. oleifera* cultivars.

## Authors' contributions

C.H., N.B.L.Q., H.N.T.H., R.S., L.M.H.D. and N.C.Q. conducted the experiments, collected the data, and participated in interpretation of results. T.H.T.H. designed experiments, prepared manuscript draft. T. H.T.H. and H.N.H. finalized the manuscript.

## Declaration of Competing Interest

Thank you for your consideration of our manuscript, entitled "Waterlogging tolerance, phenolic and flavonoid contents, and genetic diversity among Moringa oleifera self-pollinated lines" by Truong Thi Hong Hai and co-workers. We declare no competing interests and confirm that the manuscript is not currently under consideration for publication with another journal.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2023.04.012.

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