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African Journal of Biotechnology

Review

Current status of molecular tools development for cowpea [*Vigna unguiculata* (L.) Walp.] improvement

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Cowpea [*Vigna unguiculata* (L.) Walp.] is a popular tropical grain legume which is widely produced and consumed in sub-Saharan Africa (SSA). The grains are rich in dietary protein for human while the haulm is high quality fodder for livestock particularly ruminants. Compared with many other crops cowpea is a laggard in development, evaluation and deployment of different molecular markers for use in genetics and breeding. Application of DNA-based markers is of considerable significance to crop improvement. Some DNA based markers have been used to study genetic diversity, linkage and quantitative traits loci (QTL) mapping in cowpea. Results from these studies have demonstrated the extent of genetic diversity in cowpea and its relationship with other members of the *Vigna* species. In addition, genetic linkage maps have been produced and used for detection of QTLs for some desirable traits. Some of these include QTLs for seed size, seed coat and eye color, leaf shape, pod length, resistance to macrophomina, domestication-related traits such as pod length, days to flowering, etc. In view of the potential benefits of DNA markers to the development of better performing improved cowpea varieties, concerted efforts are now being devoted to develop molecular tools for the crop. The developed consensus genetic linkage map and genome sequence for cowpea will boost the application of molecular tools for its genetic improvement. A panel of 17 SNP markers have been developed for use in quality assurance and control in cowpea breeding activities. This review aims at highlighting the molecular approaches that have been used and being pursued for genetic diversity, QTL mapping of some qualitative and quantitative traits as well as marker-assisted selection leading to the development of high performing new improved lines that meet the needs of farmers and consumers.

Key words: Cowpea, molecular tools, *Vigna unguiculata,* QTL mapping, marker-assisted selection.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important grain legume of sub-Saharan Africa (SSA) that is widely cultivated and consumed. It is especially well-suited to the SSA's dry savannah and sahel regions, where some other crops would fail or perform poorly due to water stress induced by unpredictable and short-duration

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rainfall, in addition to soil quality (De Ron, 2015). The crop's world production is estimated at over 8.9 million MT per year on about 14.4 million hectares. Sub-Saharan Africa accounts for 87% of the world's production. Nigeria is the largest cowpea producer, followed by Niger, Burkina Faso, Cameroon, and Mali (FAOSTAT, 2021).

Cowpea is important for diverse reasons. This crop has good nutritional value and is a valuable cash crop in semi-arid locations (Ehlers and Hall, 1997). In the food and feed business, it plays a crucial role in human nutrition due to the high dietary value of its grain, which contains 23 to 32% quality protein and substantial amounts of minerals and vitamins (Badiane et al., 2014). Cowpea is tolerant to low soil fertility because of its ability to fix nitrogen. It is a drought-tolerant crop that grows well in drought prone areas, making it particularly popular in semi-arid regions of the tropics where other food legumes do not perform well. Even in poor soils with organic matter less than 0.2%, pH range of 4.5-9.0 and sand content greater than 85%, cowpea has a remarkable ability to perform better than many other crops due to its ability to fix nitrogen (Xiong et al., 2016).

Low agricultural yields in SSA are largely due to poor soil fertility, high temperature, drought due to irregular rainfall and lack of irrigation, growing unimproved varieties, inadequate cultural practices, diseases and pests (Enete and Amusa, 2010). Drought, low soil fertility, and heat are abiotic constraints, while insects, bacteria, fungi, parasitic weeds, and nematodes are biotic constraints (Boukar et al., 2016). However, considerable scope exists to enhance cowpea productivity. Modern molecular genetics tools and techniques can complement conventional approaches to allow breeders effectively develop improved varieties that are well adapted and capable of producing high yields. Molecular marker-assisted breeding is now being used to improve efficiency of breeding programmes for many crops. Molecular markers have been found useful in different aspects of variety development starting from genetic diversity studies, confirmation of hybrids between parental lines up to selection of the final product, that is, the newly developed variety.

Conventional breeding is time-consuming, labour-intensive and has been linked to transfer of undesirable genes with desired ones (linkage drag) especially when wild relatives are crossed with cultivars. It is therefore imperative to integrate other breeding approaches which can enable higher levels of precision with respect to gene delivery leading to better performing improved varieties. Using molecular markers can help facilitate this process remarkably well. The integration of phenotypic and molecular markers in marker-assisted breeding has the potential to reduce the number of years required for cultivar development (Nkhoma et al., 2020).

Molecular markers are useful in genetics and plant breeding (Ganal et al., 2009) as they can be used for genetic diversity studies, genetic linkage mapping, gene cloning, and marker-assisted selection (Asare et al., 2010; Egbadzor et al., 2014; Xiong et al., 2016). Among the molecular markers available to plant breeders, the ones that have been used are Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSRs), Single Nucleotide Polymorphisms (SNPs), and Diversity Arrays Technology (DArT) SNPs. The latter two marker systems are now more commonly used due to their relatively low cost per data point and high throughput procedures. With these markers (SNPs), more robust data can be generated within a short period of time.

The objective of this paper is to review the efforts that are being made with molecular markers to improve cowpea and to point out research gaps that can be exploited.

CHALLENGES TO COWPEA PRODUCTION

Cowpea productivity in SSA is being constrained by several causes, including climate change-related stresses and socio-economic restrictions (Amusa et al., 2015). The most common biotic constraints of cowpea include insect pests and diseases that attack the foliage and stems. The most common fungal diseases include anthracnose (*Colletotrichum lindemuthianum*), fusarium wilt (*Fusarium oxysporum* f.sp. *tracheiphilum),* foot rot (*Fusarium solani* Matt. Scc), rust (*Uromyces phaseoli* Pers. Wint), and scab (*Elsinore phaseoli*) (Singh et al., 2003). Aphids (*Aphis craccivora* Koch) and leafhoppers are the insects that affect cowpea during each stage of development and growth while bud thrips (*Megalulothrips sjostedti* Trybom) attack the plant during the flowering stage, and pod borers (*Maruca vitrata*) attack the pods and young shoots (Boukar et al., 2016). A complex of pod sucking bugs damage seeds in the field while seed weevil attacks seeds in storage.

Numerous viruses infect cowpea such as *Cowpea aphid-borne mosaic virus* (CABMV, genus *Potyvirus*, family Potyviridae), *Bean common mosaic virus*-blackeye cowpea mosaic strain (BCMV-BlCM, genus *Potyvirus*, family Potyviridae), *Cowpea mosaic virus* (CPMV, genus *Comovirus,* family Secoviridae), Southern *bean mosaic virus* (SBMV, genus *Sobemovirus*), *Cowpea mottle virus* (CPMoV, genus *Carmovirus*, family Tombusviridae), *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family Bromoviridae), *Cowpea mild mottle virus* (CPMMV, genus *Carlavirus*, family *Betaflexiviridae)*, as well as *Cowpea golden mosaic virus* (CGMV, genus *Begomovirus*, family Geminiviridae) (Mbeyagala et al., 2014). Bacterial blight infections caused by *Xanthomonas campestris* PV. *vignicola* and *Xanthomonas campestris* pv. *vignaeuguiculatae* cause 71, 68, and 53% losses in pods per plant, seeds per pod, and fodder yield,

respectively (Singh et al., 2003). The parasitic weed *Striga gesnerioides ,* can cause significant damage to cowpea yield (Tignegre, 2010). The most common abiotic constraints such as drought, heat, and poor soil fertility are the major causes of low crop yields. Drought and heat hurt plant growth at all stages of development and high night temperatures in particular lead to flower abortion in cowpea which affect pod formation and grain yield (Fahad et al., 2017; Lamaoui et al., 2018). Boukar et al. (2018) reported that drought at the flowering phase caused yield loss of cowpea ranging from 360 to 1000 kg ha^{-1}. To increase cowpea yield in SSA, early maturing cowpea cultivars that can withstand extreme heat and escape drought are recommended.

MOLECULAR MARKERS IN GENETIC DIVERSITY STUDIES

Genetic diversity information, which is the foundation of breeding and genetic research, is particularly significant for cowpea breeding. Accurate assessment of genetic variability is important for the conservation and utilization of germplasm resources, and improvement of cowpea. Effective breeding and genetic conservation require a well-characterized agricultural genetic resource. Phenotypic traits and molecular markers are used to measure genetic diversity.

For example, Fatokun et al. (1993) employed 44 accessions of diverse species belonging to four subgenera of the genus *Vigna* to study the taxonomic relationship between the subgenus *Ceratotropis* and other subgenera using RFLP markers. The findings revealed that the genus *Vigna* has a lot of genetic variability, with *Vigna* species from Africa having a lot more variation than *Vigna* spp. from Asia.

RAPD markers were used to analyze genetic diversity and to group genotypes based on degree of genetic relatedness because they are easy and require minimal DNA (Oikeh et al., 2012). Zannou et al. (2008) showed that the RAPD method may be used to characterize genetic variation among cowpea cultivars. The RAPD markers were employed to assess the genetic diversity of 70 cowpea accessions collected across Benin Republic. The study indicated that the genetic diversity was very large. The fixation index revealed a considerable differentiation of cowpea cultivars in Benin based on molecular variance. Malviya et al. (2012) examined the genetic diversity of ten Indian cowpea cultivars using 18 sets of RAPD markers. A total of 181 bands with an average of 15 bands per primer were obtained. Out of 181 bands, 148 showed polymorphism (81.7%). Variation in genetic diversity among cowpea cultivars using different primers ranged from 0.1742 to 0.4054. Zinov'ev and Sole (2004) investigated 26 cultivated and 30 wild cowpea species from Western, Eastern, and Southern Africa. More polymorphisms were found in wild species from Eastern Africa, which supports the submission by Padulosi and Ng (1997) that part of Africa is where the greatest diversity among wild cowpea can be found. The authors further reported that wild cowpea lines from southern Africa in particular were characterized by small pod and seeds, dehiscent pods, hairiness of plant parts, perenniality, outbreeding and bearded stigma.

Nkongolo (2003) used RAPD markers to provide information about origins, taxonomy, domestication, and patterns of genetic variation of cowpea within cowpea populations from various agro-ecological zones of Malawi and discovered that there was a general lack of agreement between clustering based on the molecular markers and morphological traits. Nagalakshmi et al. (2017) discovered a high level of polymorphism among 30 genotypes characterized using RAPD markers. A total of 30 RAPD primers were chosen at random to examine the genetic diversity of 36 cowpea accessions. Five primers (OPC 14, OPB 1, OPA 10, OPG 13, and OPA 4) were determined to be more informative based on the polymorphism information content values (PIC) which ranged from 0.597 to 0.885, with OPC 14 having the highest PIC value. Based on the PCA plot, the first component explained 18.56% variation and the second and third components explained 16.85 and 12.77%, respectively among the 36 accessions of cowpea. The first three components explained 48.21% of the total variation (Nameirakpam and Khanna, 2018).

Pidigam et al. (2019) characterized genetic variation among 28 accessions of yard-long beans collected from different states of India using 48 random amplified polymorphic DNA markers and reported polymorphic information content value ranging from 0.23 to 0.93 among the genotypes. Inter Simple Sequence Repeat (ISSR) markers were utilized by Ajibade et al. (2000) to investigate the genetic links among 18 *Vigna* spp. They reported that closely related species within each subgenus clustered together. Simple sequence repeat (SSR)/Microsatellite marker-based diversity analysis revealed considerable genetic diversity among 141 cowpea accessions collected across Ghana's nine geographical regions. The accessions were clustered into five main branches loosely associated with the geographical regions. The average PIC was 0.38, with a range of 0.07 to 0.66 (Asare et al., 2010). Badiane et al. (2012) used SSR markers to assess the genetic diversity and phylogenetic relationships among 22 local cowpea varieties and lines collected across Senegal, and developed a set of 44 polymorphic marker combinations based on cowpea genomic or expressed sequence tags, with PIC values ranging from 0.08 to 0.33. Except for 53-3, 58-53, and 58-57, all of the local varieties were found in the same group, whereas Ndoute yellow pods, Ndoute violet pods, and Baye Ngagne were found in the second. In another study involving microsatellite markers, Chen et al. (2017) reported 155 alleles and 2.9 alleles per marker, and the average polymorphic information content (PIC)

value was 0.3615 using 105 selected genotypes from the National Genebank of China at the Institute of Crop Science (ICS) and found a low level of genetic diversity among the accessions. Sarr et al. (2021) conducted a similar study to analyze the genetic diversity of 671 accessions grown in eight regions of Senegal, as well as 66 wild relatives and intermediate forms (weedy). The findings revealed a narrow genetic variation between accessions from the different regions and cultivars with genetic similarity ranging from 0.861 to 0.965 with genetic differentiation indices between 0.018 and 0.100. The wild/weedy accessions showed more diversity than the cultivated with genetic diversity of 0.480 and 0.389, respectively. Haruna et al. (2020) characterized forty-six cowpea genotypes in Ghana for resistance to *Striga gesnerioides* using SSR primers. The findings showed that genetic diversity ranged from 0.04 to 0.49 with an average of 0.29; and average allele frequency of 0.78 genetic diversity and the polymorphism information content (PIC) varied from 0.08 to 1.00 with an average of 0.55. Ohlson and Timko (2020) screened seven cowpea lines against 58 unique *S. gesnerioides* populations collected across nine West African countries using SSR markers. Lioi et al. (2019) used a total of 19 SSR markers to identify genetic diversity of 13 cowpea landraces from a small geographical area in Apulia (southern Italy) using 12 of cultivar group *unguiculata* and 1 of cultivar group *sesquipedalis*. Gomes et al. (2020) assessed the genetic variation and gene flow in 59 *V. unguiculata* (cowpea) accessions from 10 landraces spanning across six agro-ecological zones of Mozambique using nuclear microsatellite markers (nSSRs). The results showed nine microsatellites that were highly polymorphic and revealed the existence of high genetic diversity between landraces from Mozambique (Ho: 0.222-0.426; He: 0.451-0.654). Also, AFLP markers were used to assess the genetic relationships among 117 cowpea accessions including 47 domesticated cowpea (ssp. *unguiculata* var. *unguiculata*), 52 wild and weedy annuals (ssp. *unguiculat*a var. *spontanea*), as well as 18 perennial accessions of the wild subspecies *pubescens*, *tenuis* and *alba.* The findings showed that domesticated cowpea was more diversified than wild annual cowpea (Coulibaly et al., 2002).

Fang et al. (2007) explored genetic links among 60 advanced breeding lines from six West African and American breeding programs, as well as 27 landraces from Africa, Asia, and South America using AFLP markers. The results showed that the 87 cowpea accessions shared a minimum of 86% genetic similarity and the percentage of polymorphic fragments per primer set ranged from 47.8 to 70.7%.

Egbadzor et al. (2014) characterized 113 cowpea accessions, 108 from Ghana and five from other countries, using SNP markers. Their study revealed that SNP markers were more effective than morphological, seed protein polymorphism, and SSR markers in differentiating across cowpea germplasm. Using genotyping by sequencing, Xiong et al. (2016) evaluated the genetic diversity and population structure of 768 cultivated cowpea genotypes from USDA GRIN cowpea germplasm collected from 56 countries. Based on PIC values, the accessions that originated in India and East Africa are most highly diversified (3.2 and 3.0), followed by Oceania and Europe with lowest PIC (0.17).

Muñoz-Amatriaín et al. (2017) conducted whole-genome sequencing of 37 cowpea accessions and developed a Cowpea iSelect Consortium Array (Illumina, Inc.) containing 51,128 SNPs. Carvalho et al. (2017) used these 51,128 SNPs to genotype 96 cowpea accessions comprising 43 landraces, and cultivars from the Iberian Peninsula and 53 landraces collected worldwide. Four sub-populations were identified with a lower genetic diversity level in the Iberian Peninsula accessions compared to worldwide accessions and average PIC and He values of 0.25 and 0.31, respectively, were found. A more comprehensive study by Muñoz-Amatriaín et al. (2021) using a high-density genotyping with 51,128 SNP to examine the genetic diversity of the University of California, Riverside (UCR) mini core, made up of 368 international accessions of cultivated cowpea, revealed six sub-populations distinguished by cultivar group and geographic origin. Based on SNP markers, Fatokun et al. (2018) used a sub-set of 298 lines from the loosely composed mini core collection of 370 landraces collected from 50 countries. The finding revealed three major clusters with a genetic distance ranging between 0.0096 and 0.462. A set of 40,089SNPs converted to the Kompetitive Allele-Specific PCR (KASP) SNPs was used to genotype 299 cowpea accessions. A pre-core pool of 434 SNPs and 50 informative core SNPs were selected and validated for use in future genetic diversity analyses of cowpea germplasm (Wu et al., 2021). Nkhoma et al. (2020) conducted a study with 100 cowpea genotypes using SNP markers and discovered that the SNP markers were fairly polymorphic, with a mean PIC value of 0.17 for the general population and 0.21 for mutant lines.

Diversity arrays technology (DArT), a new marker platform, was recently developed as a revolutionary method for whole-genome profiling without the need for sequence information. It is a high-throughput approach that can lead to discovery of hundreds of markers in a single experiment for a low price per data point (Huttner et al., 2005). Recently, Gbedevi et al. (2021) used DArT markers for genetic diversity and population structure study of 255 cowpea accessions collected from different regions and the Agricultural Research Institute of Togo Republic. The findings showed a range of 0.19 to 0.27 of polymorphic information content (PIC) with a mean value of 0.25 among the regions while the expected heterozygosity (He) varied from 0.22 to 0.34 with a mean value of 0.31 and observed heterozygosity (Ho) varied from 0.03 to 0.07 with an average of 0.05. The variation among accessions was higher (78%) within populations

and lower between populations (7%). The mean PIC value in this study was similar to the one obtained by Seo et al. (2020) where they reported a mean PIC value of 0.287 following assessment of 229 Korean germplasm lines based on SNP markers. Similarly, Sodedji et al. (2021) examined the genetic diversity and population structure of 274 cowpea accessions from different origins viz. western and central Africa, eastern Africa, and Asia using diversity array technology (DArT) showed 7% of the variance being among the populations with genetic distances ranging from 0.005 to 0.44. A genetic diversity of *Striga gesnerioides* were recently examined by Ohlson and Timko (2020). They reported that 58 different *S. gesnerioides* populations from nine different West African nations tested against seven cowpea lines revealed that none of the cowpea lines was resistant to all *S. gesnerioides* populations, and that no one S. *gesnerioides* population could overcome the resistance of all seven cowpea lines. The single sequence repeats used to genotype the *Striga* populations revealed significant divergence, showing that genetic relatedness is more commonly a result of geographic proximity than host compatibility. This study indicates that generating broad-spectrum and durable *S*. *gesnerioides* cowpea-resistant lines requires the stacking of multiple resistance genes. Adu et al. (2021) used 9,706 silicoDArT markers to reveal genetic variation among 16 cowpea accessions collection in Ghana based on agro-morphological traits.

Most findings revealed narrow PIC and low levels of heterozygotes within the germplasm characterized, which have been explained by the fact that cowpea is a highly self-pollinated crop with a low level of out-crossing. The self-pollinating nature of cowpea has been reported as the reason for the observed low genetic variation among cowpea landraces (Wamalwa et al., 2016; Carvalho et al., 2017).

QTL MAPPING FOR AGRONOMICALLY IMPORTANT TRAITS

The mapping and identification of major quantitative trait loci (QTLs) that harbor candidate gene(s) underlying beneficial traits, as well as related molecular and genetic studies, are key steps in deploying genomics-based breeding to improve crop varieties. Here, we describe some QTLs and genetic loci/genes with prospect for improving cowpea particularly breeding for yield and its components, grain quality traits, resistance to biotic and abiotic stresses. Quantitative Trait Loci are segments of the genome that contribute to variation in a trait of interest (Oikeh et al., 2012). QTL mapping is the foundation for the generation of markers for Marker-Assisted Selection (MAS). Marker-Assisted Selection enhances the breeding program and is successful in studying the genetic regulation of complex traits (Naidoo et al. 2012).

QTL ANALYSIS FOR YIELD AND ITS COMPONENTS

The basic goal of most plant breeding initiatives is to increase yield. Breeding for higher yield has been done using both conventional and marker-assisted methods. In conventional breeding, superior genotypes are chosen based on their phenotypic performance in a variety of situations (Acquaah, 2015).

Days to flowering and maturity, grain weight, pod number per plant, pod length, number of seeds per pod, 100-seed weight, number of pods per cluster, number of clusters per plant, number of primary branches per plant, days to 50% flowering and harvest index are among the traits targeted for improvement in cowpea variety development (Meena et al., 2015; Aliyu and Makinde, 2016).

Big seed size plays a major role in consumer preference. Several genes affect seed size, which is a significant component of grain yield (Song et al., 2007). Floral induction is the first step in seed development and it is influenced by a variety of elements such as the plant's age, environmental circumstances, and dry matter accumulation, among others. According to Fery and Singh (1997) genes that control seed size in cowpea have been reported by some authors.

Fatokun et al. (1992) published the first report on QTLs for seed weight in cowpea using 188 restriction fragment length polymorphism (RFLP) markers on 58 F_2 lines derived from a cross between cultivated and wild cowpea varieties. The authors identified two major QTLs with effects on this trait which explained 32 to 36% of the phenotypic variation and are orthologous to QTLs for seed weight in mung bean (*Vigna radiata*). In another study conducted by Ubi et al. (2000) using 94 F_8 RILs derived from the inter-subspecies cross involving an improved line and a wild relative, and 77 RAPD markers, five loci for seed weight were identified. These QTLs explained between 7 and 15% of the phenotypic variation. The relationship between the QTLs for seed weight in the study by Ubi et al. (2000) and those identified by Fatokun et al. (1992) was not clarified.

Flowering time is one of the most important traits that plays a key role in the adaptation of a variety to specific agro-ecological zone. Early maturing cultivars are referred to as climate wise cultivars since they can escape drought as well as insect and disease damages that generally occur later in the cropping season. On the other hand, earliness is associated with low yield due to the shortened vegetative and reproductive stages, which may result in reduced photosynthate accumulation and grain filling (Owusu et al., 2018). Timko et al. (2013) used bi-parental lines to conduct QTL analysis study under greenhouse settings for flowering period linked traits: time of flower opening and days to flowering. Five QTLs related to time of flower opening were discovered accounting for 8.8 to 29.8% of the phenotypic variance. Three QTLs for days to first flower were mapped using

SSR markers that explained 5.7 to 18.5% of the phenotypic variance. In the genetic map published by Xu et al. (2013), one major QTL which explained 31.9% of phenotypic variation for days to first flowering was mapped on chromosome 11 (Table 1).

Andargie et al. (2014) genotyped a population of 159 F_7 recombinant inbred lines derived from a cross involving asparagus bean with SSR markers and detected QTLs for seed, pod, and flower-related traits. For seed weight, seven QTLs were mainly detected on LG1, LG2, LG3 (two QTLs each on LG2 and LG3), LG7, and LG10 accounting for 9.2% of the phenotypic variance. Three QTLs were mapped onto LG1, LG2, and LG7 and explained 18.5% of the phenotypic variance for days to flowering. One major QTL for number of pods per plant which accounts for 20.1% of the phenotypic variation was detected on LG3. Following a genome-wide association study (GWAS) using diversity panel of 299 landraces and breeding lines, Xu et al. (2017) detected 72 SNPs for pod length. The phenotypic variation explained by any single SNP varied from 4.6 to 7.1%. Transcriptomic analysis in this study suggested the involvement of sugar, gibberellin, and nutritional signalling in the regulation of pod length. Lucas et al. (2013b) identified 10 QTLs for seed weight using eight bi-parental mapping populations and 1,536 SNPs. Pan et al. (2017) conducted a study using RAD sequencing (restriction-site associated DNA) technology to discover 34,868 SNPs in the cowpea genome using 170 $F_{2:3}$ biparental lines. Eleven QTLs for yield-related traits were mapped onto LGs (LG4, 5, 6, 7, 9, 10, and 11), four QTLs for pod length, four for thousand-grain weight, two QTLs for number of grains per pod, one QTL for carpopodium length accounting for 0.05 to 17.32% of phenotypic variation. A total of 215 recombinant inbred lines was used by Lo et al. (2018) to study domestication related traits of cowpea. Sixteen QTLs for nine traits located across the eleven chromosomes were detected. Two QTLs on days to first flower were detected, one each on chromosomes 5 and 9; three QTLs for seed weight were detected with one each on chromosomes 1, 6, and 8. Pod length was analyzed as a measure of the increase in organ size, and two QTLs were identified, one each on chromosomes 3 and 8. Two QTLs for leaf width were identified on chromosomes 1 and 8 while for number of seeds per pod, two QTLs were detected, one each on chromosomes 5 and 9. Two significant QTLs were detected for pod shattering, one each on chromosomes 3 and 5. These QTLs could serve as good candidates in MAS to improve cowpea for higher yield. Four of the QTLs affecting flowering time were mapped on chromosomes 1, 4, 5 and 9 using a set of 305 F_8 recombinants derived from multi-parent advanced generation inter-cross (MAGIC) population (Huynh et al., 2018). Lo et al. (2019) reported 17 QTLs for four traits, including seed weight, length, width, and density using 51,128 single nucleotide polymorphism markers spanning a large section of cowpea genome. This study used a mini-core collection of 368 accessions, and QTLs were mapped onto chromosomes 3, 4, 5, 6, 8, 10, and 11. This information could be valuable for developing cowpea varieties.

Muñoz-Amatriaín et al. (2021) conducted a GWAS using 51,128 SNPs markers including 368 worldwide cowpea accessions evaluated during the summers of 2016 and 2017 in California (USA) under long-days at the UCR Citrus Research Center and Agricultural Experiment Station in Riverside (CA) as well as under short days at the UCR Coachella Valley Agricultural Research Station in Thermal (CA) during the autumn of 2016 and 2017 at the International Institute of Tropical Agriculture (IITA) experimental fields of Malamadori and Minjibir, near Kano, Nigeria. Among 40 significant QTLs, 26 were associated with days to first flower under short days while 14 were associated with days to first flower under long days explaining between 5 and 9% of the phenotypic variation.

Garcia-Oliveira et al. (2020) reported a total of 30 QTLs accounting for 1.8 to 13.0% phenotypic variation for pod and seed traits using DArT markers (Table 1). Some major QTLs for number of peduncles per plant (qPeN2.2), pod length (qPoL3), seed breadth (qSB4), seed length (qSL7.2), and seed thickness (qST9) were discovered on chromosomes 2, 3, 4, 7, and 9 using a bi-parental $F_{2:3}$ population. Some QTLs for these traits were clustered especially on chromosomes 5, 7, 8, 9, and 10. More recently, Angira et al. (2020) reported one major QTL (qDTF9.1) for days to first flower and one major (qPH9.1) and a minor (qPH4.1) QTLs for plant height (PH) explaining 29.3 and 29.5% of the phenotypic variation (PVE), respectively using a dense SNP linkage map.

QTL MAPPING FOR GRAIN QUALITY TRAITS

The efforts of breeders are generally focused on improving yields, both by improving the resistance to biotic and abiotic stresses and increasing the maximum obtainable yields. However, cowpea is a consumer good bought and sold along a supply chain stretching from the original producers to the end-use consumers (Langyintuo et al., 2003). Consumers are generally unaware of the constraints on production and are thus focused on other traits of interest to them, usually visible ones termed consumer-related traits. This disparity between producers and consumers regarding the preferred characteristics can result in breeders, who mostly interact with producers, developing new varieties which do not meet the preferences of consumers. This can lead to new varieties not being accepted by the public, resulting in lower adoption rates. Breeders therefore need to understand the genetic control of consumer-related traits so that they can be taken into consideration from the stage of selection of parental lines in the breeding

Table 1. QTLs affecting some traits in cowpea.

Quantitative trait	Pedigree	Type	Marker type	Chromosome location	PV%	Nb QTL	Reference
Root-knot nematode (*Meloidogine* spp.) resistance	CB27 x 24- 125B-1	RIL	SNP	LG13, LG14, LG15, LG16	-	4	
	IT84S- 2049 x UCR779	$F_{2:3}$	SNP	LG19		1	Huynh et al. (2016)
	IT93K- 503- 1 x UCR779	$F_{2:3}$	SNP	LG14		1	
Root-knot nematode (*Meloidogyne* spp.)	524B x IT84S- 2049	RIL	SNP	LG9	-	1	Santos et al. (2018)
Fusarium wilt resistance (for race 4)	IT93K-503-1 × CB46,	RIL	SNP	LG8	19-47	1	Pottorff et al. (2014)
	CB27 × 24-125B-1	RIL	SNP	LG9	32-40	1	
	CB27 × IT82E-18	RIL	SNP	LG3	18-27	1	
Striga resistance	Gorom x Tvx 3236	F2	AFLP-markers	LG1, LG6	-	2	Ouédraogo et al. (2002a)
Heat tolerance	CB27 × IT82E-18	RIL	SNP	LG2, LG7, LG6, LG10, LG3	12-18	5	Lucas et al., 2013a
Number of pods per plant	TVu2185 x TVu6642.	F_2	SNP-markers (DArT)	LG8	7.4	1	Garcia-Oliveira et al. (2020)
Number of peduncles per plant	TVu2185 x TVu6642.	F_2	SNP-markers (DArT)	LG2, LG9	10	2	Garcia-Oliveira et al. (2020)
pod length	TVu2185 x TVu6642.	F_2	SNP-markers (DArT)	LG3, LG4, LG5, LG7, LG8, LG10.	1.8-12.2	6	Garcia-Oliveira et al. (2020)
Flower and seed coat color	ZN016 × Zhijiang 28-2	RIL	SNP and SSR	LG8	-	1 each	Xu et al. (2011)
Number of pods per plant	ZN016 × ZJ282	RIL	SSR	LG3, LG2, LG4	11-20	3	Xu et al, (2013)
Days to flowering	524 B × 219-01	RIL	SSR	LG1	6-19	3	Timko et al. (2013)
Days to first flowering	ZN016 × ZJ282	RIL	SNP	LG11, LG10, LG3	10-32	3	Xu et al. (2013)
Hilum-eye type	GEC'xIT98K-476-8	RIL	SNP	LG7, LG9, LG 10	-	3	Brijesh et al. (2022)
Seed coat	MAGIC	RIL	SNP	LG7, LG9, LG10	10.1-75.9	3	Herniter et al. (2019)
Domestication related trait	(JP81610 × JP89083) × JP81610	BC_1F_1	SSR	1–11 for most traits	LG3, LG7, LG8, LG11	3–57	Kongjaimun et al. (2012)
Seed weight	IT2246-4 × TVNuI963	F_2	RFLP	LG 2 LG6	37–53	2	Fatokun et al. (1992)
Peduncle length	TVu2185 x TVu6642.	F_2	SNP-markers (DArT)	LG1, LG7, LG10	3.8- 6.3	3	Garcia-Oliveira et al. (2020)
Seed weight	524B × 219-01	RIL	SSR	LG1, LG2, LG3, LG10	8–19	6	Andargie et al. (2011)
Pod length	(JP81610 × TVnu457) × JP81610	BC_1F_1	SSR	LG1, LG2, LG3, LG4, LG5, LG7, LG8, LG9, LG11	31	9	Kongjaimun et al., 2012
Number of seeds per pod	TVu2185 x TVu6642	F_2	SNP-markers (DArT)	LG8, LG9, LG10	10.4	4	Garcia-Oliveira et al. (2020)
Maturity	IT93K503–1 × CB46	RIL	AFLP	LG7, LG8	25–29	2	Muchero et al. (2010)
100-seed weight	TVu2185 x TVu6642	F_2	SNP-markers (DArT)	LG7, LG8, LG9	3.9- 9.1	3	Garcia-Oliveira et al. (2020)
Flowering time under long-day length	CB27 X IT82E-18	MAGIC RIL	SNP	LG4, LG5, LG9, LG11	31	4	Huynh et al. (2018.)
Seed size	IT82E-18 and IT00K-1263	MAGIC RIL	SNP	LG6, LG8	-	2	Huynh et al. (2018)
Seed size	Eight different population	RILs	SNP	LG5, LG7, LG2, LG6, LG8, LG10	47	10	Lucas et al. (2013b)
Flowering time under short day length	IT84S-2049, CB27, and IT82E-18	MAGIC RIL	SNP	LG1, LG4, LG5, LG9	9-10	4	Huynh et al. (2018)
Seed length	TVu2185 x TVu6642	F_2	SNP-markers (DArT)	LG3, LG5, LG7, LG8	10.3	6	Garcia-Oliveira et al. (2020)
Seed thickness	TVu2185 x TVu6642	F_2	SNP-markers (DArT)	LG5, LG8, LG9, LG10	10	4	Garcia-Oliveira et al. (2020)
Seed breadth,	TVu2185 x TVu6642	F_2	SNP-markers (DArT)	LG4, LG5, LG8, LG10	4.8-13	4	Garcia-Oliveira et al. (2020)
Seed size	524B × 219-01	RIL	SSR	LG1, LG10	-	6	Andargie et al. (2011)

AFLP = Amplified fragment length polymorphism; F_2 = second filial generation; F_3 = third filial generation; LG= linkage group; SNP= single nucleotide polymorphism; SSR= simple sequence repeat; RIL = recombinant inbred line; PV% = phenotypic variation explained by a given QTL; DArT = Diversity Array Technology; MAGIC = Multiparent Advanced Generation Inter Cross.
Source: Authors.

programmes. This would enable the development of new varieties with high yield, biotic and abiotic resistance/ tolerance characteristics and at the same time acceptable to consumers (Herniter et al., 2019).

The seed coat colour, which has been a subject of study for decades, with the genetic elements behind their expression established, is one of the most important qualities of cowpea impacting its attractiveness and introduction to markets (Herniter et al., 2019). In cowpea, the seed coat pattern is a significant consumer-related trait. Consumers make qualitative choices about a product's acceptability, quality, and taste depending on its appearance, according to previous studies (Kostyla et al., 1978; Jaeger et al., 2018). As a result, determining the genetic regulation of seed coat pattern traits would be beneficial to breeding programmes generating novel varieties with a good chance of commercial acceptability. Cowpea displays a variety of seed coat patterns, including varied eye shapes, Holstein, Watson, and full coat pigmentation, among others (Herniter et al., 2019). There are various patterns of seed coat displayed by cowpea and the preferred colours and pigmentations are region specific (Herniter et al., 2019). Colour Factor (C), Watson (W), Holstein-1 (H-1), and Holstein-2 (H-2) are four factors that regulate seed coat pattern as reported by Spillman (1911) and Harland (1919). In a bi-parental population, however, Harland (1919) discovered two H loci, which he dubbed "H-1" and "H-2". Aside individual population-based QTL mapping, few QTL analyses of seed coat patterns have been undertaken using numerous bi-parental populations. Seed coat pattern QTL study utilizing RIL populations revealed a total of 35 SNP loci, all of which were mapped on three chromosomes (Table 1), LG7 (C locus), LG9 (H locus), and Vu10 (L locus) (W locus) (Herniter et al., 2019).

Xu et al. (2011) conducted a study to map flower and seed coat colors using 209 F7:8 RILs with 184 SSRs and 191 SNPs. They identified one locus for each trait and both loci are tightly linked with a genetic distance of 0.4 cM. A similar study on QTL analysis using a bi-parental population for hilum-eye type has been carried out. In this study, three major genes controlling different seed hilum-eye types in cowpea and their three corresponding QTLs were successfully identified. The three genes were designated as W (Watson hilum-eye type), S (Small hilum-eye type), and R (Ring hilum-eye type) and mapped onto chromosomes 7, 9, and 10, respectively (Brijesh et al., 2022). Unfortunately, numerous issues impeded the implementation of QTL-based MAS in cowpea breeding, including the lack of consistent and substantial phenotypic impacts of QTLs in heterogeneous recipient genetic backgrounds (Zhao et al., 2021).

QTL MAPPING FOR RESISTANCE TO BIOTIC AND ABIOTIC STRESSES

During their growth cycles, plants are exposed to various favorable and unfavorable environmental conditions. Such conditions include biotic stresses like insect pest attacks and disease infections, as well as abiotic stresses such as heat, cold, drought, low soil fertility, increased salt levels, and toxic metals and metalloids in the soils. The principal and most frequently encountered climatic conditions that reduce agricultural crop yields are temperature (heat or frost), drought, flood and salt.

Efforts have been undertaken on linkage maps to identify QTLs for resistance to abiotic and biotic constraints in cowpea. For example Huynh et al. (2015) conducted a study on resistance to Aphis craccivora Koch and identified one minor and one major QTLs mapped on linkage groups 1 and 7, respectively using 1,536 SNPs and 92 recombinant inbred lines explaining 5 to 13% and 61 to 66% of phenotypic variations, respectively. More recently, a combination of SSR and SNP markers was used by Kusi et al. (2017) to identify QTL regions for aphid (*Aphis craccivora*) resistance in an F_2 population backcrossing and in a bi-parental recombinant inbred line population and reported a major QTL on chromosome 10 to a position of 11.5 cM. Huynh et al. (2016) reported a major QTL related to resistance to root-knot nematodes on linkage group 11 of distinct mapping populations. To map candidate genes for root-knot nematode resistance, Santos et al. (2018) used 84 F_{10} recombinant inbred lines population and transcriptome alterations in two cowpea near-isogenic lines (NILs). A major QTL, QRk-vu9.1 was discovered on chromosome 9 at position 13.37 cM. Pottorff et al. (2012a) reported a *Fusarium oxysporum* f. sp. *tracheiphilum* race 3 resistance locus (Fot3-1) to a 1.2 cM region and discovered SNP marker 1_1107 as co-segregating during their efforts to develop resistant cowpea varieties. Research conducted by Ouédraogo et al. (2002a) revealed that the genes for *Striga* races 1 and 3 were located on linkage groups one and six of the cowpea genome using AFLP markers (Table 1).

The numbering of cowpea linkage groups changed from 2009 based on the work done by Muchero et al. (2009). Linkage groups one and six became ten and nine, respectively. Ampadu (2017) used SNP markers distributed across the cowpea genome and detected QTLs on linkage group nine associated with *S. gesnerioides* resistance spanning the length of 19.89 cM. Omo-Ikerodah et al. (2008) employed 92 bi-parental recombinant inbred lines to map QTLs for resistance to flower bud thrips using AFLP and SSR markers. In another study, Muchero et al. (2010) reported 9 QTLs accounting for 6.1 to 40.0% of the phenotypic variance (R^2) for resistance to thrips damage. Resistance to blackeye cowpea mosaic potyvirus (B1CMV) and the southern bean mosaic virus (SBMV) were mapped to LG8 and LG6, respectively, whereas resistance to the cowpea mosaic virus (CPMV) and the cowpea severe mosaic virus (CPSMV) were mapped to the opposite ends of LG3 (Ouédraogo et al., 2002b).

Agbicodo et al. (2010) reported three QTLs for bacterial

blight resistance, CoBB-1, CoBB-2, and CoBB-3 on LG3, LG5 and LG9, respectively showing that potential resistance candidate genes co-segregated with CoBB resistance phenotypes. Two of the QTLs (CoBB-1 and CoBB-2) were confirmed in the two experiments explaining 22.1 and to 17.4% of phenotypic variation for the first and second experiments while CoBB-3 was discovered for the first experiment with less phenotypic variation explained of about 10%. Miesho et al. (2019) used a cowpea linkage map of 41,948 SNP markers to identify candidate genes associated with resistance to bruchid using a set of 217 mini-core cowpea accessions. Using plant mortality data from 3 years of field experiments and disease severity scores from two greenhouse trials, Muchero et al. (2011) reported QTL associated with *Macrophomina phaseolina* resistance as Mac-1 located on LG2, Mac-2, Mac-3, and Mac-4 on LG3, Mac-5 on LG11, Mac6 and Mac-7 on LG5, and Mac-8 and Mac-9 on LG6. Ohlson et al. (2018) also used a bi-parental F_2 population and genotyped with 99 newly created allele-specific polymerase chain reaction (AS-PCR) markers for QTL analysis and found one major and three minor QTLs for resistance to brown blotch on LG2, LG3, LG6, and LG8. Similarly, Ibié et al. (2021) used parents, F_1, F_2, and BC_1F_1 progenies and discovered QTLs linked to brown blotch resistance. Ten QTLs were found using a RIL population established from a hybrid between IT93K-503-1 (tolerant) and CB46 (sensitive) that differed in their tolerance to seedling-stage drought (Muchero et al., 2009). Some of these QTLs coincided with QTLs for stem greenness (stg) and recovery dry weight (rdw) after drought stress under greenhouse and field conditions. The 10 QTLs were located on LG1, 2, 3, 5, 6, 7, 9, and 10 and accounted for between 4.7 and 24.2% of the phenotypic variance.

Using RIL population, Pottorff et al. (2012b) discovered a significant QTL that affects cowpea leaf shape which may potentially influence drought tolerance. Muchero et al. (2013) used phenotypic data from 13 experiments carried out across four countries to conduct association mapping and identified QTLs for delayed senescence, biomass and grain yield in a panel of 383 diverse cowpea accessions and a recombinant inbred line population. Lucas et al. (2013a) reported five genomic regions in a RIL population that explained 11.5 to 18.1% of the phenotypic variation controlling heat tolerance in cowpea. Similarly, Pottorff et al. (2014) discovered three QTLs (Hbs-1, Hbs-2, and Hbs-3) that influence heat stress-induced seed coat browning in cowpea from two RIL populations. The underlying candidate genes encoding ACC oxidase 2 and ethylene-responsive element-binding factor 3 (ERF3) were revealed, and the QTLs explained 9.5 to 77.3% of the phenotypic variation.

CONCLUSION

This paper provides an overview of some of the recent advances in cowpea improvement attributable to molecular markers. Molecular marker applications to cowpea improvement include characterization of its germplasm and analysis of genetic diversity, population structure, as well as QTL analyses which identified genomic regions involved in an array of economically important traits. Some of the traits for which associated molecular markers have been identified following QTL analyses are resistance to abiotic constraints such as drought, heat, and biotic constraints such as bacterial blight, root-knot nematode, viruses, striga and alectra. QTLs with effects on yield and its components as well as grain quality and domestication related traits have been reported. Compared to many other crops, molecular marker assisted breeding has only recently begun in cowpea.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antioxidant activities and GC-MS profiling of fractions of methanol extract of *Andrographis paniculata*

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This study was intended to investigate the antioxidant activities of different solvent fractions (hexane (HEX), chloroform (CHL), ethylacetate (ETHYL) and methanol (MET) fractions) of methanol extract of *Andrographis paniculata* and GC-MS profiling of the most active fraction (MET). The crude methanol extract was fractionated using vacuum liquid chromatography method. Antioxidant activity was evaluated using total phenolic content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), ferric reducing antioxidant potential (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate (ABTS), and Nitric oxide (NO) radical scavenging abilities. GC-MS was conducted to determine phytochemical present in methanol fraction. The results indicated that MET possessed significantly ($p < 0.05$) higher TPC (33.98 ±1.63 mg GAE/g), TFC (15.81 ± 0.9 5mg QUE/g), TAC (21.44 ± 0.29 mg AAE/g) and FRAP (57.87 ± 0.88 mg Fe^{2+}/g) than the other fractions. Also, MET exhibited the highest scavenging (ABTS, DPPH and NO) abilities of all the fractions. The GC-MS profiling of methanol fraction showed abundance presence of 2, 5- octadecadienoic-methyl ester and hexadecanoic acid-methyl ester. In conclusion, various fractions of *A. paniculata* have antioxidant abilities and could be used in diseases associated with free radicals.

Key words: *Andrographis paniculata*, antioxidant, solvent-fractions, GC-MS.

INTRODUCTION

Free radicals are highly unstable reactive molecules with at least single unpaired electrons. At physiological level, they play roles in detoxification pathways, phagocytosis, cell signaling and maintaining homeostasis of apoptosis and cell proliferation via oxidative coordination of DNA-transcribing proteins and cascade enzymes (Droge, 2002; Valko et al., 2007; Neupane and Lamichhane, 2020). However, a condition termed as oxidative stress occurs when the free radicals exceed the antioxidant potential of the human system. Antioxidants are molecular species that scavenge or retard the detrimental role of free radicals in the body (Soetan et al., 2018). Oxidative stress has been linked to the etiology of many diseases like malaria, diabetes mellitus consequent upon generation of excessive free radicals and this necessitated using antioxidant in the management and treatment of these diseases.

Andrographis paniculata is a yearly shrub that grows abundantly in many parts of the world. It is a member of Acanthaceae family and popularly known as “king of bitters” due to it having bitter taste and flavour (Subramanian et al., 2012). It is aboriginal to South Asian

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countries and well used in Africa and Asia for the treatment of malaria, diabetes mellitus, typhoid fever, diarrhea, cough and dysentery (Subramanian et al., 2012). The leaves from this plant have been shown to have many phytochemicals with antioxidant and antimicrobial activities (Khushboo et al., 2023). In Ayurvedic system, this plant formed at least 26 formulations and has been added to the World Health Organization (WHO) monographs on chosen herbal plants, and reported to be effective at disrupting the advancement of influenza epidemic of 1919 in India (Polash et al., 2017). In reference to WHO Monograph, *A. paniculata* should not be taken by lactating or pregnant women and those who are allergic to plant of the family of Acanthaceae. Adverse reactions of the plant include urticaria, vomiting and gastric discomfort (Worakunphanich et al., 2021). This medicinal herb has been shown to have antidiabetic, antioxidant, antimicrobial and antimalarial capacities (Hossain et al., 2014; Ahmad et al., 2020; Khushboo et al., 2023).

Gas chromatography mass spectrophotometry (GC-MS) is 2-in-1 analytical procedure comprising gas chromatography coupled with mass spectrophotometry. Gas chromatography (GC) segregates the compounds in a sample and mass spectroscopy (MS) identifies individual compound. It is widely used to determine various phytochemical components in plant extracts and essential oils (Olivia et al., 2021; Saravanakumar et al., 2021).

Several reported medicinal properties of this plant have necessitated the search for a lead component which may eventually assist in the development of drugs from this plant. Therefore, the present study evaluated the polyphenol contents and antioxidant potential of different fractions of methanol extract of *A. paniculata* and profiling of the most active fraction by GC-MS.

MATERIALS AND METHODS

Leaves of *A. paniculata* used in this work were obtained from farm garden at Ado-Ekiti and identified at Afe Babalola University Ado-Ekiti. They were dried with air at ambient temperature and ground into powdery form.

Chemicals and reagent

Chloroform, ethyl acetate, n-hexane, methane, sodium carbonate, sodium nitrite, aluminium chloride, sodium acetate, ferric chloride, ferrous sulfate and sodium nitroprusside were bought from LOBA chemie, India. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from AK Scientific, USA. Ascorbic acid, quercetin and gallic acid, were gotten from Sigma-Aldrich, USA.

Extraction of sample

Extraction of the sample was done with the protocol of Karigidi et al. (2019). 200 g of powdered *A. paniculata* were soaked in 1000 ml of methanol. The resulting solution was shaken at internal for 48 h and later filtered with filter paper. The concentration was done with rotary evaporator at 40°C, and the extract was subjected to fractionation.

Fractionation of samples

Fractionation was carried out as reported by Karigidi and Olaiya (2020). 40 g of methanol extract was adsorbed using silica gel, packed and successively eluted with hexane, chloroform, ethylacetate and methanol under pressure. The fractions were dried using rotary evaporator to give hexane (HEX), chloroform (CHL), ethylacetate (ETHYL) and methanol (MET) fractions. These fractions were used for further analyses.

Total phenolic content (TPC)

TPC of the fractions was evaluated with the procedure of Kim et al. (2003). Shortly, 1.0 ml (mg/ml) of the fractions was added to 1.0 ml (10%) of Folin-Ciocalteu phenol reagent. 5 min later, 5.0 ml of 7% Na_2CO_3 and 5.0 ml of distilled water were added and mixed together. The mixture was permitted for 90 min in the dark at ambient temperature. Spectrophotometric reading was measured at 750 nm and TPC was determined from gallic acid calibration curve as mg GAE/100 g.

Total flavonoid content (TFC)

TFC of the fractions was assayed with Park et al. (2008). Fraction, 0.3 ml (mg/ml) was added to 3.4 ml (30%) of methanol, 0.15 ml (0.5 M) of $NaNO_2$ and 0.15 ml (0.3 M) of $AlCl_36H_20$. Five minutes later, 1 ml of 1 M NaOH was introduced. The spectrophotometric reading was measured at 506 nm and TFC was quantified from Quercetin calibration graph as mg QUE/100 g.

Total antioxidant capacity (TAC)

TAC of the fractions was evaluated with phosphomolybdate method as described by Prieto et al. (1999). Fraction, 0.4 ml (mg/ml) and 4.0 ml of phosphomolybdate reagent (0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate) were added together. The mixture was put in water bath for 90 min at 95°C. The mixture was allowed to cool to ambient temperature and spectrophotometric reading was conducted at 695 nm. The TAC was determined from ascorbic acid standard graph as mg AAE/100 g.

Ferric reducing antioxidant potential (FRAP)

FRAP of the fractions was determined with the procedure of Benzie and Strain (1996). Freshly prepared FRAP reagent activated at 37°C was used. Fraction, 0.2 ml (mg/ml) was mixed with 2.80 ml of the FRAP reagent and incubated in the dark for 30 min. Spectrophotometric reading was taken at 593 nm and FRAP activity was quantified from $FeSO_4$ calibration graph as mg Fe^{2+}E/100 g.

2, 2-diphenyl-1-picrylhydrazyl scavenging activity (DPPH)

The DPPH of the fractions assayed with the Gyamfi et al. (1999) method. After reconstitution of sample, 1.0 ml (0.1 - 0.4 mg/ml) was added to 4 ml of freshly prepared DPPH solution (30 mg/l) in

Table 1. Phenolics, Flavonoids, TAC and FRAP of Hexane, Chloroform, Ethylacetate and Methanol fractions of Methanol extract of *Andrographis paniculata*.

Fraction	Phenolics (mg GAE/g)	Flavonoids (mg QUE/g)	TAC (mg AAE/g)	FRAP (mg Fe^{2+}E/g)
Hexane	9.74± 0.93^{d}	6.58±0.95^{c}	11.39±1.65^{b}	11.95±0.53^{c}
Chloroform	11.35±1.18^{c}	10.37±0.16^{b}	12.36±0.77^{b}	6.48±0.58^{d}
Ethylacetate	16.22±2.06^{b}	14.51±0.65^{a}	23.09±3.35^{a}	19.96±0.28^{b}
Methanol	39.88±1.63^{a}	15.81±0.95^{a}	21.44±0.29^{a}	57.87±0.88^{a}

Data were expressed as Mean ± SD. Numbers with different superscript across the same column are different ($p < 0.05$) significantly.
Source: Authors

methanol. The mixture was shaken and allowed to stay in unlighted area for 1800 s. Spectrophotometric reading was taken at 520 nm. The percentage inhibition was determined as:

Percentage inhibition of DPPH = {(Ab control - Ab Sample) / (Ab Control)} × 100

DPPH reagent was used as control.

2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate radical scavenging ability (ABTS)

The ABTS of the fractions was assayed with the procedure of Re et al. (1999). ABTS reagent was prepared with 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in unlit arena for 16 h and spectrophotometric reading at 734 nm was adjusted to 0.700 with ethanol. Fraction, 0.2 ml (mg/ml) was introduced to 2.0 ml ABTS reagent and permitted to incubate for 15 min. Spectrophotometric reading at 734 nm and inhibition percentage was evaluated as:

Inhibition percentage of ABTS = {(Ab control- Ab Sample) / (Ab Control)} × 100

ABTS reagent was used as control.

Nitric oxide radical scavenging ability (NO)

The NO was assayed using the protocol of Mondal et al. (2006). Sodium nitroprusside (10 mM) prepared in 10 mM, pH 7.4 phosphate buffer was added to 1.0 ml (0.1 - 0.4 mg/ml) of the fraction and incubated for 150 min at 37°C. After that, 1.0 ml of fresh Griess reagent (1% sulfanilic acid and 0.1% naphthylethylene diamine dihydrochloride in 2% phosphoric acid) was added. Spectrophotometric reading was taken at 546 nm and percentage inhibition was determined.

Percentage inhibition of NO = {(Ab control- Ab Sample)/ (Ab Control)} × 100

Reaction mixture without extract was used as control.

GC-MS chromatography

The GC-MS was assayed as described by Karigidi and Olaiya (2020). An aliquot (1 ml) of methanol was pipetted into the column with 350°C as the temperature of injector. The temperature of the oven was initiated at 60°C and held for 120 s till it got to 260°C. Holding was permitted for 9 min at a program rate 5°C min^{-1} till 280°C. The temperatures of detector and injector were put at 280 and 250°C, respectively. Temperature of ion source was maintained at 200°C. The mass spectrum of components in the fraction was derived by electron ionization at 70 eV and the detector was operated in scan mode from 45 to 450 atomic mass units (amu). A scan interval of 0.5 s and fragments from 45 to 450 Da was maintained. The accumulated processing period was 54 min. Elucidation of GC-MS mass spectrum was done with the database of National Institute Standard and Technology (NIST) showing more than 62,000 patterns. The spectrum of the component in the fraction was compared with the known components stored in the NIST library.

Statistical analyses

Results were presented as the mean ± SD of three measurements, analyzed using ANOVA, and the means were separated by least significant difference ($p < 0.05$). Pearson correlation test established correlations between polyphenols and antioxidant abilities.

RESULTS

The results of polyphenols (phenolics and flavonoids) and some antioxidant abilities (TAC and FRAP) are shown in Table 1. The phenolics content scoped from 9.74 to 39.88 mg GAE/g. The hexane fraction has the lowest phenolics content while the methanol has the highest content. The differences are significant ($p < 0.05$) when compared with each other. Also, the flavonoids content of the various fractions ranged from 6.58 to 15.81 mg QUE/g. The trend is the same as phenolics but no significant ($p < 0.05$) difference was noted between ethyl acetate and methanol fractions. The results of TAC and FRAP were presented in Table 1. The TAC ranged between 11.39 and 23.09 mg AAE/g, ethyl acetate fraction has the highest activity while the hexane fraction has the least activity; no significant difference was noted between ethyl acetate and methanol fraction. The results of antioxidant ability (ABTS, DPPH and NO) of different fractions of *A. paniculata* are presented in Figures 1, 2 and 3, respectively. The fractions inhibited the ABTS radical in concentration dependent manner (Figure 1) and their IC_{50} calculated (Table 2). In this study, all the fractions inhibited DPPH in a concentration-dependent order and the IC_{50} was calculated (Table 2). The highest

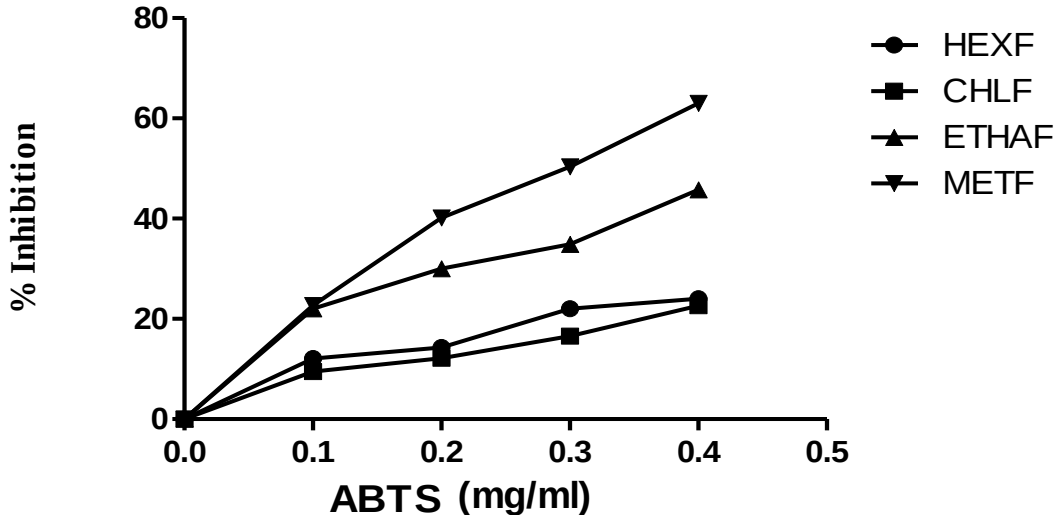

Figure 1. ABTS scavenging activities of fractions of methanol extract of *A. paniculata.*
Source: Authors

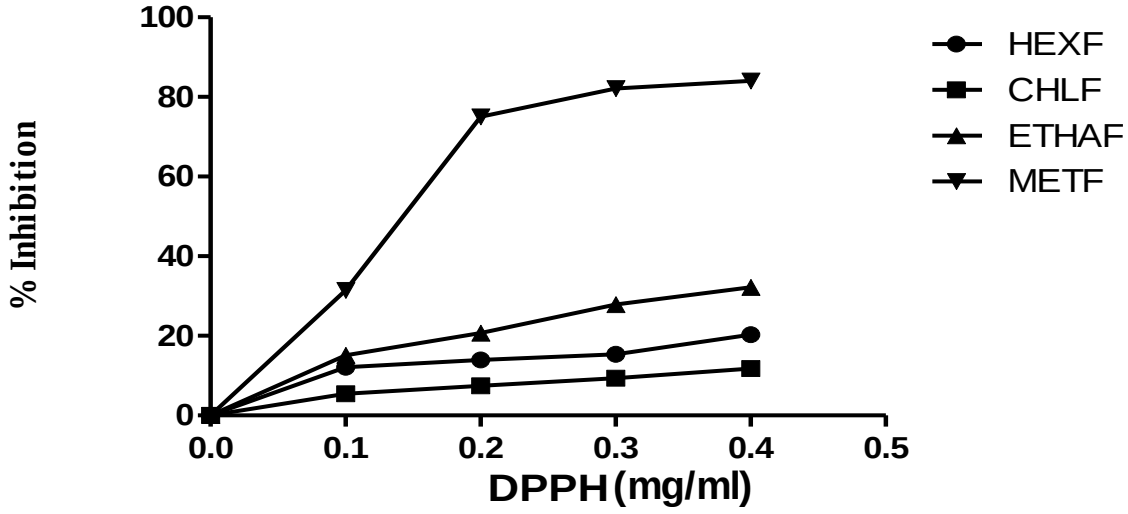

Figure 2. DPPH scavenging activities of fractions of methanol extract of *A. paniculata*.
Source: Authors

inhibition was found in methanol fraction while the lowest inhibition was found in the hexane fraction. The potential of the fractions to retard the nitric oxide radical generation is presented in Figure 3. The methanol fraction has the lowest IC_{50} (0.22 mg/ml) while hexane fraction has the highest IC_{50} (0.55 mg/ml). Pearson correlation was done to establish the association between antioxidant abilities and polyphenol (Table 3). There was positive and significant ($p < 0.05$) relationship between total phenolics and all the antioxidant assays. The same trend was observed for total flavonoids except that the relationship was not significant for DPPH. The GC-MS profiling of the methanol fraction of *A. paniculata* indicated abundant presence of 2-Monolinolenin, 2TMS derivative, p-Cresyl glycidyl ether and allyl acetate (Table 4 and Figure 4).

DISCUSSION

Phenolics compound are one of the secondary metabolites of plant; they are derived from phenylalanine and tyrosine. They exhibit various biological functions through their antioxidant capacity. They are very effective as chain breaking agent and this antioxidant-like activity is consequent upon the presence of their reactive phenol ring. The value of phenolics obtained in this study is

Table 2. IC_{50} (mg/ml) of the Hexane, Chloroform, Ethylacetate and Methanol fractions against ABTS, DPPH and NO scavenging activities.

Fraction	ABTS	DPPH	NO
Hexane	0.89 ± 0.09^{c}	1.25 ± 0.17^{d}	0.55 ± 0.05^{c}
Chloroform	0.94 ± 0.05^{c}	1.60 ± 0.10^{c}	0.46 ± 0.04^{c}
Ethylacetate	0.51 ± 0.02^{b}	0.60 ± 0.05^{b}	0.30 ± 0.02^{b}
Methanol	0.30 ± 0.01^{a}	0.15 ± 0.01^{a}	0.22 ± 0.02^{a}

Data were expressed as Mean ± SD. Numbers with different superscript across the same column are different ($p < 0.05$) significantly.
Source: Authors

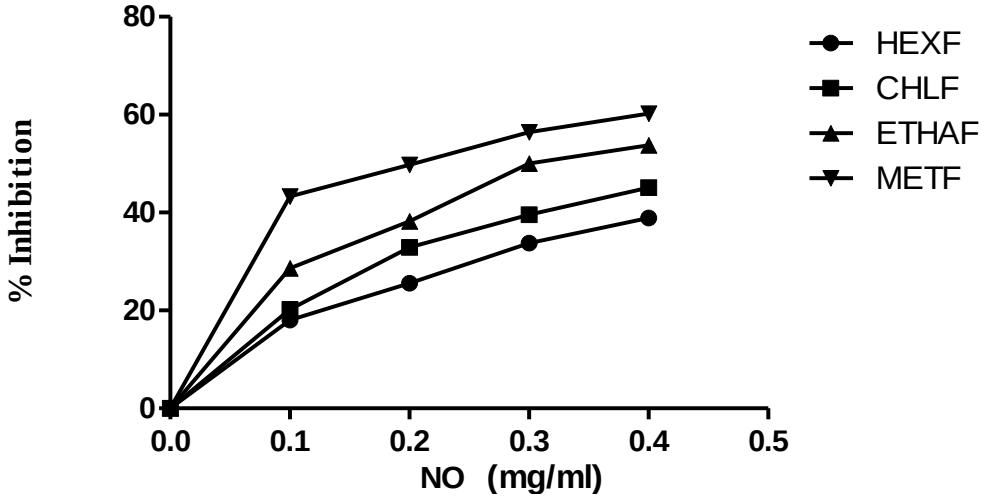

Figure 3. NO scavenging activities of fractions of methanol extract of *A. paniculata*.
Source: Authors

Table 3. Correlations among TP, TF, TAC, FRAP, ABTS, DPPH and NO.

Correlation	TP	TF	TAC	FRAP	ABTS	DPPH	NO
TP	1						
TF	0.63*	1					
TAC	0.60*	0.74*	1				
FRAP	0.98*	0.55*	0.63*	1			
ABTS	0.70*	0.54*	0.62*	0.71*	1		
DPPH	0.85*	0.43	0.52*	0.89*	0.88*	1	
NO	0.74*	0.60*	0.60*	0.72*	0.93*	0.78*	1

Correlation is significant at the 0.05 level.
Source: Authors

lower than the ones reported by Ahmad et al. (2020) for methanol and acetone extracts of the plant.

Flavonoids are polyphenolic component of plant widely found in human diets; they have prominent antioxidant capacities with many physiological benefits (Adetuyi et al., 2018). The presence of hydroxyl group at position C-3 and C-4 of the B ring of this phytocompounds is responsible for its antioxidant potentials. The flavonoid contents of the present study are lower when compared with what was reported by Borgohain and Kakoti (2019) for the methanol extract of *A. paniculata*.

Antioxidants are molecular entities that inhibit or slow down the actions of reactive nitrogen species (RNS) and reactive oxygen species (ROS) that lead to chronic and

Table 4. Chemical profiling of Methanol fraction of *A. paniculata*.

RT	Compound detected	MF	MW	PA
17.50	Acetic acid, methyl ester	$C_3H_6O_2$	74	1.51
34.50	Allyl acetate	$C_5H_8O_2$	100	11.48
38.00	2-(2-Hydroxyethoxy) ethyl acetate	$C_6H_{12}O_4$	148	2.12
41.00	2-Monolinolenin, 2TMS derivative	$C_{27}H_{52}O_4Si_2$	496	17.66
41.61	p-Cresyl glycidyl ether	$C_{10}H_{12}O_2$	164	14.24
42.00	2,6- Dimethoxy-4-(2-propenyl)- phenol	$C_{11}H_{14}O_3$	194	6.41
43.25	Oleic acid	$C_{18}H_{34}O_2$	282	7.91
44.50	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	5.78
45.50	Phytol	$C_{20}H_{40}O$	296	3.49
46.52	4-Ethylbenzoic acid, 4-hexadecyl ester	$C_{25}H_{42}O_2$	374	6.23
48.98	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	7.07
49.36	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	4.54
53.26	2H-Pyran,2-(7- heptadecyny-loxy) tetrahydro-	$C_{22}H_{40}O_2$	336	4.59
53.50	2,5- Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	290	6.76

MF= Molecular formular; MW= molecular weight; PA= percentage abundance.
Source: Authors

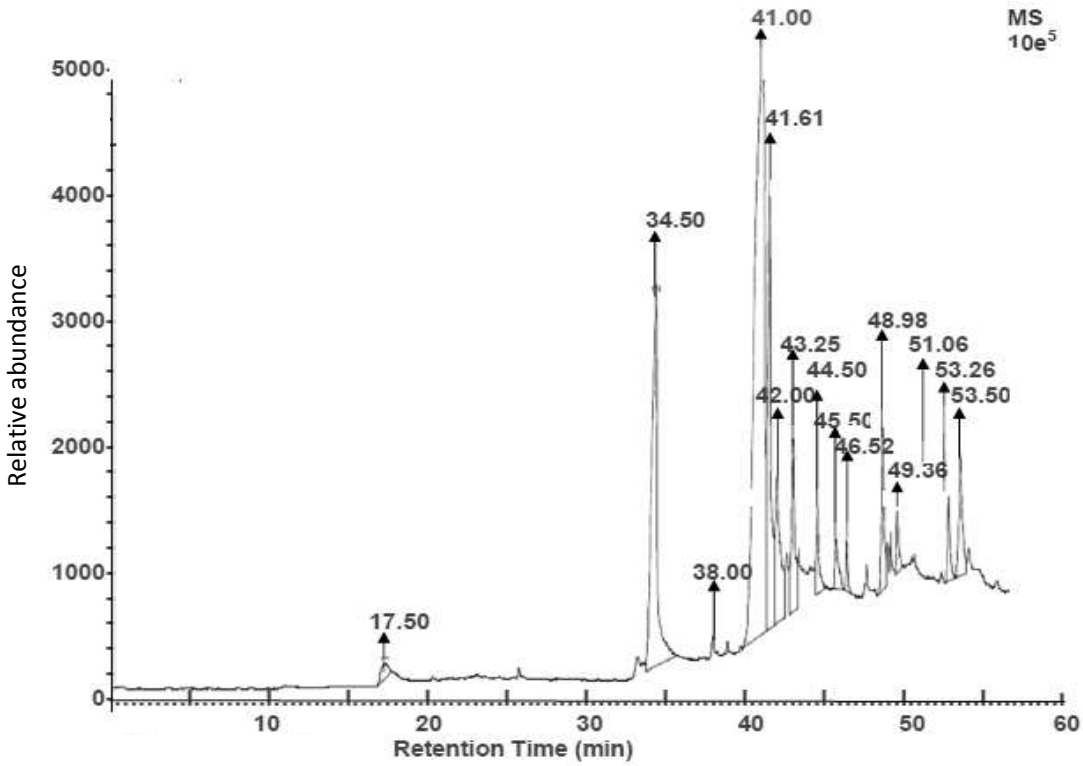

Figure 4. GC-MS chromatogram of methanol fraction of methanol extract of *A. paniculata*.
Source: Authors

degenerative diseases (Soetan et al., 2018). Antioxidant assays are usually of two types; hydrogen atom transfer (HAT) and single electron transfer (SET) assays (Adetuyi et al., 2018). The HAT assays are kinetics based while SET assays measure the antioxidant capacity as a function of colour change when oxidant is reduced.

In this study, SET (ABTS, DPPH and NO) assays are used because of their popularity and accuracy in *invitro*

studies. The TAC relies on the reduction of Molybdate (VI) to Molybdate (V) by an antioxidant species with the development of green phosphomolybdate (V) chromogen. The result obtained in this study had lower values compared to the ones reported by Adetuyi et al. (2018) for aqueous and methanol extract of *Ageratum* conyzoides.

The FRAP is based on the capacity of an antioxidant compound to attenuate iron from the ferric (Fe^{3+}) state to the ferrous (Fe^{2+}) state. The methanol fraction has significantly higher activities than other fractions. The FRAP activity of methanol extract was higher than the one reported for *Rhododendron arboreum* flowers (Kashyap et al., 2017).

The ABTS antioxidant assay measures the loss of the blue-green chromophore of generated $ABTS^{\cdot}$ when antioxidant is added to it. The loss of colour is proportional to the antioxidant potential of the extract (Alam et al., 2013). The IC_{50} values of both ethyl acetate and methanol fractions were lower than what was reported for aqueous extract of *A. paniculata* by Ismail et al. (2017).

The DPPH scavenging assay evaluates the potential of a reductant to donate hydrogen atom leading to the subsequent loss of its violet colour (Alam et al., 2013). The IC_{50} of the ethyl acetate and methanol fractions were lower while hexane and chloroform fractions were higher than the ones reported by Abdul Rahman et al. (2017) for *A. paniculata* extract.

Nitric oxide radical has been one of the striking founts of oxidative stress in cardiac diseases. It is a reactive radical that charade as oxidative signaling molecule in several biological processes, which include blood pressure regulation and neurotransmission, (Valko et al., 2007). In this study, NO• is developed at physiological pH from sodium nitroprusside. The IC_{50} obtained in this study is greater than the ones reported for different extracts of *A. paniculata* (Borgohain and Kakoti, 2019). This is an indication that the fractions of *A. paniculata* might prevent generation of nitric oxide in the body.

Pearson correlation enabled the determination of the association between polyphenol and antioxidant abilities (Table 3). There was positive and significant ($p < 0.05$) relationship between the total phenolics and all the antioxidant assays. The same trend was observed for total flavonoids except for non-significant relationship observed for DPPH.

The presence of 2, 5- octadecadienoic-methyl ester and hexadecanoic acid-methyl ester might be responsible for the activity of the fraction as Ukwubile et al. (2019) as linked it their presence to anticancer potential.

Conclusion

Fractions of *A. paniculata* possess polyphenolic compounds with antioxidant properties. Moreover, methanol fraction has the highest polyphenols and exhibited the highest level of antioxidant ability among the considered fractions. The profiling of the methanol fraction done with GC-MS revealed abundant presence of 2, 5- octadecadienoic-methyl ester and hexadecanoic acid-methyl ester.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genome-wide association analysis identifies resistance loci for bacterial blight in diverse East African rice germplasm

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***Xanthomonas oryzae pv. oryzae (Xoo),* the causal agent of rice bacterial blight disease has been extensively characterized, and loci against different races identified. Many rice cultivars have been developed and utilized to combat the disease, however, due to the rapid evolution of *Xoo*, several resistances have broken down. The continuous challenge of ever-evolving *Xoo* and the breakdown of resistance in cultivated rice varieties make it even more important to discover new loci to enable sustainable durable deployment of broad-spectrum resistance genes in elite breeding lines. African germplasm can be exploited as reservoirs of useful genetic variation for bacterial blight (BB) resistance. This study was conducted to identify loci associated with BB resistance and new genetic donors for the breeding program. To identify candidate sources of resistance for advancing breeding, four virulent strains of *Xoo* (PXO99, MAI1, BAI3, and *Xoo*3-1) were used to screen 78 East African accessions by genome-wide association studies. The diverse accessions' core genetic base exhibited high resistance to the *Xoo* strains. 50.63% of the accessions were highly resistant to the Philippines strain PX099, while 20.25% were highly susceptible to the virulent West African strain MAI1. Two novel resistant loci significantly associated hotspots were identified using 1901 SNPs. The two hits were located on chromosome 12 (*Xa25*) and Chr. 6 (*Xa7, Xa27, Xa33*). Novel loci were identified that gives a useful basis for more investigation and a wide core genetic pool of high resistance for broad-spectrum resistance for genetic improvement.**

Key words: Genome-wide association, *Oryza sativa,* bacterial blight (BB), *Xanthomonas oryzae*, disease resistance.

INTRODUCTION

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating and economically important diseases of rice (*Oryza sativa* L.) all over the world (Savary et al., 2019). Rice resistance

against BB can be generally divided into two main categories; the qualitative resistance controlled by major resistance (*R*) genes, and the quantitative resistance conferred by multiple minor genes or quantitative trait loci (QTLs) (Ramalingam et al., 2003; Deng et al., 2012; Bossa-Castro et al., 2018).

So far, over 40 R genes that confer qualitative resistance to BB has been identified (Jiang et al., 2020) and 11 of them (*Xa1, Xa3/Xa26, Xa4, xa5, Xa10, xa13, Xa21, Xa23, xa25, Xa27, xa41*) have been cloned successfully by using map-based cloning strategy or knowledge-based molecular screening (Yoshimura et al., 1998; Han et al., 2014; Hutin et al., 2015; Wang et al., 2015; Ji et al., 2018). The *R* genes with comparatively broader spectra of resistance such as *Xa3*, *Xa4*, *Xa7*, *xa13*, *Xa21,* and *Xa23*, have been widely used in rice breeding programs, and many resistant rice cultivars have been released (Huang et al., 1997; Han et al., 2014; Wang et al., 2015; Zhang et al., 2015; Hu et al., 2017; Jiang et al., 2020).

Although the disease resistance conferred by a single *R* gene is usually effective against certain races of the *X. oryzae* pv. *oryzae* pathogen, the resistance is easily breakdown due to greater selection pressure on pathogen evolution. Conversely, the quantitative resistance mediated by QTLs is presumably non-race-specific and is considered more durable (Liu et al., 2016). Thus, it has attracted more attention in the past decades and more than 70 QTLs for BB resistance have been identified (Li et al., 2006; Han et al., 2014; Djedatin et al., 2016; Dilla-Ermita et al., 2017; Zhang et al., 2017; Bossa-Castro et al., 2018).

Deployment of broad-spectrum resistant rice cultivars is considered the most effective and environmentally friendly way to control bacterial blight (Zhang et al., 2017), quantitative resistance has been considered as a preferred strategy to achieve durable resistance although marker-assisted selection has not been effectively used for the improvement of BB resistance in rice. This issue is attributed to the polygenic nature of the trait and each QTL has a small effect. It is therefore difficult to accumulate multiple QTLs with small effects in breeding. In addition, most of the QTLs for BB resistance were identified and mapped using bi-parental population QTL analysis in the past decades. Because of limited molecular markers used and fewer recombinants in a primary mapping population, most of the QTLs for BB resistance are mapped to a region of 10 ~ 30 cM (Yang et al., 2021). Since a prerequisite for successful marker-assisted selection (MAS) is the availability of markers that are closely linked with the target gene, the inaccuracy of QTL mapping hinders the application of MAS. Therefore, the discovery of the large-effect QTLs and the use of a more powerful approach for the genetic dissection of complex traits are crucial to address this issue challenge in rice production.

Genome-wide association studies (GWAS) present another alternative with two main advantages: (1) it can use a nature population instead of a bi-parental population. The rice varieties used in GWAS contain much more genetic diversity than the bi-parental lines used in segregation populations. Because using diverse germplasm for QTL mapping in GWAS, favors the identification of large-effect and novel QTLs; (2) most GWAS can result in a relatively high mapping resolution due to the existence of numerous historical recombination events (Takeda and Matsuoka, 2008) and using plenty of SNPs for association mapping. Therefore, GWAS provides a powerful tool for large-scale and precise identification of QTLs for complex traits like BB resistance in germplasm (Zhao et al., 2011; Han and Huang, 2013; Zhang et al., 2017; Zhai et al., 2018).

In this study, we identified the distribution of BB resistance genes of the East African accessions that are distributed and bred in the regions. We detected two loci on chromosomes 6 and 12 that carry the genes (*Xa7, Xa27, Xa33*) and (*Xa25*), respectively.

METHODOLOGY

Plant materials

The selection of diverse panel breeding lines, varieties, and landraces including accessions of 78 genotypes was collected from accessions obtained from National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda. The diverse germplasm collection was obtained from the East African countries of Uganda, Kenya, Tanzania, Rwanda, and Burundi. The field studies were carried out at Africa Rice Centre Mbe station Cote D'Ivoire in accordance with institutional, national, and international guidelines and legislation. The germplasm used in this research was non-GMOs.

BB resistance screening

The experiment was carried out in a contained screen house facility to prevent the spread of inoculum and was done using RCBD split-plot with nested subplot design. The four *X. oryzae* pv. *oryzae* strains were the main plots and, in each plot, a sub-plot of 78 genotypes was nested into early maturing and medium maturing Genotypes.

The BB resistance screening was performed 6 weeks after sowing. A total of four strains representing different races of *X.*

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Table 1. Four strains of *Xanthomonas oryzae* pv. *oryzae* used in this study.

Race	Strain[a]	Gene conferring Resistance[b]
6	PXO99	*Xa13, Xa21, Xa23, xa24, Xa30, Xa32*
A3	MAI1	*Xa4, Xa5* and *Xa7*
A2	BAI3	*Xa4, Xa5* and *Xa7*
A1	Xoo3.1	*Xa12*

[a]African and Philippine strains to specific *Xoo* races. [b]Naturally occurring *Xa* genes known to confer resistance against specific *Xoo* races based on a review.
Source: Nino-Liu et al. (2006) and Verdier et al. (2011).

oryzae pv. *oryzae* were used, namely: *Xoo*3.1, BAI3, MAI1, and PXO99.

Inoculation of the rice plants was done by cutting 1 to 2 cm of the leaf tip with a pair of scissors dipped in bacteria suspension (Kauffman et al., 1973). The screening was replicated two times over time. For each replicate, ten leaves of two plants per entry were inoculated with the *X. oryzae* pv. *oryzae* strains. Lesion lengths were measured 14 days after inoculation. Genotypes having lesion lengths ranging from 1 to 5 cm were rated resistant (R), 5 to 10 cm were rated as medium-resistant (MR), 10 to 15 cm were rated as medium-susceptible (MS), and those having greater than 15 cm were rated as susceptible (S).

Association mapping

The East African accessions comprising 78 germplasm samples were selected and phenotyped by measuring the lesion length of leaves inoculated with BB races Xoo3.1, BAI3, MAI1, and PXO99.

Linkage analysis and QTL mapping

TASSEL version 5.2 software (Bradbury et al., 2007) was used for manipulating and filtering SNPs for genome-wide association analysis. The initial dataset was filtered based on MAF 1 and 95% call rate. A unified mixed-model approach was deployed to account for population structure and familial relatedness (Yu et al., 2006; Price et al., 2006). A compressed mixed linear model (MLM) was used to analyze association, considering population structure (Q) and relatedness or kinship (K) to reduce spurious associations (Yu et al., 2006).

RESULTS

BB resistance screening and population structure

Four diverse strains representing four different races of *X. oryzae* pv. *oryzae* (Table 1) were used to screen 78 genotypes. The lesion length (LL) distribution in the 78 accessions inoculated with four *Xoo* strains (PX099, MAI1, BAI3, and *Xoo*3.1) showed large phenotypic variation (Table 2). Among the inoculated cultivars, five accessions were highly resistant to all four strains with LL< 5 cm, and only highly susceptible to all four strains with LL≥15 cm (highlighted in white and red, respectively in (Figure 1)). Two accessions from Tanzania (*Afaa Milela* and *Mbawa Mbili*) conferred high resistance to all four strains from the Philippines (PX099), West Africa (MAI1 and BAI3), and the East African strain (Xoo3.1)

Based on the LL of all the accessions, the four *X. oryzae* pv. *oryzae* strains were divided into three groups, namely the West African strain group (BAI3, MAI1), East African (Xoo,3.1), and Philippines strain group (PX099) (Figure 1). According to classification by LL, a large proportion of the accessions were resistant with 39.24, 35.44, 40.51 and 50.63% for strains Xoo3.1, BAI3, MAI1, and PX099 respectively (Figure 1).

The sources of resistant East African germplasm from different backgrounds can provide valuable material for facilitating breeding for BB resistance.

Identification of resistance loci against

To dissect genome-wide associated resistance loci for two West African *X. oryzae* pv. *oryzae* strains, one East African *X. oryzae* pv. *oryzae* strain, and one Philippines *X. oryzae* pv. *oryzae* strain, we performed GWAS with a mixed linear model of the TASSEL ver. 5.2 program, using 2,612 high-quality SNPs and LL as genotype and phenotype data, respectively (Figure 2). Based on the effective number of independent markers, the threshold of significant *P*-value was estimated to be 3.0E-5 by the Bonferroni correction method. In total, we identified 2 QTL within 1901 unique SNPs associated with BB resistance to four strains.

Significant resistance genes hotspots

GWAS results from this study indicated chromosomes 6 and 12 as hotspots for BB resistance. SNPs consistently associated with resistance against several strains were identified by GWAS. Known genes and loci (*Xa*) conferring resistance to specific *Xoo* races (Table 1) were overlaid with the Manhattan plots. Among the known *Xa* loci, the regions on chromosome 6 (*Xa*7, *Xa*27, *Xa*33(t)) and *Xa*25 on chromosome 12 (Wang et al., 2001; Blair et

Table 2. Phenotypic reactions of the East African accessions to four strains of bacterial blight.

Reaction	*Xoo 3.1*		*BAI3*		*MAI1*		*PX099*	
	Count	%	Count	%	Count	%	Count	%
MR	21	26.58	24	30.38	15	18.99	24	30.38
MS	17	21.52	12	15.19	16	20.25	6	7.59
R	31	39.24	28	35.44	32	40.51	40	50.63
S	10	12.66	15	18.99	16	20.25	9	11.39

Source: Authors

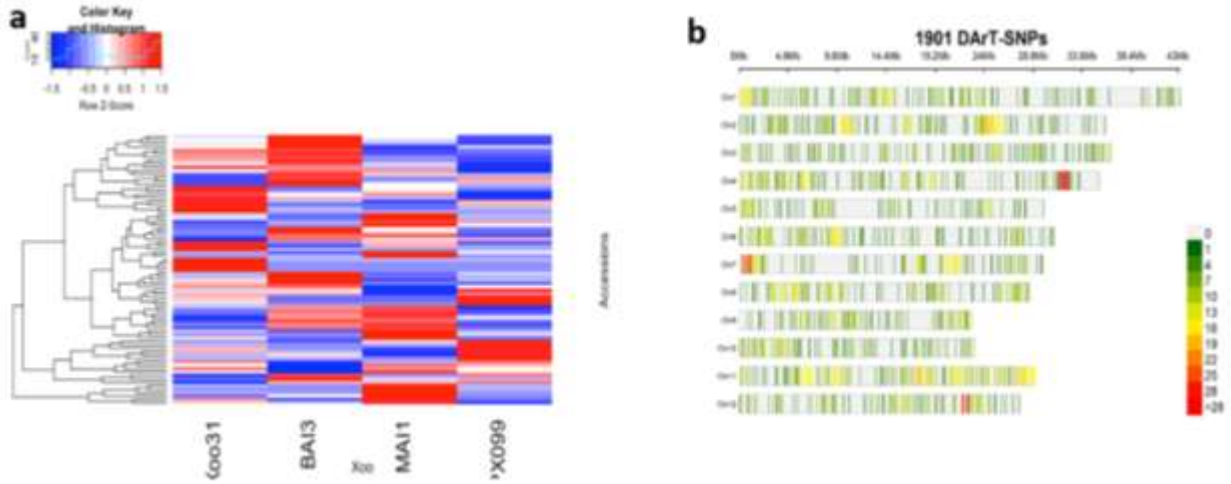

Figure 1. Bacterial blight resistance evaluation of 78 rice accessions inoculated with representative *Xoo* strains from Philippines, West Africa, and Uganda. (a) Hierarchical cluster of accessions and strains based on lesion length (LL). (b) Summary of genotypic SNPs used in the analysis.
Source: Authors

al., 2003; Chen et al., 2008; Chu et al., 2006a, b; Bao et al., 2010; Song et al., 1995; Chen et al., 2002) were identified as overlapping with significant SNPs in this study.

DISCUSSION

This study was conducted to screen and identify disease-resistant rice cultivars, as well as key functional genes applicable for breeding new varieties with broad-spectrum BB resistance. Bacterial blight is one of the most diseases of rice, causing significant yield losses in rice-growing ecologies throughout Africa (Kim, 2018). The current BB management strategies are not effective due to the rapid development of virulent *Xoo* strains (Kim et al., 2015). In Africa where rice bacterial blight outbreaks can be epidemic, new Xoo strains need to be identified and characterized.

In the present study, we identified two accessions from Tanzania (*Afaa Milela* and *Mbawa Mbili*) that are highly resistant to all four *X. oryzae* pv. *oryzae* strains (PX099, MAI1, BAI3, and *Xoo*3.1). The data generated in this study regarding these BB-resistant accessions will be utilized for rice breeding programs.

Application of QTL in rice breeding

Chromosome 11 is known as an important and complex region of the rice genome with respect to BB resistance, containing mapped or finely-mapped BB *R* genes *Xa22(t), Xa30(t), Xa32(t), Xa35(t), Xa36(t), Xa39, Xa40, xa41(t), Xa43(t)* and *xa44(t),* and cloned genes *Xa3/Xa26, Xa4, Xa10, Xa21,* and *Xa23* (https://shigen.nig.ac.jp/rice/oryzabase/).

Recently, many *R* genes for BB were successfully incorporated into both elite inbred varieties and parental lines of hybrid rice to control the disease using MAS (Chukwu et al., 2019; Jiang et al., 2020; Li et al., 2020). Markedly, a few of those *R* genes, such as *Xa3, Xa4, Xa7*, and *Xa21*, have been widely utilized in rice

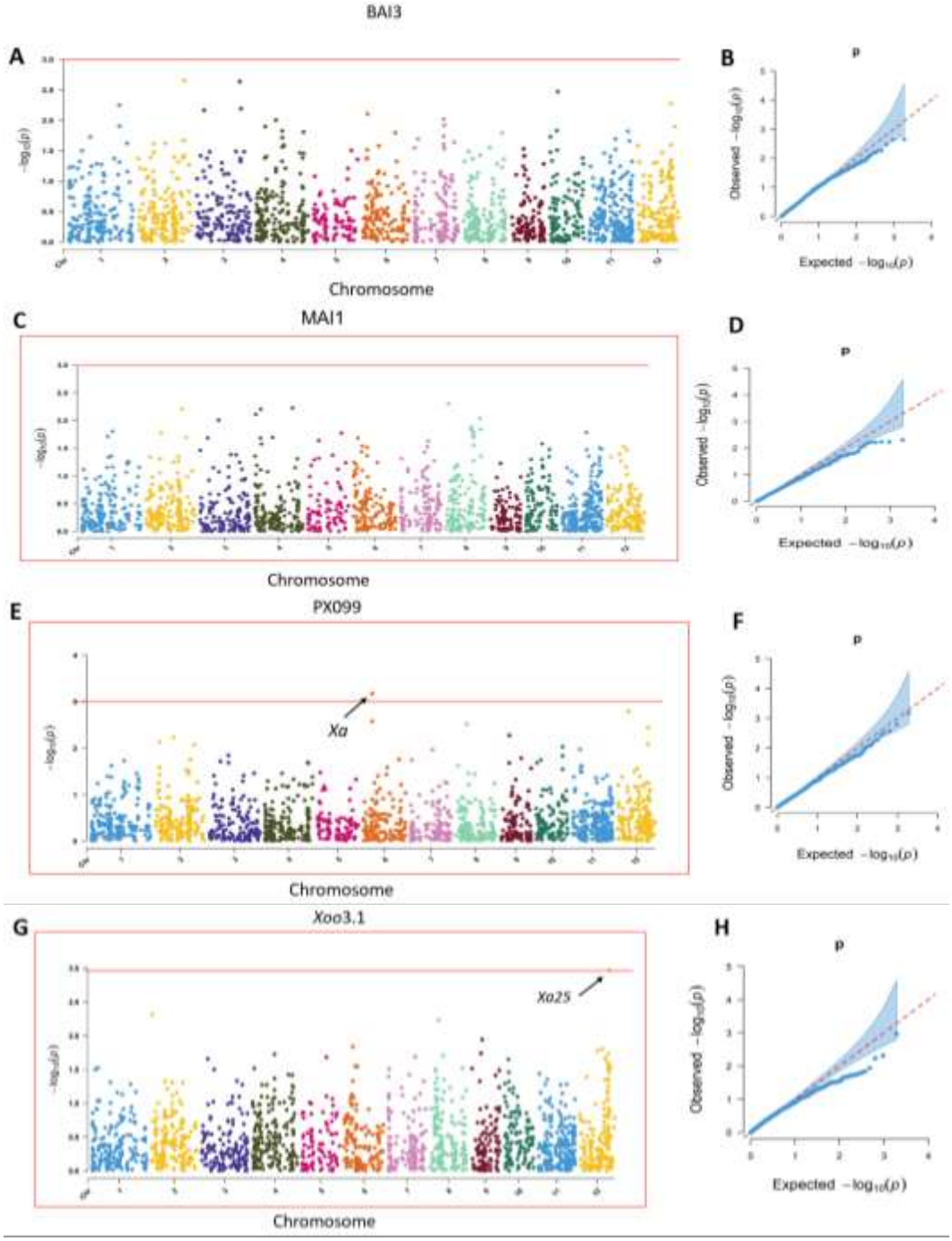

Figure 2. Genome-wide association study of rice resistance to four *Xoo* strains. A, C, E, G Manhattan plots of GWAS results for strain BAI3, MAI1, PX099, and *Xoo*3.1, respectively. B, D, F, H Quantile plots of expected and observed -$\log_{10}$ (*P*-value) for strains BAI3, MAI1, PX099, and *Xoo*3.1, respectively. The horizontal blue line indicates the significant P-value threshold of 3.0E-5. The arrow indicates the reported bacterial blight resistance genes.
Source: Authors

resistance breeding since the 1980s (Deng et al., 2006; Zhang, 2009; Chen et al., 2011; Luo et al., 2012). With the wide deployment of *R* genes and *Xoo*-Rice coevolution, elite *R* genes are being overcome by the

newly emerged *X. oryzae* pv. *oryzae* strains (Zeng et al., 2002; Zhang, 2005).

Conclusion

The results from this GWAS have pinpointed resistance loci conferring differential resistance to the four representative strains of *X. oryzae* pv. *oryzae* in the East African accession. Two resistant loci were identified through this analysis. Using efficient phenotypic data and SNPs from genotyping is a powerful tool to decipher disease resistance in rice. The SNPs associated with *X. oryzae* pv. *oryzae* resistance would be helpful in the development of SNP markers for marker-assisted selection and tracking of known *Xa* genes. Effective monitoring of resistance genes in the breeding pipeline guides breeders on which varieties to deploy to specific areas depending on the *Xoo* population. The full potential of the novel loci identified loci in the East African germplasm will be unraveled through expression profiling. The genotypes that have exhibited a high degree of resistance but have no resistance alleles for specific *Xa* QTLs serve as new sources of resistance loci to plant breeders to diversify the genetic base of core breeding sets. Further analysis of the East African germplasm is recommended to identify potentially novel loci which were not detected in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

BB, Bacterial blight; **GWAS,** Genome-wide association study; **LD,** Linkage disequilibrium; **MAF,** Minor allele frequency; **MLM,** Mixed linear model; **PCA,** Principal component analysis; **QTL,** Quantitative trait loci; ***Xoo*****,** *Xanthomonas oryzae* pv. *oryzae*

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Full Length Research Paper

Expression of bioactive compounds in different pepper cultivars (*Capsicum annuum* L.) in response to different fertilizer treatments

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This research aimed at evaluating four cultivars of *Capsicum annuum* L. with different nutrient sources to ascertain the nutrient source that would improve the soil characteristic and promote the accumulation of carotenoids and flavonoids in peppers. Four nutrient sources: poultry manure, pig manure, goat manure, and nitrogen: phosphorus: potassium (NPK) were used for the study. The experiment was conducted as a 4 × 5 Factorial in a Complete Randomized Design (CRD) at the Botanical Garden of the University of Nigeria, Nsukka. The soils characteristic features were analyzed by standard methods. The fruits biochemical content was quantified with the aid of a High-Performance Liquid Chromatography at the National Research Institute for Chemical Technology, Zaria, Nigeria. The α-carotene level (333.48±0.27 mg/L) was the highest in "Nsukka yellow pepper" fruits grown on soil mixed with goat manure while β-carotene (45.56±0.29 mg/L) was the highest when grown on soil mixed with poultry manure. "Tatase" cv. planted on soil mixed with poultry manure expressed the highest fruit capsanthin level while lutein was highly expressed when grown on soil mixed with goat manure. In conclusion, growing "Nsukka yellow pepper" with goat manure could increase the production of α-carotene, while poultry manure will increase the production of β-carotene.

Key words: Biochemical, inorganic fertilizer, micronutrient deficiency, organic manure, pepper.

INTRODUCTION

Deficiencies in nutrition and their resultant diseases remain prevalent in both the developed and developing world (Popkin et al., 2012). There are reports that about one-third of the world's population suffer deficiencies of vitamins mainly vitamins A and C and essential minerals (such as iodine, iron, and zinc) (Global Nutrition Report, 2014; Abu et al., 2019a) which results in health effects that range from mild to severe. More often than not, these deficiencies often go unnoticed and not tackled until its associated medical condition manifests itself. Because of their invisibility, such deficiencies are widely referred to as "hidden hunger". Hidden hunger poses serious effects because people often do not realize that they are suffering from hidden hunger (Abu et al., 2019a). Pregnant women and young children who show rapid growth and development are the most susceptible to deficiencies of micronutrient and thus, suffer the maximum effects which are usually unpleasant (Meshram et al., 2012; Abu et al., 2019a). However, the production of pepper fruits of high nutritional value will contribute immensely in meeting up with the daily allowances of micronutrients as pepper fruits are consumed by every household either fresh or

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dried but the fresh form is mostly used in preparing delicacy (Abu et al., 2019a).

Pepper (*Capsicum annuum*) fruit is valued for its antioxidant capacity and high composition of bioactive molecules. However, it is ranked worldwide among the most popular fresh vegetables due to its combination of colour, flavour and nutritional value (Blanco-Ríos et al., 2013; Abu et al., 2019b). Pepper fruits are excellent sources of health-promoting molecules, such as ascorbic acid (vitamin C), antioxidants, provitamin A, sugars, carotenoids, and polyphenols (Jadczak et al., 2010). They are also repositories of various phenolics, flavonoids, and carotenoids (Materska and Perucka, 2005). Kelly and Boyhan, (2009) reported that one medium green pepper can provide up to 180% of vitamin C, 2% of iron, 8% of the Recommended Daily Allowance of vitamin A, and 2% of calcium. Lycopene, which protects against cancer is also present in red peppers (Perez-Lopez et al., 2007).

Genes responsible for the expression of nutritional traits are inherent in the DNA of pepper cultivars. However, for optimal expression of these genes, the perfect environment, and agronomic practice must be maintained. Liaven et al. (2008) found that amounts and characteristics of pepper fruits from plants cultivated in soil supplemented with organic manure were generally better than those from plants grown in soil only. Organic manure application has been one of the agronomic practices adopted by farmers to ensure optimal production. Organic fertilizers are more environmentally friendly as compared to chemical fertilizers. For a material to be eligible as an organic fertilizer, the material ought to occur in nature naturally. Generally, organic fertilizer is normally derived from single ingredients, thus, the types of organic fertilizers are derived from either plant, animal, or mineral sources. The organic fertilizers may supply nutrients to the soil but, a different type of source of fertilizer can give some different effects on the plant (Khandaker et al., 2017). Most often, landraces cultivated by farmers are poor in micronutrients due to poor agronomic practices such as nutrients application, planting date, postharvest handling among others. Understanding the best organic treatment that can help boost micronutrients composition in crops (pepper) is indispensable. Therefore, this study seeks to evaluate the biochemical composition of four cultivars of *C. annuum* fruits (“Shombo”, “Tatase”, “Ataragu” and “Nsukka yellow pepper”) grown with different nutrient sources.

MATERIALS AND METHODS

Plant

The fruits of the four cultivars of *C. annuum*: "Shombo", "Tatase", "Ataragu" and "Nsukka yellow pepper” were obtained from the germplasm of Dr. Mrs. N. E. Abu from the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The seeds were extracted by excising the fruits and the extracted seeds were dried under the sunlight.

Preparation of planting medium

Polypots were prepared by filling polythene bags which were perforated at the base to avoid water logging with 12 kg of the different nutrient medium. The different nutrient medium for the experiment was prepared by mixing topsoil with runoff sandy soil and different organic sources (poultry droppings, pig dung, and goat dung) separately at a ratio 3:2:1, respectively and allowed to cure for a period of seven days. While the polypots for nitrogen, phosphorus and potassium (NPK) fertilizer and the control were filled with topsoil and run off sandy soil (3:1) without mixing the organic source. Nevertheless, 30 g of NPK 20:10:10 was applied two weeks after transplanting using the ring method of application (Olatunji and Agele, 2015).

Planting

The seeds of the different cultivars were nursed separately in nursery baskets filled with topsoil mixed with poultry manure and run off sandy soil (3:2:1) and watered daily (Ojua et al., 2019; Abu et al., 2019a). After a period of 6 weeks, the seedlings were then transplanted into different poly pots filled with soil mixed with different organic manure. The plants were grown in the Botanical Garden of the University of Nigeria, Nsukka using a 4 × 5 factorial experimental laid out in a completely randomized design (CRD) with 15 replications for every treatment. The factors were the cultivars and the different nutrient medium.

Soil (planting medium) analysis

Following standard procedures, the five nutrient medium (soil) were analyzed for both the chemical {pH in water (H_2O) and potassium chloride (KCl) (McLean, 1982), organic matter (Nelson and Sommers, 1996), exchangeable cations (calcium, magnesium, sodium, potassium) (Chapman, 1965), exchangeable acidity (H^+), available phosphorus (Olsen and Sommers, 1982), total nitrogen (Bremner and Mulvaney, 1982), cation exchange capacity (CEC) (Hendershot and Duquette, 1986), and base saturation (Mclean, 1982)} and physical {Percentage particle size of clay, silt, fine sand, and coarse sand were determined by the Bouyoucos hydrometer method after destroying organic matter using hydrogen peroxide (H_2O_2) and dispersing the soil with sodium hydroxides (NaOH) in place of Calgon {sodium hexametaphosphate ($NaPO_3$)} (Bouyoucos, 1951; AOAC, 2005)} properties at the Laboratory of the Department of Soil Science, University of Nigeria, Nsukka.

Carotenoids and flavonoids quantification

At maturity, fully ripened fruits were harvested and bulked into separate groups according to the cultivars grown on the different medium for biochemical analysis. The bulked groups were further divided into 3 sets, deseeded and analyzed for carotenoids and flavonoids composition. The characterization and quantification were performed with the aid of a High-Performance Liquid Chromatography (HPLC) at the National Research Institute for Chemical Technology (NARICT) Zaria, Kaduna State, Nigeria.

Carotenoids extraction from fresh fruit samples was done according to the methodology of Bureau and Bushway (1986). Pepper fruit samples were chopped into small pieces, and a 10 g subsample was immediately combined with 1 g of magnesium carbonate ($MgCO_3$), 5 g of sodium sulphate (Na_2SO_4), and 125 mL tetrahydrofuran (THF) stabilized with 0.01% butylated hydroxytoluene (BHT). Each sample was homogenized with a Tekmar tissumizer (Cincinnati) for 5 min on medium speed. Samples were vacuum filtered (Whatman #42), and the residue was

Table 1. Physical and chemical characteristics of soil and nutrient mixtures used in this research.

Physio-chemical properties	Poultry mixture	Pig mixture	Goat mixture	NPK mixture	Control
Clay (%)	7	7	7	7	7
Silt (%)	4	4	4	4	4
Fine sand (%)	30	34	36	48	45
Coarse sand (%)	59	55	53	41	44
Textured class	Sandy, clay, loam (SCL)	SC L	SCL	SCL	SCL
Soil pH (in H_2O)	9.1	8.1	7.3	7.6	7.5
Soil pH (in KCl)	8.2	7.2	6.5	6.5	6.8
Total carbon (%)	0.890	0.890	1.122	0.697	0.658
Total organic matter (%)	1.535	1.535	1.935	1.201	1.134
Total nitrogen (%)	0.154	0.126	0.154	0.154	0.070
Exchangeable sodium (meq/100 g of soil)	0.03	0.03	0.05	0.03	0.03
Exchangeable potassium (meq/100 g of soil)	0.06	0.06	0.08	0.06	0.06
Exchangeable calcium (meq/100 g of soil)	2.00	2.20	2.80	1.80	2.20
Exchangeable magnesium (meq/100 g of soil)	1.40	1.20	1.20	0.60	0.60
Exchangeable aluminum (meq/100 g of soil)	-	-	-	-	-
Exchangeable hydrogen (meq/100 g of soil)	1.20	1.00	1.40	2.00	1.20
Cation exchange capacity (meq/100 g of soil)	8.40	6.40	15.20	14.80	4.80
Base saturation (%)	41.55	54.53	27.17	16.82	60.21
Available phosphorus (ppm)	40.02	53.16	72.75	60.62	51.30

Source: Authors

re-extracted with an additional 125 mL THF. A 20 mL portion of the sample was concentrated under nitrogen gas and stored at -20°C until HPLC analysis where the samples were resuspended in 0.4 mL THF and vortexed.

All samples were filtered through a nylon Magna 0.22 µm filter into HPLC vials for injection. The HPLC (Waters C 18 column, Shimadzu, Japan) analysis was done to confirm the presence and quantity of specific carotenoids and flavonoids. The column conditions were HPLC column of waters C18 symmetry, column (4.6 × 250 mm), and waters 600 pump 7725 rheodyne 7725 injector, waters 2487 dual-wavelength UV absorbance Detector 230n m. The mobile phase components (acetonitrile: water) was used in a gradient form, which varied with a change in time and a flow rate of 1.2 mL/min.

The column temperature was set at 40°C. The range on the photodiode array detector was also set at 380 to 550 nm, with maximum detection at 454 nm. The carotenoids were identified by their absorption spectra captured by the photodiode array detector, and HPLC retention times in comparison with authentic standards. Also, samples were spiked with standards (Sigma-Aldrich, European reference standard) to verify the identity of sample peaks with similar retention times.

Total carotenoids were quantified as capsanthin equivalents and β-carotene by using an authentic standard. All standards were handled under low light on the ice. Standard solutions of β-carotene were prepared in 20 mL THF, and capsanthin standards in methanol: acetonitrile (1:1). Aliquots were diluted in methanol: acetonitrile (1:1) to provide standard concentrations ranging from 2 to 10 µg/L with a detection limit of 0.1 µg/L.

Statistical analysis

Data collected from the research work were analyzed using Genstat Discovery Edition 4 to get the Analysis of Variance (ANOVA) and Least Significant Difference (LSD) was used to separate the means at $P \leq 0.05$ level of significance.

RESULTS AND DISCUSSION

The percentage clay and silt composition of the nutrient medium were similar while soil mixed with poultry manure and NPK had a higher percentage of fine and coarse sand. Similarly, the soil pH in both water and potassium chloride, exchangeable magnesium was higher in poultry manure mixed soil, while the goat manure had the highest percentage concentration of total carbon, organic matter, exchangeable sodium, potassium, calcium, available phosphorus and cation exchange capacity (Table 1). On the whole, the organic nutrient sources were better in the available organic carbon, organic matter, exchangeable calcium, magnesium, and pH when compared with the NPK and the control. This is an indication of a sufficient supply of essential nutrient elements by the organic nutrient sources to bridge the deficiency gap in the soil used (Baiyeri et al., 2016). Following the recommendation of Baiyeri et al. (2016) that higher soil pH can help bridge the putative nutrient deficiency gap of the soil, poultry, and pig manure would be more useful in bridging the nutrient deficiency gap in the soil than the commercial NPK fertilizer. The total nitrogen recorded in poultry and goat manure was comparable to NPK fertilizer. Olatunji and Agele (2015) had earlier linked the increase in plant agro-

Table 2. Effect of *Capsicum annuum* L. cultivars and nutrient sources on mean value of fruits carotenoids.

Cultivar	Nutrient sources	α-carotene (mg/L)	β-carotene (mg/L)	Capsanthin (mg/L)	Lutein (mg/L)	Total carotenoids (mg/L)
Atarugu	Poultry	$0.91^{g} \pm 0.03$	$0.97^{e} \pm 0.01$	$0.46^{f} \pm 0.01$	$0.00^{f} \pm 0.00$	$11.14^{l} \pm 0.12$
	Pig	$0.42^{ghi} \pm 0.02$	$0.48^{fg} \pm 0.02$	$0.15^{f} \pm 0.08$	$0.00^{f} \pm 0.00$	$5.10^{l} \pm 0.03$
	Goat	$0.63^{gh} \pm 0.01$	$0.86^{ef} \pm 0.02$	$0.39^{f} \pm 0.01$	$0.00^{f} \pm 0.00$	$13.78^{l} \pm 0.01$
	NPK	$0.00^{i} \pm 0.00$	$0.00^{i} \pm 0.00$	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$	$2.28^{l} \pm 0.05$
	Control	$0.00^{i} \pm 0.00$	$0.00^{i} \pm 0.00$	$0.00^{f} \pm 0.00$	$0.00^{f} \pm 0.00$	$190.13^{j} \pm 3.95$
Shombo	Poultry	$26.89^{d} \pm 0.02$	$0.16^{ghi} \pm 0.09$	$2.32^{f} \pm 0.02$	$0.09^{ef} \pm 0.05$	$70.36^{k} \pm 1.00$
	Pig	$0.12^{hi} \pm 0.01$	$0.02^{hi} \pm 0.003$	$0.13^{f} \pm 0.003$	$0.07^{ef} \pm 0.01$	$0.67^{l} \pm 0.01$
	Goat	$0.10^{hi} \pm 0.003$	$0.4^{gh} \pm 0.29$	$0.16^{f} \pm 0.01$	$0.09^{ef} \pm 0.003$	$1.47^{l} \pm 0.02$
	NPK	$137.88^{b} \pm 0.78$	$0.23^{ghi} \pm 0.03$	$164.19^{b} \pm 0.54$	$0.23^{def} \pm 0.13$	$933.08^{g} \pm 7.07$
	Control	$0.04^{hi} \pm 0.03$	$0.22^{ghi} \pm 0.06$	$0.20^{f} \pm 0.12$	$0.08^{ef} \pm 0.04$	$915.72^{g} \pm 0.76$
Tatase	Poultry	$76.06^{c} \pm 0.38$	$0.41^{gh} \pm 0.25$	$213.13^{a} \pm 0.66$	$0.08^{ef} \pm 0.03$	$571.6^{i} \pm 0.39$
	Pig	$0.18^{hi} \pm 0.03$	$0.04^{hi} \pm 0.02$	$82.55^{cd} \pm 28.45$	$0.35^{de} \pm 0.19$	$839.29^{h} \pm 3.59$
	Goat	$0.07^{hi} \pm 0.03$	$25.09^{c} \pm 0.09$	$168.07^{b} \pm 0.42$	$255.4^{a} \pm 0.26$	$928.94^{g} \pm 0.47$
	NPK	$0.00^{i} \pm 0.00$	$0.01^{i} \pm 0.01$	$100.72^{c} \pm 0.79$	$0.06^{ef} \pm 0.03$	$2184.21^{a} \pm 41.7$
	Control	$0.13^{hi} \pm 0.06$	$2.55^{d} \pm 0.05$	$67.74^{d} \pm 0.81$	$0.08^{ef} \pm 0.04$	$2053.28^{b} \pm 10.74$
Nsukka yellow	Poultry	$7.53^{f} \pm 0.17$	$45.56^{a} \pm 0.29$	$93.74^{c} \pm 0.16$	$45.20^{c} \pm 0.14$	$1247.78^{c} \pm 11.26$
	Pig	$9.08^{e} \pm 0.04$	$29.56^{b} \pm 0.29$	$46.32^{e} \pm 0.18$	$0.49^{d} \pm 0.09$	$1081.32^{d} \pm 4.11$
	Goat	$333.48^{a} \pm 0.27$	$0.26^{ghi} \pm 0.13$	$5.60^{f} \pm 0.16$	$185.16^{b} \pm 0.26$	$955.51^{f} \pm 0.71$
	NPK	$0.06^{hi} \pm 0.03$	$0.09^{ghi} \pm 0.07$	$9.67^{f} \pm 0.17$	$0.06^{ef} \pm 0.03$	$1000.53^{e} \pm 1.13$
	Control	$0^{i} \pm 0$	$0.05^{hi} \pm 0.03$	$0.03^{f} \pm 0.01$	$0.03^{f} \pm 0.03$	$923.77^{g} \pm 0.46$
LSD		0.59	0.39	18.21	0.30	19.15

Values are presented as mean ± standard error and significant means are separated with different alphabets on the same column using Least Significant Difference Test (F-LSD) at P≤ 0.05.
Source: Authors

morphological traits to the availability of nitrogen that helps in plant growth and development. Therefore, poultry and goat manure would be a reliable replacement of the inorganic fertilizer for the supply of nitrogen required for plant growth and development.

Significant (P ≤ 0.05) variations were observed across the nutrient sources and cultivars for the different carotenoids concentration (Table 2). While α-carotene was the highest in Nsukka yellow planted on soil mixed with goat manure, β-carotene was the highest in Nsukka yellow planted on soil mixed with poultry manure. Tatase cultivar planted on soil mixed poultry manure expressed the highest Capsanthin level while Lutein was highly expressed in Tatase cultivar planted on soil mixed with goat manure. This could be an indication that the availability of some nutrient sources supported the synthesis of some of the carotenoids in some of the cultivars. These observations were in harmony with the works of Antonius et al. (2014), which reported that concentration of carotenoids and antioxidant content in the fruits of *C. annuum* varied and were significantly dependent on soil treatment. Ha et al. (2007) and Guzman et al. (2010) also indicated that concentrations of carotenoids in pepper fruits are highly dependent on soil nutritional factors, growth stage of fruit and also the colour of fruits, while Sarafi et al. (2018) asserted that, the concentration of carotenoid for a given cultivar depends mainly on the morphological and physiological characteristics of that cultivar in addition to certain growth factors. From the results of this research, the organic nutrient sources especially the poultry and goat nutrient source was generally better than the inorganic and control in enhancing the production of different carotenoids. This observation conforms with the work of Wu et al. (2013), who reported antioxidant activity under organic fertilization were higher than under mineral fertilization. Therefore, the presence of various major and minor elements in organic fertilizers may have contributed to the increase in secondary metabolites and antioxidant potentials as compared to the case of mineral fertilizers that contain only three basic minerals which include; Nitrogen, Potassium, and Phosphorous (Ibrahim et al., 2013).

Variations in the concentration of different flavonoids such as myricetin, quercetin, kaempferol, and luteolin, including the total flavonoids across the cultivars and

Table 3. Effect of *Capsicum annuum* L. cultivars and nutrient sources on mean value of fruits flavonoids.

Cultivar	Treatment	Myricetin (mg/L)	Quercetin (mg/L)	Kaempferol (mg/L)	Luteolin (mg/L)	Total flavonoids (mg/L)
	Poultry	$721.10^{a} \pm 4.55$	$0.11^{de} \pm 0.06$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$727.12^{b} \pm 0.11$
	Pig	$460.86^{b} \pm 0.34$	$285.97^{a} \pm 0.95$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$724.80^{b} \pm 0.46$
Atarugu	Goat	$165.30^{c} \pm 0.21$	$0.19^{de} \pm 0.10$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$179.35^{h} \pm 0.39$
	NPK	$0.05^{f} \pm 0.04$	$0.14^{de} \pm 0.01$	$24.13^{c} \pm 0.16$	$3.13^{b} \pm 0.06$	$54.38^{l} \pm 0.29$
	Control	$0.00^{f} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$91.84^{i} \pm 0.20$
	Poultry	$0.08^{f} \pm 0.04$	$0.03^{e} \pm 0.02$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$527.89^{e} \pm 2.66$
	Pig	$0.002^{f} \pm 0.0003$	$0.004^{e} \pm 0.0003$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$285.49^{g} \pm 1.16$
Shombo	Goat	$0.00^{f} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$54.77^{l} \pm 0.24$
	NPK	$0.00^{f} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$415.09^{f} \pm 0.13$
	Control	$0.001^{f} \pm 0.001$	$0.29^{de} \pm 0.02$	$0.00^{d} \pm 0.00$	$6.95^{a} \pm 0.39$	$895.55^{a} \pm 2.11$
	Poultry	$6.34^{e} \pm 0.17$	$0.0003^{e} \pm 0.0003$	$42.31^{a} \pm 0.34$	$0.00^{d} \pm 0.00$	$693.97^{c} \pm 0.53$
	Pig	$72.20^{d} \pm 0.21$	$181.73^{b} \pm 0.53$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$599.42^{d} \pm 1.59$
Tatase	Goat	$0.00^{f} \pm 0.00$	$0.002^{e} \pm 0.0001$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$81.46^{k} \pm 0.63$
	NPK	$0.002^{f} \pm 0.001$	$0.59^{de} \pm 0.02$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.61^{o} \pm 0.01$
	Control	$0.00^{f} \pm 0.00$	$0.82^{d} \pm 0.02$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$3.69^{n} \pm 0.07$
	Poultry	$5.19^{e} \pm 0.04$	$0.002^{e} \pm 0.001$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$6.15^{n} \pm 0.09$
	Pig	$0.003^{f} \pm 0.0003$	$0.14^{de} \pm 0.03$	$25.03^{b} \pm 0.04$	$0.28^{c} \pm 0.01$	$84.96^{j} \pm 0.41$
Nsukka yellow	Goat	$5.5^{e} \pm 0.25$	$0.31^{de} \pm 0.04$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$10.08^{m} \pm 0.14$
	NPK	$0.002^{f} \pm 0.001$	$9.22^{c} \pm 0.26$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$9.51^{m} \pm 0.12$
	Control	$0^{f} \pm 0$	$0.001^{e} \pm 0.001$	$0.03^{d} \pm 0.006$	$0.03^{cd} \pm 0.003$	$5.29^{n} \pm 0.09$
LSD		2.93	0.72	0.24	0.26	2.63

Values are presented as mean ± standard error and significant means are separated with different alphabets on the same column using Least Significant Difference Test (F-LSD) at P≤ 0.05.
Source: Authors

nutrient sources were observed (Table 3). Myricetin concentration was significantly the highest in Atarugu cultivar grown with poultry manure; quercetin was the highest in the same cultivar grown with pig manure, while kaempferol was the highest in Tatase grown with poultry manure (Table 3). Generally, the nutrient sources affected the flavonoids concentrations differently. More so, the organic nutrient sources favoured the synthesis of more flavonoids than the control and NPK fertilizer. This report corroborates the works of Mohd et al. (2013) that phenolics and flavonoids were enhanced by 12 and 22%, respectively in treatment with organic fertilizer as compared to inorganic fertilization. Zhang and Hamauzu (2003), Marinova et al. (2005) and Navarro et al. (2006) also emphasized that the concentration of flavonoids and phenols depends greatly on cultivation, ripeness, storage, and soil salinity. The positive influence of organic fertilizers in producing crops with enhanced phytochemicals and bioactive compounds was also asserted in the reports of Mitchell et al. (2007). They observed that organic crop management practices led to the increase in flavonoids content in tomatoes and also an increase in the levels of antioxidant as was analyzed in strawberry. The variations recorded among the cultivars in this research are an excellent indication of the variations abounds in the concentration of different flavonoids in different cultivars of pepper. This observation could be attributed to the genotypic variation inherent in the DNA of these pepper cultivars. Araceli et al. (2011) reported similar findings, that apart from the fruit shape and appearance, pepper fruits also vary in their content of vitamin C, phenols, flavonoids, beta-carotene, capsaicin, and dihydrocapsaicin depending on the ripeness stage. This report is also in agreement with the works of Sarafi et al. (2018), who opined that genetic and abiotic stress may be responsible for the overall phytochemical concentration in peppers. Therefore, genotypes respond differently to an organic treatment.

Within the scope of this research, it could be established that growing crops with organic nutrient sources would be more productive; they also cost less amount in acquisition when compared with the inorganic fertilizer and most importantly, they contribute to a safer environment with less pollution than the inorganic nutrients. Invariably, consumption of pepper fruits with high concentration of the micronutrients most especially

the carotenoids and flavonoids would help combat a lot of latent but dangerous diseases that could result to serious health challenges because pepper fruits with high concentration of these micronutrients will help provide the Recommended Daily Allowances (RDA) of these micronutrients when consumed in our diets.

Conclusion

Poultry and goat manure were found to increase soil nutrients and could be reliable replacement of the inorganic fertilizer for the supply of nitrogen required for plant growth and development. The manure sources influenced the production of bioactive compounds in peppers. On the average, poultry manure supported the synthesis of bioactive compounds more. Growing Nsukka yellow with goat manure increased the production of α-carotene, while poultry manure increased the production of β-carotene. Growing Tatase on poultry manure increased the production of capsanthin levels while goat manure increased the production of lutein. Poultry manure favoured the production of myricetin in Atarugu, while pig manure increased the production of quercetin and kaempferol was the highest in Tatase grown with poultry manure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Contact activities of *Piper guineense* (Schum and Thonn) and *Eugenia aromaticum* (L). (Merril and Perryl) extracts against larvae of hide beetles, *Dermestes maculatus* (Degger) (Coleoptera: Dermestidae)

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Contact activities of *Piper guineense* (Schum and Thonn) and *Eugenia aromaticum* (L). (Merril and Perryl) extracts in the control of *Dermestes maculatus* larvae infesting stored fish (*Clarias gariepinus*) were investigated under laboratory condition (28±3°C and 65±5% RH). The extracts were tested by application of 2.0 µL each to ten third instars larvae using micro pipette at a concentration of 6.00, 10.00 and 20.00% each of the plant extracts (methanol, n-hexane and ethyl acetate). Mortality was recorded at 1, 2, 3 and 7 days of post treatments. The observed mortality was dose and exposure-dependent. All the extracts significantly enhanced larval mortality (P>0.05) when compared with the control. The n-hexane and ethyl-acetate extracts of *P. guineense* at 20% concentration induced the highest mortality of 86.66%, lowest mortality of 56.66% was observed on methanol fraction treated larvae after 7 days of post treatments. The n-haxane of *E. aromaticum* extracts recorded highest mortality (80.0%), followed by ethyl-acetate (76.66%) and methanolic (7.00%) fractions treated larvae at 20% concentration after 7 days of post-treatments. The results showed strong insecticidal activity in control of larvae of hide beetles infesting dried fish.

Key words: Plant extracts, *Piper guineense*, *Eugenia aromaticum*, *Dermestes maculatus.*

INTRODUCTION

Dermestes maculatus (Hide Beetle, Degeer) (Coleoptera; Dermestidae) is one of the most destructive insect pests of stored smoked-dried fish in Nigeria (Tejumade, 2019). These pests generally infest dried fish during storage, transportation and marketing, thus responsible for extensive damage to marketed fish leading to enormous weight loss ranging from negligible amount to 50% weight loss (Don-Pedro, 1989; Amadi and Dimkpa, 2018).

Tejumade (2019) reported *D. maculatus* account for about 71.5% of the observed infestation with substantial loss of about 43 to 62.7% in dry weight depending on the length of storage.

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The control of this pest in Nigeria is primarily dependent on repeated application of synthetic chemicals such as chloripyripos-methyl, permetrin, cypermetrin, BHC, and “Otapiapia” (locally formulated) onto fish carton for protection against insect pest (Igene et al., 1998; Abolagba et al., 2011). Although many synthetic chemicals are effective the general use of such chemicals to protect stored fish has been hampered by the report of health hazard, high cost of purchase, lack of availability, illiteracy of fish handler for right application and less susceptibility of Dermestid larvae (Booke et al., 2001; Amusan and Okorie, 2002). With these demerits of synthetic chemicals, currently world-wide interest is centered on search for alternative pesticide to stored product by the use of botanical pesticide.

Botanical pesticide tends to have broad spectrum activity relatively specific in their mode of action and easy to process and use (Viglianco et al., 2008). To minimize use of synthetic pesticide, several plants’ extracts have been reported as effective against *D. maculatus* on dried fish by several researchers (Fasakin, 2003; Akinwumi et al., 2006, 2007; Akpotu and Adebote, 2013; Olayinka, 2014). These extracts provide a solution to the problem emanating from the use of synthetic chemicals. The present studies have been chosen to investigate the effects of methanolic extracts fractions of *Piper guineense* and *Eugenia aromatica* against *D. maculatus* larvae as an alternative strategy to synthetic chemicals method of pest control.

MATERIALS AND METHODS

Collection, identification of plant material and preparation of plant powders

The sample of dried fruit of *P. guineense* and *E. aromatica* was obtained from Sokoto Central Market, Nigeria. The plants were identified and authenticated in the Herbarium of Biological Sciences Department of Usman Danfodiyo University, Sokoto. Voucher specimens (UDUH/ANS/0258 and 0221) were deposited. Samples were milled into fine powders using mortar and pestle, sieved with 0.2 mm mesh following the methods of Adedire and Lajide (2000), Akinwumi et al. (2006) and Jose and Adesina (2004). Each of the plant powders was labeled and kept in a separate plastic container and placed in a cool dry place prior to use.

Preparations of feed

The samples of dried fish *Clarias gariepinus* were purchased from fish mongers at Sokoto Central Market, identified and authenticated in Hydrobiology Laboratory, Biological Sciences Department, Usmanu Danfodiyo University, Sokoto. The fish samples were disinfected by heat treatment in the laboratory-drying cabinet at 60°C for 1 h and allowed to cool at room temperature as adopted by Onu and Baba (2003).

Collection of hide beetle and maintenance of insect culture

Different stages of hide beetle were obtained from naturally infested fish collected from Sokoto Central Market fish stalls. Several adult pairs of *D. maculatus* were obtained and kept in transparent plastic containers (19.0 cm height and 21.2 cm in diameter) fed with dried fish. The containers were covered with Muslin cloth and tightened with rubber band. Wet cotton wool was supplied in each jar to provide water requirements for oviposition as suggested by Hill (1990). The adult laid eggs were hatched into larvae and changed into pupae, which were picked up and transferred into separate containers to obtain newly emerged adult, which were used for regular supply of larvae for the experiment in line with Akinwumi et al. (2006).

Preparation of methanol extracts and solvent fractionation

Four hundred grams of *E. aromatica* and *P. gueneense* were homogenized with 95% methanol (1 L) in plastic container and kept at room temperature for 24 h and filtered. The methanol crude extract was collected and concentrated almost to dryness in drying cabinet at 40°C for 48 h. The dried extracts were stored in freezing medium until used for fractionation (Akinwumi et al., 2006).

The dried methanol crude extract of *E. aromatica* (19.47 g) and *P. guineense* (26.17 g) were suspended in distilled water and then partitioned with 500 ml of n-Hexane, ethyl acetate and water in increasing order of polarity, following the method of Bakele et al. (2016).

Effect of extract fractions on *D. maculatus*

Effects of each plant extract fraction were conducted according to Talukder and Howse (1994). 20% stock solution was prepared for each solvent (methanol, n-haxane and ethyl acetate). Lower concentrations (6 and 10%) were obtained from dilution of the stock solution with distilled water. Ten third instars larvae were chilled for 5 min to immobilize them and then picked up individually by the use of camel hair brush and 2 ul of each of the solution was applied to the dorsal surface of the larvae. Experiments were in three replicates (each replicate contains ten treated larvae). In addition, the same number of larvae (10) was treated with distilled water only as control. After treatment, insects were transferred into transparent plastic containers (19.0 cm height and 21.2 cm in diameter) containing dried fish. Observations were made daily and those that did not move or respond to gentle touch were considered dead. Mortality was recorded at 1st, 2nd, 3rd and 7th days of post treatment.

Data analysis

Data obtained were subjected to one-way analysis of variance (ANOVA) using General Linear Model (SPSS, 2019) and means found to be significant were separated using Duncan multiple range test at 5% level of significance ($p<0.05$).

RESULTS

Effects of methanol fractions on mortality of *D. maculatus* larvae

The effect of *P. guineense* methanolic extracts applied to *D. maculautus* larvae by topical application is presented in Table 1. All the three extracts of *P. guineense* exhibited insecticidal activity against *D. maculatus* larvae as dose and time-dependent variables. At day 1, all the

Table 1. Mortality among *D. maculatus* larvae by topical application with *P. guineense* methanolic fractions.

Fraction	No. of Larvae introduced (n)	Mean larval mortality ± SE					
		Period of exposure (in days)					
		Concentration (%)	1st	2nd	3rd	7th	Mortality (%)
Methanol	10	20.00	2.33±0.88^{bcde}	3.33±0.88^{bc}	4.33±0.33^{cd}	5.66±0.88^{cd}	56.60
	10	10.00	2.33±0.33^{bcde}	3.33±0.88^{bc}	4.33±1.20^{cde}	5.66±0.33^{cde}	56.60
	10	6.00	1.33±0.66^{cde}	1.66±0.88^{cd}	2.00±1.73^{ef}	3.33±1.20^{ef}	33.30
N-hexane	10	20.00	4.66±0.33^{b}	6.33±0.33^{ab}	8.00±0.57^{ab}	8.66±0.66^{bc}	86.60
	10	10.00	4.33±1.45^{b}	6.00±2.08^{abc}	7.00±2.08^{abc}	8.00±2.00^{abc}	80.00
	10	6.00	1.00±0.57^{cd}	2.00±0.57^{cd}	3.66±0.33^{cde}	5.66±0.33^{de}	56.60
Ethyl-acetate	10	20.00	6.67±0.88^{a}	8.00±0.57^{a}	8.33±0.66^{ab}	8.66±0.33^{bc}	86.60
	10	10.00	4.33±0.33^{b}	5.66±0.33^{ab}	5.66±0.33^{bcd}	7.33±0.33^{abc}	73.30
	10	6.00	3.66±0.88^{bc}	4.00±1.15^{bc}	5.66±0.33^{bcd}	6.3388^{bc}	63.30
Cypermetrin	10	0.05	3.00±0.57^{bcd}	6.33±0.33^{ab}	9.33±0.33^{a}	9.66±0.33^{a}	96.60
Control	10		0.00±0.00^{e}	0.33±0.46^{d}	1.33±0.33^{e}	1.33±0.33^{e}	13.30

Means that have the same super script within a column are not significantly different at 5% level using Duncan's multiple range test.
Source: Authors.

three extracts of *P. guineense* showed less than 50% mortality of larvae except ethyl-acetate extract at highest concentration (20%) which caused 66.70% mortality of larvae. At 2nd and 3rd days, mortalities in all the treatment at all concentrations increased as compared to day 1 of exposure. The ethyl-acetate extract at 20% concentration remains the highest (80%) morality of larvae. However, at the 7th day of exposure, all the three extracts at all concentrations except 6% concentration of methanol showed a significant ($p<0.05$) mortality of larvae when compared with the control. The ethyl-acetate extract recorded the highest mortality range of 63.30 to 86.66%, followed by n-Hexane (56.60-86.6%) and methanol extracts (33.30-56.60%).

The contact activity of *E. aromatica* methanolic extracts fractions applied *D. maculatus* larvae are presented in Table 2. All treatments except 6% concentration of methanol and 10 and 6% of n-Hexane were significantly more toxic than control at 1st day of exposure. Efficacy was dosage-dependent with significant higher mortality occurring with increase in dosage. No mortality occurred in the control (0.00%). Highest mortality was recorded in ethyl-acetate extract at 20% concentration with a percentage mortality of 63.30%. At the 2nd day of exposure, only 6.0% concentration of methanol extract was statistically similar ($p>0.05$) with the control; all other treatments showed significant mortality of larvae when compared with the control. Highest mortality was recorded in ethyl-acetate (63.33%) followed by n-Hexane (56.60%) and methanol (53.30%). However, at the 3rd day of exposure, mortality in all the treatments followed a similar trend with the 2nd day of exposure with ethyl-acetate extract which was the highest with a mortality range of 50 to 70%. At the 7th day of exposure, the highest mortality of 80.00% was recorded from n-Hexane fraction at 20% concentration. Other concentration of n-Hexane also showed higher mortality of larvae of 53.30 and 46.66%. In addition, the ethyl acetate and methanol extract fraction recorded mortality of larvae ranging from 53.30 to 76.60% and 43.30 to 70.00%, respectively.

DISCUSSION

In this study, the three extracts of *P. guineense* demonstrated contact efficacy to *D. maculatus* larvae. The results indicated that insecticidal activity of *P. guineense* extract fraction varied depending on the organic solvent used for extraction, concentration and exposure time. Among the extracts applied, ethyl-acetate fractions were the most toxic against *D. maculatus* larvae followed by n-Haxane and methanolic fractions. The efficacy of the ethyl acetate and n-Haxane could be attributed to their oily appearance, in contrast to the solid methanolic extracts, which is similar to the finding of Ajayi and Peter (2016) who reported extract of *P. guineense* yielded oil which has been reported to be very effective in the control of stored product pest. The use of plant extract and other forms of plant materials as insect pest control and management of stored food products have been reported by several researchers (Fasakin, 2003; Adebote et al., 2006; Akinwumi et al., 2007; Olayinka, 2014; Fasakin, 2003) and it has been shown that oil extracts obtained from *P. guineense*, *Monodora myristica*,

Table 2. Mortality among *D. maculatus* larvae by topical application of *E. aromaticum* methanolic fractions.

Fractions	No. of Larvae introduced (n)	Mean larval mortality ± SE					
		Period of exposure (days)					
		Concentration (%)	1st	2nd	3rd	7th	Mortality (%)
Methanol	10	20	3.33±o.33^{ed}	5.33±0.33^{abc}	6.00±1.15^{b}	7.00±1.15^{bcd}	70.00
	10	10	4.44±0.66^{bc}	4.00±0.57^{bcd}	5.66±0.33^{b}	6.33±0.66^{bcd}	63.30
	10	6.0	1.33±0.33^{efg}	1.33±0.33^{ef}	2.66±0.66^{cd}	4.33±0.66^{e}	43.30
N-Hexane	10	20	5.33±0.33^{ab}	5.66±0.33^{ab}	6.33±0.33^{b}	8.00±1.00^{ab}	80.00
	10	10	1.00±0.66^{fg}	3.33±0.66^{cd}	4.66±0.66^{bc}	5.33±0.88^{cde}	53.30
	10	6.0	1.66±0.88^{defg}	2.33±0.88 ^{de}	2.33±0.88^{d}	4.66±0.33^{de}	46.60
Ethyl-acetate	10	20	6.33±0.66^{a}	6.33±0.57^{ab}	7.0±1.15^{b}	7.66±0.57^{abc}	76.66
	10	10	4.33±1.20^{bc}	6.00±0.57^{ab}	6.33±0.33^{b}	7.00±0.33^{bcd}	70.00
	10	6.0	2.00±0.57^{def}	4.33±1.20^{abcd}	5.00±1.15^{b}	5.33±0.88^{cde}	53.30
Cypermetrin	10	0.05	3.00±0.57^{cde}	6.33±0.33^{a}	9.66±0.33^{a}	9.66±0.33^{a}	96.60
Control	10	0.00	0.00±0.00^{g}	0.33±0.33^{f}	0.66±0.33^{d}	1.33±0.33^{f}	13.30

Means that have the same super script within a column are not significantly different at 5% level using Duncan's multiple range test.
Source: Authors.

and *African melegueta* were highly effective in controlling various stages of *D. maculatus* on smoked fish during storage. The oil extracts of these plants were 100% effective when compared with 16.7% in untreated (control) smoked fish. Adebote et al. (2006) observed that 0.025 ml g^{-1} of oil from *Detarium microcarpum* seed produced 85.56 to 96.67 mortality of *Dermestes* larvae within 24 and 96 h of post treatment. Okonkwo and Okoye (2001) reported 100% mortality of larvae of *D. maculatus* when treated with extracts of *Dennettia tripetala* and *P. guineense* at a dosage lower than powder. Akinwumi et al. (2007) reported that ethanol extract of *D. tripetala* and *P. guineense* resulted in 100% mortality of *D. maculatus* larvae after 3 days of post treatment. Ajayi (2015) stated that acetone extract is more effective in reducing oviposition and adult emergence of *Callosobrunchus maculutus* than methanolic and ethanolic extracts of the same plant, while methanolic and ethanolic extracts were significantly more effective than aqueous extract.

In the current study, higher activity of ethyl acetate fraction observed might be due to the presence of polar and no polar bioactive component against larval stage of *D. maculatus*, as ethyl acetate is a semi polar solvent that had the ability to extract polar and nonpolar compounds in the extract of *P. guineense.* Variation in the bioactivities of different solvent fractions observed in the study confirmed the finding of Sun et al. (2001). That crude extract that was screened with ethyl acetate, n-butyl alcohol and water fractions of alcoholic extract of leaves and stem of *Vanilla fragrans* against *Culex pipiens* larvae showed that n-butyl alcohol and ethyl-acetate fractions were active in the bioassay, while the water fraction appeared to contain no substance that inhibited the larval growth (Overgaard et al., 2014). The mortality rates of mosquitoes declined with increasing polarity of the solvent, the water extract of *Zanthoxylum heitzii* (Rutaceae) produced the lowest adult mortalities whereas its ethyl-acetate and hexane extracts produce high mortalities against *Anopheles gambie.*

Furthermore, phytochemical compound contained in the fractions might be responsible for insecticidal actions. Lale and Alaga (2000) reported *P. guineense* extract is known to contain atleast three different alkaloids responsible for its insecticidal activity (piperine, chavicine and piperidine).

The result of the study also revealed the efficacy of *E. aromatica* extracts in which all three extract fractions gave high mortalities of larvae which could be due to its important secondary metabolite such as tarpenes, linoleic acid and oleic identified as the main active compound in *E. aromatica* (Golob et al., 1999). This supports the finding of Akinwumi (2010) who reported 100% mortality of *D. maculatus* adults when 1 ml of oil is mixed with 10.00 g of powder of *E. aromaticum* after seven days of post treatment. The finding also supports the work of Ajayi (2015) who reported that clove and west African black pepper were significantly more toxic to adult of *Tribolium castaneum* than ginger at dosage of 100 mg/50 of seed. Clove and West African black pepper and ginger oil caused 96.3, 100 and 13.2% adult mortalities, respectively and 65.7 and 9.6 larval mortalities, respectively. Akinwumi et al. (2007) also reported 0.5 g of *E. aromatica* recorded 50.00% larval mortality and

51.67% adult mortality and concentration of 1.0, 2.0 and 2.5 g recorded 100% larval and adult mortalities.

Conclusion

The study demonstrated the contact toxicity *of P. guinense* and *E. aromatica* against *D. maculatu*s (larvae). The maximum mortality was recorded at the highest dose of ethyl acetate fractions of *P. guineense* and *E. aromatica*. This is followed by n-Haxane, while methanolic extract recorded the least activity. Hence, ethyl-acetate extract of both plants has potential insecticidal activity against *D. maculatus* larvae.

Recommendations

The finding revealed that both methanolic fractions of *P. guineense* and *E. aromatica* could be used as fish protect ant against *D. maculatus* infestetations.

Therefore, the use of these extracts is recommended as potential dry fish ant protector in the control of *D. maculatus* infestation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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