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African Journal of Plant Science

Full Length Research Paper

Potential and measures for sustaining *Prunus africana* (Hook.F.) Kalkman (Rosaceae) in Tchabal Mbabo forest (Mf), Adamaoua Cameroon

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***Prunus africana* (Hook. F.) Kalkman is a medicinal plant for which the bark is used to treat benign prostate hypertrophy. *P. africana* is listed in Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Its exploitation has been regulated in Cameroon through the settlement of some management standards. This paper aims to assess the potential of *P. africana* in the Tchabal Mbabo forest (MF), vast of 25,671 ha in view to formulate guidelines for sustainable harvesting. Data collection took place from October 1st to 28th 2021. This consisted of conducting forest management inventories at a sampling rate of 0.72%. Three hundred and fifty-eight (358) stems of *P. africana* were counted on a surface area of 57.5 ha, which gives a density of 6.23 stems/ha. The average values of diameter, height and bark thickness of the stems (unharvested and harvested sides) are respectively 48.17 ± 19.8 cm; 7 ± 4.26 m; 14.1 ± 4.72 mm and 10.39 ± 4.22 mm. The average annual bark increment is 1.3 mm/year for a bark regeneration rate of 60%. A half rotation of 7 years was obtained for a dry bark quota of 164.6 tons/year. It would be wise to take into account the dynamic elements to review the calculation of the quota for the second rotation.**

Key words: *Prunus africana*, standards, parameters, sustainable management, Tchabal Mbabo.

INTRODUCTION

Cameroonian forests with 22.5 million hectares are among the vast and rich forest massifs in the Congo basin (Wete, 2022). During the last decade of the twentieth century, the role of forest was again redefined

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by include additional parameters such as carbon sequestration, biodiversity, regeneration status, and Non-Timber Forest Product (NTFP) (Tewari, 2016). The NTFP from these forests bring together food plants, medicinal plants and service products (Betti et al., 2016). The use of plants for therapeutic purposes has been known since the dawn of time (Lehmann, 2013). Today, the effectiveness of herbal medicine is proven and its undeniable benefits (Bene et al., 2016). Among the health problems listed, the prostate cancer is an important public health problem (Shenouda et al., 2007). According to Diarra (2006), apart from cancer proper, other prostate conditions are generally associated with it, amongst them we have the benign prostate hypertrophy (HBP). It is a non-cancerous prostate enlargement (Nyamai et al., 2016) which causes discomfort in elderly men, present in more than 50% of men over 60 years (Bodeker et al., 2014). Medicine by plants becomes daily (Bene et al., 2016) because,The conventional methods lead to severe side effects including erectile dysfunction and gynecomastia. So, people prefer to opt for phytotherapy for the management of the condition to avoid these adverse effects (Nyamai et al., 2015).

The use of *Prunus africana* in traditional African medicine (ATM) to treat prostate cancer and related conditions is not a new phenomenon in various communities in Africa (Ochwang'i, et al., 2014; Komakech et al., 2017). Mutuma et al. (2020) confirms that the dichloromethane stem bark extract of the *P. africana* presented anti-inflammatory activity, hence a possible candidate for extraction of active anti-inflammatory compounds. Its importance resides in the curative properties of its bark extracts used for the manufacture of more than 19 drugs, sold on the European and American markets for the treatment of benign prostatic hypertrophy (Cunningham et al., 2002). These extracts are mainly made up of of pentacyclic triterpenoids, ferulic esters of long-chain fatty alcohols, and phytosterols contained in bark and find in certain drugs, for e.g., Tadenan, Bidrolar and Pygenil (Nyamai et al., 2016; Komakech et al., 2017). The worldwide demand for these barks is estimated at 4000 tons (Simons et al., 1998). This high demand has therefore led to irrational harvesting of the plant species in the various production areas. Thus, trees have been felled abundantly and frequently before the maturation stage or debarked completely at the trunk level (Randriambololona, 1994; Rasoanandrasana, 2010). As a result of this intensive exploitation and poor harvesting methods, *P. africana* has become very scarce, the rootstock has disappeared and its natural regeneration has been reduced (Dawson and Rabevohitra, 1996; Sven and Rakotonirina, 1995; Rasoanandrasana, 2010; Momo et al., 2016). All this led to the listing of the species in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1995 (Tonye et al., 2000). In Cameroon the majority of *P. africana* populations are found in the North West, South West and Adamaoua regions where they have been widely exploited for their bark since 1980 (Momo et al., 2016).

A key requirement of CITES for any species listed in appendix II is the establishment of a non-detriment findings made by the Scientific Authority of the range State prior to export, certifying that export is not detrimental to the survival of the species. This requires information on the location, stocking, growth and condition of the species and on its ecology, regeneration and subsequent protection. Such information is often lacking, incomplete or imprecise making a proper evaluation of the sustainable levels of utilization and conditions attached to be difficult. The Scientific Authorities also face obstacles due to inadequately trained and resourced staff. Following irregularities observed in some range of countries, a conference was organized by CITES in September 2008 in Lima, Peru with a view to deciding on the management methods of *P. africana* in exporting countries. During this conference, some countries, such as Cameroon, were asked to voluntarily consider a zero-export quota before December 31st, 2008, in order to conduct forest inventories and develop the management plan for *P. africana*. Failure to comply with these recommendations could lead to an embargo on trade in this species from these countries (Ingram et al., 2009). In the meantime, this deficiency observed in the management of *P. africana* in Cameroon led the European Union (EU) to suspend exports from Cameroon in 2008 (Akoa et al., 2010). The exploitation of *P. africana* resumed in Cameroon only in 2010 after the first results of the management inventories which led to the development of a non-detriment finding document (NDF) for this species in the North-West Region, Cameroon (Akoa et al., 2010).

This work, carried out within the framework of the joint program of the International Tropical Timber Organization (ITTO) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), known as "The ITTO-CITES program", was extended in the southwest (Akoa et al. 2011a) and then in Adamaoua (Akoa et al., 2011b) regions and allowed Cameroon to be granted an annual quota of 630 tons of dry bark of *P. africana* in 2011 and distributed as follows: Adamaoua (350 tons), North-West (150), and South-West (170). All these interventions have enabled Cameroon to take ownership of the management mechanisms of CITES species. However, the reports of certain NGOs and in particular the Deutsche Gesellschaft fur Internationale Zusammenarbeit GmbH (GIZ) always mentioned the unsustainable management of the Pygeum, so, CITES called on Cameroon on this subject and in particular on the reduction of export quotas (https://cites.org/sites/default/files/fra/com/pc/22/ExSum/F-PC22-SR.pdf). Until September 2017, Cameroon had not yet provided clear answers to all CITES recommendations, especially on the real potential of each

production unit of *P. africana.* The state of the art on the management/harvesting of *P. africana* in Cameroon revealed that, for the Adamawa Region, forest inventories carried out by trade companies themselves were not conducted in fair manner, using approved standards settled by the Cameroon forest management guidelines. Furthermore, the management parameters used to estimate the annual quota is based on studies conducted in the North west and South west regions of Cameroon (Wete, 2022). The commercialization of medicinal plants needs to account for the limits of the resource to ensure the sustainable, i.e., the long-term and continued, harvesting of the species (Bodeker et al., 2014).The present work attempts to assess the potential of *P. africana* with the view to ensure sustainable exploitation of the bark of this species in the Tchabal Mbabo forest (MF). The specific objectives are: (1) to analyze the sampling effort in mountain forests (2) to appreciate the structural parameters (density, diametric structure, basal area, carbon stock) (3) to characterize the response of *Prunus* trees to the harvesting (sanitary statement, bark regeneration) (4) and to propose sustainable management measures for bark harvesting (rotation, harvesting zones, harvesting techniques).

MATERIALS

Study site

Tchabal Mbabo is geographically located in the Adamaoua Region, on either side of the Departments of Mayo Banyo and Faro and Déo, in three subdivisions including Banyo in the Mayo Banyo Division, Galim Tignère and Kontcha in the Faro and Déo Division (Hiol Hiol, 2021). It culminates at an altitude of 2240 meters (MINFOF, 2018). The work was conducted in 4 localities namely: Foungoi with coordinates (07°14'04.51"N, 12°02'56.96"E), Yangare (07°13'42.47"N, 12°07'57.40"E), Botendji (07°14'49.41"N, 12°10'07.29"E) and Horé garba (07°19'22.98"N, 12°14'02.45"E). The human population of the area comprises diverse ethnic groups including Mbororos, Foulbes, Nyem Nyems and Hauossas. These groups are divided into traditional chiefdoms or Lamida and different native languages (Unsongo, 2019). The main vegetation of Tchabal Mbabo is made up of forest galleries, grassy savannas, dry altitude forests, and wooded savannas. Forest galleries are known to be predilection sites for *P. africana* (Wete, 2022). The North and south of the Adamaoua plateau is made up of metamorphic rocks and granitoids of African panic age. It is crossed in the north and east by basalt flows and cones, domes and trachyte-colored domes and phonolitis of mio-plicen ages (Fagny et al., 2017). Tchabal Mbabo hosts 294 bird species, 22 species restricted to the afro montane ecosystem, 10 of which are endemic to the mountain chain. The area is also known to harbour some Critically Endangered and Endangered reptiles and amphibians (Usongo, 2019). The hottest months are April and May with average daily temperatures of up to 30°C. Annual temperatures on the plateau average 18°C with daily amplitude of 13-15°C (Herrmann et al., 2007). Figure 1 present the ombrothermic diagram of the zone.

Description of the species

P. africana grows well in the sub-mountain and mountain forests at an altitude of 800-3000 m. In Cameroon, the plant is largely found in five regions including Adamaoua, North West, Littoral, South west, and West (Betti and Ambara, 2013). The description made by Vivien and Faure (2011) is as follows: Its base is a simple wheelbase or thick 8 -10 cm thick, deviating at 1 m of the tree at 1 m in height. It was straight with a crown with tortuous branches eased obliquely with young reddish twigs. It's very dark brown bark (1.5 cm) formed large or less square platelets in the aged trees; It tender, fibrous, pink turning brown, with characteristic odor of bitter almond little differentiated, pinkish white (3 cm). Its wood is brown pink in light with leaves persistent, alternate, simple (6-15 x 3-6 cm), with a tender and shiny then tough and matt blade, on a crenellated edge with a small black gland at each point, sometimes 1 or 2 glands at the base of the blade, At 6-12 pairs of side veins. Its fruit is a drupe with 2 red lobes, with a small point at the top.

METHODS

The method used is a combination of surveys and forest inventories. The surveys were conducted using the participatory method to get an idea of the actual production sites of *P. africana* in the Tchabal Mbabo forest. Discussions were conducted with administrative officials, including forestry services, community representatives, traditional chiefs or Djaouro, village populations (elderly and non-elderly), and field-based staff of companies that harvest *P. africana* in Cameroon. *P. africana* inventories were carried out in areas previously identified as *P. africana* production sites from October 1st to 28th 2021. The method used is, the classical forest inventory method standardised (arêté n° 222) for management inventories (MINEF, 2001).

Sampling design

The surveys carried out made it possible to map and delimit the potential sites of predilection of *P. africana* in altitudes ranging from 1,400 to 2,100 m. Maps were made in three stages ,including: (1) document review and exploration of photos and images (2) identification of areas to be surveyed, and (3) refinement of the data. The BING and (Open Street Map) OSM aerial funds were used for this purpose. The elevation factor proved to be of primary importance in the spatial distribution of *P. africana* in the inventory area, so we made a Digital Terrain Model (DTM) on which the information layers were draped. The DTM was made by downloading a LANDSAT 8 image of March 2021 of the inventory area and then processed on QGIS 3.0. For this inventory, "layons" were oriented in the direction perpendicular to the general direction of slope, according to the national standards (MINFOF, 2019), the sampling is systematic and stratified to 1 degree when the statistical unit is the plot. The samples or plots of 0.5 ha (250 m long × 20 m large) are distributed systematically throughout the entire population and not by stratum (forest type). The stratification is done definitively after the sampling. The systematic disposal of plots allows the assumption that the intensity of sampling for each stratum is proportional to its area in the forest. Results of the inventory and their accuracy are calculated for each stratum. In practice, sampling is carried out along straight and continuous axes called "layons" or transects. These "layons" are oriented along a predetermined magnetic direction but are systematically arranged in such a way that they are mostly parallel, equidistant and perpendicular to the general direction of both drainage and slopes. In Cameroon, the sampling intensity for management inventories, the surveys conducted have estimated the useful area of *P. africana* in the Tchabal Mbabo to be 28,456 ha. For this work, a total of four villages were selected because of (1) their accessibility and (2) of the consent of the village leaders. The total area selected is 8,000 ha distributed as follows in the four villages: Foungoi (1,846.48 ha), Yangaré (3,018.97), Botendji (1,011.14) and Horé

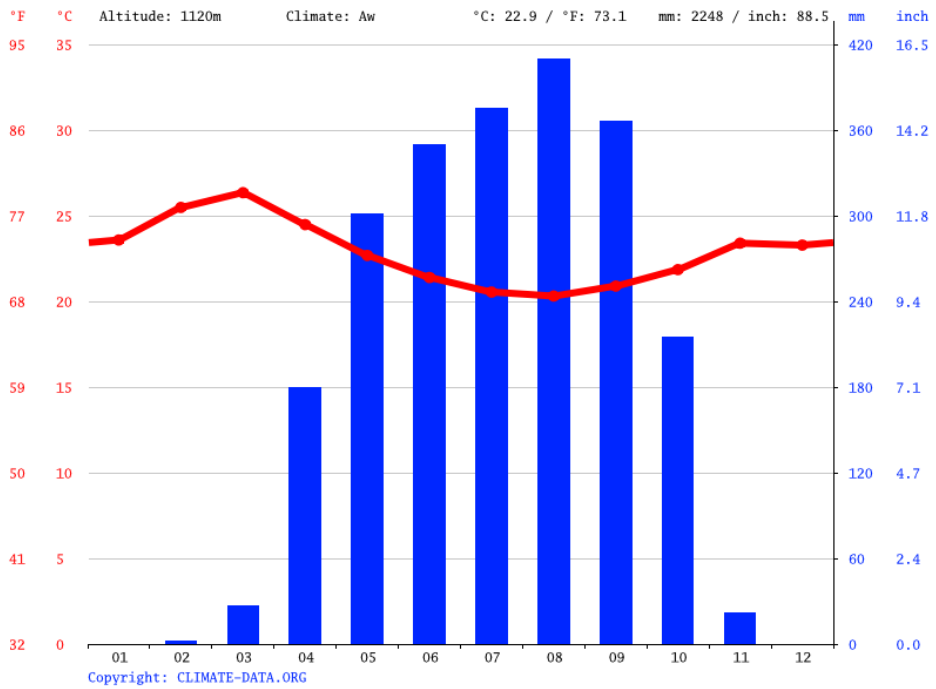

Figure 1. Ombrothermic diagram.
Source: Authors

Garba (2,123.41). A provisional sampling rate of 2% was designed. Figure 2 illustrates the sampling design.

Implementation of the sampling design

The implementation of the sampling design (or the inventory) consists of two steps: “layons” or line opening/transect cutting and counting. Line opening/transect cutting: This step consists of opening or cutting according to a defined magnetic direction, corridors or alleys of 1.5 m wide. These corridors are clearly cleaned by cutting shrubs, vines and branches that obstruct the passage. They are then identified by marks. “Layons” constitute the reference system which will be used by the subsequent counting team. It is during the “layons” opening that details on topography, habitat types, rivers and the corrected horizontal distance of the “layon” (after reading the slopes) are given. It is also during this stage that the sample plots are identified and numbered. The data collected are recorded on specific sheet. Counting: The counting step includes all operations relating to dendrological and dendrometric records. During the counting, several operations are made including: Identification of stems of *P. africana*, the measurement of stems with diameter at breast height (dbh = 1.30 m)>= 5 cm, appreciation of the health state of the tree in three classes (dead trees, damaged trees, and living trees). The appreciation of the healthy status of the tree is mainly based on the health of the leaves and number of dried branches. Lines and plots are identified and numbered with their geographical coordinates and altitudes. For each *P. africana* stem encountered, the height, diameter at breast height, bark thickness was measured. The thickness was measured for each exploited stem on the face of the bark not yet exploited as well as on the face of the bark already exploited, that is, the bark in regeneration. For this purpose, a square or rectangular bark core was taken and the thicknesses of the four sides were measured. The average thickness of the four sides was taken as the bark thickness of this tree and for the indicated side. The harvesting techniques used were described and the year of harvesting was noted.

Data analysis

The processing of the collected data allowed highlighting density, basal area, diametric structure, bark reconstitution rate, carbon stock, and half-rotation. The formulas used are the following:

Density is expressed as:

$D = Ni / Ss$

with *Ni*: number of stems counted; *Ss*: area surveyed.

Basal area is $G = \sum (\pi D^2/4)$

with *D*: stem diameter and $\pi = 3.14$.

The average annual increment of reconstituted bark is:

$AAMEr = EMCe / t$

where *EMCe*: average thickness on logged side; *t*: time between logging and collection date.

The rate of reconstitution in thickness of the exploited bark is:

$TREee = EMCe / EMCne$

with *EMCne*: average of the thicknesses on the non-exploited side.

The evolution of the average annual increase in thickness of the unharvested side over time was assessed from the ratio of the

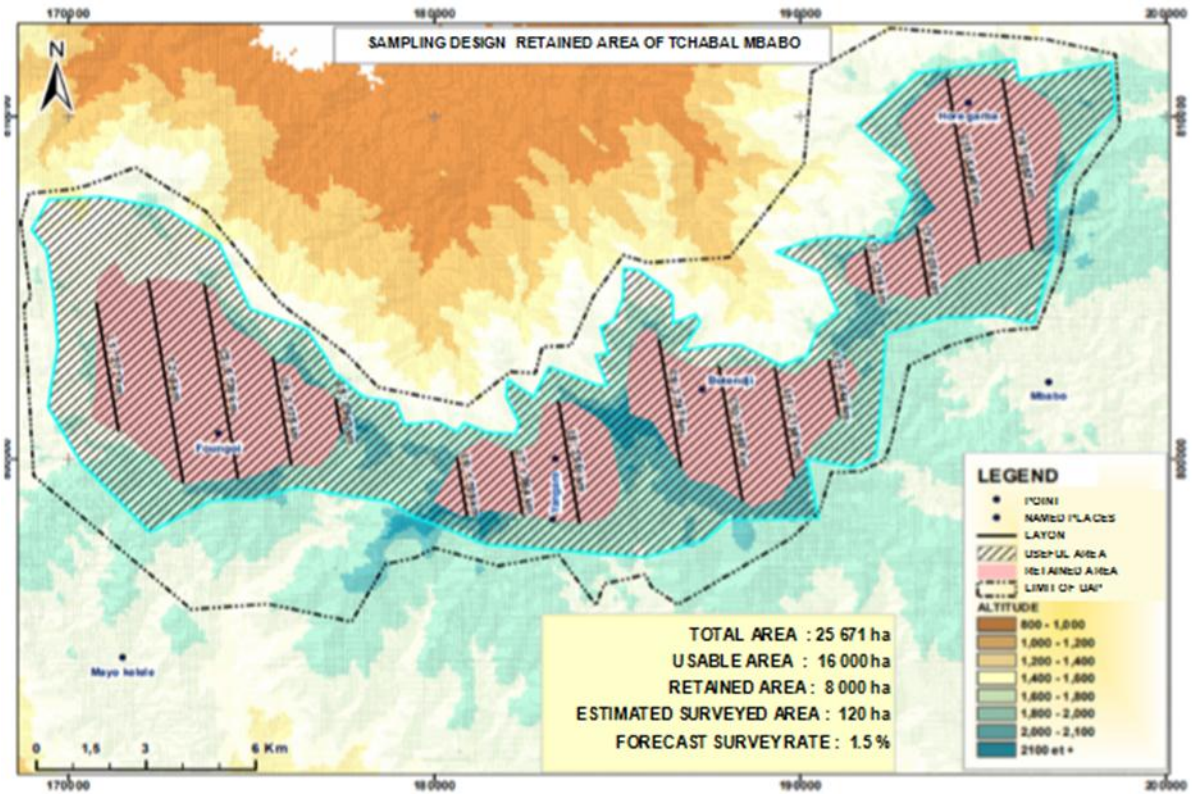

Figure 2. Sampling design.
Source: Authors

thickness of the unharvested side to the diameter. *RCneD = EMCne / MDhp* with *MDhp*: mean diameter at breast height. The Ho assumption here is that the average annual increase in thickness of the unharvested bark increases with diameter over the same period of time. The formula for calculating quotas is: $Qa = De \times R \times Su / T$ where *Qa*: Annual harvesting quota; *De*: Density of harvestable stems; *R*: Productivity or yield in kilograms of dry stem bark from a stem harvested using the 2/4-opposite technique; *Su*: Useful area; *T*: Demi-rotation. The average productivity of a stem (*R*) is as determined by Betti and Ambara (2013); it is equal to 30 kg of dried bark for the 2/4 opposite sides harvesting technic. For carbon stock assessment, the allometric equation developed by Chave et al. (2014) was used for epigenetic biomass estimation: $AGB = exp[-1.803-0.976E+0.976ln(\varphi) + 2.673ln(D)-0.0299$ (ln(D)2] with *AGB*: estimated epigenetic biomass in kg; *E*: environmental index that depends on the geographical coordinates of each plot; φ: the specific gravity of the wood in g/cm^3; *D*: diameter at breast height in cm. Thus, the carbon stock (in t C / ha) is deduced from the biomass according to the following formula: $(AGBx0.47)/1000$ (Zapfack et al., 2013); then it will be converted into ton of carbon per hectare. The graphs and curves were made with Excel 2013, the statistical analyses used are the chi^2 test of independence, one-factor ANOVA, and simple linear regressions using R software version 3.3.2.

RESULTS

Sampling effort

The inventory conducted required 3 production teams. A production team is the one which combines both lining and counting. Each team is composed of six members including 1 GPS operator, 2 botanists, 2 macheters, and one secretary. The three teams counted *P. africana* trees on 23 km for an average sampling effort of 1.9 ≈ 2 km per day per team (Table 1). Twenty-four forest galleries were inventoried by the teams in the four selected villages. A total of 115 plots were delimitated and inventoried, representing a sampling area of 57.5 ha and a sampling rate of 0.72%. The observation made is that the final sampling rate is somewhat different from the theoretical sampling rate designed in advance. Table 2 gives the overall results from the implementation of the sampling design**.**

Structural features

Stem density

Table 3 presents the densities of *P. africana* stems counted by locality/village. The average density of all villages is 6.23 stems/ha. The density of harvestable stems is 4.8 stems/ha. The sites with the highest density of harvestable stems were Hore Garba (6.93 stems/ha), Foungoi (5.16) and Yangaré (4.61). The total basal area of the Mbabo forest is 57.35 m^2/ha. The most covered

Table 1. Work effort for the inventory conducted in Tchabal Mbabo

Team	Employees	Plots	Length (km)	Period (j)	Work effort (km/j)
1	6	27	5.4	4	1.4
2	6	52	10.4	5	2.1
3	6	36	7.2	3	2.4
Total	**18**	**115**	**23**	**12**	**1.9**

Source: Authors

Table 2. Characteristics of the inventory conducted in Tchabal Mbabo.

Locality	Botendji	Foungoi	Horé Garba	Yangaré	Total MF
Total area	3244.74	5925.34	6814	9687.85	25671.95
Surveyed area (ha)	2022.28	3692.96	4246.82	6037.94	16000
Useful area retained (ha)	1011 .14	1846.48	2123.41	3018.97	8000
Estimated area surveyed (ha)	15.17	27.7	31.85	45.28	120
Predicted sampling rate	0.1	0.5	0.2	0.7	1.5
Number of plots	8	38	15	54	115
Area surveyed (ha)	4	19	7.5	27	57.5
Sampling rate	**0.4**	**1.03**	**0.35**	**0.89**	**0.72**
Sampling effort	**3.3**	**15.64**	**6.17**	**22.22**	**47.33**

Source: Authors

Table 3. Density and basal area of *P.africana* stems in different localities of Mbabo.

Locality	Surveyed area (ha)	Diameter of stems < MDE	Diameter of stems ≥ MDE	Total stems	Density of stems diameter < MDE	Density of stems diameter ≥ MDE	Total density	Basal area
Botendji	4	16	10	26	4	2.5	6.5	1.74
Foungoi	19	41	98	139	2.16	5.16	7.32	17.32
Horé Garba	7.5	5	52	57	0.67	6.93	7.6	16.98
Yangaré	27	20	116	136	0.85	4.61	5.04	21.31
Total MF	**57.5**	**82**	**276**	**358**	**1.48**	**4.8**	**6.23**	**57.35**

Source: Author

locality is Yangaré (21.31 m^2/ha) and the least covered is Botendji (1.74). The average basal area per stem is 0.16 m^2/ha. Figure 3 shows that basal area increases with tree size (diameter at breast height) and not with stem density per unit area.

Distribution of P. africana stems by diameter class

The distribution of the 358 stems recorded by diameter class is illustrated in Figure 4. The general shape of the distribution of the species is bell-shaped with a modal class located in the 30-39 cm range, indicating a limited renewal capacity of the species. Note the presence of a few young stems (10-20 cm in diameter) which are represented at 7.8% of the total number. Ninety-one stems have a diameter smaller than the minimum exploitability diameter (MED) which is 30 cm. Those non-harvestable trees represent 25.42%. The average diameter of the stems is 48.17 ± 19.8 cm. The average height of the stems is 7 ± 4.26 m while the average thickness of the bark on the unharvested side is 14.1 ± 4.72 mm. There were significant differences in these three parameters between the four locations with respective probabilities of 5.68E-13, 0.000293 and 0.000224 for diameter, height and bark thickness (Table 4).

Assessment of epigeous carbon stock

The epigeous woody biomass of the Mbabo forest is

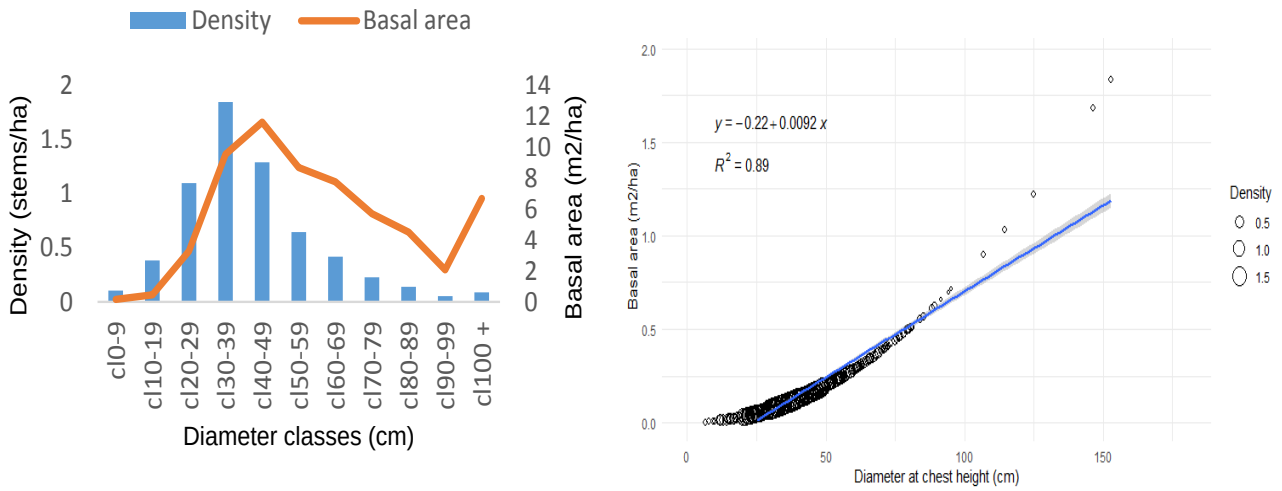

Figure 3. Evolution of basal area per unit area.
Source: Author

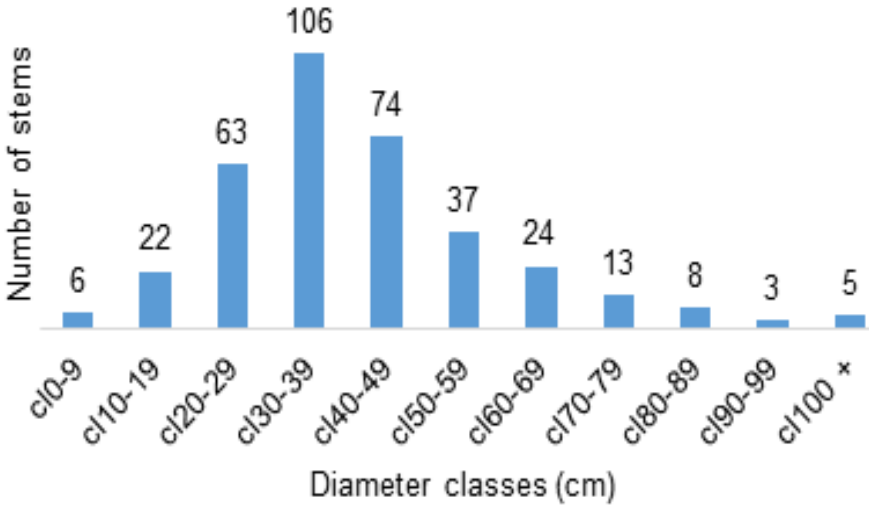

Figure 4. Diameter structure of *Prunus africana* from MF.
Source: Author

Table 4. Average harvestable diameter, average height and average thickness of unharvested bark at Tchabal Mbabo.

Locality	Number of stems	Diameter average (cm)	Height average (m)	Average thickness_not exploited (mm)
Botendji	26	37.3	5.8	15.04
Foungoi	139	43.7	6.2	12.8
Horé Garba	57	60.5	6.7	15.4
Yangaré	136	47.3	8.2	14.9
Average MF	-	48.17	7	14.1
F value	-	21.77	6.444	6.648
Pr (>F)	-	**5.68e-13*** **	**0.000293*** **	**0.000224*** **

Source: Author

Table 5. Sanitary status and mortality rate of MFM.

Sanitary status	Causes	Stems (%)	Rate M (%)
Alive	-	342 (95.53)	-
Dying	Previous exploitation, disease	3 (0.84)	-
Dead	Previous exploitation	4 (1.12)	3.63
	Other reasons	9 (2.51)	-

Source: Author

estimated at 1432.75 kg/ha, for an overall epigeous carbon stock (for stems ≥10 cm) of 67.3 ± 0 .0015t C /ha. This corresponds to an estimated financial value of 1,749,800 FCFA/ha (USD 2 859.54).

Response of *Prunus* trees to harvesting

Harvesting technique

Of the 358 stems counted, 94 have been previously harvested, representing 26.25%. Figure 7 shows that several harvesting techniques have been used, including 1/2 (1 over 2) opposite sides, 1/4 (1 over 4) side, 2/4 (2 over 4) opposite sides, 3/4 (3 over 4) sides and 4/4 (total debarking). The technique known as two quarters (2/4) opposite is the most used (79.65%).

Sanitary state of the stems and mortality rate

The majority of the stems exploited or not is alive with a percentage of 95.53%. The few dead and dying stems represent only 4.47% of the total, i.e. a density of 0.28 stems/ha. Mortality due to exploitation is 1.12%, while mortality induced for all other reasons (natural, bush fire, felling, broken tree) is 2.51%. This results in a global mortality rate of 3.63% (Table 5).

Growth parameters and dynamics

The average annual increment of regenerated bark varies among localities depending on the years of harvesting. The average annual increment is 1.3 mm/year, and the significant difference was noted between the four localities (F value = 3.597; Pr(>F) = 0.0166). The greatest is recorded in Botendji (1.48 mm/year). On the other hand, although Botendji has the highest rate of thickness recovery (70%), there is no general difference between the four localities (F value = 2.365; Pr(>F) = 0.0763) (Table 6). A relationship exists between the thickness of unharvested bark and the diameter at breast high (dhp) (X-squared = 4544.9, df = 4224, p-value = 0.0003206). The average annual increment in unharvested bark thickness is assessed here on the basis of the ratio: bark thickness by diameter. This shows that for a mature stem, the growth (size) of the unharvested bark is 3.7% of that of the diameter for the same growth period (Table 7). Figure 8 illustrates this relationship. It can be deduced that the growth rate of unharvested bark decreases with the growth in diameter during the same period. This is explained by the exponential equation $y = 10.05e^{-0.209x}$ with a correlation coefficient $R^2 = 0.9542$.

Management measures

Distribution of P. africana according to the elevation and distinction of suitability zones

Figure 5 illustrates the distribution of stems by altitude or elevation class. Most of the *P. africana* stems are found in the 1700-2100 m altitude classes, representing 90%. Ten percent are between 1500-1700 m and no stems were found above 2100 m. One significant difference is noted (F value = 11.31; P(>F) = 4.19e-07) between these spatial positions of stems in the 4 surveyed localities. The analysis of the distribution of *P. africana* stems according to their elevation allows to estimate its density by zone including: the low altitude zone (below 1300 m) where the species is almost absent, the altitude zone between 1300 and 1700 m where *P. africana* exists but at low densities, the zone between 1700 and 2100 m considered as the occupation zone per excellence (high densities) and the zone above 2100 m, marked by the absence of *P. africana* but colonized most often by grassy meadows Figure 6 illustrates the spatial distribution of *P. africana* densities in the four villages. The analysis of the distribution of *P. africana* stems by altitude class makes it possible to propose a map of activities to be carried out specifically in relation to *P. africana* (Figure 9) in the four altitude zones identified:

1. Altitude zone below 1400 m (the species is almost absent): no activities related to *P. africana* should be carried out;
2. Zone of altitude between 1400 and 1700 m (the species exists but at low densities): zone with favorable conditions for the development of *P. africana* which could

Table 6. Annual growth and recovery rate of harvested stems at Tchabal Mbabo.

Locality	Year	Period	Stems	Height	Dhp	EMCne	EMCe	AAMEr	TREee
Botendji	2011	10	9	7.22	31.68	21	14.78	1.48	70
Foungoi	2011	10	13	5.47	44.11	13.17	8.62	0.86	65
	2013	8	26	5.87	38.23	18.73	8.88	1.11	47
Average Foungoi	2012	9	39	5.73	40.2	15.95	8.75	0.97	56
Hore garba	2015	6	22	6.73	64.92	16.82	8.45	1.41	50
Yangare	2014	7	24	8.92	48.08	15.79	9.58	1.37	61
Average MF	2013	8	94	7.15	46.22	17.39	10.39	1.3	60
F value				4.359	9.686	3.274	6.486	3.597	2.365
Pr (>F)				0.00649**	1.32e-0.5***	0.0247*	0.000504***	0.0166*	0.0763

EMCne: Average bark thickness on the unharvested side (mm); EMCe: Average bark thickness on the harvested side of harvested stems (mm); AAMEr: Average annual increment of regenerated bark (mm/year); TREee: Thickness recovery rate of logged bark (%).
Source: Author

Table 7. Ratio of unmined bark thickness to diameter in the wild.

Locality	Stems	EMCne	Dhp	RCneD
Botendji	26	15	292.03	5.2
Foungoi	143	12.81	365.88	3.5
Hore garba	57	15.37	574.61	2.7
Yangare	135	14.89	426.29	3.5
Average MF		14.53	414.70	3.7
F value				3.343
Pr (>F)				0.0194*

EMCne: Average bark thickness on the unmined side (mm); Dhp: Diameter at breast height (mm); RCneD: Ratio of unmined bark thickness to Dhp (%).
Source: Author

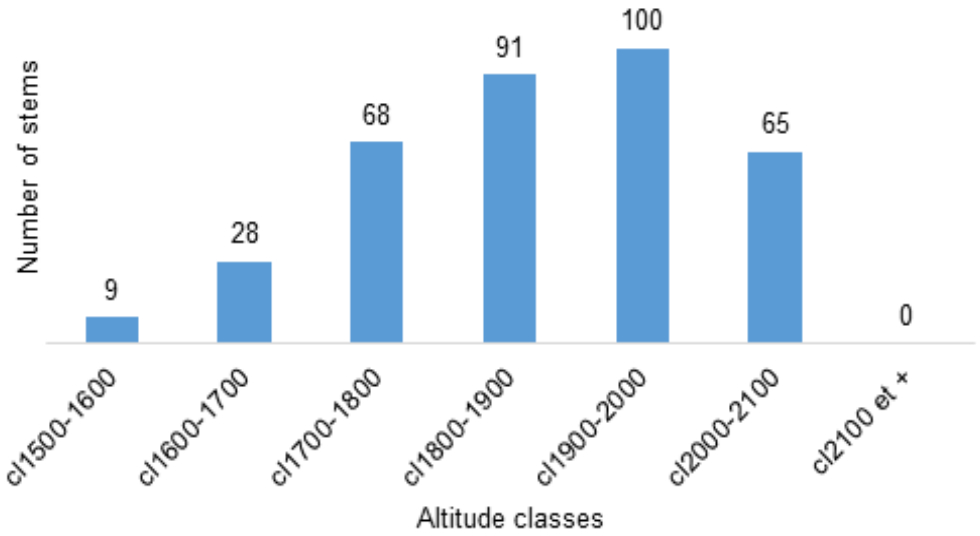

Figure 5. Distribution of *P. africana* stems according to altitude classes.
Source: Author

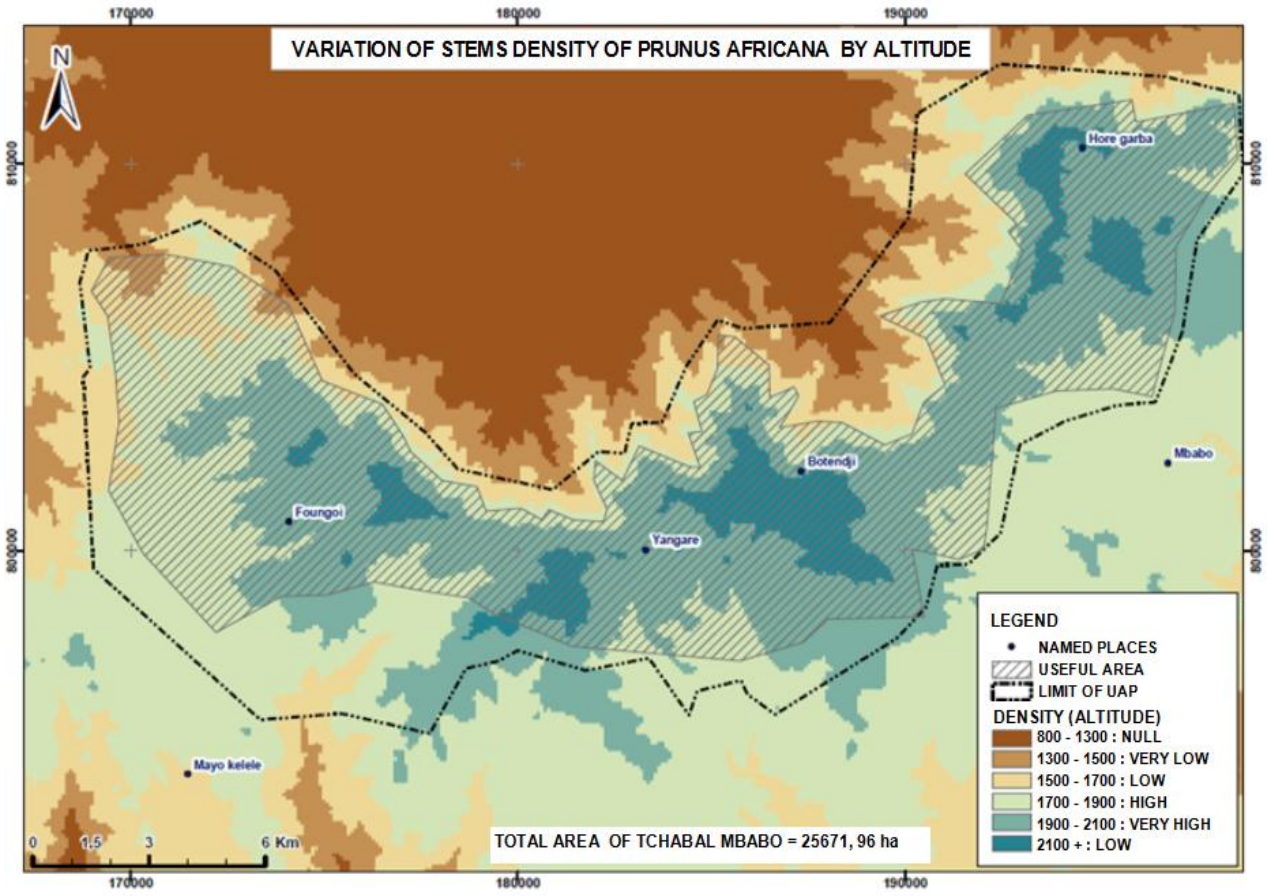

Figure 6. Relative suitability zones of *P. africana* in the MF.
Source: Author

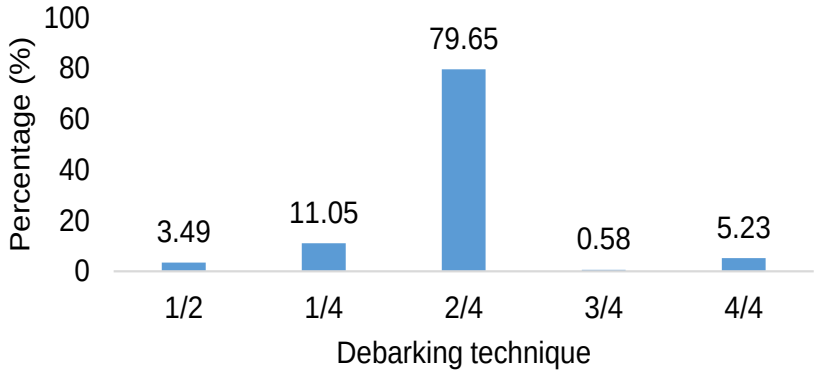

Figure 7. Identified harvesting techniques.
Source: Author

constitute a favorable area for the establishment of *P. africana* plantations (agroforestry). There should be no exploitation of natural populations of *P. africana* in this area;

3. Zone between 1700 and 2100 m (high densities of the species): zone where the bark of *P. africana* should be harvested in the wild and where agroforestry is still possible, but using enrichment practices;

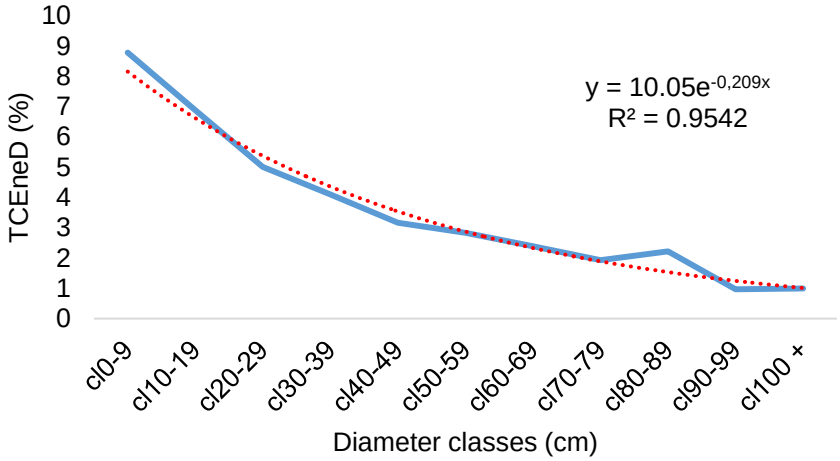

Figure 8. Growth of unharvested bark as a function of diameter
Source: Author

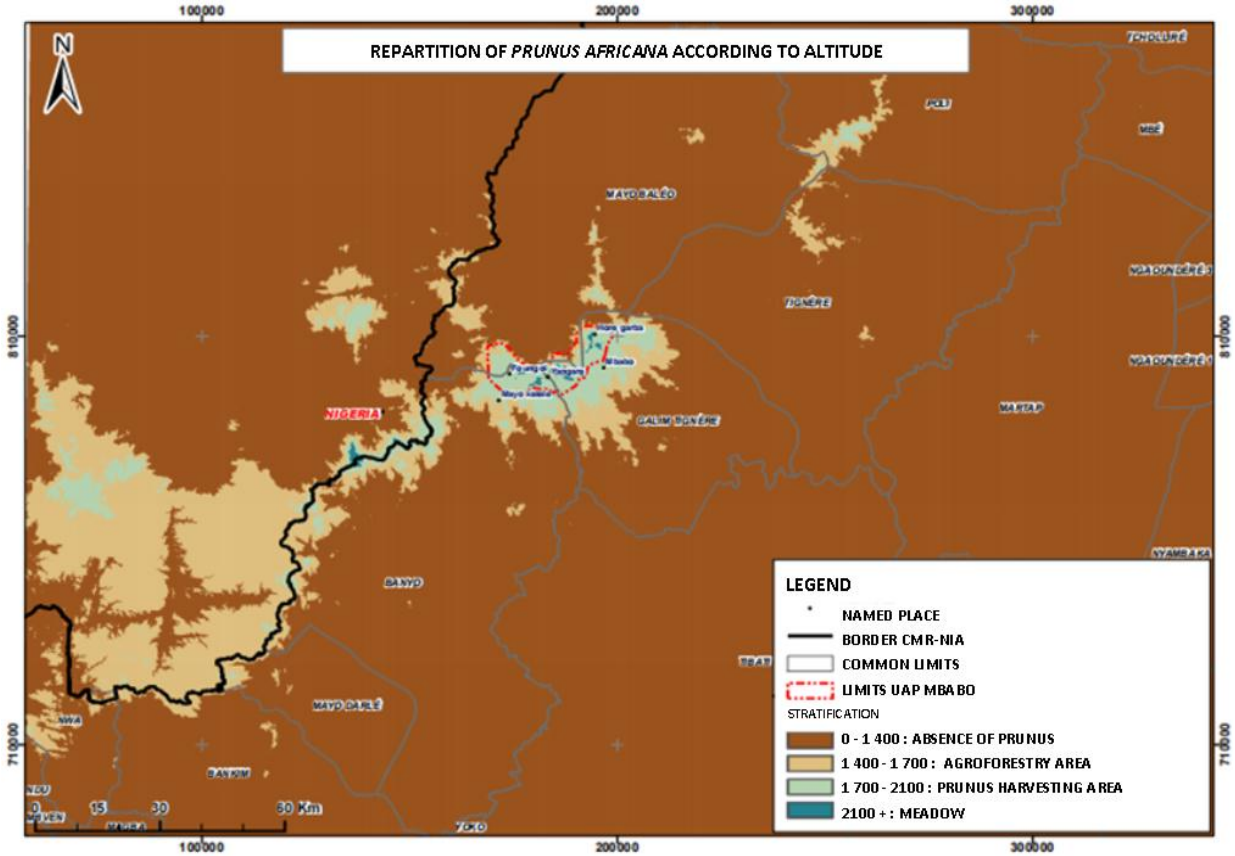

Figure 9. Activities by area based on stem density variations by altitude.
Source: Author

4. Zone of altitude more than 2100 m (almost null or accidental presence of the species): zone colonized most often by the grassy meadows.

Debarking techniques

Several debarking techniques guarantee the regeneration

Table 8. Unharvested bark growth as a function of diameter in the wild.

MF	EMCne	AAMEr	Rotation	Half-rotation
	17.39	1.3	13.39	6.69

EMCne: Average bark thickness on the unharvested side (mm); AAMEr: Average annual increment of regenerated bark.
Source: Author

of the bark after the exploitation. Taking into account the ageing aspect of the stems, the weak regeneration recorded in this massif, for practical reasons on the ground and to facilitate the follow-up of the rotation; two techniques can be proposed to know:

1. Two opposite 1/ 4 or (2/4) for stems between 30 and 70 cm;
2. Four opposite 1/8 or (4/8) for stems over 70 cm

Rotation

The time required for a second harvest is presented in Table 8. It is obtained from the AAMEr (Average Annual Increase in the Regenerated Bark). To harvest bark from the same strip of regenerated bark at Tchabal Mbabo, it would require an average time of 13.39 years or 14 years. This means that to come back on the same tree but on the remaining opposite side, the harvester will need to wait 6.69 ≈ 7 years, representing the half rotation.

Annual possibility or annual quota (case of localities)

The annual exploitable quota in dry tons of *P. africana* bark is presented by explored locality in Table 9. This quota is 164.6 tons for the entire Tchabal Mbabo Forest.

DISCUSSION

Sampling effort

A total of 115 plots were scanned by the inventory teams representing a sampling area of 57.5 ha. The survey rate is 0.72%. The actual sampling data obtained in the field, are somewhat different from the theoretical sampling data designed. This discrepancy is due to the difficulties encountered in the field, the most significant of which are those related to the excessively uneven terrain in some places. Nevertheless, the sampling rate obtained, which is not too far from the 1% proposed by the forest inventory standard (MINFOF, 2019) is still very high, at least time 6 higher compared to similar inventory campaigns conducted in previous years in the same area including the sampling rate of 0.1% realized in 2004 by ONADEF (2005), 0.12% in 2011 by ANAFOR under the ITTO-CITES Program (Akoa et al., 2011b), and 0.04% in 2021 by TRAFFIC International (Hiol Hiol, 2021). At Lume (North Kivu) in the Democratic Republic of Congo, *P. africana* inventories were carried out at a sampling rate of 1.2

Structural features

The average density of all stems is 6.23 stems/ha. This density is lower than 7.94 stems/ha found in Mbabo (Akoa et al., 2011b), 7.18 found in Bioko Island/Equatorial Guinea (Navarro-Cerrillo et al., 2008; Muñoz et al., 2006) and 7.2 stems/ha in Ethiopia (Chaffey, 1978). However, our density is higher than the 3.43 stems/ha found in Mount Cameroon (Betti et al., 2011). On the other hand, the exploitable stem density is 4.8 stems/ha, which is much higher than 1.79 stems/ha and 2.68 stems/ha obtained respectively at Tchabal Ngandaba and Tchabal Mbabo (Akoa et al., 2011b). This shows that the growth of future stems inventoried in 2011 is effective. The low density of young stems shows that Tchabal Mbabo suffers from regeneration problems. The *Prunus* basal area is 57.35 m^2/ha. The average basal area for a stem is 0.16 m^2/ha, which is lower than the 0.94 m^2/ha obtained in the Bioko Island (Navarro-Cerrillo et al., 2008). Basal area increases with diameter at breast height, not with stem density. Betti et al. (2021) found for *Pericopsis elata* that the stand basal area increases with both tree density and diameter at breast high. Contrary to Yankam, (2013) in Mount Cameroon and Ronoh et al. (2018) in South west Mau Forest (Kenya) found an inverted "j" structure, the distribution of the 358 stems surveyed is bell-shaped. This clearly shows that *P. africana* suffers from some regeneration problems in Mbabo. *P. africana* is a light demanding tree species. For this reason, the number of small stems is often low in primary or non-perturbed forest, due to the lack of light (Durieu de Madron, Forni and Meok, 1998), which tends to suggest that forest logging can be a tool for sustaining ligh demanding tree species. The very low presence of young stems (10-20 cm in diameter) at 7.8% could be due to the death of shoots by competition linked to the absence of light, or to bush fires. In Gabon, Bayol and Borie (2004) present the bell-shaped as the dominant structure of highland forests. Damage caused to the regeneration of stands can constitute a risk for the future of the sustainability of stands (Ellis and Putz, 2019) because the future of forests depends on adequate and safe natural regeneration (Cruz et al., 2021). These regeneration problems can also be explained by its low rate of propagation by its seeds due to its long flowering cycle and its recalcitrant seeds (Komakech et al., 2020). In Mbabo, the average stem diameter is 48.17 ± 19.8 cm and the average height is 7 ± 4.26 m. These are respectively lower than the 101.17 cm and 23.87 m

Table 9. Distribution of annual quota in the different localities explored.

Localities	Useful area (ha)	Useful area of Mbabo (ha)	Density of stems diameter≥ MDE	Average productivity of a stem in dry matter weight (Kg)	Half-rotation	Annual quota (Tons)
Botendji	1011.14		2.5	30	7	
Foungoi	1846.48		5.3	30	7	
Horé Garba	2123.41		6.9	30	7	
Yangaré	3018.97		4.4	30	7	
Total/Mean	**8000**	**16000**	**4.8**	**30**	**7**	**164.6**

Source: Author

obtained in Kenya in the South Nandi forest (Koros et al., 2016). Betti and Ambara (2013) obtain an average diameter of 68.07 cm in Mbabo. In Equatorial Guinea (Pico de Basilé and Moca) stems reach 24 m in height (Muñoz et al., 2006). When the trees reached 50 cm in diameter, they reached their maximum height (Namuene and Egbe, 2022). In three Mugaga plantations in Kenya (Nyamai et al., 2015) obtained an average diameter of 34.73 ± 13.51 cm for stem 8.36 ± 1.22 m in height.

In Mbabo, the average thickness of the bark on the unharvested side is 14.1 ± 4.72 mm. This value is high than the 12.03 mm found by Lekefack (2016) in Mount Cameroon. The high value of the thickness of the bark obtained in Mbabo may be link to the presence of frequent bush fires in the area compared to North west and South west regions. Shafer et al. (2015) demonstrated that large bark thicknesses are correlated with the time necessary for Cambium to reach dead temperatures and, thus, constituted a strong predictor of resistance from the rod to the bush fire.

Response of trees to harvesting

In Mbabo, 95.53% of the stems, whether harvested or not, are alive, showing a mortality rate of 3.63%. Stewart (2001) estimated the annual mortality of adult trees in *P. africana* natural populations at 1.5% in Mount Oku, North west Cameroon. The bush fires is one of the main causes of mortality *of P. africana* stems in Mbabo area. The exploitation of tree bark very often has a very high impact on biodiversity because an intense exploitation of bark exposes the sapwood to the attacks of pests. This weakens the biological functioning of the tree and can cause their death and this, especially in the rainy season (Bayoi et al., 2021). Across the Mbabo forest, the average annual increment in regenerated bark thickness is 1.3 mm/year, with a significant difference between the 4 localities (F value = 3.597; Pr(>F) = 0.0166). This increment is much higher than the 0.61 mm/year in Kumbo plantations (Betti et al., 2019). However, it is smaller than the 1.85 mm/year found at Mount Cameroon (Lekefack, 2016) and also the 2.15 mm/yr obtained from Fundong plantations (Betti et al., 2019). Interestingly, tree size has an effect on bark recovery rate for *P. africana* (Momo et al., 2016). Nkeng et al. (2010) found that for the first two years after debarking, increment is 0.15 cm/year and this decreases over time at 0.06 cm/year. Solefack and Kinjouo (2017) show in their study in the South west Cameroon that, the bark levy causes a sharp reduction or a slowdown in the growth in thickness of the rod for the benefit of the renewal of the bark surface taken. The reconstitution rate of regenerated bark in thickness is 60%. This rate of over 50% shows that the species has a great capacity for regeneration. However, this regeneration can be influenced by the harvesting period, the spatial position (altitude) or by the diameter of the tree. According to Momo et al., (2016), the rate of bark recovery is influenced by several factors: season and intensity of landing, tree size and altitude. In the montane southern Cape forests of South Africa, the differences in the regeneration process observed at the levels of species and individuals are associated with the intensity of tissue damage and the cutting season (Beltrán et al., 2021). The correlation between unharvested thickness and diameter shows that the growth rate of unharvested bark is 3.7% of diameter growth. The growth rate of the bark decreases over the years with the growth of the diameter of the tree. The younger is the tree, the more important is the growth rate. Betti et al. (2019) found that the thickness of the bark increases with the diameter of the tree beyond a certain level before it starts decreasing. The average bark thickness varies from one diameter class to another. Changes in diameter classes result in variations in bark thickness of *P. africana* (Tadjuidje, 2011). Light also could play an important role in this report. It is an environmental factor that influences plant growth because it is a crucial requirement for photosynthesis (Nyamai et al., 2015).

Management measures

Four major zones of variable Prunus density can be

identified between 1400 and 2100 m. It is practically in this altitude interval (1400 to 2500 m) that the stems are found in Equatorial Guinea (Navarro-Cerrillo et al., 2008). In Tchabal Mbabo, 90% of *P. africana* stems are found at altitudes of 1700 to 2100 m (very high density). This can be explained by the fact that it is in this interval where the majority of forest galleries are present, which favors a low temperature climate (favorable environment for the species). In the same line, Awono et al. (2015) stated that the distribution is significantly influenced by altitude, temperatures, rainfall and cloud cover. For the area below 1300 m the temperature is higher, thus not favoring the development of the species. Given these regeneration problems and its low density, it would be interested in turning to the agroforestry or plantations. A study at the center of the Uganda noted that 73% of the population (farmers) around the forests received and granted a favorable opinion to the domestication of the species (Galabuzi et al., 2021). Five harvesting techniques are identified: 1/2, 1/4, 2/4, 3/4 and 4/4. The technique known as two quarters (2/4) opposite sides is the most used (67.8%). This result is close to that of Betti et al. (2019) where six harvesting methods were recorded in northwestern agrosystems. In both of these cases, the 2/4 opposite technique was also the most used. Wete (2022) noted that two legal techniques including 2/4 and 4/8, and two illegal techniques 3/6 and total debarking were used in the Mount Cameroon production site. The so-called legal techniques resulted in the death of 4.12% of stems and the decline of 20.58% of stems. In the North-west Region, three harvesting techniques were identified in community forests and six techniques in agroecosystems. This observation of several harvesting techniques may be justified by the incompetence of the harvesters, or by their desire to increase the amount of the bark harvested in order to earn more money. However, precious efforts have been made to establish good standardization, as well as specific guidelines for good practices of harvesting medicinal plants (Pandey and Das, 2013). Betti et al. (2016) reveal during a socioeconomic study in Mount Cameroon and the state of the exploitation measures of *Prunus africana* in the South west that operators do not regularly apply the recommended methods because of the very damaged relief for some and the price per kilometer very low for others. Geudje et al. (2016) evaluated the capacity of *Garcinia kola* trees to regenerate their bark after the harvest. A debarking species can survive if it can overcome the trauma of the targeting and develop new tissues (hardening and thickening of the epidermis) which allow it to ensure its survival (Solefack and Kinjouo, 2017). The rate of stand recovery is influenced by the natural diametric growth rate of the tree. During a half rotation there are stand dynamics, including stem recruitment and bark growth. If stem recruitment is not high, this will have a negative impact on the harvestable quota for the new half rotation. Wete (2022) obtained an annual diameter increment for *P. africana* of 0.42 cm/year in Mount Cameroon. For plantations in the Northwest the increment is 0.91 cm/year (Betti et al., 2019). Bark regrowth is more reliable and accurate in some areas with favorable climatic conditions than others (Cunningham et al., 2016). However, aside from environmental conditions, poor harvesting practice could increase mortality rate or diameter growths, which are also key factors in recovery/recruitment. Damage to young trees has a long -term impact on the regeneration process (Danilović et al., 2015), and diameter growth can be reduced by 10 to 20 % due to surface injuries (Yilmaz and Akay, 2008). Using the productivity of a stem which is 30 kg (Betti and Ambara, 2013), the annual exploitable quota of dry matter of *P. africana* bark is 164.6 tons for the 8 000 ha of useful forest delimitated in the four villages inventoried. This represents about 0.021 tones/ha. This quota could be important if the useful area was even greater. But Bodeker et al., (2014 notes a great decrease in the geographic distribution in sub -Saharan Africa of this species of canopy caused by farming, slash-and-burn agriculture and the construction of habitats. This is also the case with Tchabal Mbabo. All the same, this quota is higher, times 8 than the 473.245 tons obtained on 120 994,08 ha for a half rotation of 5.5 years in Mbabo in 2011 by ANAFOR (Akoa et al., 2011b) and which represents 0.003 tons/ha for the same half rotation (7 years). This could be explained by the fact that our inventory focused on sites indicated by local people as production sites of *P. africana.*

Conclusion

Forest inventories conducted in the Mbabo forest reveal that *P. africana* has a good stem density (6.23 stems/ha) with a basal area of 57.35 m^2/ha. The average bark thicknesses are 14.1 ± 4.72 mm and 10.39 ± 4.22 mm for the unharvested and harvested sides respectively. The annual bark growth is 1.3 cm/year for a recovery rate of 60%. The surveys and forest inventories conducted have made it possible to estimate a first annual exploitation quota for *P. africana* of 164.6 tons/year in Tchabal Mbabo for a half rotation of 7 years. This quota is to be harvested from a useful area of 8000 ha between the altitudes of 1700 and 2100 m. This massif suffers from regeneration because young trees are not very abundant. Agroforestry would be beneficial for this forest in areas where the species is present but at low density to increase its potential, at the altitude 1 300-1 700 m to be precise.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

AKNOWLEDGEMENTS

This work was carried out within the frame of the project

"projet de plan d'action et d'actualisation de l'avis de commerce non prejudiciable en vue de la gestion durable de Prunus Africana (Rosaceae), espece d'arbrelistee en annexe ll de la CITES au Cameroun" of the CITES Endangered Tree Species Program (CTSP) coordinated by the CITES Secretariat General and funded by the European Union Commision. The University of Douala, local communities, AFRIMED and SGP leaders for allowing them use their facilities, for their active participation, advices and cooperation.

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Genetic diversity in pepper (*Capsicum annum L.*) germplasms using SSR markers

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Analysis of pepper genetic diversity and genetic relationship is important in selecting genetically diverse parental lines drawn from several genetic populations, and also helps to implement effective conservation strategies. For this purpose, 25 pepper genotypes comprising both accessions and improved varieties were examined using 16 SSR markers. The markers were polymorphic and showed a mean PIC value of 52% with a range of 8 to 80%, and generated a total of 67 alleles, with an average of 4.19 alleles per marker. The gene diversity ranged from 0.09 to 0.82, with an average of 0.57. Interestingly, pairwise genetic dissimilarity was the highest (1.00) between PBC-731 and Acc-22, and the lowest (0.25) between Acc-13 and Acc-11 genotypes. This is expected because improved varieties are genetically far from accessions than accessions are from each other. Neighbor-joining (NJ) tree produced three major clusters consisting of C1=100% accessions, C2= 67% improved varieties, and 33% accessions, whereas C3= 50% accessions and 50% improved varieties. The principal coordinate analysis (PCoA), showed a scatter plot with a wide dispersion of the genotypes in all the quadrants without forming a clear cluster, and some genotypes like PBC-731, Acc-45, Acc-9, and Acc-22 are plotted far from the central axis. The population structure generated an optimal groups of ΔK=4 with a high level of admixtures. The analysis of molecular variance (AMOVA) both based on STRUCTURE results and grouping into the accessions and improved varieties partitioned the total variance into 9% among groups, and 91% among individuals in the groups. The high level of genetic diversity found in Ethiopian pepper genotypes in the present study will help breeders to utilize the genotypes for further improvements in pepper germplasm.

Key words: *Capsicum annum*, genetic diversity, hybridization, pepper, SSR markers.

INTRODUCTION

Pepper (*Capsicum annum* L.) is one of the species from the Solanaceae family and genus Capsicum. *Capsicum* comprises around 38 recognized species believed to have originated in the tropical South American Regions,

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of which only five are domesticated and cultivated, namely *C. annum*, *C. baccatum*, *C. chinense*, C. *frutescens* and *C. pubescent* (Bosland et al., 2012; Moscone et al., 2007). *C. annum* is a self-pollinating diploid crop having a varied chromosome number of pungent type (2n=2x=24 and non-pungent (2n=2x=26) with comparatively large genome size (Moscone et al., 2007; Kim et al., 2014). The Pungent (chilli or hot pepper) and non-pungent (sweet pepper) variants of *Capsicum annuum* L. are the most popular vegetable and spices, with worldwide commercial distribution. Pepper is one of the ancient crops which has been domesticated for thousands of years contributing great importance to human welfare (Bosland et al., 2012).

In Ethiopia, it was first introduced by the Portuguese in the 17th century and subsequently from all over the world and it has since been cultivated for centuries and adapted to varied agro-ecological regions in the country (Geleta et al., 2005). The complex geographic environment and climatic conditions helped abundant germplasms of pepper to be evolved with different features, such as fruit type, pungency, and pests and disease resistance (Marame et al., 2009). For instance, there are reports on which Ethiopian origin small-fruited and pungent *C. annum* to be the most important and persistent source of powdery mildew (*Leveillula taurica*) (Jo et al., 2017) and wilt disease resistance (Woubit et al., 2021). Pepper in Ethiopia is grown in different agro-ecologies at altitudinal range from 1400 to 2120 m under rained and irrigated conditions mainly in South Nation Nationalities and Peoples, central (Eastern and Southern Shoa), western, north-western (Wellega, Gojjam) (Nigussie and Zewdia, 2021). According to the FAOSTAT report, Ethiopia produces 4511 metric tons regarded as one of the top five pepper (hot Pepper) producing country in Africa (FAOSTAT, 2016). The total estimated area covered under green pepper and red pepper is 11,409 and 174,463.62 ha, respectively, which is about 73% of the total vegetable production of the country (CSA, 2019), contributing an important role in the national economy. Pepper is a popular vegetable and spice crop in Ethiopia, and it is consumed in different forms. It is widely used in the Ethiopian diet, mainly used in traditional foods known locally as “Karia’’ the green fruit, eaten raw as a salad and dried red fruit grounded into powder, named ‘‘berbere” added as a sauce to “wot”. Pepper consumption is strongly ingrained in Ethiopian dietary habits, with an average daily consumption of 15 g by Ethiopian adults, which is higher than eating of other vegetable crops (Woubit et al., 2021). It is an excellent source for bioactive compounds, vitamins, dietary fibers and some essential minerals (Bosland et al., 2012). In addition, pepper has a wide variety of uses in pharmaceuticals, cosmetics, natural coloring agents, and as an ornamental plant. Despite its wide range of possible applications, Ethiopia's average pepper yield is low as compared to the global scenario (CSA, 2019). The scarcity of appropriate high-yielding varieties, the use of unknown seed sources and low-quality seeds, a poor irrigation system, insufficient knowledge about soil fertility, and the prevalence of fungal, bacterial, and viral diseases are some of the yield-limiting factors (Belay et al., 2019). As a result, pepper breeding goals are focused on overcoming those constraints in order to enhance national production and productivity. In Ethiopia, promising efforts are made to develop improved varieties, and some enhanced cultivars are under production (MANR, 2016).

Crop improvement heavily relies on a comprehensive understanding of the genetic variability and their genetic relationships, which could then be used in breeding programs. The information on genetic relationships has been used to estimate the genetic distance between the genotypes/species evaluated, allowing the species to be classified into distinct groups based on their genetic similarity (Ve and Palloix, 2013). This in turn necessitates the selection of genetically diverse parental lines drawn from several genetic populations and the varietal identification and purity test (Shapturenko et al., 2014). Whenever divergent parents are used in crossing programs, the progenies are expected to have a lot of heterosis (Jagosz, 2011) and increase the chance of obtaining superior segregants in advanced generations, and is important to enhance the genetic base. In addition, knowing the genetic resources of crops is crucial for implementing effective conservation strategies (Gollin, 2020).

The use of molecular markers for plant genetic diversity analysis is thought to be an appropriate tools (Collard and Mackill, 2008), because they are independent of environmental factors and can detect differences in alleles or changes in DNA sequence. Various molecular marker systems such as RAPD (Bhadragoudar and Patil, 2011; Devi et al., 2018), AFLP (Geleta et al., 2005; Aktas et al., 2009), ISSR (Patel et al., 2011; Alayachew et al., 2017; López Castilla et al., 2019) and SSR (Nagy et al., 2007; Dhaliwal et al., 2014; Christov et al., 2021; Woubit et al., 2021), have been utilized to examine the genetic diversity and phylogenetic relationships of pepper germplasms. The use of polymorphic, multi-allelic, reproducible, and widely distributed microsatellite markers in pepper accessions could assist in the selection of traits of interest and potential breeding materials for introgression through the use of molecular marker-assisted breeding and germplasm conservation (Mimura et al., 2012). Therefore, a more accurate analysis employing co-dominant microsatellite (SSR) markers is required to determine the genetic diversity and to infer the genetic relationship of Ethiopian peppers. To date, SSR markers have only been used in a few studies in Ethiopian pepper germplasms to assess the genetic diversity (Rabuma et al., 2020; Woubit et al., 2021) and the improved pepper cultivars have not been studied using SSR markers. Thus, the study aims are to identify and characterize *Capsicum* spp., as well as to capture

Table 1. Lists of pepper genotypes used for the study.

S/N	Accession number and/ genotype names	Local name	Area collected	Source
1	Acc-3	Mi-Alaba2	Kuleto (Halaba)	Halaba market
2	Acc-9	Mi-Alaba1	Halaba- (Adama)	Adama market
3	Acc-10	Ha-Bedessa	Bedessa (W/Hararghe)	W/Hararghe market
4	Acc-11	Tad-Halaba	Ansia (Halaba)	Halaba market
5	Acc-12	Tad-Ybale (Agarfa)	Agarfa (Bale)	Bale market
6	Acc-13	Wfo-Gojam	Finote-Selam (Gojam)	Gojam market
7	Acc-17	Har-Milkay	Mechara(W/Hararghe)	W/Hararghe market
8	Acc-21	Marko-Kumo Almin8	Marko (Gurage)	Hawasa Research
9	Acc-22	Marko-Didamidore9	Marko (Gurage)	Hawasa Research
10	Acc-24	Awa-Dalle1	Awassa-Dalle2	Hawasa Research
11	Acc-26	Awa-Gello2-	Gello-Argessa	Hawasa Research
12	Acc-40	Turu-11	Bati-Futo	Farmers seed lot
13	Melka Awaze	Improved variety	-	MARC
14	Melka shola	Improved variety	-	MARC
15	Melka Oli	Improved variety	-	MARC
16	PBC 602	Improved variety	-	MARC
17	Mareko Fana	Improved variety	-	MARC
18	PBC 731	Improved variety	-	MARC
19	Melka Zala	Improved variety	-	MARC
20	Melk Dhera	Improved variety	-	MARC
21	Melka Eshete	Improved variety	-	MARC
22	Melka Shote	Improved variety	-	MARC
23	Acc-41	Walga-2	Abishege	Farmers seed lot
24	Acc-8	Na-Ybale	Bale2 (Adama)	Adama market
25	Acc-45	Assossa-2	Benishangule	Assossa Research

Source: Aklilu et al. (2016) and Melkassa Agricultural Research Center (MARC).

the potential genetic divergence and genetic relationships among pepper accessions along with the improved varieties.

MATERIALS AND METHODS

Plant materials

A total of twenty-five (25) pepper genotypes (*Capsicum annum L.*) were used for this study, which comprises of fifteen (15) accessions and ten (10) improved varieties (Table 1). Seed samples were obtained from Melkassa Agricultural Research Center (MARC).

Genotyping

Seeds from each genotype were sown in seedling raising tray in the greenhouse at National Agricultural Biotechnology Research Center (NABRC), Holeta. Young healthy leaves from a single seedling at the 2 to 3 leaf stage were collected in an Eppendorf tube and immediately dried using liquid nitrogen. The dried leaves were then pulverized using a Geno grinder (MM-200, Retsch) at 25 rpm for 3 min. Genomic DNA was extracted following plant DNA extraction protocol (DARTs, 2000) with minor modifications. The quality and quantity of the isolated DNA were checked by gel-electrophoresis using 0.8% agarose at 100 constant voltages for 45 min. The gels were visualized under UV light and photographed with a camera mounted on the UV Transilluminator. The quality and concentration of the DNA were further confirmed by a spectrophotometer (8 pedestal, Nano drop) at 260/280 nm wavelength absorbance. Good-quality DNA from each sample was used for PCR analysis after normalization to approximately 50 ng/µl (the normalization was carried out based on the concentration of each sample from Nano drop result).

A set of 16 SSR markers previously reported by Nagy et al. (2007), Dhaliwal et al. (2014) and Sharmin et al. (2018) were obtained and used for the final genotyping of pepper collections (Table 2). Prior to whole sample amplification, gradient PCR was applied to each primer pair on BIO-RAD T100^{TM} thermal cycler to get an optimum annealing temperature and other PCR setup. The polymerase chain reaction (PCR) was then carried out in a 12.5 µl final reaction volume containing 6.25 µl of Taq DNA polymerase, 0.5 µl of each forward and reverse primers, 3.25 µl of nuclease-free water, and finally 2 µl of gDNA. The PCR condition was adjusted at initial denaturation of 95°C for 5 min, 36 cycles of denaturation 94°C for 45 s, annealing varied with the primers (Table 2) for 45 s, extension 72°C for 90 s, and final extension 72°C for 10 min.

The amplified products stained with 6X loading dye-containing gel red, were separated by 3% agarose gel electrophoresis with 1×TAE buffer at 100v constant voltage run for 2:30 hrs. A 100 and 50 bp DNA ladder (SMOBIO, DM2100 and DM1100) was used to estimate the molecular weight of the fragments. The gels

Table 2. Lists of SSR markers used for the study and their detail information.

S/N	Markers	Forward sequence (5'-3')	Reverse sequence (5'-3')	Expected size (in bp)	Ta (^{o}C)	Reference
1	*AVRDC PP-18*	GCTAGGCTTGATCCTTCACC	CGCTTGAAATCATGCTCACT	83-113*	47.9	Dhaliwal et al. (2014)
2	*AVRDC PP-32*	ATGGAGGATTACCTCGCAAC	CATGATGACCATCCATCCAT	102-177*	46	>>
3	*AVRDC PP-65*	GTGAGGCCGAGAATGAAGAT	AACGACCATGTGTGGTTGA	425-562*	48.2	>>
4	*AVRDC PP-167*	TCATCTTACACGGCTTGCTC	AGCTCCTCAACTGCCTTTTA	254-341*	55.7	>>
5	*AVRDC PP-67*	TATTCCTTCTTCACCCCTCC	GAAAGAGGCGCTAACTGGAC	153-310*	55.5	>>
6	*CAMS-806*	GCTAGGCTTGATCCTTCACC	CGCTTGAAATCATGCTCACT	170-210	54.3	Sharmin et al. (2018)
7	*GPMS8*	TGATGATAAGGCCATGATAAAATG	CCAGATTCTTTAGCAAGGTTTACC	159-229	54.3	Nagy et al. (2007)
8	*GPMS6*	CAGAGCACTTGACATGCCTT	GATCTTTATAGTAGCTCATCAATA	122-172	52.5	>>
9	*GPMS112*	TCCCTCAGCAGCAACAATTT	GTCGGGCTCTTTGATTGTGT	203-280	54.3	>>
10	*GPMS117*	GATGTTAGGTCCGTGCTTCG	AAGCCCCATGGAAGTTATCC	111-177	53.2	>>
11	*GPMS178*	GATTTTTGACATGTCACATTCATG	AACGTTGAAAAATAAAGTAAGCAAG	230-261	58.2	>>
12	*GPMS197*	GCAGAGAAAATAAAATTCTCGG	CAATGGAAATTTCATCGACG	272-344	54.2	>>
13	*EPMS303*	AAAACTCCAAACTACCCCTGG	TTAAGCGTAGCGCTTGTGTG	292-330	53.2	>>
14	*EPMS331*	AACCCAATCCCCTTATCCAC	GCATTAGCAGAAGCCATTTG	97-107	53.2	>>
15	*EPMS376*	ACCCACCTTCATCAACAACC	ATTTGTGGCTTTTCGAAACG	235-259	53.2	>>
16	*EPMS418*	ATCTTCTTCTCATTTCTCCCTTC	TGCTCAGCATTAACGACGTC	178-210	54.8	>>

*= observed fragment size in the present study; the others were reported in previous studies.
Source: Author

were visualized under UV light and image capture was done by a gel documentation system (UV Transilluminator).

Data scoring and analysis

The fragment sizes detected by each SSR region were scored using PyElph 1.4 software package (Pavel and Vasile, 2012) with respect to the size marker. For a single locus, fragments with the same mobility were treated as the same fragment size and treated as the same allele, while bands of differing molecular weight were treated as distinct alleles. To determine gene diversity (GD), observed heterozygosity (Ho), the number of alleles (Na), and polymorphic information content (PIC) in each marker, Power marker v3.25 software (Liu and Muse, 2005) was used. The PIC value for each primer was estimated using the formula:

$$PIC = \frac{1 - \sum Pij^2}{j=1}$$

Where Pij is the frequency of j^{th} allele in the i^{th} primer and summation extends over 'n' patterns. The genetic relationships within and among pepper genotypes, simple matching pairwise dissimilarity across each genotype, and a biplot display of principal coordinate analysis (PCoA) were estimated using DARwin ver 6.0.21 software (Perrier and Jacquemoud-Collet, 2006). The pairwise dissimilarity was calculated based on the following formula:

$$dij=1-1/L\sum_{i=1}^{L} ml/\pi$$

Where, dij=dissimilarity between units i and j, L=number of loci, π= ploidy level, ml= number of matching alleles for locus L.

Analysis of molecular variance (AMOVA) to estimate population genetic differentiation of among and within pepper accessions and improved varieties were computed by GenAlex 6.5 software (Peakall and Smouse, 2012).

A model-based population structure analysis was carried out with STRUCTURE ver.2.3.1 Software (Pritchard et al., 2000) using the admixture model with correlated allele frequencies. The number of possible *K* was set from 1 to 10 with 20 runs for each K, and each run had a burn-in period of 250,000 and 500,000 MCMC iterations. The optimum value of *K* was determined using ΔK simulation (Evanno et al., 2005) implemented in the web-based Analysis tool STRUCTURE HARVESTER v6.93 (Earl and vonHoldt, 2012).

RESULTS AND DISCUSSION

Gene diversity and markers polymorphism

16 SSR markers were successfully implemented

Table 3. Summary of gene diversity indices at 16 polymorphic loci in 25 Pepper genotypes.

Marker	MAF	Na	Ho	GD	PIC (%)
AVRDC PP-18	0.48	6	0.00	0.69	65
AVRDC PP-32	0.52	5	0.74	0.60	53
AVRDC PP-65	0.52	5	0.59	0.66	62
AVRDC PP-167	0.50	2	1.00	0.50	38
AVRDC PP-67	0.32	9	0.00	0.82	80
CAMS-806	0.40	4	0.54	0.71	66
GPMS8	0.61	4	0.00	0.57	53
GPMS6	0.95	2	0.00	0.09	8
GPMS112	0.93	2	0.14	0.13	12
GPMS117	0.56	4	0.00	0.55	47
GPMS178	0.46	4	0.00	0.66	60
GPMS197	0.52	3	0.00	0.54	44
EPMS303	0.30	4	0.00	0.74	70
EPMS331	0.50	4	0.12	0.64	58
EPMS376	0.32	6	0.00	0.79	75
EPMS418	0.77	3	0.00	0.38	34
Mean	**0.54**	**4.19**	**0.20**	**0.57**	**52**

MAF=major allele frequency, Na=number of alleles, GD=gene diversity, Ho= observed heterozygosity, PIC=polymorphic information content.
Source: Author

in 25 pepper genotypes to evaluate the gene diversity and levels of polymorphism within and among the accessions and improved varieties. The gene diversity indices varied across the entire markers in tested genotypes. The 16 SSR markers generated a total of 67 alleles, ranging from 9 for marker *AVRDC PP-67* to 2 for markers *AVRDC PP-167*, *GPMS6,* and *GPMS112* with a mean of 4.19 alleles per marker (Table 3).

Various results have been reported from similar studies on varying number of genotypes and markers. For instance, Rabuma et al. (2020) reported a lower mean number of alleles (2.2) in 32 Ethiopian and Indian accessions using 14 SSR markers. In contrast, a higher number of alleles were reported by Woubit et al. (2021) that identified a mean of 8.54 alleles in 75 Ethiopian pepper germplasms using 13 SSR markers. Similarly, a mean number of alleles of 6.9 are reported from a large *Capsicum annum* collection in 179 individuals from six countries, other than Ethiopian origins using 21 SSR markers (Christov et al., 2021). A relatively lower (2.7) mean number of alleles were reported in 64 Indian pepper accessions using 27 polymorphic SSR markers. Obviously, the number of alleles detected in the germplasm or population is influenced by the species' genetic backgrounds and the molecular markers difference deployed.

The major allele frequency (MAF) ranged from 30 to 95% with a mean of 54%. A mean value of 0.20 was obtained for observed heterozygosity (Ho) with the highest record (1.00) was attained by marker *AVRDC PP-167* and no (0.00) observed heterozygosity by the ten markers (Table 3). The lowest Ho observed in most of the markers in our study is directly correlated with the fact that the majority of improved varieties and accessions are being homozygous. This can also be attributable to the high level of inbreeding that improved varieties are expected to exhibit. A variation in the levels of markers polymorphism has been observed (Figure 1), with a PIC value ranging from 8 to 80% and a mean of 52% (Table 3).

Molecular markers with PIC values of >0.50 (50%) are considered to be highly informative for genotyping studies (Botstein et al., 1980). Except for the two markers (*GPMS6* and *GPMS112),* the others showed high PIC values, implying the presence of immense genetic diversity among studied genotypes and the very suitability of the markers for molecular characterization of pepper genotypes. Similar results were obtained with a mean PIC value of 0.57 using different SSR markers (Rabuma et al., 2020).

A gene diversity index is one of the most important measures of genetic divergence and is useful for determining the amount of diversity in the genotypes. The gene diversity (GD) in this study ranged from 0.09 to 0.82 with an average of 0.57 (Table 3), indicating the presence of high genetic diversity among Ethiopian pepper genotypes. The highest GD value (GD=0.82) was recorded for marker *AVRDC PP-67* while the lowest

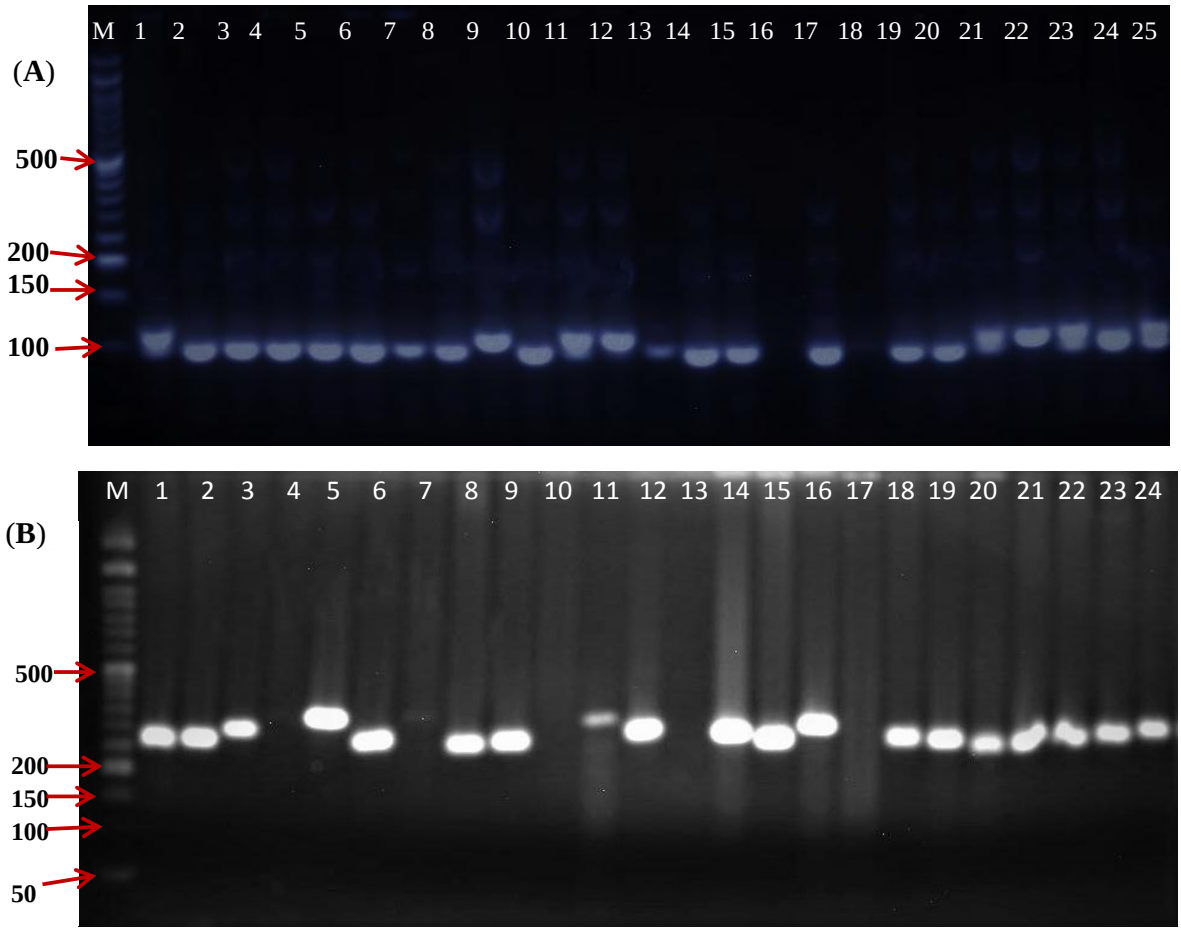

Figure 1. PCR amplification profile of pepper genotypes with a marker *AVRDC PP-18* (A) and *EPMS376* (B), M; represents DNA ladder (50 bp, SMOBIO, DM1100) and lane 1-25/24 are pepper genotypes.
Source: Author

(GD=0.09) was for marker *GPMS6.*

Genetic dissimilarity and phylogenetic relationshionship

The pairwise genetic dissimilarity coefficient determines the genetic relatedness among the genotypes. The highest genetic distance value of 1.00 was observed between PBC-731 and Acc-22 and the lowest (0.25) was between Acc-13 and Acc-11 genotypes (Table 4). The dissimilarity coefficient value of 1.00 indicates that the two genotypes are genetically different; while, the the value 0.25 indicates that the two genotypes have a higher genetic similarity. In general, most of the pairwise dissimilarities obsereved were higher across the 25 genotypes implying a broad range of genetic variability basis among the tested pepper genotypes.

Several evolutionary factors influence genetic diversity among and within species, including seed dispersal, gene flow, natural selection, geographic range, and the diversity center (Sork, 2016). Cluster analysis was used to find the best possible grouping based on genetic distance. In the present study, a neighbor-joining (NJ) tree was built to determine the genetic relationship of the 25 pepper genotypes using 16 SSR markers, and the analysis deployed all of the genotypes into three major clusters and formed different sub-clusters (Figure 2). The first cluster (C1) comprised of 9 (100%) genotypes all of them are accessions, while the second cluster (C2) contained 12 genotypes of which 8 (67%) are improved varieties and 4 (33%) are accessions. The third cluster (C3) is composed of 4 genotypes, 2 (50%) are improved varieties while the other 2 (50%) are accessions. Although the first cluster comprises the majority of the accessions, some of the accessions were found dispersed in all of the other clusters, depicting the presence of a high genetic distance between accessions. Likewise, most of the improved varieties fell in the second cluster; while, some were in the third cluster, showing presence of considerable genetic distance between varieties though most seems relatively close. The current

Table 4. A pairwise genetic dissimilarity across the 25 pepper genotypes.

	Acc-3	Acc-9	Acc-10	Acc-11	Acc-12	Acc-13	Acc-17	Acc-21	Acc-22	Acc-24	Acc-26	Acc-40	Melka awaze	Melka shola	Melka oli	PBC 602	Mareko fana	PBC 731	Melka zala	Melka dera	Melka eshete	Melka shote	Acc-41	Acc-8	Acc-45
Acc-3	***																								
Acc-9	0.56	***																							
Acc-10	0.56	0.41	***																						
Acc-11	0.47	0.50	0.34	***																					
Acc-12	0.44	0.44	0.44	0.31	***																				
Acc-13	0.31	0.41	0.34	**0.25**	0.28	***																			
Acc-17	0.69	0.59	0.69	0.72	0.63	0.72	***																		
Acc-21	0.50	0.44	0.47	0.50	0.38	0.31	0.66	***																	
Acc-22	0.44	0.59	0.41	0.47	0.53	0.38	0.75	0.47	***																
Acc-24	0.47	0.59	0.47	0.50	0.47	0.41	0.59	0.34	0.53	***															
Acc-26	0.59	0.81	0.69	0.72	0.53	0.53	0.81	0.50	0.59	0.44	***														
Acc-40	0.47	0.72	0.53	0.56	0.50	0.47	0.72	0.47	0.50	0.47	0.34	***													
Melka awaze	0.50	0.72	0.56	0.53	0.50	0.47	0.53	0.53	0.63	0.34	0.50	0.38	***												
Melka shola	0.69	0.75	0.56	0.72	0.56	0.56	0.81	0.56	0.63	0.59	0.47	0.44	0.63	***											
Melka oli	0.53	0.72	0.69	0.63	0.47	0.56	0.59	0.56	0.75	0.53	0.56	0.38	0.41	0.56	***										
PBC 602	0.81	0.75	0.84	0.78	0.72	0.75	0.69	0.78	0.75	0.81	0.72	0.78	0.72	0.69	0.72	***									
Mareko fana	0.66	0.84	0.72	0.69	0.59	0.66	0.75	0.53	0.69	0.53	0.47	0.47	0.56	0.44	0.47	0.59	***								
PBC 731	0.88	0.66	0.88	0.81	0.84	0.88	0.75	0.78	**1.00**	0.81	0.88	0.75	0.72	0.81	0.81	0.84	0.75	***							
Melka zala	0.63	0.66	0.53	0.59	0.59	0.50	0.75	0.63	0.69	0.47	0.59	0.69	0.59	0.47	0.66	0.69	0.69	0.78	***						
Melka dera	0.66	0.81	0.56	0.59	0.56	0.53	0.78	0.47	0.63	0.38	0.44	0.53	0.50	0.50	0.56	0.72	0.47	0.78	0.28	***					
Melka eshete	0.53	0.78	0.69	0.75	0.59	0.69	0.66	0.59	0.56	0.56	0.47	0.34	0.50	0.47	0.41	0.78	0.53	0.75	0.63	0.41	***				
Melka shote	0.34	0.72	0.69	0.63	0.59	0.56	0.53	0.63	0.53	0.47	0.50	0.44	0.47	0.69	0.44	0.72	0.53	0.75	0.66	0.56	0.28	***			
Acc-41	0.69	0.72	0.59	0.66	0.63	0.63	0.69	0.53	0.44	0.63	0.63	0.47	0.72	0.53	0.59	0.78	0.63	0.94	0.78	0.63	0.50	0.59	***		
Acc-8	0.50	0.69	0.66	0.63	0.63	0.59	0.66	0.50	0.66	0.41	0.63	0.56	0.47	0.69	0.56	0.78	0.59	0.75	0.56	0.47	0.34	0.31	0.66	***	
Acc-45	0.81	0.78	0.81	0.75	0.66	0.75	0.88	0.66	0.84	0.72	0.72	0.81	0.81	0.78	0.88	0.97	0.81	0.56	0.78	0.78	0.84	0.81	0.81	0.63	***

Source: Author

study did not split the genotypes in to only accessions and improved varieties. Besides, the cluster of the accessions was not based on their geographic proximity. Most likely, this could be due to seed mixture as some genotypes were collected from local markets (Aklilu et al., 2016). Additionally, lack of a formal seed system in the country (Abebe and Lijalem, 2011) may contribute to seed exchange across different geographic locations.

Phylogenetic analysis is useful not only for estimating the genetic distance of genotype collections but also for selecting crossing parental lines. Varieties with a greater genetic distance are generally recommended as parents to produce

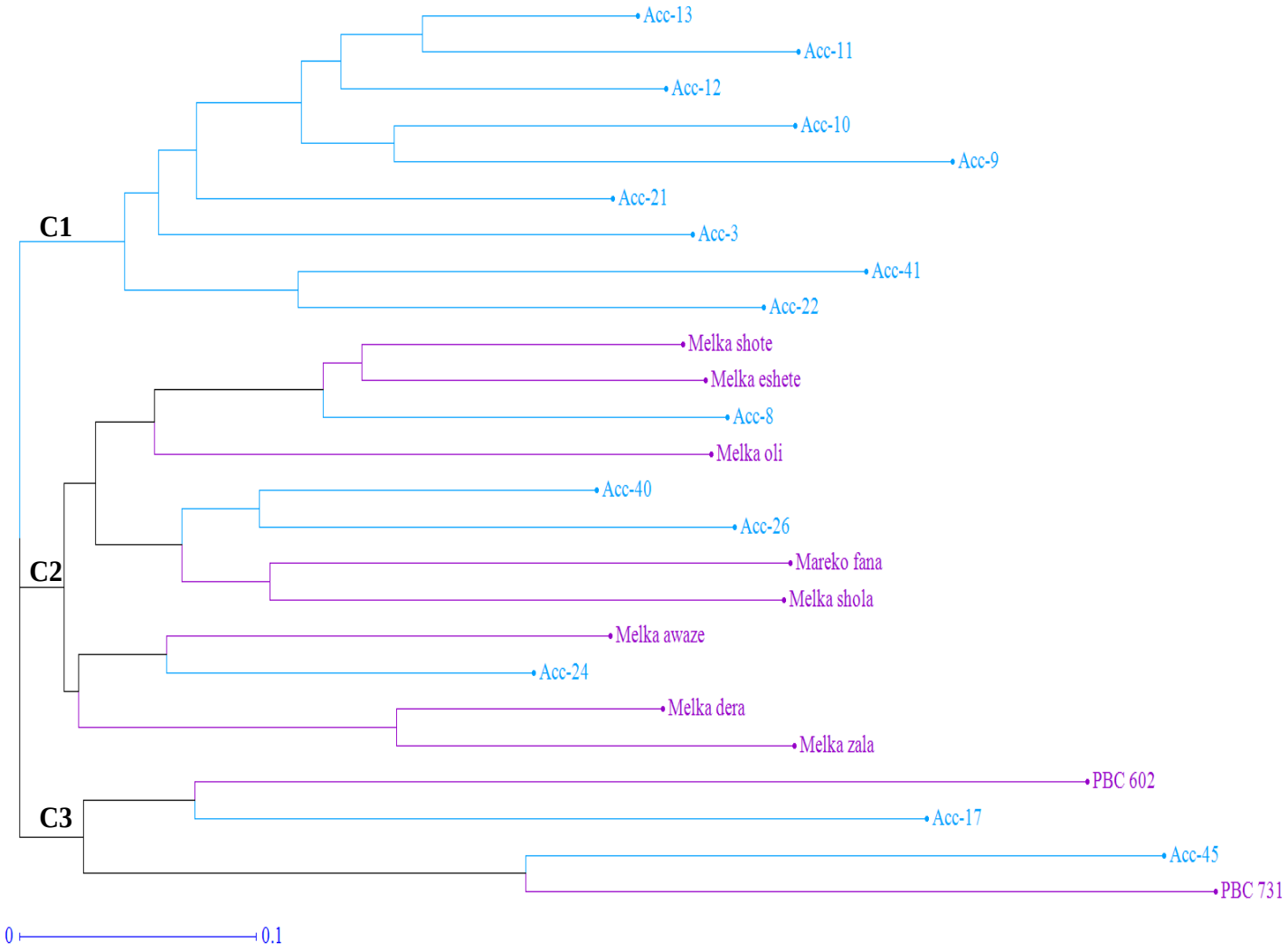

Figure 2. Phylogenetic relationship of 25 Pepper genotypes using 16 SSR markers. The colors, light blues are accessions and purple are improved varieties.
Source: Author

progeny with a heterosis effect. Indeed, the wide range of diversity in our tested genotypes could be important for broadening the genetic base because it enhances the chances of discovering more unique genes. Moreover, it provides the opportunity of hybridization between distant genotypes which helps in the production of heterozygous individuals with desirable traits.

A two-dimensional display of principal coordinate analysis (PCoA) was also performed to further investigate the genetic relationship of pepper genotypes and the result showed the first three principal coordinate axes explained 44.33% of the total genotypic variation in the studied genotypes. The first and the second explained 17.01 and 16.06% of the total variation, respectively. The PCoA displayed a scatter plot with a wider dispersion of the genotypes in all the quadrants without forming a clear cluster, and some genotypes like PBC-731, Acc-45, Acc-9, and Acc-22 are plotted far from the central axis, indicating the individuals' genetic distance among pepper genotypes and such kind of genotypes are highly recommended for future pepper breeding (Figure 3). In most cases, even though the genotypes are displayed scattered across all quadrants in the PCoA, the majority of the accessions and improved varieties are somewhat separated and formed three clusters (C1, C2, and C3) based on their genetic background, as similar as the dendrogram.

Population structure and analysis of molecular variance

The population structure analysis was inferred on the 25 genotypes (15 accessions and 10 improved varieties).

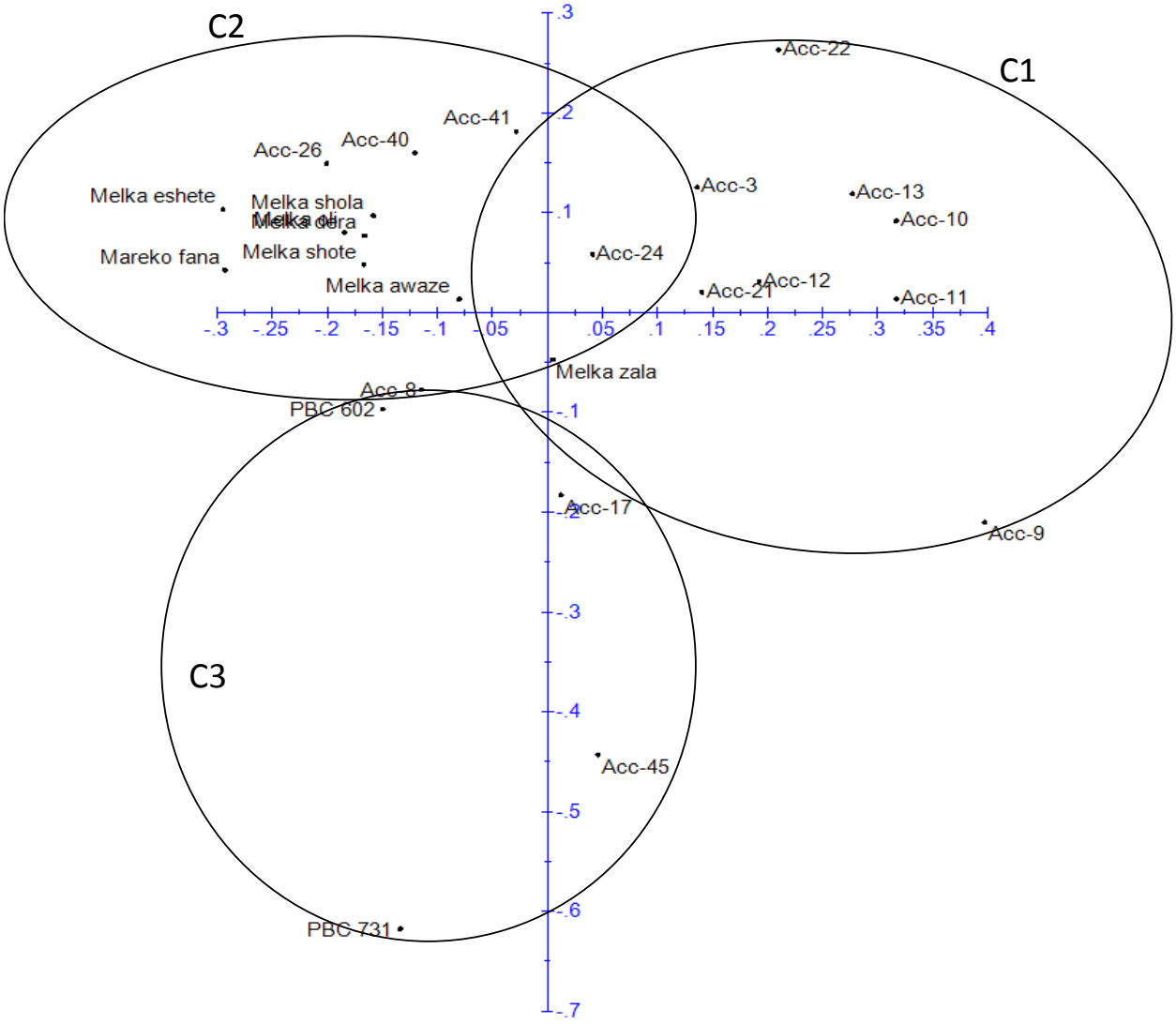

Figure 3. A biplot display of the axis 1 and 2 of the principal coordinate analysis based on the dissimilarity matrix of 16 SSR markers for the 25 Pepper genotypes.
Source: Author

The highest value of delta K (ΔK) was obtained for K=4, revealing the existence of four genetic groups (Group I, Group II, Group III and Group IV) of pepper genotypes (Figure 4A and B). Group I comprised 9 genotypes, of which 5 were improved varieties and 4 were accessions. Group II and Group III consists of 8 and 1 accessions, respectively. Whereas Group IV constituted 7 genotypes, 5 of which are improved varieties while 2 are accessions. However, this structure analysis displayed that the group I and IV, and genotypes from the predicted genetic groups had a high level of admixture (Figure 4C). We speculate that the reason for this is maybe pepper genotypes were acquired from the different gene pools with a high-level of mixture, as reflected by a high level of gene flow (Nm=2.4) and low genetic differentiation between groups (Table 5). The grouping at K=4 showed less concordance with that of the dendrogram and PCoA, this is because the very few distantly related genotypes could have contributed for the less concordance. Reports from population structure analysis in other pepper diversity panel indicated the existence of well-differentiated population groups (Solomon et al., 2019; Rabuma et al., 2020). The same is true in the present study except we used relatively small number of genotypes.

Analysis of molecular variance (AMOVA) was used to quantify the genetic divergence within and among groups. We partitioned the total molecular variance based on the K=4 from STRUCTURE result and the priory grouping information into accessions and improved varieties (Table 5A and B). As a result, the total variation was partitioned in to 9% among the four groups, and 91% within groups.

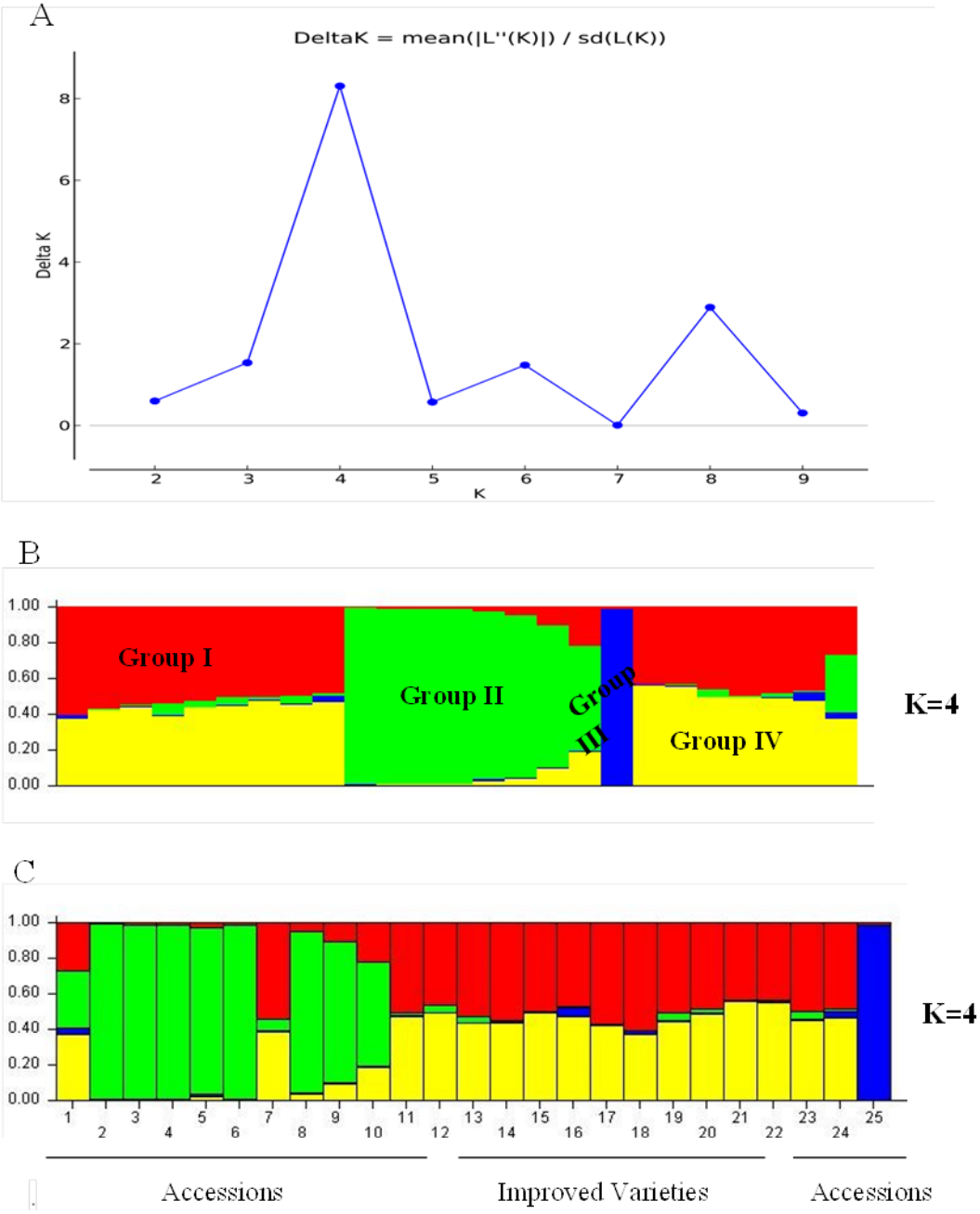

Figure 4. Population structure analysis of 25 pepper genotypes (A) inferred optimal Δ*K* based on the Evanno method (Evanno et al., 2005), (B) bar plot showing four of the groups (*k=4*) and their estimated membership built by *Q matrix,* (C) bar plot of the individual genotypes and their admixture. Each accession is represented by a vertical bar. Each color represents one ancestral group, and the length of each colored segment of each vertical bar represents the group contributed by ancestral groups.
Source: Author

Apparently, the high genetic differentiation has been demonstrated among individuals (Fis=1.00) as it is evident from Table 5. Almost the same result is obtained from the AMOVA based on the grouping with priory information in to improved varieties and accessions (Table 5B). Except for the negligible difference in estimated variance and F-statistics, exactly identical % of variations were found for among population (9%) and among individuals (91%) sources. This result may suggest that the ΔK based grouping of the genotypes is somehow related to grouping in to the accessions and improved varieties which are genetical grouping as well.

Table 5. Analysis of molecular variance (AMOVA) based on grouping from STRUCTURE results and a priory information into accessions and improved varieties.

Source	df	SS	MS	Est. Var.	% of variation	F-statistics	P-value	Gene flow (Nm)
A. Grouping based on structure analysis results; K=4								
Among Pops	3	43.742	14.581	0.470	9%	Fst=0.094		2.4
Among individuals	21	189.778	9.037	4.519	91%	Fis=1.00	0.001	
Total	24	233.520	23.618	4.989	100%			
B. Grouping based on a priory information in to accessions and improved varieties								
Among Pops	1	20.653	20.653	0.475	9%	Fst=0.093	0.001	2.4
Among individuals	23	212.867	9.255	4.628	91%	Fis=1.00		
Total	24	252.280	29.908	5.436	100%			

Df, degree of freedom; SS, sum square; MS, mean square; Est. Var., estimated variance.
Source: Author

Several variable results have been reported from previous studies conducted by various authors. Woubit et al. (2021) reported a partitioning of the total molecular variance in to 7% among eight-geographic groups, 63% among individuals, and 26% within individuals of Ethiopian pepper accessions. Similarly, in 32 Ethiopian and Indian accessions grouped in 9 populations by SSR markers, Rabuma et al. (2020) reported 32% of the total variation among populations and 68% within populations of the total variation. In another study conducted by SSR markers on Mexican pepper populations, of the total molecular variance among the population, 10% was among wild, landrace, and hybrids, 15% was among individuals within populations, and 74% was within individuals in the populations (Pacheco-Olvera et al., 2012). In a nut shell, levels of molecular variations explained by sources of variations in diversity studies are a function of the grouping compositions (varieties, landrace accessions, hybrids, advanced breeding lines etc.) the number of individuals, polymorphic power of the markers used etc.

Conclusion

Conclusively, the genetic diversity of Ethiopian pepper accessions and improved varieties are effectively investigated using SSR markers. Our result revealed that the SSR markers used were polymorphic suggesting their potential use for genetic studies of pepper collections. The markers detected a high genetic diversity in the studied pepper genotypes, which could be used as a source for breeding and genetic improvements. The results can aid breeders in effectively selecting genetically distant parents and applying hybridization. It is recommended that a large number of collections from all over the country have to be studied using efficient marker tools to generate more comprehensive information.

CONFLICT OF INTERESTS

The authors declare that they have no any conflict of interest.

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Review

Alleviation of Huanglongbing disease in citrus by foliar application of microelements

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Huanglongbing (HLB), also referred to as citrus greening disease, ranks high among the most destructive diseases in citrus plants worldwide. This disease is caused by the Gram-negative bacterium Candidatus Liberibacter species. As a strategy for the appropriate management of this disease has not been established yet, economic cultivation of citrus in the diseased areas has mostly ceased. One of the most conspicuous phenotypic characteristics of this disease is the chlorosis caused by bacterial plaques in the plant phloem systems due to microelement deficiency. Therefore, the effects of the disease may be mitigated with sufficient supply of these nutrients. This may in turn lead to the establishment of a strategy to manage the disease symptoms, even though trees might not completely recover. Such management would at least enhance the longevity of trees and contribute to an increase in their yield. Thus, an approach of reviewing microelement function might provide insights that can be translated into strategies for HLB management.

Key words: Citrus, HLB, nutrient, micronutrient, pathogen.

INTRODUCTION

Citrus huanglongbing (HLB) or citrus greening disease, caused by the Gram-negative, phloem-limited Alphaproteobacteria Candidatus Liberibacter species is the most destructive citrus pathosystem across the globe (Jagoueix et al., 1994; Bové, 2006; Duan et al., 2009; Gottwald, 2010; Ghosh et al., 2018). This pathogen comprises three species, that is, '*Candidatus* Liberibacter asiaticus' (CLas), '*Candidatus* Liberibacter africanus', and '*Candidatus* Liberibacter americanus', which are distinguished using 16 rDNA sequencing (Bové, 2006). These bacteria are vectored by hemipteran insects, that is, *Diaphorina citri* Kuwayama (Grafton-Cardwell et al., 2013; Tabatchnick, 2015) or *Trioza erytreae* Del Guercio (Rasowo et al., 2019; Aidoo et al., 2021). HLB caused by these pathogens is distributed in more than 40 countries including major citrus-producing areas, such as China, Brazil, USA, and India (Gottwald, 2010). This disease seems to have potential to expand into unaffected areas

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(Ajene et al., 2020). If curative measures are not developed, the citrus industry could be destroyed globally. In the USA, the disease was first reported in 2005 in Florida, and then spread to Louisiana, South Carolina, Georgia, Texas, California, and Arizona (Gottwald, 2010). It is estimated that the production costs were 40% greater for the management of the vector insect and HLB after the spread than before (Irey et al., 2008). So far, about 80% of citrus trees in Florida were infected with the HLB pathogen, compared to before HLB pandemic, and the average percentage yield loss reaches 41% (Singerman and Useche, 2016). In Asia, HLB was reported in South China in 1943 and in Taiwan in 1951. This destructive citrus disease continued to spread in Southeast Asia and reached Japan in 1988 (Miyakawa and Tsuno, 1989).

Presently, HLB management strategies are limited with respect to both efficiency and efficacy (Bassanezi et al., 2020; Li and Feng, 2020; Zapata et al., 2021). In some countries where HLB is limited to small areas or where HLB invasion is in a relatively early stage, the removal of HLB-infected trees is used as a strategy to eradicate the disease (Bové, 2012; Bassanezi et al., 2013). However, this strategy is laborious and cannot be carried out in large areas where vector invasions are unavoidable or uncontrolled. Another strategy is the use of antibiotics (Zhang et al., 2014; Hu et al., 2018; Chanvatik et al., 2019; McKenna, 2019; Yang et al., 2020), which may reduce the pathogen load in trees, resulting in the disappearance of symptoms. Nonetheless, “re-appearance” or “re-infection” does occur in response to the surviving bacteria or repeated transmission of the pathogen by vectors (Aubert and Quilici, 1984; Zhang et al., 2014; Hu et al., 2018). However, the use of antibiotics is restricted or has been completely eliminated in agriculture. Recently, Huang et al. (2021) revealed that stable antimicrobial peptides from *Microcitrus australasica* killed HLB bacteria and consequently prevented HLB infections (Huang et al., 2021). However, as this technology is still in its initial stages, it cannot be used for HLB management yet. Thus, although some of the measures reported so far may reduce the occurrence of the disease, they are either expensive or labor-intensive. Therefore, practical management measures for citrus growers need to be developed.

Foliar application of nourishing materials including micronutrients enhances citrus tree vigor against HLB (Wang, 2019; Bassanezi et al., 2011). Recently, two pioneer papers have reported data that can be used to develop practical management strategies for HLB. While one paper reported the curative effects of manganese (Mn) on HLB, particularly the disappearance of the pathogen from the HLB-affected trees (Zambon et al., 2019), the other reported the reduction of both the pathogen population and symptomatic appearances on the plant body (Inoue et al., 2020a). As typical HLB symptoms, such as yellowing of leaves, resemble symptoms that are attributed to micronutrient deficiency (Ohtsu et al., 1998), it may be possible that a sufficient supply of these elements could mitigate the disease symptoms. Although the use of these nutrients to treat the disease is debatable, it is worth reviewing the functions of these elements as potential remedial agents in terms of their interaction with the pathogen and with respect to plant physiology. These elements have a relatively lower cost of use and might be included in strategies used for HLB management. Here, we discuss micronutrients that have been reported in relation to citrus physiology and determine whether they are effective with respect to stemming HLB infection, and their potential for disease management.

HLB TREATMENT BY Mn APPLICATION

In plants, Mn plays an important role in physiological function, that is, acting as an enzyme co-factor or as a metal with catalytic activity in biological processes. As HLB infection lowers the pH in the leaves of satsuma mandarin (*Citrus reticulata*) (Masaoka et al. 2011, Zambon et al. 2019), foliar application of $MnSO_4$ resulted in 45% more yield from HLB-affected trees. This effect of Mn is concentration dependent. A higher concentration, that is, 2-times more, reduced the yield by 25% (Morgan et al., 2016), whereas foliar application of $Mn_3(PO_3)_2$ in a phosphate form resulted in a 25% reduction (Morgan et al., 2016). These results suggest that Mn mitigates the effects of HLB if applied in an appropriate compound form and at an appropriate concentration.

The improvement in physiological conditions in response to Mn application has been reported in other crops. For example, the foliar application of Mn as $MnSO_4$ in cowpea reduced 42.7 and 42.0% of the disease severity caused by *Rhizoctonia solani* and *Rhizoctonia bataticola*, respectively (Kalim et al., 2003). In a sugarcane variety (*Saccharum* species), which is susceptible to orange rust (*Puccinia kuebnii*), a single spray of Mn at a concentration of 0.5 or 1.0% reduced the percentage of diseased leaf area by 2.2 and 0.9%, respectively, which was much lower than the 15% observed in untreated plants (Mesquita et al., 2019). In coffee, the foliar application of $Mn_3(PO_3)_2$ suppressed the coffee rust (*Hemileia vastatrix*)-induced bean damage (Pérez et al., 2020). Chaves et al. (2021) reported that Mn reduced the symptoms of white-mold disease (causative agent, *Sclerotinia sclerotiorum*) in tomato. No physiological and biochemical roles of Mn have been distinguished. The adverse effects of *S. sclerotiorum* infection on photosynthesis have been reported to be mitigated by $MnPO_4$, as evidenced upon the evaluation of

the net carbon assimilation rate, stomatal conductance in water vapor, transpiration rate, maximal photosystem II quantum yield values, and concentrations of photosynthetic pigments (Chaves et al., 2021). Excess Mn in plant leaves induces oxidative stress, resulting in toxicity by disruption of photosynthetic electron flow in chloroplasts (Fernando and Lynch, 2015). In addition, *in-vitro* assays showed that $MnPO_4$ inhibited the growth of *S. sclerotiorum* in a dose-dependent manner, indicating that $MnPO_4$ directly affects pathogen growth, in addition to allowing the plant to develop resistance against the disease. TigerSul manganese+, a solution containing 0.16% Mn, has been reported to reduce the pathogenicity/virulence of CLas using quantitative PCR of sweet orange leaves (Zambon et al., 2019); its root application increased the yield by 45% and lowered the HLB-pathogen load to below a qPCR detectable level. However, the therapeutic effects of a mixture of Mn (0.16%) and boron (0.44%) have not been confirmed. No explanations have been provided for the loss of Mn effects when Mn is applied in the form of this mixture. A possible reason is that micronutrients applied in combination including Mn can reduce the acquisition of CLas by *D. citri*, thereby reducing the disease infection (da Silva et al., 2020). We are await further studies on the effects of Mn on citrus HLB.

ALLEVIATION OF HLB SYMPTOMS BY Fe IN BIOAVAILABLE FORMULATIONS

The mechanism of Fe absorption systems in plants are divided into two strategies, strategy I and strategy II (Römheld and Marschner, 1986). Under Fe deficiency, graminaceous plants secrete Fe chelate compounds from their roots, mugineic acids family to uptake Fe(III)-mugineic acids as a complex formulation (Römheld and Marschner, 1986). Non-graminaceous plants secrete reductants or chelate compounds from their roots into the rhizosphere, enhancing proton excretion and increasing their ferric reduction capacity in the root surface and the transport of Fe(II) across the plasma membrane by Fe(II) transporters (strategy I) (Mori, 1999). In contrast, some dicot plants are poorly adapted for Fe limited soil. Citrus plants utilize strategy I, and some plants in the citrus species are susceptible to Fe limited calcareous soil; citrus trees with many commercial rootstocks perform poorly in high-carbonate soils (Castle et al., 2009).

The HLB pathogen causes interveinal chlorosis in leaves, which reduces the activity of basic chemical reactions in the photosynthesis of plants. Masaoka et al. (2011) compared the composition of metal elements in leaves between healthy trees and HLB-infected ones in two mandarin plants: satsuma mandarin in Japan (*Citrus unshiu* Marc.) and Siem in Indonesia (*C. reticulata*). They revealed similar deficiency of Fe, Zn, and Mn in HLB-infected trees, especially Fe (Nwugo et al., 2013; Manzanilla-Ramírez et al., 2019; Zambon et al., 2019). These studies suggested that HLB led these three elements to be reduced in plant leaves. Therefore, supplying these citrus bioavailable microelements may help overcome HLB disease symptoms.

Fe deficiency of citrus may have traits similar to those of HLB disease resistance. As Fe in an insoluble form (Fe_2O_3) cannot be used directly by plants grown on neutral to alkaline soils, these plants suffer from Fe deficiency and experience disorders in essential physiological reactions in their body such as photosynthesis, respiration, oxygen transport, and gene regulation (Marschner, 2011). Therefore, plants have evolved Fe acquisition strategies, such as strategy I and II (Marschner 2011), of which citrus plants use strategy I (Wulandari et al., 2014). Graham et al. (2017) reported that symptoms of HLB developed rapidly in citrus grown on high-pH soils in Florida, where Fe precipitated easily into the soil. Similarly, HLB-infected trees are not found in low-pH soils and are common in high-pH soils in Tokunoshima, Kagoshima Prefecture, Japan (Inoue et al., 2020b), suggesting that citrus trees in alkaline soil are more vulnerable to HLB pathogenicity. In addition, cultivars resistant to HLB, such as *Murraya exotica* (Ramadugu et al., 2016), have higher root Fe reductase activity than susceptible cultivars, such as *Poncirus trifoliata* (Wulandari et al., 2014). In summary, citrus plants that can make efficient use of Fe are resistant to HLB disease.

Physiological functions of Fe in the plant body have been reported for their antagonistic effects on plant diseases. Foliar spray of Fe reduces the pathogenicity of the disease, resulting in the disappearance or paling of symptoms (Aznar et al., 2015; Peris-Peris et al., 2017; Nobori et al., 2018). The expression of Fe reductase oxidase genes (*FROs*), which turn ferric ion into highly active ferrous ion that is involved in Fe acquisition, were partially suppressed in HLB-affected citrus (Zhong et al., 2015). In other words, if FROs are activated by supplying bioavailable Fe in the plant body, then the plant may recover from HLB or the effects of the disease. Among these Fe chelate solutions, the most effective for cure HLB-affected tree can sustain the divalent Fe state via X-ray absorption fine structure analysis (Inoue et al., 2020a). The possible Fe impact on pathogen survival is supported by experiments on the model plant *Arabidopsis thaliana* in which a wide variety of siderophores secreted by the pathogenic *Pseudomonas syringe* pv. tomato DC3000 could be controlled by divalent Fe (Nobori et al., 2018). The authors suggest a competitive function of Fe with the microorganisms in the plant, assuming that microorganisms may be able to use Fe in their own biological processes, in turn raising the competition for Fe uptake between plants and pathogens.

Adverse effects of extra-applied Cu due to ionization tendency over the other metals

Copper (Cu) is an essential element in plants used as a

growth stimulant. Camp and Fudge (1939) first revealed the nutritional role of Cu. However, Cu overdose has been recognized as being toxic by citrus growers. Nevertheless, Cu has been used as a nutritional element or a fungicide over the past 80 years in Florida (Driscoll, 2004). Extra Cu applied on the plant provides no hints on the aerial parts of the plant body but causes serious damage in the subterranean systems, especially on the fine root growth (Adrees et al., 2015). The expression of overused Cu might be due to the competitive behavior of this metal with others in the soil or by prevention of physiological functions of other elements in the leaf.

Owing to its lower ionization tendency, excess Cu is precipitated out of soil in the form of a cation and leaches out of the soil, while other metals remain ionized in the soil. Thus, the chemical interaction of Cu results in the deficiency of the elements in the plant (Marschner, 2011; Kopittke and Menzies, 2006). Similar interactions of Cu may occur in leaves, and the overuse of Cu results in high concentrations of Cu reducing yields of citrus production (Bakshi et al., 2013; Behlau et al., 2010; Fan et al., 2011). The overuse of Cu leads to the reduction of microelements in HLB-affected trees of *Citrus sinensis*, although not statistically significant (Ebel et al., 2019). These effects of Cu may be seen in Florida, where the land suffered from severe deficiency in micronutrients due to excessive Cu application (Driscoll, 2004). This is partly explained by the following reasons: Cu has a lower ionization tendency than other heavy metals, which promotes ionization of other metals. Therefore, it is considered that a deficiency of metals other than Cu is caused by yield reduction of citrus fruits. Gottwald et al. (2012) succeeded in removing the Cu effects on micronutrient deficiency and increased citrus fruit yield by supplying the deficient elements through foliar applications or soil drench. Therefore, excessive supply of Cu may have adverse effects on citrus plants.

ZINC TRANSPORT SYSTEM OF HLB-AFFECTED CITRUS MAY BE HIJACKED BY HLB-BACTERIA FOR ITS PATHOGENICITY

Zinc (Zn) that is trivially absorbed through root is indispensable in plants. According to comprehensive reviews on the nature and biochemistry of elements by Broadley et al. (2007), Haydon and Cobbett (2007), and Marschner (2011), HLB-affected citrus trees appear to have much higher Zn requirements than healthy trees. After one year, HLB-affected citrus showed typical HLB symptoms and significantly reduced Zn concentrations in leaves. Micro-XRF imaging of Zn and other nutrients showed that preferential localization of Zn is observed in the stems and leaves collected from healthy grapefruit plants, but lower signal is from HLB-affected samples. Zn concentration in the phloem of veins in healthy leaves is more than 10 times higher than that in HLB-affected leaves (Tian et al., 2014). Albrecht and Bowman (2008) revealed differential expression of the Zn transporter Z1P1 (AT3G12750 in the AGI number system) in the microarray of healthy or HLB-infected *C. sinensis* trees. The *ZIP1* gene was up-regulated by 13.2-fold in HLB-affected trees compared to that in healthy trees. Aritua et al. (2013) reported that the Zn transporter ZIP1 and putative Zn transporter genes were upregulated by 3.76- and 1.48-fold, respectively (in the value of a digit Log2), in HLB-infected trees. Shahzad et al. (2020) performed RNA-seq analyses and suggested that the expression of Zn transporter genes in sweet orange was homologous to the genes. The authors confirmed the expression of Zn transporter10 (ZIP10 orange 1.1g018585) by real-time PCR. Their results suggested that HLB-affected citrus trees had an increased requirement for Zn according to the gene expression level. Treatment of HLB-infected trees with Zn thus augments the pathogenicity of the bacteria in the trees (Zhang et al., 2016). This indicates that the pathogen is hardly controlled by the application of Zn.

Zn is an essential micronutrient for bacteria (McDevitt et al., 2011) and modifies the function of about 100 different proteins including enzymes (Ma et al., 2009). The genome sequence analyses of HLB-infected trees revealed a high-affinity Zn in the uptake system (Duan et al., 2009; Vahling-Armstrong et al., 2012). Molecular studies showed that the Zn cascade encoded by znuABC in plant cells regulated Zn metabolism by importing the element in insufficient amounts due to HLB infection (Vahling-Armstrong et al., 2012). In HLB-infected trees, higher levels of Zn are observed, which may be due to plants physiological changes or Zn related gene expression above (Razi et al., 2011; Zhang et al., 2021). The shortage of Zn may be caused by the Zn uptake by HLB bacteria that overrules a number of plant functions for their survival in the plant (Zhang et al., 2015; Shi et al., 2016). Therefore, the bacteria are compared to a hijacker in the metal transport system of the plant, which consequently develops virulence to the host.

CONCLUSION

Plants treated with Mn are protected from severe attacks by pathogens with recessing HLB disease symptoms (Zambon et al., 2019; Kwakye et al., 2022). This report does not refer to the changes in Fe dynamics in plants by Mn application. The mutual or antagonistic relationships between Mn and Fe, particularly their synergic functions in disease therapeutics, need further study. Other elements may be involved in the interaction of the two microelements. An application of zinc sulphate in combination with manganese sulphate can enhance the vigor and quality of citrus fruits against citrus greening disease (Hussain et al., 2022). The mechanism of element usage can contribute to the development of HLB

control. Although the costs associated with the use of these agents must be taken into account for the establishment of HLB management, the application of micro nutritional elements has not been studied so far. Therefore, this review could facilitate future research to address these issues.

CONFLICT OF INTERESTS

The authors have not declared conflict of interests.

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