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African Journal of Biotechnology

September 2019
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

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Vol. 18(29), pp. 766-773, September, 2019
DOI: 10.5897/AJB2019.16912
Article Number: D93E30F61812
ISSN: 1684-5315

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African Journal of Biotechnology

Full Length Research Paper

Molecular identification of acetic acid bacteria isolated from fermented mango juices of Burkina Faso: 16S rRNA gene sequencing

Assiètta OUATTARA[1*], K. Marius SOMDA[1], A. T. Cheik OUATTARA[2], Bassirou N'DOYE[3], S. Alfred TRAORE[2] and S. Aboubakar OUATTARA[1]

[1]Laboratory of Microbiology and Microbial Biotechnology, Research Center in Biological Food and Nutrition Sciences (CRSBAN), Department of Biochemistry and Microbiology, University Joseph KI-ZERBO, Burkina Faso.
[2]Laboratory of Food Technology, Research Center in Biological Food and Nutrition Sciences (CRSBAN), Department of Biochemistry and Microbiology, Joseph KI-ZERBO, Burkina Faso.
[3]University of Sine Saloum El Hadji Ibrahima Niass (USSEIN), Sing-Sing, BP 55, Kaolack, Senegal.

Received 7 July 2019; Accepted 19 August, 2019

Acetic acid bacteria are known for their ability to oxidize ethanol to acetic acid. This study investigated to identify dominant strain acetic acid bacteria involving in fermented juice of mango and capable to produce Vinegar, total of 4 bacteria (CRSBAN-BVA1, CRSBAN-BVK1, CRSBAN-BVK2, CRSBAN-BVI1) bacteria strains were preselected for the analyses. The strains were examined with biochemical, physiological and phenotypical methods such as Gram die, catalase and oxidase test, ethanol oxidation to acetic acid, and over to CO_2 and H_2O and also metabolism of carbohydrate was tested, for their affiliation to the genera of acetic acid bacteria. Subsequently, genotypic identification was conducted by sequencing the gene coding for 16S rRNA of one targeted strain and phylogenetic analysis was realized throughout 16S rRNA sequences. The results showed that one of the isolated strain (CRSBAN-BVA1) present 99. 90% of similarity in the sequence 16S rRNA region with *Acetobacter tropicalis*. It demonstrated that bacterial diversity in the mango vinegar is dominated by *A. tropicalis*. Therefore this strain is potentially useful for its utilization as a starter in vinegar production.

Key words: Fermented juice, acetic acid bacteria, 16S rRNA gene sequence, *Acetobacter tropicalis*.

INTRODUCTION

Acetic acid bacteria (AAB) are important organisms in food and beverage industries etc. It is known that they adapt well to sugary and alcoholized fluid (Muramatsu et al., 2009). AAB are Gram negative, rod shape and obligate aerobic bacteria with the ability to oxidize ethanol to acetic acid (Moryadee et al., 2008; Maal et al., 2010). Belonging to the family of *Acetobacteriaceae*, AAB are classified in twelve genera: *Acetobacter*, *Gluconobacter*,

*Corresponding author. E-mail: ouattaraassietta@yahoo.fr. Tel: +226) 76 19 72 20.

Acidomonas, Gluconacetobacter, Asaia, Kozakia, Swaminathania, Saccharibacter, Neoasaia, Granulibacter, *Tanticharoenia* and *Ameyamaea* (Sengun et al., 2011). They are ubiquitous organisms that are well adapted to sugar and ethanol rich environments (Bartowsky et al., 2008). Vinegar, from the French vinaigre, meaning sour wine, can be made from almost any fermentable carbohydrate source, including apples, dates, grapes, pears, coconut, honey, mangos etc (Johnston and Gaas, 2006). Burkina Faso, as in other Sahelian countries, fruits production is dominated by mango. The production of fresh mangoes is estimated at around 337101 ton per year (CEFCOD, 2013). However, factors such as: lack of control over harvesting, packaging and storage standards, poor road infrastructure, poor commercialized on the local market and inadequacy of processing infrastructures, inflict enormous losses which handicap this sector. The resulting annual losses are estimated at about 30-40% of mango production (Ngamo et al., 2010; Vayssieres et al., 2013). However, mango is a substrat rich in fermentable substances sugars.

Thus, transforming them via biotechnological processes to obtain exotic products like vinegar by the local strain of AAB would be interressant. In Burkina like the other majority countries of West Africa, the most of vinegar consumed comes from the dilution of acetic acid of chemical origin because of lack of AAB strain. To raise this challenges two *Acetobacter* strains, *Acetobacter tropicalis* and *Acetobacter pasteurianus* were isolated Dolofrom mango fruit (*Mangifera indica*) in Senegal and (local beer obtained by fermenting cereal product) in Burkina Faso respectively (Ndoye et al., 2006).

This study aimed to isolate an AAB strain whose features make them applicable for biological vinegar production. Thus, we first isolated, identified and characterized AAB strains of mangos fruits. A recent classification of the AAB includes the genera of *Acetobacter, Acidomonas, Ameyamaea, Asaia, Gluconacetobacter, Gluconobacter, Granulibacter, Kozakia, Neoasaia, Neokomagataea, Saccharibacter, Swaminathania* and *Tanticharoenia* (Yamada and Yukphan, 2008; Mamlouk and Gullo, 2013).

AAB are generally found in nature because they can use a variety of substrates (Sharafi et al., 2010) and these bacteria have been isolated from alcoholic beverage, vinegar, fruits and fruit juice, flowers, honey, sugar cane, soil and water (Klawpiyapamornkun et al., 2015). Mango waste has a high carbohydrate and organic acid content that creates an acidic niche (Ouattara et al., 2018). Therefore, fermented juice of mango is a good source for isolation of AAB (Ouattara et al., 2018). The methods of identification based on the analysis of the phenotypical characteristics of the bacteria of non precise acetic acid and also very long do not have is not enough with the identification to the species. To optimize their use, it is necessary to determine their 16S rRNA gene sequences to understand their taxonomic positions.

MATERIALS AND METHODS

Sampling

One kilogram of mango samples were collected from four sites in Burkina Faso (Bobo-Dioulasso, Banfora, Orodara and Ouagadougou) (Figure 1). Six different varieties (Amelie, Kent, Sauvage, Brooks, Lippens and Springfield) of mangos were collected. A total of 80 mangos samples of different varieties were collected in May-July 2016 and 2017. After collection, they were subsequently crushed aseptically and were stored for spontaneous fermentation at room temperature.

Screening of acetic acid bacteria (AAB)

Screening of potentially AAB was performed on GYEA modified medium. Prefermented mango were transferred in a GYEA enrichment medium containing of glucose 2% (w/v), yeast extract 1% (w/v), ethanol 2% (v/v) and acetic acid 1% (v/v). Samples were incubated under agitation (120 rpm) at room temperature (30°C) for one week (Mounir et al., 2016). A volume of 100 µl from different dilutions were inoculated in GYC solid medium (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar, pH 6.8) supplemented with 100 mg l^{-1} of Cycloheximide and nystatine were to inhibit the growth of fungi and lactic acid bacteria, respectively (Sharafi et al., 2010; Kadere et al., 2008). This antibiotic was added to the culture medium from the stock solution after the medium had been sterilized. Plates dish were incubated at 30°C for 2-3 days under aerobic conditions. Only isolates which were able to produce clear halos around the colonies, caracteristic fundamental associates a colony to the group of acetic bacteria were further characterized (Cleenwerck and De Vos, 2008). *Acetobacter* and *Gluconobacter* were distinguished from each other on Carr medium in the presence of bromocresol green. *Acetobacter* turns the media color to yellow and then to green while *Gluconobacter* turns it into yellow.

Phenotypic characterization of acetic acid bacteria (AAB)

Biochemical and morphological identification tests were performed to confirm that the selected isolates belong to *Acetobacter* genera. Morphology of bacteria, including their shape, size, arrangement, Gram and motility, was characterized using cells grown on GYC at 30°C under aerobic conditions (Cleenwerck et al., 2002). Tests, such as catalase, oxidase, and growth in varying concentrations of ethanol and glucose, were employed according the method of Conventional biochemical. Other biochemical tests such as carbohydrate assimilation was performed on presumed *Acetobacter* strains.

DNA preparation

DNA extraction was performed according method by Ruiz et al., (2000) with the following modifications. Cells were grown overnight in 5 ml medium, centrifuged at 4000 g and washed twice with 5 ml of water. The pellet was suspended in 300 ml of 3% (w/v) SDS-TE buffer (10 mmol l^{-1} Tris-HCl, pH 7.5; mmol l-1EDTA) and incubated for 10 min at room temperature. After addition of 200 ml TE buffer, 500 ml of phenol-chloroform-isoamyl alcohol (25: 24: 1, v/v), the

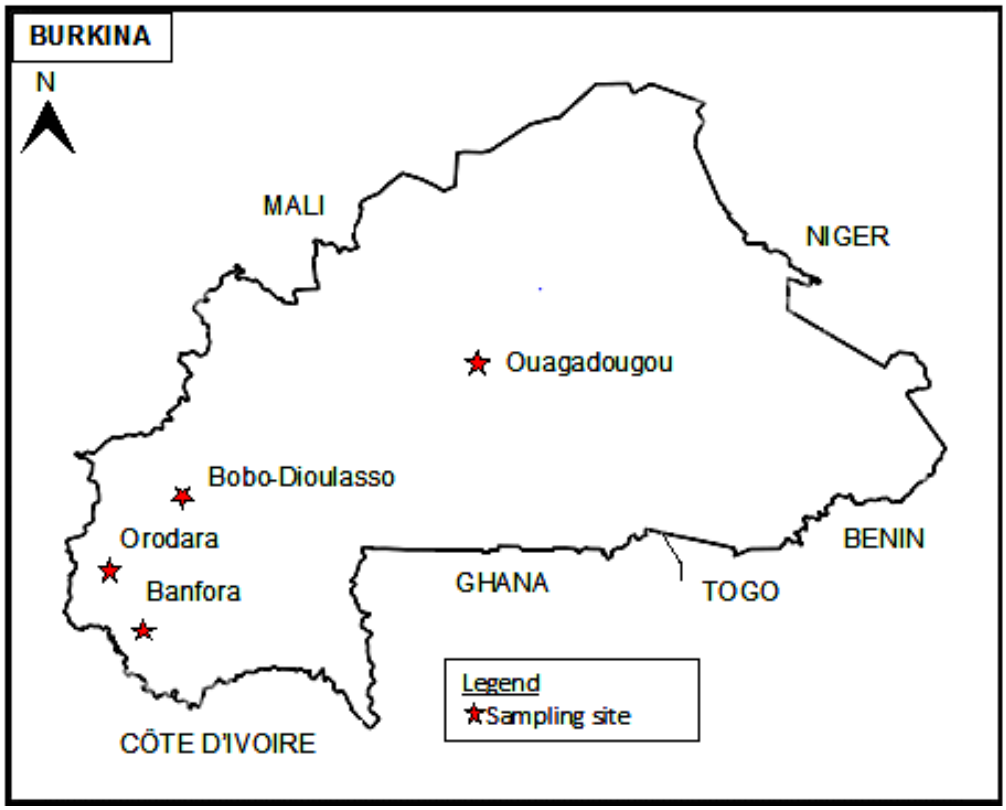

Figure 1. Chart presenting sampling site.

aqueous phase was separated by centrifugation for 10 min at 10 000 g. The DNA was precipitated with isopropanol and washed with 70% (v/v) ethanol. Finally, the DNA was resuspended in 20 ml TE buffer to a final concentration of 1150 ng/ml and stored at -20°C.

PCR amplification, sequencing, and phylogenetic analysis of 16S rRNA genes

DNA for 16S rRNA gene sequencing was extracted by the method of Wilson (2001) with minor modifications (Cleenwerck et al., 2002). The 16S rDNA genes of both community bacteria were amplified by PCR using the pair of 16S rDNA gene universal primers. Forward primer (5-AGAGTTTGATCCTGGCTCAG-3) and Reverse primer (5-ACGGCTACCTTGTTTACGACTT-3) which is targeted to bacterial 16S rDNA gene was used. The forward and reverse 16S rDNA gene universal primers generate a 1.5 kb fragment. The polymerase chain reaction (PCR) reaction was performed in 0.5 ml microcentrifuge tubes (Eppendorf, UK) with 25 ml of reaction mixture: 12.5 ml¨Go Taq Green˝master mix (2.5 units Taq DNA polymerase, 1X Qiagen PCR buffer and 200 µM of each dNTP), 0.5 µl forward primer (10 µM), 0.3 µl reverse primer (10 µM) and 1.5 µl RNA template, and made up to 25 µl with 10.2 µl of nuclease-free sterile distilled water. The PCR protocol consisted of an initial denaturation step of 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 44°C for 30 s and elongation at 72°C for 2 min, final holding at 73 °C for 4 min. PCR reaction was performed in a 20 well block thermocycler (TECHGENE, UK). Sequence blast, alignment and phylogenetic trees were obtained from the website le BIBI https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi. The topology of the trees was evaluated using bootstrap method with 1000 replicates. Alignments of 16S rDNA sequences from the GenBank database were screened to select the most suitable primers to use in the detection and identification of AAB.

RESULTS

Phenotypical identification of strains

The examination of the primary screened such as macroscopic, microscopic and biochemical of isolated strain showed that this strain related to AAB group. Overoxidation in Carr medium resulted in conversion of blue color of medium to yellow after 24-48 h and then reconversion of yellow color to blue after 72-96 h (Figure 2). Also utilization of $CaCO_3$ and creation of transparent zones around the colonies in Frateur medium confirmed that the isolated strain was AAB. All strains were able to produce acid from following sugars such as: glucose, mannose, melibiose and mannitol and were unable to produce acid from arabinose, galactose, fructose, lactose, maltose, sucrose and saccharose. The preliminary identification on the basis of biochemical and physiologique tests (Table 1) brought about the possibility of having the *Acetobacter*. Hence the 16s rRNA technique was further employed to confirm the isolate.

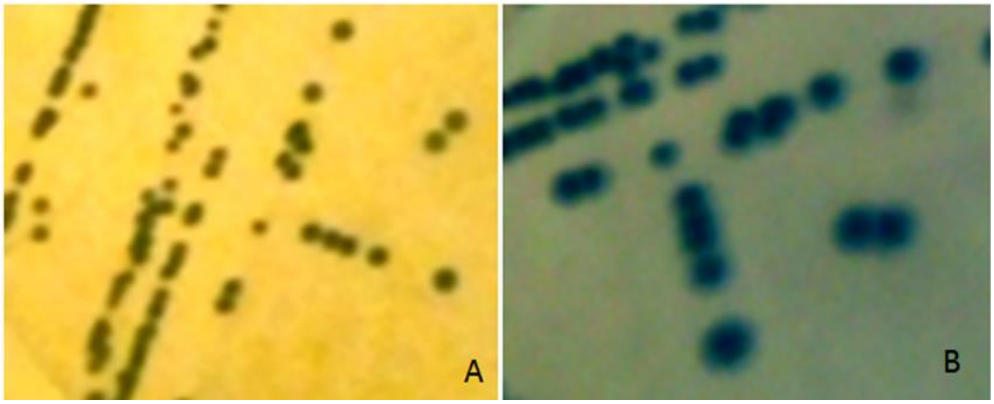

Figure 2. Color of colonies in Carr medium after 24h (A) and after 96h (B).

Table 1. Bio-chemical and physiological characters of the obtained *A. tropicalis.*

Biochemical/physiologic test	Strains			
	***Acetobacter tropicalis* CRSBAN-BVA1**	**CRSBAN-BVK1**	**CRSBAN-BVI1**	**CRSBAN-BVK2**
Gram	-	-	-	-
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	-	-	-	-
Ketogenesis of glycerol	-	-	-	-
Cellulose production	+	+	-	-
Peroxydisation	+	+	+	+
Saccharose	+	+	+	+
Glucose	+	+	+	+
Sucrose	-	-	-	-
Fructose	-	-	+	+
Lactose	-	-	-	-
Arabinose	-	-	-	-
Meliobiose	+	+	+	+
Mannitol	+	+	+	+
Galactose	-	-	-	-
Maltose	-	-	-	-
Mannose	+	+	+	+

Symbols: + (positive), - (negative).

Identification of *A. tropicalis*

The phylogenetic affiliation of strain was based on 16S rRNA gene sequence analysis, where it was shown to belong to the genus *Acetobacter*. The phylogenetic analysis of the strains (Figure 3) was carried out using leBiBi software to determine similarity and close relationship of isolate. The phylogenetic tree analysis revealed that sequence was closely related to *Acetobacter* species. Figure 3 showed that strain *A. tropicalis* (CRSBAN-VBA1) belonged to the stable subcluster containing *A. orleanensis, A. malorum, A. cerevisiae, A. farinalis, A. persici, A. indonesiensis, A. tropicalis, A. senegalensis, A. cibinongensis, A. orientalis, A. musti, A. oeni, A. estunensis, A. sicerae, A. aceti, A. thailandicus, A. oryzifermentans, A. ascendens, A. pasteurianus, A. pomorum A. suratthaniensis, A. syzygii, A. lambici, A. okinawensis A. fabarum, A. lovaniensis* and *A. ghanensis*. The 16S rRNA gene sequence similarities obtained by pairwise alignment with the Bio Numerics 4.5 software package between strain *A. tropicalis* (CRSBAN-BVA1) and the type strains or

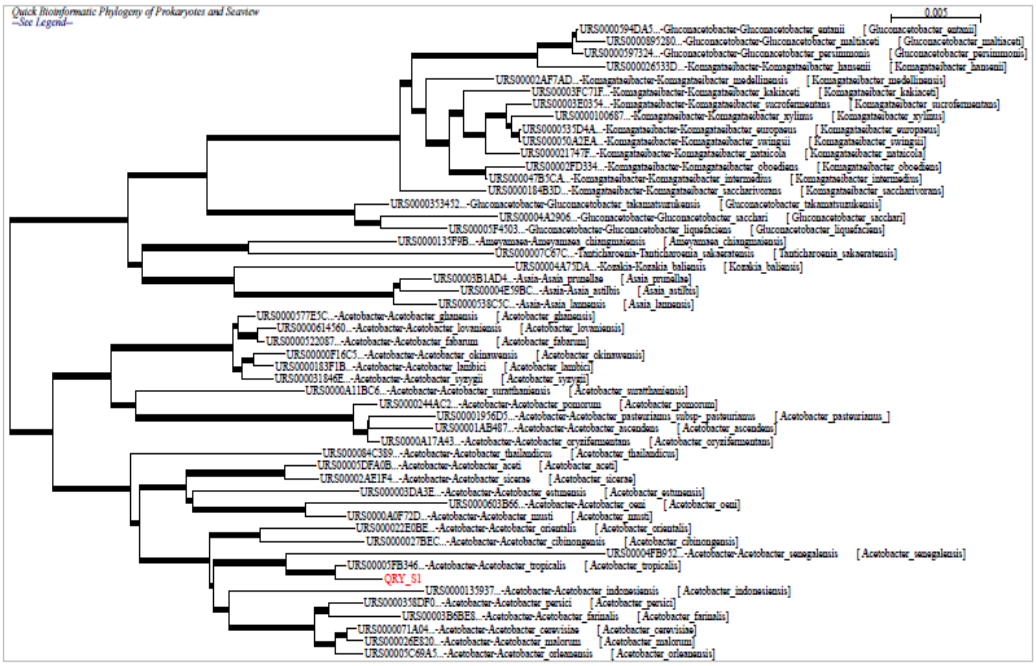

Figure 3. Phylogenetic analysis of the sequence *Acetobacter tropicalis* (CRSBAN- BVA1).

another strains of recognized *Acetobacter* species were represented in Table 2.

DISCUSSION

Acetic acid bacteria are characterized by the ability to oxidize alcohols or sugars incompletely, and a common feature to most of strains their capacity to oxidize ethanol to acetic acid. Although strains of AAB that are generally isolated with GYC plates showed distinct clear zones (Trcek, 2005). The methods based on the presence of a clear zone was not completely believable because other strains, such as some lactic acid bacteria, could also form distinct clear zones (Trcek, 2005). The result of biochemical tests showed that the isolated strains from fermented juice of mangos belonged to genus of *Acetobacter* or *Gluconacetobacter*. In addition to their ability to oxidise ethanol, *Acetobacter* and *Gluconacetobacter* species can further oxidise acetic acid to CO_2 and H_2O, generating the so-called acetate overoxidation, that is carried out by the tricarboxylic acid cycle (TCA) when there is a high level of dissolved oxygen and no ethanol in the medium. According to Sakurai et al., (2013), strains of *Gluconobacter* are not able to overoxidise because of non-functional α-ketoglutarate dehydrogenase and succinate dehydrogenase of tricarboxylic acid cycle; they can only oxidize ethanol to acetic acid (Du Toit and Pretorius, 2002). Hence the presence of the ethanol in the medium represses the activity of TCA enzyme cycles in *Acetobacter* genus. The results of acid production with different sugars showed also that strains produced acid with some sugars and did not have the capacity to produce acid with other sugars. These results are slightly comparable to those found by Lisdiyanti et al. (2000) and kadere et al. (2008). According to Lisdiyanti et al., (2000) this slight difference is a variability between strains of *Acetobater* genus.

On the Carr medium, the production of the acid in the medium by strain can be seen in form of clearing of opacity of medium or the colour change of bromocresol green confirms that the isolate is *Acetobacter* species. Sharafi et al., (2010); Mounir et al., (2016) had reported that color change of the indicator bromocresol green in the medium from green to yellow confirm that the isolate is *Acetobacter.* The production of acid acetic of this strains was previously determined by Ouattara et al., (2018), where it was shown that these strains had the capacity to produce a high concentration of acetic acid and the highest was found with strain CRSBAN-BVA1. Molecular characterization will confirm our results. Molecular techniques have been employed by PCR-amplified fragment of the gene coding for 16S rRNA to confirm that the isolate is *Acetobacter.*

The fast molecular detection was proven to be efficient and accurate According 16S rRNA sequencing and phylogenetic tree analysis, the isolate was further proven to be *A. tropicalis*. The comparison of 16S rRNA gene sequence of the strains with the total nucleotide collection in the LEBIBI nucleotide database was used to assign the bacterial name with ≥ 99% similarity. The greatest

Table 2. Alignments of *16S rRNA* sequences grouped by closest sequence.

Closest species based on 16S rRNA sequence	Acession number of 16SrRNA ref seq.	% of 16s rRNA similarity	Closest species based on 16S rRNA sequence	Acession number of 16SrRNA ref seq.	% of 16s rRNA similarity
Acetobacter orleanensis	URS00005C69A5	98.38	*Acetobacter lovaniensis*	URS0000614560	96.56
Acetobacter malorum	URS000026E820	98.47	*Acetobacter ghanensis*	URS0000577E5C	96.85
Acetobacter cerevisiae	URS0000071A04	98.68	*Asaia lannensis*	URS0000538C5C	95.93
Acetobacter farinalis	URS00003B6BE8	97.86	*Asaia astilbis*,	URS00004E59BC	95.75
Acetobacter persici	URS0000358DF0	98.27	*Asaia prunellae*	URS00003B1AD4	95.95
Acetobacter indonesiensis	URS0000135937	97.48	*Acetobacter lovaniensis*	URS00004A75DA	96.14
Acetobacter tropicalis	URS00005FB346	99.90	*Acetobacter ghanensis*	URS000007C67C	95.73
Acetobacter senegalensis	URS00004FB952	97.87	*Asaia lannensis*	URS0000135F9B	95.95
Acetobacter cibinongensis	URS0000027BEC	98.07	*Asaia astilbis,*	URS00005F4503	95.96
Acetobacter orientalis	URS000022E0BE	98.38	*Asaia prunellae*	URS00004A2906	95.96
Acetobacter musti	URS0000A0F72D	98.08	*Gluconacetobacter takamatsuzukensis*	URS0000353452	96.14
Acetobacter oeni	URS0000603B66	96.97	*Komagataeibacter saccharivorans*	URS0000184B3D	95.85
Acetobacter estunensis	URS000003DA3E	97.36	*Komagataeibacter intermedius*	URS000047B5CA	95.96
Acetobacter sicerae	URS00002AE1F4	97.66	*Komagataeibacter oboediens*	URS00002FD334	95.75
Acetobacter aceti	URS00005DFA0B	97.57	*Komagataeibacter nataicola*	URS000021747F	95.75
Acetobacter thailandicus	URS000084C389	97.25	*Komagataeibacter swingsii*	URS000050A2EA	95.83
Acetobacter oryzifermentans	URS0000A17A43	97.25	*Komagataeibacter europaeus*	URS0000535D4A	95.65
Acetobacter ascendens	URS00001AB487	95.95	*Komagataeibacter xylinus*	URS0000100687	95.95
Acetobacter pasteurianus	URS00001956D5	95.95	*Komagataeibacter sucrofermentans*	URS00003E0354	95.65
Acetobacter pomorum	URS0000244AC2	96.26	*Komagataeibacter kakiaceti*	URS00003FC71F	95.83
Acetobacter suratthaniensis	URS0000A11BC6	95.93	*Komagataeibacter medellinensis*	URS00002AF7AD	95.85
Acetobacter syzygii	URS000031846E	96.54	*Komagataeibacter hansenii*	URS000026533D	95.83
Acetobacter lambici	URS0000183F1B	96.54	*Gluconacetobacter persimmonis*	URS0000597324	95.95
Acetobacter okinawensis	URS00000F16C5	96.44	*Gluconacetobacter maltiaceti*	URS0000895280	95.85
Acetobacter fabarum	URS0000522087	96.44	*Gluconacetobacter entanii*	URS0000594DA5	95.65

similitude (99.90%) was obtained with the species *A. tropicalis* and another similitude which various between 95.75 to 98.68% with other species such as *Acetobacter*, *Asaia, Gluconacetobacter* and *Komagataeibacter* were represented in Table 2. *A. tropicalis* was identified as the only predominant group. The phylogenetic tree reflects the results obtained in Table 2. Lisdiyanti et al., (2000) in their study on the diversity of AAB in Indonesia, Thailand and the Philippines, have isolated *A. tropicalis* from fermented foods (palm wine and rice wine), fruits (lime, orange, guava, coconut), and coconut juice whose similarity is in a range of 96.5 to 98.9% between the type strain of A. *tropicalis* and the type strains of other *Acetobacter* species. Others researchers as Kounatidis et al., (2009) have isolated *A. tropicalis* with in similitude 99.7% from fermented wine. In Senegal *A. tropicalis* with in similitude 99.3% was isolated from mango fruit (*Mangifera indica*) (Ndoye et al., 2007). These similarities are

slightly lower than our which was 99.90%. Hence the identity of the isolates were confirmed by the 16s rRNA method, as *A. tropicalis*

Conclusion

In this study, AAB were isolated and identified from fermented juice of mangos–sing of 16S rRNA gene sequence has allowed differenciation between species and could represent a tool for a rapid and low cost effective preliminary profiling of Acetic Acid Bacteria genera. The molecular technique can also be useful to highlight the phylogenetically closely related species. This first knowledge of the acetic acid bacteria will serve as a guide in selecting starter for the production of vinegar in Burkina Faso.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEGEMENTS

The authors need to thank Laboratory Microbial Biotechnology then Laboratory of Food Technology (Department of Biochemistry and Microbiology, University of Joseph KI-ZERBO), Ministry for the Woman, national Solidarity, the Family and the humane Action for their help and Laboratory of Genoscreen for genotyping. We also thank the International Sciences Programme (ISP-Sweden).

REFERENCES

Bartowsky EJ, Henschke PA (2008). Acetic acid bacteria spoilage of bottled red wine-Areview. International Journal Food of Microbiology 125(1):60-70.DOI: 10.1016/j.ijfoodmicro.2007.10.016.

Centre d'Etude, de Formation et de Conseil en Développement (CEFCOD) (2013).Situation de référence des principales filières agricoles au Burkina Faso. Rapport version finale, Ministère de l'Agriculture et de la Sécurité Alimentaire 74 p.

Cleenwerck I, De Vos P (2008). Polyphasic taxonomy of acetic acid bacteria: An overview of the currently applied methodology. International Journal of Food Microbiology 125(1):2–14. DOI: 10.1016/j.ijfoodmicro.2007.04.017

Cleenwerck I, Vandemeulebroecke K, Janssens D, Swings J (2002). Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae sp*. nov., and *Acetobacter malorum sp*. nov. International Journal of Systematic and Evolutionary Microbiology 52(5):1551-1558. DOI: 10.1099/00207713-52-5-1551.

Du Toit WJ, Pretorius IS (2002). The occurrence, control and 12 esoteric effect of acetic acid bacteria in winemaking. Annals of Microbiology 52(2):155-179. DOI:10.1.1.454.5620.

Johnston CS, Gaas CA (2006). Vinegar: medicinal uses and antiglycemic effect. Medscape General Medicine 8(2):61. PMID:16926800.

Kadere TT, Miyamoto T, Oniang R K, Kutima PM Njoroge SM (2008). African Journal of Biotechnology 7(16):2963-2971. DOI: 10.5897/AJB08.390.

Klawpiyapamornkun T, Bovonsombut S, Bovonsombut S (2015). Isolation and Characterization of Acetic acid Bacteria from Fruits and Fermented fruit juices for Vinegar Production. Food and Applied Bioscience Journal 3(1):30-38. DOI: https://www.tci-thaijo.org/index.php/fabjournal/article/view/78118.

Kounatidis I , Crotti E, Sapountzis P, Sacchi L, Rizzi A, Chouaia B, Bandi C, Alma A, Daffonchio D, Mavragani TP, Bourtzis K (2009). *Acetobacter tropicalis* Is a Major Symbiont of the Olive Fruit Fly (Bactrocera oleae). Applied and Environmental Microbiology 75(10):3281-3288. DOI:10.1128/AEM.02933-083281–3288.

Lisdiyanti P, Kawasaki H, Seki T, Yamada Y, Uchimura T, Komagata K (2000). Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter Indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov., and *Acetobacter estunensis* (Carr 1958) comb. nov. Journal of General and Applied Microbiology 46(3):147-165. PMID:12483588.

Maal KB, Shafiei R, Kabir N (2010). Production of Apricot Vinegar Using an Isolated *Acetobacter* Strain from Iranian Apricot. International Journal of Food nutrition and food Engineering 4(11):177-180. DOI: 10.5281/zenodo.1080223.

Mamlouk D, Gullo M (2013). Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. Indian Journal of Microbiology 53(4): 377-384. DOI: 10.1007/s12088-013- 0414-z.

Moryadee A, Pathom-Aree W (2008). Isolation of Thermotolerant Acetic Acid Bacteria From Fruits for Vinegar Production. Journal of Microbiology 3(3): 209-212. DOI: 10.3923/jm.2008.209.212.

Mounir M, Shafiei R, Zarmehrkhorshid R, Hamouda A, Ismaili AM, Thonart P (2016). Simultaneous production of acetic and gluconic acids by a thermotolerant *Acetobacter* strain during acetous fermentation in a bioreactor. Journal of Bioscience and Bioengineering 121(2):166-171. DOI: 10.5897/AJB2016.15323.

Muramatsu Y, Yukphan P, Takahashi M, Kaneyasu M, Malimas T, Potacharoen W, Yamada Y, Nakagawa Y, Tanticharoen M, Suzuki K (2009). 16S rRNA gene sequences analysis of acetic acid bacteria isolated from Thailand. Microbiology and Culture Collections 25(1): 13-20. PMID:19590153.

Ndoye B, Weekers F, Diawara B, Guiro AT, Thonart P (2007). Survival and preservation after freeze-drying process of thermoresistant acetic acid bacteria (TAAB) isolated From tropical products of Subsaharan. African Journal Food Engineering 79(4):1374-1382. DOI: 10.1099/ijs.0.64678-0.

Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro TA, Kere C, Diawara B,Thonart P (2006). Thermoresistant properties of acetic acid bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme and Microbial Technology 39(4):916-923.DOI: 10.1016/j.enzmictec.2006.01.020.

Ngamo TL, Ladang D, Vayssieres JF, Lyannaz JP (2010). Diversité des espèces de mouches des fruits (Diptera :Tephritidae) dans un verger mixte dans la localité de Malang (Ngaoundéré, Cameroun). International Journal of Biological and Chemical Sciences 4(5):1425-1434. Available online at http://ajol.info/index.php/ijbcs.

Ouattara A, Somda KM, Ouattara ATC, Traore SA, Ouattara SA (2018). Production of acetic acid by acetic acid bacteria using mango juice in Burkina Faso). International Journal Biological and Chemical Science 12(5): 2309-2317. http://www.ifgdg.org.

Ruiz A, Poblet M, Mas A, Guillamon JM (2000). Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. International Journal of Systematic and Evolutionary Microbiology 50(6):1981-1987. DOI:10.1099/00207713-50-6-1981.

Sakurai K, Yamazaki S, Ishii M, Igarashi Y, Arai H (2013). Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*. Journal of Bioscience and Bioengineering 115(1):32-6. DOI: 10.1016/j.jbiosc.2012.07.017.

Sharafi SM, Rasooli I, Beheshti-Maal K (2010). Isolation, characterization and optimization of indigenous acetic acid bacteria and evaluation of their preservation methods. Iranian journal of Microbiology 2(1):38-45. PMID: 22347549.

Sengun IY, Karabiyikli S (2011). Importance of acetic acid bacteria in food. Industry Food Control 22(5):647-656. DOI: 10.1016/j.foodcont.2010.11.008.

Trcek J *(*2005) .Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S-23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. Systematic and Applied Microbiology 28(8):735-745. DOI:10.1016/j.syapm.2005.05.001.

Vayssieres JF, Sinzogan A, Adandonon A, Van Mele Paul, Korie S (2013). Ovipositional behaviour of two mango fruit fly species (Diptera Tephritidae) in relation to *Oecophylla* cues (Hymenoptera Formicidae) as compared to natural conditions without ant cues. International Journal of Biological and Chemical Sciences 7(2):447-456. Available online at http://ajol.info/index.php/ijbcs.

Wilson K (2001). Preparation of genomic DNA from bacteria. In Current Protocols in Molecular Biology 56(1):2. 4.1-2.4.5. https://doi.org/10.1002/0471142727.mb0204s56.

Yamada Y, Yukphan P (2008). Genera and species in acetic acid bacteria. International Journal Food of Microbiology 125(1):15-24. https://doi.org/10.1016/j.ijfoodmicro.2007.11.077.

Vol. 18(29), pp. 774-782, September, 2019
DOI: 10.5897/AJB2018.16617
Article Number: 8EB743061814
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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Phylogenetic analysis of bluetongue virus serotype16 based on genome segment 5 (encoding NS1)

Molalegne Bitew[1,2*], Sukdeb Nandi[2], Chintu Ravishankar[2] and Asit Sharma[2]

[1]Ethiopian Biotechnology Institute (EBTI), Ethiopia.
[2]Virus Laboratory, Center for Animal Disease Research and Diagnosis (CADRD), Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh-243122, India.

Received 10 August, 2018; Accepted 25 October, 2018

Bluetongue virus (BTV) is the 'type' species of the genus *Orbivirus* within the family *Reoviridae*. The BTV genome is composed of ten linear segments of double-stranded RNA (dsRNA), each of which codes for at least one of ten distinct viral proteins. Phylogenetic analysis based on VP2, VP3, VP5, VP7 and NS3 gene has been advocated by different researchers around the world. However, very little information about the phylogenetic analysis based on the NS1 gene of BTV-16 isolates is available. In this study, a partial sequence of segment 5 (Seg 5) from the BTV-16 Hisar isolate was sequenced. Sequence analysis of the Seg 5 of the BTV-16 Hisar isolate revealed highest nucleotide sequence identity of 98.9% (BTV-21), -96.4% (BTV-9) with sequences of NS1 genes of Indian isolates and 97.8% (BTV-3),-93.4%(BTV-1) isolates from other countries. The lowest sequence identity detected was 91.2% between sequences of NS1 gene of JQ924824 of BTV 16 Indian isolate and JX861502 BTV-1 of France. The study also assessed the comparative identity from the deduced amino acid sequences and found maximum homology (100% identity) with Indian isolates and 95.2 to 98.8% identity with Western isolates with only three amino acid difference. Phylogenetic analysis of the NS1 gene of the BTV-16 Hisar isolate with others around the world has revealed two major clusters which includes the Indian (Eastern) linage and Western linage separately. The BTV-16 Hisar isolate was found to be closely related with the BTV-9 and 21 Indian isolates based on its NS1 gene. Phylogenetic analysis of segment 5 is highly conserved among the different serotypes however, NS1 of BTV-3 of USA clustered with the Eastern lineage indicating introduction of Western BTV strains and a possible reassortment between Eastern and Western field strains in India.

Key words: Bluetongue virus, genome, NS1, *Orbivirus,* phylogeneticanalysis, segment-5, serotype.

INTRODUCTION

Bluetongue is an arthropod-transmitted hemorrhagic disease of wild and domestic ruminants. It is enzootic between approximately 45 to 53°N and 35°S, in many tropical, subtropical and some temperate regions, including much of the Americas, Africa, southern Asia and northern Australia, and coincides with the geographic

*Corresponding author. E-mail: molalegne23@yahoo.com. Tel: +251911735280.

distribution and seasonal activity of competent *Culicoides* vector insects (Purse et al., 2005, 2009).

Bluetongue virus (BTV) is the prototype of the genus *Orbivirus* in the family *Reoviridae* (Mertens et al., 2004; Bitew et al., 2017; Abera et al., 2018). Currently, there are 27 recognized serotypes with recent additions of the 25th serotype ("Toggenburg orbivirus") from Switzerland in goat and 26th serotype from Kuwait in sheep and goat (Hofmann et al., 2008; Maan et al., 2011, 2012c; Batten et al., 2013) and 27th from Corsica, France (Schulz et al., 2016). Bluetongue virus is a non-enveloped virus, approximately 90 nm in diameter, with a triple-layered icosahedral protein capsid. Its genome consists of 10 double-stranded (ds) RNA segments coding for 7 structural proteins (VP1–VP7) and 4 non-structural proteins (NS1, NS2, NS3/NS3a, NS4) of approximately 19.2kb pairs in total (Singh et al., 2005; Sperlova and Zendulkova, 2011; Ratinier et al., 2011; Bitew et al., 2017; Abera et al., 2018).

The outer capsid of the virion is composed of 60 trimers of VP2 and 120 trimers of VP5 (Zhang et al., 2011) and differences within this outer capsid define the 26 BTV serotypes which have been described so far (Hofmann et al., 2008; Maan et al., 2007). The outer capsid proteins, VP2in particular, stimulate virus neutralizing antibodies which in general protect only against the homologous serotype (DeMaula et al., 2000). The internal core is formed by two layers, constituted by VP3 (subcore) and the immunodominant VP7 (intermediate layer) (Grimes et al., 1998). Three minor enzymatic proteins, VP1 (RNA dependent RNApolymerase), VP4 (capping enzyme and transmethylase) and VP6(RNA dependent ATPase and helicase) are contained within the core that is transcriptionally active in infected cells (Mertens and Diprose, 2004; Sutton et al., 2007; Roy, 2008; Noad and Roy, 2009; Boyce et al., 2004; Wehrfritz et al., 2007; Singh et al., 2005). The BTV genome encodes also 4 non-structural proteins: NS1, NS2, NS3/NS3a and NS4 (Ratinier et al., 2011). NS1 and NS2 are highly expressed viralproteins and their multimers are morphological features of BTV infected cells. Multimers of the NS1 protein form tubules (approximately 50 nm in diameter and up to 1000 nm in length) that appear to be linked to cellular cytopathogenicity (Owens et al., 2004; Ratinier et al., 2011), while NS2 is the major component of the viral inclusion bodies. NS2 plays a key role in viral replication and assembly as it has a high affinity for single stranded RNA (ssRNA) and possesses phosphohydrolase activity (Horscroft and Roy, 2000; Ratinier et al., 2011; Belhouchet et al., 2011). NS3/NS3a is glycosylated proteins involved in BTV exit. There are two isoforms of NS3: NS3 and NS3a with the latter lacking the N-terminal 13 amino acid residues (Celma and Roy, 2009; Han and Harty, 2004; Boyce et al., 2008). It has been recently identified that BTV expresses a fourth non-structural protein (NS4) encoded by an open reading frame in segment 9 overlapping the open reading frame encoding VP6. NS4 is 77 to 79 amino acid residues in length and highly conserved among several BTV serotypes/strains (Ratinier et al., 2011; Belhouchet et al., 2011).

BTV-16 was first isolated in 1960 in Pakistan (Maan et al., 2012a). It is the most prevalent virus in India. It has been isolated from sheep in Madhya Pradesh, Maharashtra, Tamil Nadu, Uttar Pradesh and Jammu and Kashmir. BTV-16 field strains from China and Australia have been fully sequenced (Boyle et al., 2012; Yang et al., 2012), as well as reference strain RSArrrr/16 and BTV-16 strain Goat/10/Ind/ABT/Hisar (Maan et al., 2012a; Minakshi et al., 2012). Phylogenetic analysis of VP2, VP3 and VP7 genes have been recommended to determine the relationship between the orbiviruses. Phylogenetic analysis based on VP2, VP3, VP5, VP7 and NS3 gene has been advocated by different researchers around the world (Dash et al., 2005; Singh et al., 2005; Maan et al., 2007; Maan et al., 2012b, d). However, little information about the phylogenetic analysis based on NS1 gene of BTV-16 isolates is available. This study was carried out with the objectives to study the extent of nucleotide and the amino acid sequence variation in the BTV-16 Hisar isolate in comparison with 23 other BTV serotypes isolated from different geographical locations of the world and to study the phylogenetic analysis of the BTV-16 Hisar isolate based on partial sequence of segment 5 (NS1) genes.

MATERIALS AND METHODS

Virus

The BTV-16 Hisar isolate used in this study was acquired from the India Network Programme on Bluetongue (AINP-BT) project repository, Virus Laboratory, Center for Animal Disease Research and Diagnosis (CADRAD), Indian Veterinary Research Institute (IVRI), Izatnagar, U.P. (243122), India. The BTV 16 prototype virus was propagated in baby hamster kidney (BHK)-21 cell cultures. Two 75 cm^2 cultures of BHK-21cells at 70 to 80% cell confluency were infected with 0.1 multiplicity of infection (MOI) per cell, and infected cells were harvested and pelleted by centrifugation (2000 g for 10 min at 4°C). RNA extraction was performed when approximately 80% of the infected cells showed cytopathology (approx. 72 hpi).

Viral dsRNA extraction

Total RNA was extracted from 250 µl infected BHK-21 cell suspension with 750 µl Trizol-LS reagent (Life Technology, USA), according to the manufacturer's recommended method. RNA was precipitated with isopropanol and washed with 70% ethanol. Double-stranded RNA (dsRNA) was purified by 2M LiCl differential precipitation of ssRNA as described previously (Wilson et al., 1990). The dsRNA was dried in a dry bath and resuspended in 30 µl DEPC treated water. The quality of the dsRNA was assessed by 1% agarose gel electrophoresis (7 V/ cm^{-1} for 1 h) in Tris acetate EDTA (TAE) buffer containing 0.5 mg ethidium bromide ml^{-1}. The concentration of dsRNA was determined using a nanophotometer by measuring absorbance at 260 nm. Extracted RNA was stored at -80°C until complimentary DNA (cDNA) synthesis was performed.

cDNA synthesis and amplification bypolymerase chain reaction (PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) was done with two steps as described by Wade-Evans et al. (1990). Reverse transcription was performed in a final volume of 20 µl from BTV dsRNA using segment 5 (NS1) gene specific primers [Forward primer (FR) 5'- GTTCTCTAGTTGGCAACCACC-3'(nt.=10-30) and reverse primer (RP) 5'- AAGCCAGACTGTTTCCCGAT-3'(nt. 264-283)] and MMLV-RT enzyme (Promega, USA) according to manufacturer's protocols. The primers were custom synthesized by M/s Eurofins, Bangalore. The cDNA mix containing 4 µl of BTV dsRNA (500 ng), 1 µl of forward primer (20 pmol), 1 µl of reverse primer (20 pmol) and 3 µl of DPEC treated water was incubated at 94°C for 4 min for denaturation of the dsRNA and to remove secondary structures, and then chilled on ice for the primer annealing step. A master mix was prepared containing 4 µl of 5× reaction buffer (50 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$ and 75 mM KCl) (Thermoscientific, USA), 1 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 2µl DTT (0.1 M), 1.5 µl of DEPC water, 0.5µl of Ribolock^{TM} (Ribonuclease-inhibitor, 40 U/µl)(Fermentas, USA) and 1 µl of reverse transcriptase (Revert Aid H minus, 200 units/µl)(Thermoscientific, USA). The master mix was added to each reaction mixture tube in 10 µl aliquots and mixed. Reverse transcription was carried out by incubating the reaction mixture at 42°C for 60 min and the MMLV-RT inactivated by incubating the tube at 70°C for 10 min.The cDNA was stored at -20°C until PCR was performed. The PCR master mix consisted of 2.5 µl of 10Xmagnesium free PCR buffer (10mM Tris-HCl, 50mM KCl, 0.1% Triton X), 1.5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM each of four dNTP, 1 µl each of (20pmol) forward and reverse primers, 0.5 µl of 1 U/µl Taq DNA polymerase (Promega, USA), 1 µl of cDNA and nuclease free water (NFW) was added. The PCR was carried out by the GeneAmp® PCR system 9700 thermal cycler machine (Applied biosystems®, USA). The cycling conditions were initial denaturation at 95°C for 5 min, 35 cycles of 94°C/30 s denaturation, 58°C/30 sprimer annealing and 72°C/30 s extension, followed by a final extension of 72°C for 10 min. The RT-PCR product was resolved on a 1.5% agarose gel in 1X TAE buffer. The size of RT-PCR products was determined by a 100 bp DNA ladder (Fermentas, USA) run simultaneously. Gel was stained with ethidium bromide (Life technology, USA) and visualized by UV illumination using the Gel Documentation System (Gel Doc ^{TM} XR^+, imaging system, BIORAD, USA).

Cloning of the amplified NS1 partial gene in pJET1.2/blunt vector

RT-PCR product was resolved on a 1.2% low melting point agarose gel and 274 bp fragments were cut for elution. The 274 bp PCR product was purified by using GeneJET gel extraction kit (Fermentas, Lithuania) and then cloned into the pJET1.2/blunt vector using Clone- JET PCR cloning kit (Fermentas, Lithuania). The purified PCR product was ligated in pJET1.2/blunt vector by using Clone- JET PCR cloning kit (Fermentas, Lithuania) in 1:1 ratio. *Escherichia coli* strain DH5α cells were prepared employing the $CaCl_2$ method prior to ligation and stored at -70°C. Ligation mixtures were transformed in chemically competent *E. coli* strain DH5α cells by heat shock method according to the manual protocol (Sambrook and Russell, 2001). After transformation, 50 µl of transformed material was inoculated onto a Petri dish containing LB agar with 100 µg/ml ampicillin. The colonies were screened for the recombinant plasmid DNA. Re-circularized pJET1.2/blunt vector (vector without the insert) expresses a lethal restriction enzyme after transformation and hence bacteria cells are not propagated. As a result, only recombinant clones containing the insert grow on culture plates. Boiling followed by RT-PCR from supernatant was used as a screening tool. Plasmid DNA was isolated from selected colonies using GeneJet Miniprep plasmid isolation kit (Fermentas, Lithuania).

Sequencing of partial NS1 gene

The recombinant plasmids containing the 274 bp insert were sequenced by M/s Eurofins Genomics India Private Limited, Bangalore. Automated DNA sequencing system (ABI 3700) from applied Biosystems was used along with ABI PRISM genetic analyzer (Applied Biosystems, Foster City, California, USA) using BigDyeTM terminator cycle sequencing kit according to manufacturer's instructions. The BTV-16 NS1 partial gene was sequenced by using T7 promoter primer. The chromatograms received after sequencing were analyzed using Chromas Lite v2.01 software (http://www.technelysium.com.au) and sequence reading errors if any were edited and assembled using the *Bioedit, Edit Seq* and *mega Align* module of lasergene-5software package (DNASTAR Inc, USA) and compared with NS1 gene sequences reported in GenBank public database.

Nucleotide sequence data assembly and phylogenetic analysis

Homology studies were done through standard nucleotide and protein BLAST software available at NCBI web site (http://www.ncbi.nlm.nih.gov/Blast).The consensus sequence of the 274 bp fragment was obtained out of 7 clones used to obtain the genetic sequence for phylogenetic and sequence analysis. The sequences were analyzed along with the sequences of 23 selected other Indian and International BTV isolates belonging to various serotypes (Table 1). The multiple sequence alignment was done using the CLUSTALW program integrated in MEGA v5.1 software and Open Reading Frame (ORF) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Phylogenetic analysis was done by MEGA v5.1 software (Tamura et al., 2011). The percentage of nucleotide identity and diversity was done by *MegAlign* module of lasergene-5software package (DNASTAR Inc, USA).The evolutionary history was inferred using the Neighbor-Joining (N-J) method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.23054784 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 274 positions in the final dataset for nucleotide analysis. For deduced protein phylogenetic analysis, 249 positions were included, because ORF starts from position 26 with total of 83 amino acids (Tamura et al., 2011).

RESULTS

The prototype BTV-16 virusHisar isolate having titer of $10^{7.2}$ ml^{-1} was propagated in baby hamster kidney (BHK)-21 cells in 75 cm^2 cell culture flask. Cells were infected by using 0.1 multiplicity of infection (MOI), and revealed distinct cytopathic effects (CPE) characterized by appearance of foci of rounded, refractile and increasing

Table 1. BTV isolates and published Segment 5 (NS1 gene) sequences used for phylogenetic analysis.

S/N	Name of virus isolate	Serotype	Area of isolation	Gene Bank Accessionnumber
1	Hisar isolate	BTV-16	India (This study)	KC706456
2	isolate KMN07/05	BTV-21	India	JF747592
3	isolate K8	BTV-9	India	JF443168
4	strain IND1988/02	BTV-23	India	JQ771827
5	isolate IND1982/01	BTV-2	India	JQ713560
6	prototype 600565	BTV- 3	USA	FJ713338
7	Mehaboobnagar9/2002	BTV-9	India	DQ521598
8	IND/Goat/2010/16/HSR	BTV-16	India	JQ924824
9	BTV9BBF	BTV-9	India	JF443163
10	prototype 600579	BTV-19	USA	FJ713350
11	BTV11_MQ	BTV-11	Brussels, Belgium	JQ972865
12	prototype 600578	BTV-18	Laramie, USA	FJ713349
13	prototype 600567	BTV-5	USA	FJ713340
14	isolate USA	BTV-2	USA	M97680
15	USA2010	BTV-2	USA	JQ822252
16	FRA2007/18	BTV-1	France	JX861492
17	FRA2008/24	BTV-1	France	JX861502
18	IT(L)	BTV-2	Italy	JN255866
19	8341/00	BTV- 2	Italy	DQ017956
20	BTV-11_DE	BTV-11	Germany	JQ972855
21	BTV-11_REF	BTV-11	Brussels, Belgium	JQ972835
22	BTV-11_VAC	BTV-11	Brussels, Belgium	JQ972845
23	prototype 600604	BTV-12	USA	FJ713345
24	prototype 600558	BTV-1	Laramie, USA	FJ713336

the number of granular cells at 24 hpi. This was followed by aggregation of enlarged and vacuolated cells, foamy degeneration and death of infected cells resulting in detachment of cell monolayer from the surface after 72 hpi. Control cells did not reveal any cytopathic effect. RNA extraction was performed when approximately 80% of the infected cells showed cytopathology (approx. 72 h).

Synthesized cDNA was used as a template in PCR amplification, using gene specific primers, designed to amplify 274 bp fragment of segment 5 (NS1) genes. Annealing temperature was optimized at 58°C *via* gradient PCR and *Pfu* polymerase was used to amplify the product to reduce the error rate in the amplification process. Total volumes of RT-PCR products were resolved on 1.5% agarose gel along with 100 bp molecular weight marker.

Sequence analysis

In this study, the nucleotide and amino acid sequences of the NS1 gene of BTV-16 strains were compared with several other BTV serotypes isolated from different geographical locations of the world. The nucleotide sequence of the insert obtained by sequencing (274bp) and the deduced amino acid (83aa) have been submittedtoNCBI GenBank and assigned Accession number KC706456. The sequence was analyzed along with the sequences of 23selectedother Indian and global BTV isolates belonging to various serotypes (Table 1). Multiple sequence alignment of BTV-16 for this study revealed highest nucleotide sequence identity [98.9% (BTV-21, JF747592) -96.4% (BTV-9, JF443163)] with sequences of NS1 gene of Indian isolates, and isolates from other countries [97.8% (BTV-3, FJ713338)-93.4%(BTV-1,FJ713336)]. However, there were 3 nucleotide changes between BTV-16 for this study and Indian isolates and many nucleotide changes have been detected in Western isolates (Figure 1). The lowest sequence identity detected between other sequences was 91.2% between sequences of NS1 gene of JQ924824 of BTV 16 Indian isolate and JX861502 BTV-1 of France. The study also assessed the comparative sequence identity from the deduced amino acid level and found maximum homology (100% identity) with Indian isolates and 95.2-98.8% identity with Western isolates

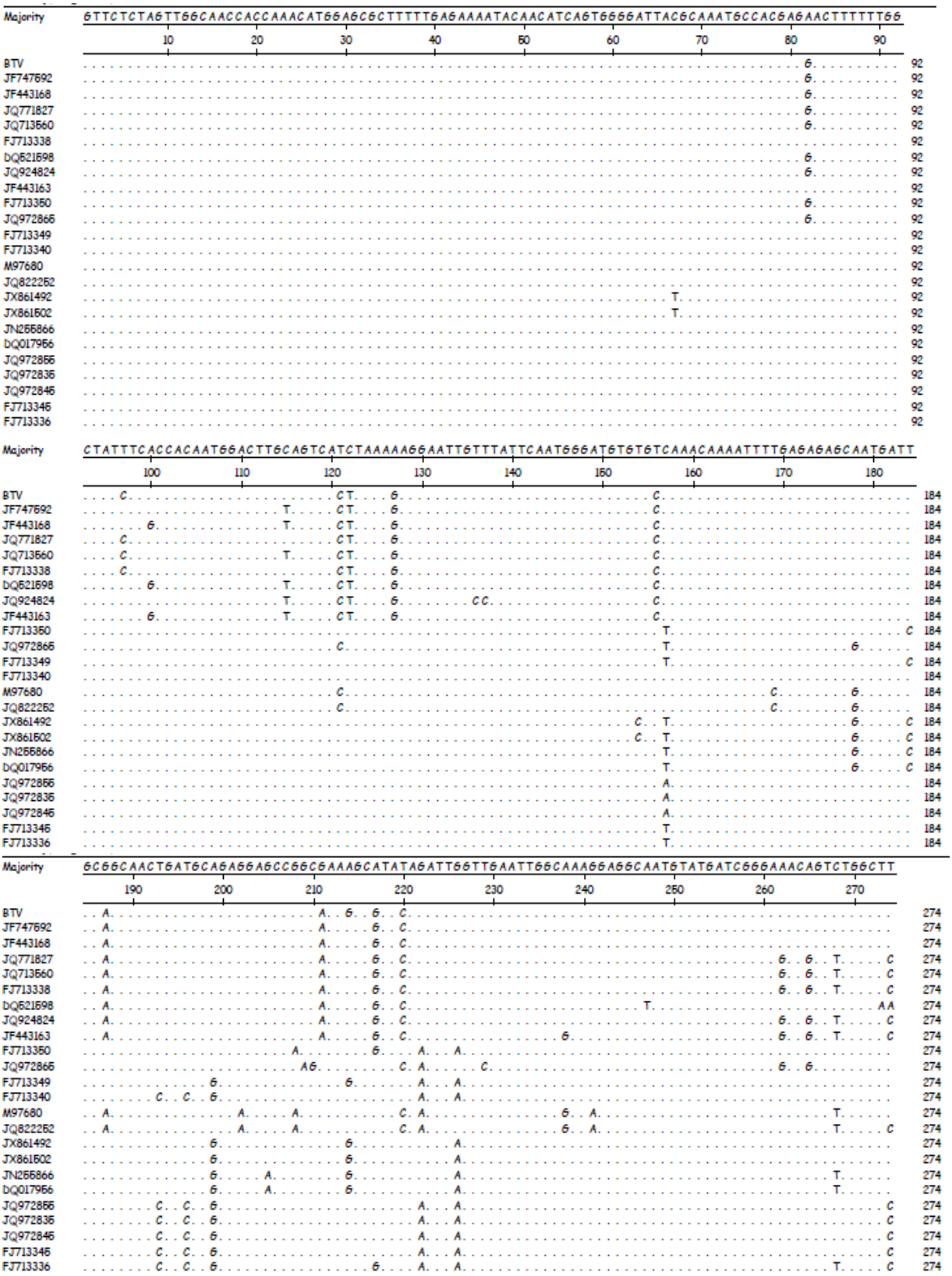

Figure 1. Multiple sequence alignment of 274 nucleotides of segment 5 (NS1) Gene (1770 nts.) corresponding to the nucleotide 10-283 of BTV-16 virus (Gene Bank Acc. No: KC706456). Dot (.) indicates nucleotide similarities with BTV-16 for this study; Dash (-) indicates either sequence not available, deletion of nucleotide or gap in the alignment.

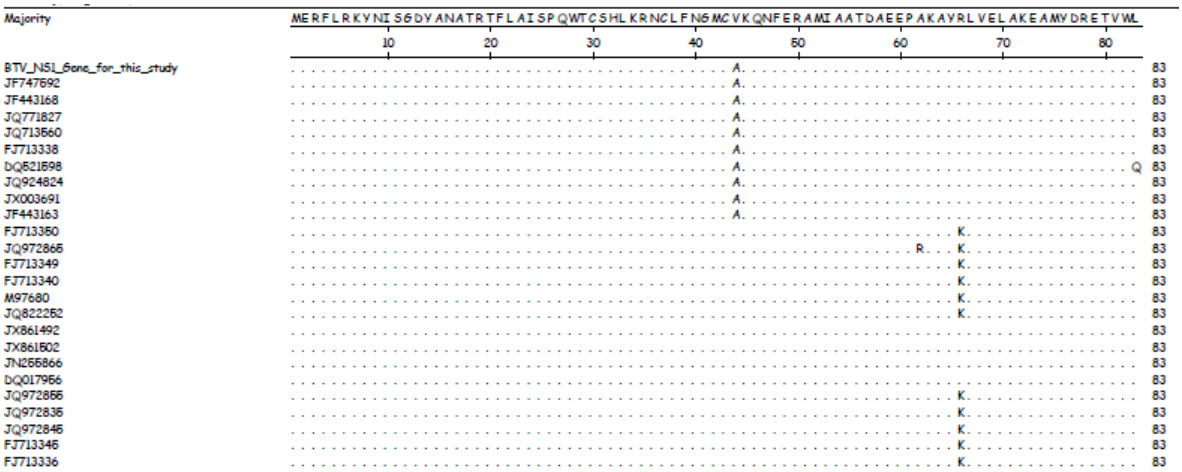

Figure 2. Multiple sequence alignment of deduced amino acid (aa) corresponding to the nt. 35-283 of segment 5(NS1) gene of BTV-16 virus (Gene Bank Acc. No: KC706456). Dot (.) indicates amino acid similarities with BTV-16 for this study; Dash (-) indicates either sequence not available, deletion of amino acid or gap in the alignment.

with only three amino acid difference. The conservation of critical amino acids was seen in this region (Figure 2).

Phylogenetic analyses

Phylogenetic analysis of NS1 gene of BTV-16 Hisar isolate with others around the world has revealed two major clusters consisting of the Indian (Eastern) linage and Western linage, with the exception of BTV-3 FJ713338 of the USA which clustered within the Indian isolate group (Figure 3). BTV-16 NS1 gene was found to be closely related with BTV-9 and 21of Indian isolates. Similarities apparent at both NS1 gene and amino acid level demonstrate that some related serotypes have sequences in common, indicating a relatively closer common ancestry. These relationships appear to depend on the geographical origin of individual isolates and serotypes (Figure 3).

DISCUSSION

Bluetongue is endemic in most parts of India (Dash et al., 2005; Singh et al., 2005; Maan et al., 2007). Twenty one out of the possible 27 serotypes of BTV are found circulating in different parts of India (Singh et al., 2005; Bitew et al., 2013). Phylogenetic analysis of VP2, VP3 and VP7 genes have been recommended to determine the relationship between the orbiviruses (Singh et al., 2005). However, molecular phylogenetic analysis including VP5 and NS3 genes have also been promoted by different researchers around the world (Dash et al., 2005; Singh et al., 2005; Maan et al., 2007, 2012a). Segment 5 (Seg-5) encodes the NS1 protein (553 amino acids), which accumulates in the cytoplasm of infected cells as tubules (Ratinier et al., 2011); however, the function of NS1 is unknown. Previous phylogenetic analysis of Seg-5 identified distinct Western and Eastern topotypes (Boyle et al., 2012). However, little information about the phylogenetic analysis of NS1 gene of BTV-16 isolates is available. In this study nucleotide and the amino acid sequence of the NS1 gene of BTV-16 isolates were compared with several other BTV serotypes isolated from different geographical locations of the world. A bluetongue virus isolated from Hisar, India was used in this study. The BTV-16 propagated in BHK21 cells revealed characteristic cytopathic effect. The extraction of dsRNA by Trizol method following differential Lithium Chloride precipitation resulted in pure BTV dsRNA. RT-PCR amplification resulted in 274 bp amplicons and this product was cloned and sequenced. This result was in line with different authors (Maan et al., 2012a; Minakshi et al., 2012; Boyle et al., 2012; Yang et al., 2012).

The comparison of nucleotide sequence of different portion of the NS1 gene revealed a few number of nucleotide substitutions among various BTV serotypes. The percent nucleotide and amino acid sequences homology among various BTV serotypes, varies from 96.4 to 98.9% and 95.2 to 100% respectively. This confirms the fact that the NS1 gene is highly conserved among the different serotypes. The result is in agreement with Maan et al. (2012a) who sequenced all genome segments of the BTV-16 reference strain (RSArrrr/16) and showed >99% sequence identity to the BTV-16 vaccine strain (RSAvvvv/16) that was derived from it and to the Chinese BTV-16 (strain BN96/16) isolated from a sheep in Yunnan province during 1996. In contrast, the genome segments of RSArrrr/16 show lower levels of identity (90 to 95%) with the BTV-16 from Australia (strain DPP96), indicating that it represents a distinct virus

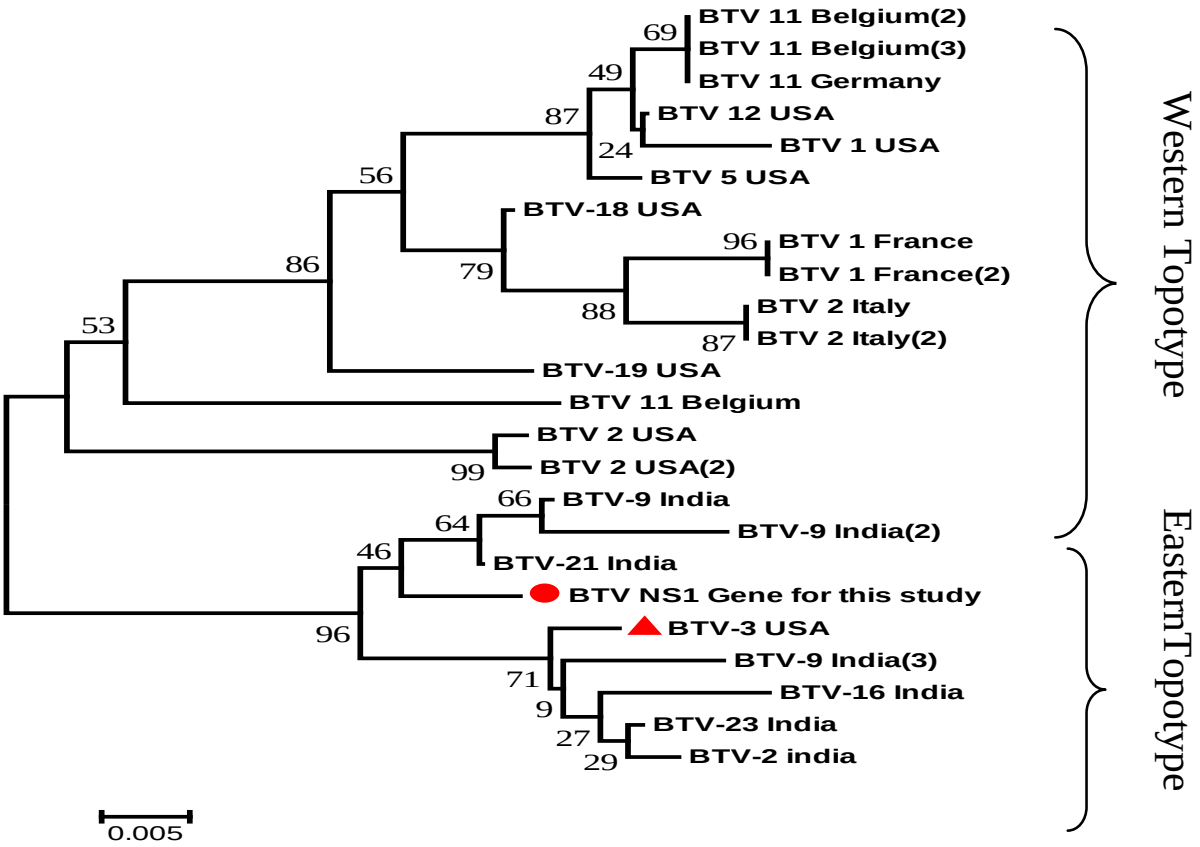

Figure 3. Neighbour-joining tree showing phylogenetic relationship between segment 5 (NS1) Gene of BTV-16 Hisar isolate and different strains (reference and field isolates) isolated worldwide. The tree is based on the 274 nucleotides of segment 5 (NS1) corresponding to the nt. 10-283 of BTV-16 virus (Gene Bank Acc.No: KC706456). Bootstrap values are indicated at the major branches.

strain/lineage, although still within the major Eastern topotype. In contrast to this, Dash et al. (2005) found that 39 to 73% and 25 to 82% nucleotide and amino acid sequences homology respective among various BTV serotypes based on L2 gene which is the variable segment of BTV.

Phylogenetic analysis of NS1 gene of BTV-16 Hisar isolate with others around the world has revealed two major clusters which includes the Indian (Eastern) linage and Western linage separately. However, BTV-3, FJ713338 of USA clustered within the Indian isolate group. This suggests the introduction of Western BTV strains and re-assortment between Eastern and Western field strains in India. BTV-16 has shown to be closely related with BTV-9 and 21of Indian isolate with its NS1 gene. This is in line with the works of Boyle et al. (2012) and Maan et al. (2011) who identified distinct Western and Eastern topotypes with previous work on phylogenetic analysis of Seg-5 on (BTV 1, 7, 9, 20, and 21) and BTV-26 respectively. Phylogenetic analyses of BTV-3 (IND2003/08 isolate) by Maan et al. (2012c) show that nine genome segments belong to an Eastern lineage/topotype, with Seg-2 and Seg-6 (encoding outer capsid proteins VP2 and VP5) sharing the highest identity (90 and 91%, respectively) with earlier Eastern isolates of BTV-3 (Japanese strain ON-6/B/98). However, Seg-5/NS1 of IND2003/08 showed up to 99% identity with the Western topotype viruses (prototype 600565 strain), providing further evidence for the introduction of Western BTV strains and reassortment between Eastern and Western field strains in India (Maan et al., 2012d, b; Wilson et al., 2009). Western topotype or reassortant BTV strains may be at least partly responsible for the increased virulence of bluetongue outbreaks seen in India, in indigenous sheep breeds. Seg-5 of IND2003/08 showed only 89% identity with a Western BTV-10 vaccine strain detected in India (Maan et al., 2012b) and is therefore unlikely derived from this source. Singh et al. (2005) also revealed the level of genetic diversity in genome segment 5, between different BTV serotypes, as well as between isolates of the same serotype. The BTV-3 isolate from USA is genetically closely related in genome segment 5 (NS1 encoding region), to strains from India, but is quite distinct from those from other

isolates from USA and Europe. Singh et al. (2005) also reported the same result in his work on phylogenetic analysis based on segment 6 of BTV-3.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This work was supported by the all India network program on bluetongue disease, Indian Council of Agricultural Research (ICAR), New Delhi, India. The authors would like to appreciate the Director, Indian Veterinary Research Institute for providing necessary facilities to carry out this study.

REFERENCES

Abera T, Bitew M, Gebre D, Mamo Y, Deneke Y, Nandi S (2018). Bluetongue Disease in small ruminants in south western Ethiopia: Cross-sectional Sero-epidemiological study. BMC Research Notes 11(1):112.

Batten CA, Henstock MR, Steedman HM, Waddington S, Edwards L, Oura AL (2013). Bluetongue virus serotype 26: Infection kinetics, pathogenesis and possible contact transmission in goats. Veterinary Microbiology 162:62-67.

Belhouchet M, Mohd Jaafar F, Firth AE, Grimes JM, Mertens PPC, Attoui H (2011). Detection of a Fourth Orbivirus Non-Structural Protein. PLoS ONE 6(10):e25697. doi:10.1371/journal.pone.0025697

Bitew M, Nandi S, Ravishankar C, Sharma A, Chander V. (2017). Humoral immune response and protective efficacy of Binary ethylenimine (BEI) inactivated pentavalent bluetongue vaccine after challenge with homologous virus in sheep. International Journal of Virology 13(1):43-52.

Bitew M, Nandi S, Ravishankar C., Somvanshi R. (2013). Serological and molecular evidence of bluetongue in sheep and goats in Uttar Pradesh, India. African Journal of Biotechnology 12(19):2699-2705.

Boyce M, Celma CC,Roy P (2008). Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. Journal of Virology 82:8339-8348.

Boyce M, Wehrfritz J, Noad R, Roy P (2004). Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. Journal of Virology 78:3994-4002.

Boyle DB, Bulach DM, Amos-Ritchie R, Adams M M, Walker PJ, Weirb R (2012). Genomic sequences of Australian bluetongue virus prototype serotypes reveal global relationships and possible routes of entry into Australia. Journal of Virology 86(12):6724.

Celma CC, Roy P (2009). A viral nonstructural protein regulates bluetongue virus trafficking and release. Journal of Virology 83:6806-6816.

Dash PK, Nandi S, Pande A, Mondal B, Bandyopadhyay SK (2005). Comparative phylogenetic analysis of bluetongue virus Based on sequencing of two different regions of L2 gene. Indian Journal of Comparative Microbiology Immunology and Infectious Diseases 26(2):79-85.

DeMaula CD, Bonneau KR, MacLachlan NJ (2000). Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. Virus Research 67:59-66.

Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Grimes JM, Burroughs JN, Gouet P, Diprose JM, Malby R, Ziéntara S, Mertens PP, Stuart DI (1998).The atomic structure of the bluetongue virus core. Nature 395:470-478.

Han Z, Harty RN (2004). The NS3 protein of bluetongue virus exhibits viroporin-like properties. Journal of Biological Chemistry 279:43092-43097.

Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G (2008). Genetic characterization of toggenberg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerging and Infectious Disease 14:1855-1861.

Horscroft NJ, Roy P (2000). NTP binding and phosphohydrolase activity associated with purified bluetongue virus non-structural protein NS2. Journal of General Virology 81:1961-1965.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

Maan NS, Maan S, Guimera M, Nomikou K, Morecroft E, Pullinger G, Belaganahalli MN, Mertens PPC (2012b). The genome sequence of a reassortant bluetongue virus serotype 3 from India. Journal of Virology 86(2):6375-6376.

Maan NS, Maan S, Nomikou K, Guimera M, Pullinger G, Singh KP, Belaganahalli MN, Mertens PPC (2012a). The genome sequence of bluetongue virus type 2 from India: evidence for reassortment between Eastern and Westerntopotype field strains. Journal of Virology 86:5967-5968.

Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, Samy AM, Reda AA, Al-Rashid SA, Batel M El, Oura CAL, MertensPPC (2011). Novel bluetongue virus serotype from Kuwait. Emerging and Infectious Disease 17:886-889.

Maan S, Maan NS, Pullinger G, Nomikou K, Morecroft E, Guimera M, Belaganahalli MN, Mertens PPC (2012d). The genome sequence of bluetongue virus type 10 from India: evidence for circulation of a Western topotype vaccine strain. Journal of Virology 86: 5971-5972.

Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PPC (2007). Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. Journal of General Virology 88:621-630.

Maan S, Maan NS, Singh KP, Belaganahalli MN, Guimera M, Pullinger G, Nomikou K, Mertens PPC (2012c).Complete Genome Sequence Analysis of a Reference Strain of Bluetongue Virus Serotype 16. Journal of Virology 86(18):10255.

Mertens PP, Diprose J (2004). The bluetongue virus core: a nano-scale transcription machine. Virus Research 101:29-43.

Mertens PP, Diprose J, Mann S, Singh KP, Attoui H (2004). Bluetongue virus replication, molecular and structural biology. VeterinariaItaliana 40:426-437.

Minakshi P, Singh R, Ranjan K, Kumar P, Joshi CG, Reddy YKM, PrasadG (2012). Complete genome sequence of bluetongue virus serotype 16 of goat origin from India. Journal of Virology 86(15):8337.

Noad R, Roy P (2009). Bluetongue virus replication and assembly. In: Mellor P, Baylis M, Mertens P, eds. Bluetongue. Amsterdam: Academic Press pp. 53-76.

Owens RJ, Limn C, Roy P (2004). Role of an arbovirus nonstructural protein in cellular pathogenesis and virus release. Journal of Virology 78: 6649-6656.

Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ (2008). Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. Revue scientifique et technique 27:427-442.

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PP, Baylis M (2005). Climate change and the recent emergence of bluetongue in Europe. Nature Review Microbiology 3:171-181.

Ratinier M, Caporale M, Golder M, Franzoni G, Allan K, Nunes FS, Armezzani A, Bayoumy A, Rixon F, Shaw A, Palmarini M (2011). Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathogens 7(12):e1002477. doi:10.1371/journal.ppat.1002477.

Roy P (2008). Bluetongue virus: dissection of the polymerase complex. Journal of General Virology 89:1789-1804.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Schulz C, Bréard E, Sailleau C, Jenckel M,Viarouge C, Vitour D, Palmarini M, Gallois M, Höper D, Hoffmann B, Beer M, Zientara S (2016). Bluetongue virus serotype 27: detection and characterization

of two novel variants in Corsica, France. Journal of General Virology 97(9):2073-2083.
Singh KP, Maan S, Samuel AR, Rao S, Meyer A, Mertens PP (2005). Phylogenetic analysis of bluetongue virus genome segment 5 (encoding NS1) from different serotypes. VeterinariaItalaliana 40:479-483.
Sperlova A, Zendulkova D (2011). Bluetongue: a review.Veterinary Medicine 56:430-452.
Sutton G, Grimes JM, Stuart DI, Roy P (2007). Bluetongue virus VP4 is an RNA-capping assembly line. Nature Structural and Molecular Biology 14:449-451.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28:2731-2739.
Wade-Evans AM, Mertens PPC, Bostock CJ (1990). Development of the polymerase chain reaction for the detection of bluetongue virus in tissue samples. Journal of Virological Methods 30:15-24.
Wehrfritz JM, Boyce M, Mirza S, Roy P (2007). Reconstitution of bluetongue virus polymerase activity from isolated domains based on a three-dimensional structural model. Biopolymers 86:83-94.
Wilson WC, Hindson BJ, O'Hearn ES, Hall S, Tellgren-Roth C, Torres C, Naraghi-Arani P, Mecham JO, Lenhoff RJ (2009). A multiplex real-time reverse transcription polymerase chain reaction assay for detection and differentiation of Bluetongue virus and Epizootic hemorrhagic disease virus serogroups. Journal of Veterinary Diagnostic Investigation 21:760-770.
Yang T, Liu N, Xu Q, Sun E, Qin Y, Zhao J, Feng Y, WuD (2012). Complete genomic sequence of bluetongue virus serotype 1 from China. Journal of Virology 86(2):1288.
Zhang X, Boyce M, Bhattacharya B, Zhang XK, Schein S, Roy P, Zhou ZH (2010).Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. Proceedings of the National Academy of Sciences of the United States of America 107:6292-6297.

Vol. 18(29), pp. 783-796, September, 2019
DOI: 10.5897/AJB2019.16918
Article Number: D39771B61816
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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Cytogenetic and molecular assessment of some nanoparticles using *Allium sativum* assay

Mona S. Al-Ahmadi

Department of Biology, College of Science, University of Imam Abdulrahman Bin Faisal, P. O. Box 1982, Dammam 31441, Kingdom of Saudi Arabia.

Received 15 July, 2019; Accepted 26 August, 2019

One of the primary objectives in agriculture is providing high-quality crops to consumers. Multiple techniques and methods are utilized to achieve this objective, including nanotechnology that depends on the use of very small materials, which will help in decreasing the amounts usually used with similar effects. Nanomaterials are used as fertilizers and also as component of nano-pesticides for plants. Despite their benefits, however, studies have noted their potential for cytotoxicity and genotoxicity. In this study, five nanoparticles (NPs) were tested to assess their effects on plants. The chromosomal aberration assay was used. The results showed that some NPs decreased the mitotic index (MI) significantly, which indicates the NPs' potential cytotoxicity. In addition, different NPs' treatments caused different types of chromosomal abnormalities e.g., chromosomes stickiness and disturbance of the metaphase and anaphase, lagging chromosomes, bridges, disturbed poles, micronuclei, s-metaphase, s-telophase, c- metaphase and bi-nucleus cells. All treatments had significant effects at p≤005. Treatments with NPs concentrations for 24 h affected the DNA content, AlO_2 and Fe_3O_4 NPs' increased the DNA content, while CeO_2, TiO_2 and Ag NPs' decreased it. High concentrations of the tested NPs decreased the DNA content. The study results showed that CeO_2 was the most harmful NP compared to the control and other NPs. Some types of chromosome abnormalities such as lagging chromosomes, bridge, and micronuclei indicate potential genotoxicity for these NPs. Despite of the positive effects, they also had negative side effects such as decreasing the MI and increasing the occurrence of different types of chromosomal abnormalities.

Key words: Cytotoxicity, genotoxicity, nanoparticles, mitotic chromosomes abnormalities.

INTRODUCTION

Nanotechnology has been used in many fields. In agriculture, one of these technologies involves the use of different elements in nano sizes, which can give satisfactory results using a low amount of the element compared with its natural size. Nanomaterials are used in various applications such as plants protection, nutrition and of farm practices management due to their small size, high surface-to-volume ratio, and unique optical properties (Ghormade et al., 2011).

Nanoparticles (NPs) interact with plants, causing many

E-mail: nsadeghi@sina.tums.ac.ir. Tel: +98-21-66954713. Fax: +098-21-66461178.

Table 1. Nanoparticles Concentrations and their description.

Nanoparticles	Description	Concentration %		Reference
		Low	High	
AlO_2 NPs	Form nano powder particle size <50 nm (TEM); surface area >40 m^2/g (BET); mp 2040°C (lit.)	20 mg	40 mg	Lee et al. (2010)
Fe_3O_4 NPs	Form nano powder particle size 50-100 nm (SEM); surface area >60 m^2/g; mp 1538°C (lit.)	0.025 g	5.9 g	Sheykhbaglou et al. (2010)
CeO_2 NPs	Form nano powder particle size <100 nm; mp >400°C	0.012 g	0.024 g	Ma et al. (2013)
TiO_2 NPs	Form, nano powder primary particle size 21 nm (TEM); surface area 35-65 m^2/g (BET); mp 1850°C >350°C (lit.)	10 mg	20 mg	Song et al. (2012)
Ag NPs	Form nanoparticles contains sodium citrate as stabilizer concentration 0.02 mg/ml in aqueous buffer particle size 10 nm (TEM); density 0.997 g/mL at 25°C	0.0005 mg	0.001 mg	Salama (2012)

morphological and physiological changes depending on their properties (Khodakovskaya et al., 2012). Chen and von Mikecz (2005) demonstrated that some NPs can enter cell nuclei and may directly affect the structure and function of the DNA genome. The efficacy of NPs depends on their concentrations, and these concentrations differ from plant to plant (Siddiqui et al., 2015). Also, NPs can have positive and negative impacts in higher plants (edible plants) and on their consumers in the food chain (Rico et al., 2011).

The minute size of NPs, smaller than cells and cellular organelles, allows them to penetrate those basic biological structures, disrupting their normal function (Buzea et al., 2007). Zheng et al., (2005) concluded that the up-take efficiency and effects of NPs on growth and metabolic function vary among plants. The concentrations of NPs affect processes like germination and plant growth. Babu et al., (2008) also suggested that the NPs' size gives them free entry inside cells, where they can interfere in normal cell function. Landsiedel et al., (2009), Kovacic and Somanathan, 2010 and Siddiqui et al., (2015) suggested that the ability of NPs to penetrate cells easily allows them to affect the intercellular organelles and nucleic acids. NPs characteristics such as their small size, their shape and their large surface-area-to-mass ratio, and their propensity to cross cell barriers and their interaction with intercellular contribute to potential cellular and genetic toxicity caused by the induction of oxidative stress. Hunt et al., (2013) assessed the effects of nano silver on *Caenorhabditis elegans* by measuring the 8-OH guanine levels and found that the silver induced oxidative damage in DNA. A similar result was found by Çekiç et al., (2017) in tomato plants. Cobalt oxide NPs were investigated by Faisal et al., (2016) to assess their effect on eggplant DNA. The results indicated that cobalt oxide NPs induced DNA strand breaks and apoptosis. Also, NPs cause chromosomal aberrations as several researchers have discussed in their study of these effects in higher plants (Kumari et al., 2009; Ghosh et al., 2010; Landa et al., 2012; Mukherjee et al., 2016; Debnath et al., 2018).

Higher plants are recognized as being excellent indicators of the cytogenetic and mutagenic effects of environmental chemicals. The study of these plants is also useful for detecting environmental mutagens indoors and outdoors. These plants are highly reliable bioassays for monitoring and testing for genotoxins because of their high sensitivity (Grant, 1999).

In this study, five NPs were tested to estimate their cytotoxicity and genotoxicity using a chromosomal aberration assay and to determine their effect on the DNA content of *Allium sativum*.

MATERIALS AND METHODS

Tested materials

Table 1 shows NPs and different concentrations chosen depending on previous studies that found out that treatment with these concentrations had a positive effect on root length, yield and quality, biomass, and plant growth without serious harm on plants.

Sample preparations

A. sativum, common name (garlic) 2n = 16, gained from local markets were used as testing material. The loose outer scales and

old roots were scraped and suspended in small beaker with distilled water.

Treatments

A. sativum were suspended in a small beaker (50 ml) with distilled water to encourage the root tips to grow until they reached 0.5 to 1 cm in length; they were then transferred to another beaker containing freshly prepared solutions of tested NPs, Aluminium oxide, Ferric oxide, Cerium oxide, Titanium oxide and Silver. Low concentrations (20 mg, 0.025 g, 0.012 g, 10 mg, 0.0005 mg) and high concentrations (40 mg, 5.9 g, 0.024 g, 20 mg, 0.001 mg) sequentially, and left for different periods of time (6, 16, and 24 h). One bulb of garlic was used for each treatment. The negative control was root tips treated with distilled water only, used as a qualified sample to compare for the effects of tested materials.

Slides preparation

The treated roots tips and negative control (untreated) were detached, fixed in freshly prepared 3:1 (v/v) ethanol alcohol: glacial acetic acid for 24 h. The root tips of *A. sativum* were hydrolyzed in 1N HCL at 60°C for 8 min. The root tips were then washed with distilled water several times and stained with 1% acetocarmin. Five temporary slides were prepared using the squash technique. Two root tips on each slide were examined for the effects of NPs on the mitotic index (MI). The same slides were analyzed for the types and frequencies of chromosomal abnormalities produced by the examined NPs.

DNA studies

Fisher bioreagents Sure-prep RNA/DNA/Protein purification kit was used to extract the genetic material from plant tissues following the instruction of HiPuraTM product. The plant DNA isolation was done using the CTAB method. The concentration of the isolated DNA was measured by Scan drop (Analytik Jena) device.

Scoring of slides and data analysis

Studying slides

The slides were viewed under light microscope (Phenix P H 50 DB047VU) using the 40X objective lens immersion. The demonstrative slides for each physical aberration were photo-graphed using Phenix micro Image analyzer Software 2008 EnV2, 2.

Mitotic index

On one slide for each treatment, a total of 2000 cells, were scored. The mitotic index (MI) was expressed as the number of dividing cells per total cells scored, as per the following equation:

Mitotic index (MI) = (Total number of dividing cell/Total number of cell examined) × 100

Cytotoxicity

The mitotic index of the treated cells was compared with that of the negative control sample.

Genotoxicity test

Chromosomal aberration per dose of each NP was examined; the percentage of cells with aberrations of each dose for each NP was scored and compared with that of the negative control as per the following equation:

Chromosomal aberration frequency (CF) = Total number of abnormal cell/Total number of normal cell

Statistical analysis

A two-way analysis of variance was used for determining the significance of difference at $p \leq 0.05$ (SPSS 16.0 for Windows statistical package).

RESULTS

Mitotic index (MI)

The NPs effects on the MI of A. sativum root tip cells

Table 2 and Figures 1 and 2 show the treatment results; it appears that the AlO_2 NPs 20 and 40 mg concentrations decreased the MI after treatment for 24 h. This treatment was insignificant at $p \geq 0.05$, while treatment with a low concentration of Fe_3O_4 for 24 h was significant at $p \leq 0.05$. Also, CeO_2 NPs decreased the MI after treatment with a low concentration for 6 h and high concentration for 24 h. This result was insignificant at $p \geq 0.05$. The TiO_2 NPs treatment with a low concentration and a high concentration for 24 h decreased the MI. This result was significant at $p \leq 0.05$. Treatment with a high concentration of Ag NPs for 16 h decreased the MI. This result was significant at $P \leq 0.05$. Some treatments with a low concentration of NPs increased the MI. The AlO_2 low concentration for 16 h, high and low concentrations of Fe_3O_4, also high and low concentrations of CeO_2 for 16 h, and a low concentration of Ag for 6 h increased the MI compared to the control. These results were insignificant $p \geq 0.05$.

Chromosomal aberrations (CA)

Examining the cytological aberrations in plants is an excellent way to detect genetic hazards that environmental substances may pose (Grant, 1978).

Table 3a-b and figures 3, 4, and 5 show the types of abnormalities found in the mitotic chromosomes of *A. sativum* root tip cells after treatment with different concentrations of NPs.

All tested materials affected the chromosomes and increased chromosomal aberrations compared to the control, and the results were significant at $p \leq 0.05$. The most harmful concentrations were AlO_2 NPs after treatment with a high concentration of 40 mg for 6 and 16 h (0.2) compared to the control (0.04), Fe_3O_4 NPs after

Table 2. Effects of different concentration of some nano-particles for different periods of time on mitotic index and chromosomal aberrations frequency.

Material	Concentration (%)	Time of duration (h)	No. of total cells	Mutant cells	Mitotic index	CA
Distilled Water	Distilled Water	6	2139	6	8	0.04
	Distilled Water	16	2054	5	7	36
	Distilled Water	24	2037	3	9	2
ALO_2 NPs	20 mg	6	2182	14	8	0.1
	20 mg	16	2126	18	8	0.12
	20 mg	24	2067	21	8.8	0.12
	40 mg	6	2305	30	8	0.2
	40 mg	16	2160	22	7	0.2
	40 mg	24	2201	17	7	0.1
Fe_3O_4 NPs	0.025g	6	2254	16	6	0.1
	0.025g	16	2178	21	7	0.1
	0.025g	24	2060	5	5	0.1
	0.05g	6	2122	27	7	0.2
	0.05g	16	2135	14	8	0.1
	0.05g	24	2125	18	6	0.1
CeO_2 NPs	0.012 g	6	2021	16	7	0.1
	0.012 g	16	2144	13	8	0.1
	0.012 g	24	2054	14	8	0.1
	0.024 g	6	2022	17	9	0.1
	0.024 g	16	2087	19	8	0.1
	0.024 g	24	2068	16	7	0.1
TiO_2 NPs	10 mg	6	2189	14	7	0.1
	10 mg	16	2317	17	7	0.1
	10 mg	24	2191	17	6	0.1
	20 mg	6	2120	11	7	0.1
	20 mg	16	2214	13	7	0.1
	20 mg	24	2262	13	7	0.1
Ag NPs	0.0005 mg	6	2019	18	10	0.1
	0.0005 mg	16	2089	17	7	0.1
	0.0005 mg	24	2076	16	8	0.1
	0.001 mg	6	2237	12	7	0.1
	0.001 mg	16	2054	5	5	0.1
	0.001 mg	24	2244	32	8	0.2

treatment with a high concentration of 0.05 g for 6 h (0.2) and Ag NPs (0.2) after treatment with a high concentration 0.001 mg for 24 h compared to the control (0.02).

The types of chromosomal abnormalities scored after treatment with different NPs were chromosomal disturbance and stickiness during metaphase and anaphase, sticky telophase, chromosomes bridges during the anaphase and telophase, micronuclei, lagging chromosomes, star metaphase and star telophase, bi-nucleus cells, and disturbed poles during anaphase. Specific types of chromosome aberrations were scored after treatment with some NPs and they were C-metaphase, lagging chromosome in the telophase stage micronuclei, bi-nucleus cells, after treatment with AlO_2 and Fe_3O_4 NPs, furthermore AlO_2 NPs caused the formation of abnormal anaphase poles. Treatment with CeO_2 NPs caused the formation of Star-metaphase, ring chromosome, C-metaphase and telophase bridge. The types of the chromosomal abnormalities scored after treatment with TiO_2 NPs were S-metaphase, lagging chromosome during anaphase stage, S-telophase,

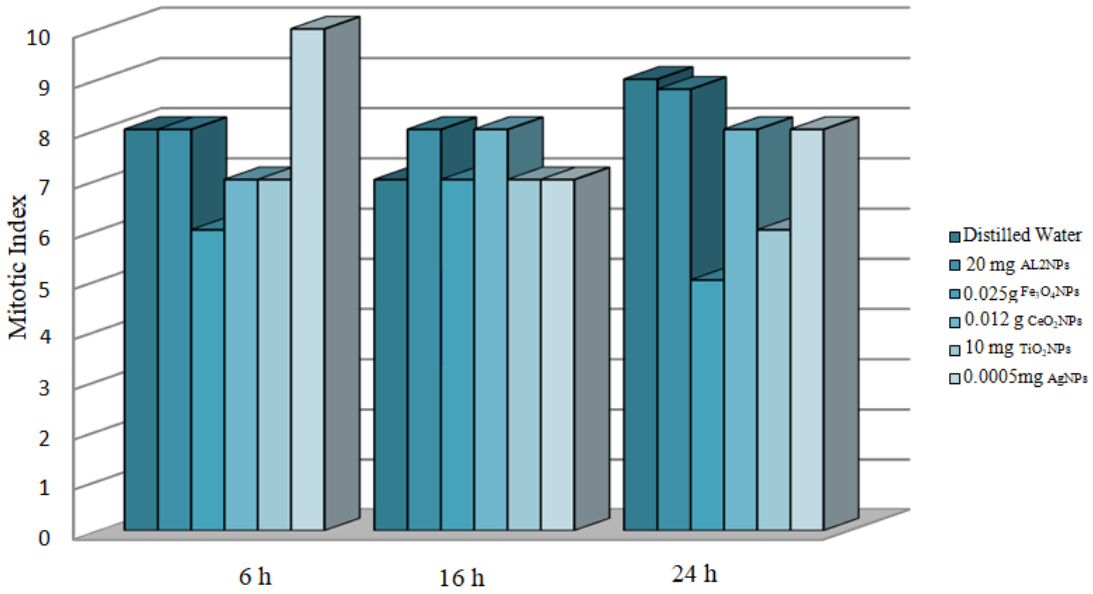

Figure 1. Effects of low concentrations of some nano-particles for different periods of time on mitotic index of *Allium sativum.*

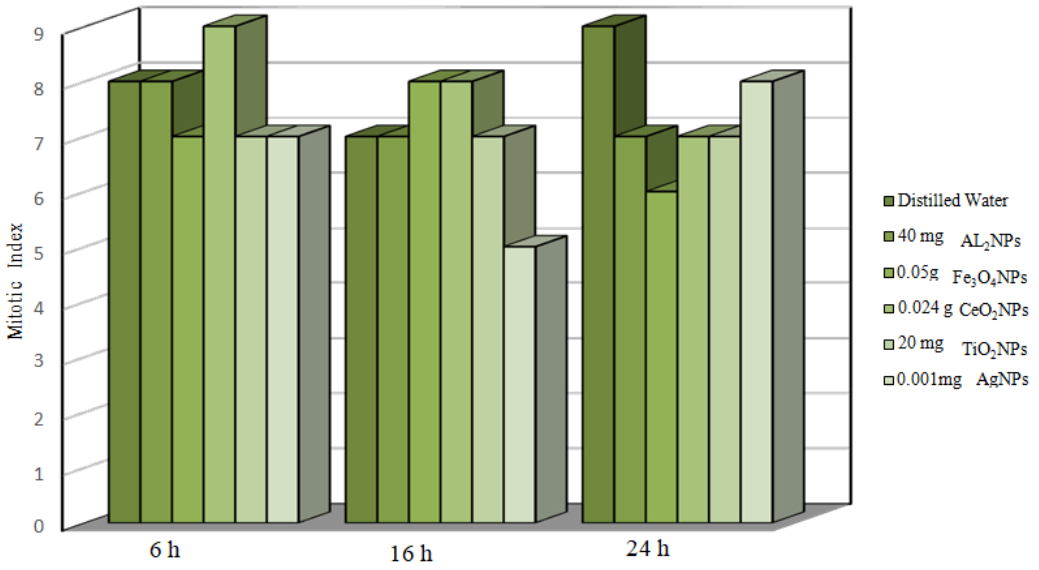

Figure 2. Effects of high concentrations of some nano-particles for different periods of time on mitotic index of *Allium sativum.*

bi-nucleus cells and micronuclei. Ag NPs produced lagging chromosome, S- anaphase, abnormal pole of anaphase stage, chromosomes bridges and bi-nucleus cells. Some types of chromosome abnormalities indicated the potential genotoxicity of tested NPs, e.g., micronuclei, lagging chromosomes, and the chromosome bridges during anaphase and telophase.

DNA content

Table 4, 5 and figures 6 and 7 show the effect of different concentrations of NPs (AlO_2, Fe_3O_4, CeO_2, TiO_2, Ag) on DNA content after 24 h.

All the tested NPs affected the DNA content. Specifically, the content decreased after treatment with

Table 3. Types of chromosomal aberrations scored after treatment with different concentrations of Nano-particles for different periods of time on root tip cells of *Allium sativum*.

Tested material		Control			ALO_2 NPs						Fe_3O_4 NPs					
					20 mg			40 mg			0.025 mg			0.05 mg		
Type of CA		**6**	**16**	**24**	**6**	**16**	**24**	**6**	**16**	**24**	**6**	**16**	**24**	**6**	**16**	**24**
Sticky	Metaphase	-	0.007	0.006	0.01	0.02	0.02	0.04	0.09	0.04	0.034	0.06	0.009	0.007	0.02	0.01
Disturb	Metaphase	-	0.03	-	-	0.04	0.03	0.05	0.007	0.03	-	-	0.03	0.04	0.03	0.0008
Lagging	Metaphase	-	-	-	-	-	-	-	-	-	-	-	-	0.007	-	-
Fragments	Metaphase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0008
c-metaphase	Metaphase	-	-	-	-	-	-	-	-	0.006	0.007	-	-	-	-	-
Sticky	Anaphase	-	-	-	-	0.006	-	-	0.007	0.006	-	0.013	-	-	-	-
Disturb	Anaphase	-	-	-	0.01	0.006	-	-	-	-	0.014	-	-	-	0.006	0.02
S. anaphase	Anaphase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lagging	Anaphase	-	-	-	-	-	-	-	-	-	-	-	-	0.007	-	-
Bridge	Anaphase	0.04	-	0.01	0.045	0.01	0.04	0.05	0.05	0.02	0.014	0.04	0.009	0.06	0.002	0.05
Sticky disturb	Telophase	-	-	-	-	-	-	-	-	-	0.02	0.013	-	-	-	-
Lagging bridge	Telophase	-	-	-	-	-	0.005	-	-	-	-	0.006	-	-	0.006	0.0008
Dis polar	Telophase	-	-	-	-	0.006	-	-	-	-	-	-	-	-	-	-
Fragment	Telophase	-	-	-	-	-	0.005	-	-	-	-	-	-	-	-	-
Bi-nucleate		-	-	-	0.01	0.006	-	0.017	-	-	0.007	-	-	-	-	-
Micronuclei		-	-	-	-	-	-	0.005	-	0.006	0.014	0.006	-	-	0.006	0.02
%		0.04	0.036	0.02	0.08	0.12	0.12	0.2	0.15	0.11	0.1	0.1	0.05	0.18	0.08	0.1

low concentrations of Ag, TiO_2 and CeO_2 NPs (50.65, 55.32 and 97.63 ng/µl, respectively), and the results were significant at $P \leq 0.05$. AlO_2 NPs and Fe_3O_4 NPs increased the DNA concentration (391.34 and 234.07 ng/µl, respectively) and these results were significant at $P \leq 0.05$ compared to the control (144.73 ng/µl). Treatments with high concentrations affected the DNA content. The NPs Fe_3O_4, Ag and CeO_2 (130.37l, 124.65, 119.33 ng/µl, respectively) decreased the DNA concentration, and these results were significant at $p \leq 0.05$; the results showed that CeO_2 NPs were the most harmful and that TiO_2 NPs were the least harmful followed by AlO_2 compared to the control (144.73 ng/µl).

DISCUSSION

Mitotic index (MI)

The NPs treatments reduced the MI. The decrease of MI might have resulted from the effect of the NPs during S-phase which inhibited the DNA synthesis. The decrease might also be due to the activation of enzymes by decreasing or inhibiting the enzymes, particularly the enzymes that involved in DNA replication or cell division (Sudhakar et al., 2001).

AlO_2 NPs caused decreased MI. This effect may be due to the blockage at GI stage, which disturbs the DNA synthesis (Mohandas and Grant, 1972). A similar result was found by Rajeshwari et al., (2015).

The effect of Fe_3O_4 NPs on cells was as reported by Alarifi et al., (2014), that is, the cell

Table 4. Types of chromosomal aberrations scored after treatment with different concentrations of Nano- particles for different periods of time on root tip cells of *Allium sativum*

Tested material		Control			ALO_2 NPs						Fe_3O_4 NPs						Ag NPs					
					20 mg			40 mg			0.025 mg			0.05 mg			0.0005 mg-l			0.001 mgl-1		
Type of CA		6	16	24	6	16	24	6	16	24	6	16	24	6	16	24	6	16	24	6	16	24
Sticky		-	0.007	0.006	0.03	0.01	0.05	-	0.02	0.04	0.03	0.03	0.04			0.01	0.02	0.03	0.02	0.007	0.009	0.06
Disturb		-	0.03	-	0.03	0.006	-	0.02	0.02	0.03	0.007	0.01	0.02	0.04	0.03	0.007	0.005	0.03	0.03	0.03	0.03	0.06
Lagging	Metaphase	-	-	-	-	-	-	0.006	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fragments		-	-	-	-	0.006	-	-	-	0.006	-	-	-	-	-	-	-	-	-	-	-	-
c-metaphase		-	-	-	-	0.006	-	-	0.006	0.006	-	0.006	-	-	0.006	-	-	-	-	-	-	-
Sticky		-	-	-	-	-	0.01	0.006	0.006	-	-			-	0.007	0.005	-					
Disturb		-	-	-	0.007	0.006	0.006	0.006	-	-	0.007	-	0.007	0.02	0.006	0.01	-	0.01	-	0.03	-	0.01
S. anaphase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.006	-	-	0.006
Polar	Anaphase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.006	-	-	-
Lagging		-	-	-	-	-	-	0.01	0.006	-	0.007	0.006	0.007	-	-	-	-	-	0.006	-	0.009	-
Bridge		0.04	-	0.01	0.04	0.04	0.03	-	0.06	0.03	0.05	0.03	0.05	0.007	0.03	0.05	0.04	0.05	0.02	0.04	-	0.05
Sticky		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	0.006
bridge		-	-	-	-	-	-	0.006	-	-	-	-	-	-	-	-	-	-		-	-	-
S. anaphase	Telophase	-	-	-	-	-	-	-	-	-	-	0.006	-	-	-	-	-	-		-	-	-
Bi-nucleate		-	-	-	-	-	-	-	-	-	-	0.02	-	-	-	-	-	-	0.01	-	-	-
Micronuclei		-	-	-	-	-	-	-	-	-	0.007	-	-	0.007	-	-	-	-	-	-	-	-
%		0.04	0.036	0.02	0.1	0.07	0.09	0.1	0.1	0.1	0.1	0.1	0.12	0.08	0.08	0.09	0.09	0.11	0.1	0.08	0.05	0.2

death mediated by the reactive oxygen species (ROS) triggered mitochondrial pathway as evidenced by the cleavage of caspase-3 activity and caused an imbalance between the production and degradation of ROS and induced oxidative stress. NPs may change the production of ROS and affect antioxidation defense and so induce oxidative stress (Srinivas et al., 2011). More explanations of iron oxide reaction were reported by Zhongwen et al., (2012), that the cytotoxicity ability of iron oxide, iron oxide trapped in acidic lysosomes of the cell, and they catalyze decomposition of H_2O_2 to produce hydroxyl radicals through peroxidase-similar activity.

The cytotoxicity of CeO_2 may be due to the oxidative stress (Jezek and HIavata, 2005). Park et al., (2008) found that CeO_2 caused cytotoxicity because of the introduction of ROS, and that the free radical species produced by CeO_2 NPs significantly reduce the levels of cellular antioxidants. Also, Sendra et al., (2016) suggested that the toxicity of CeO_2 NPs may be due to their photocatalytic properties. Similar results were demonstrated by Liman et al., (2019).

The TiO_2 NPs decreased the MI compared to the control. Pakrashi et al. (2014) found that TiO_2 NPs increased ROS and that this was the main contribution to the toxic effects. Castiglione et al., (2011) produced similar results in a study of the effect of TiO_2 NPs on *Vicia faba* and *Zea mays*, while Klien and Godnic (2012) in a study of the effect of TiO_2 NPs on rodents.

Ag NPs decreased the MI compared to the control. Patlolla et al., (2012) explained that the decrease in MI after treatment with different concentrations of Ag NPs might be due to a lower

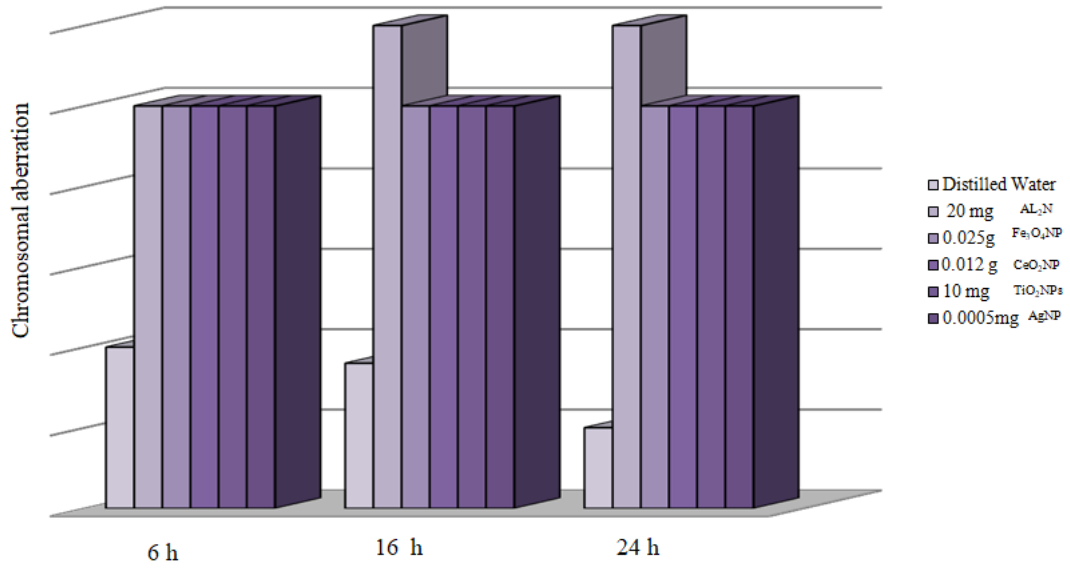

Figure 3. Effects of low concentrations of some nano-particles for different periods of time on chromosomal aberrations of *Allium sativum.*

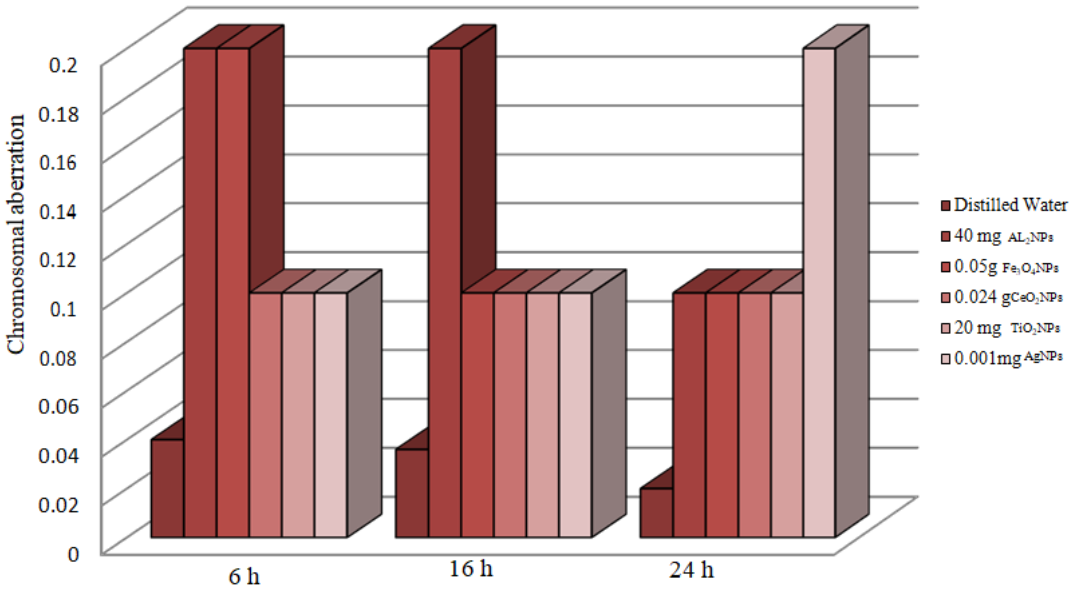

Figure 4. Effects of high concentrations of some nano-particles for different periods of time on chromosomal aberrations of *Allium sativum* root tip cells.

progression of cells from S-phase to M-phase of the cell cycle. Babu et al., (2008) suggested that Ag NPs might affect the DNA synthesis during the S-phase cell cycle, leading to mitodepressive effects and cytotoxicity. These NPs might also cause slower development of cells from the S-phase (DNA synthesis) to the M-phase (mitosis) of the cell cycle as a consequence of silver NPs exposure (Kumari et al., 2009). Similar results were found by Pulate et al. (2011).

Some treatments had no effect on the MI while others increased it. This variance might be due to the intrinsic plant detoxification mechanism of NPs when the plants are exposed to nanotoxicity. Free metal radicals, formed during oxidative stress, function as signaling molecules that later activate the ROS detoxification and antioxidant defense mechanisms in plants to deal

Table 5. Effects of low and high concentrations of different Nano-particles after 24 h of treatment.

Treatment	Control	ALO_2 NPs	Fe_3O_4 NPs	CeO_2 NPs	TiO_2 NPs	Ag NPs
Low CON.	Distilled Water 144.73 ng/µl	20 mg 391.34 ng/µl	0.025 mg 234.07 ng/µl	0.012 mg 97.63 ng/µl	10 mg 55.32 ng/µl	0.0005 mg 50.65 ng/µl
High CON.	Distilled Water 144.73 ng/µl	40 mg 136.2 ng/µl	0.05 g 130.37 ng/µl	0.024 g 119.33 ng/µl	20 mg 141.9 ng/µl	0.001 mg 124.65 ng/µl

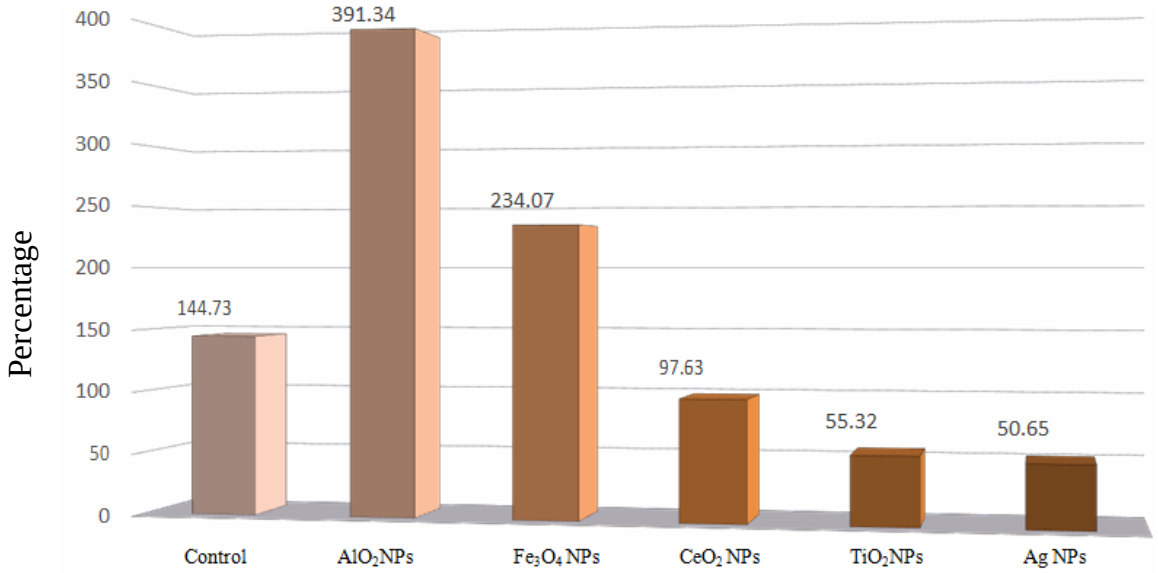

Figure 5. Effects of low concentrations of some nano-particles for 24 h on DNA content of *Allium sativum.*

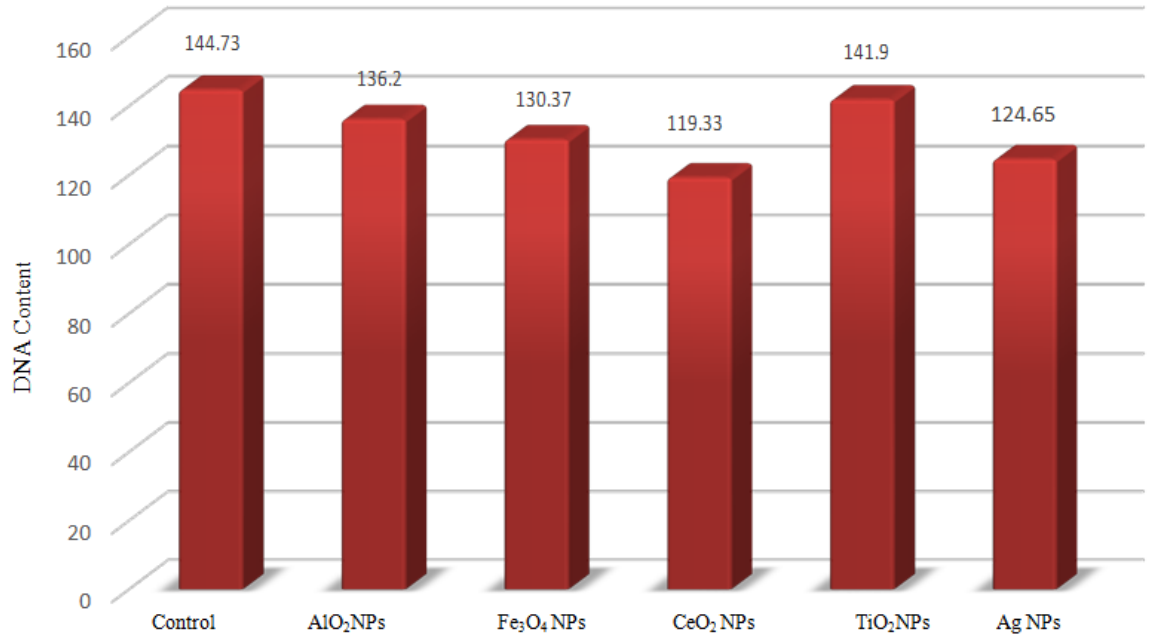

Figure 6. Effects of high concentrations of some nano-particles for 24 h on DNA content of *Allium sativum.*

with NPs toxicity (Zia-ur-Rehman et al., 2018).

Chromosomal aberrations (CA)

Treatments with different concentrations of NPs cause several types of chromosomal aberrations. Rajeshwari et al., (2015) found that AlO_2 NPs decreased the MI and increased the chromosomal aberration in root cells of *Allium cepa* due to the ROS generated by the interaction of AlO_2 NPs and root-tip cells.

The effects of Fe_3O_4 NPs were explained by Rajiv et al., (2015). They found that the metal-oxide NPs caused DNA damage and chromosomal aberrations due to the generation of ROS, which leads to cell death.

CeO_2 NPs also produce chromosomes abnormalities. In this respect, Benameur et al., (2015) demonstrated that chromosomal aberrations are consistent with cellular ROS production. Similar result was found by Liman et al., (2019).

Treatment of *A. sativum* with different concentrations of TiO_2 NPs for different time periods causes different types of chromosomal abnormalities; Ghosh et al., (2010) concluded that treatment with TiO_2 NPs caused chromosomal aberration due to the generation of superoxide radicals that sequentially resulted in lipid peroxidation in the cells. Trouiller et al., (2009) found that TiO_2 NPs are capable of causing oxidative bursts, resulting in DNA damage and the occurrence of micronuclei. Tavares et al. (2014) have the same effect of TiO_2 NPs in human lymphocytes.

Ag NPs also cause chromosomal abnormalities. Kumari et al., (2009) suggested that Ag NPs could penetrate plant system and may impair stages of cell division, causing chromosomal aberrations. Similar results were found by Pulate et al. (2011) and Patlolla et al. (2012).

The presence of disturbance, S-metaphase, S-anaphase, S-telophase, lagging chromosomes, abnormal anaphase poles, and sticky chromosomes of metaphase and telophase revealed that NPs affected spindle fibers. Several studies concluded that NPs cause chromosomal aberration by affecting the spindle fibers. These aberrations alter the direction of chromosomes during different stages of mitotic division. This may be due to the interaction of NPs with mitotic spindle apparatus, centrioles or their associated proteins leading to the loss or gain of chromosomes in daughter cells (Kuriyama and Sakai, 1974; Babu et al., 2008; Magdolenova et al., 2014).

The formation of chromosome stickiness involves the matrix of chromatin material which makes the chromosome stick or clump (Patil and Bhat, 1992). Klasterska et al., (1976) suggested that the stickiness of chromosomes arises due to the effect of NPs on nucleic acids, which causes polymerization and chromosomes stickiness. The formation of chromosomes bridges during anaphase and telophase may be due to chromosomal stickiness (EL-Khodar et al., 1990). Micronuclei being acentric fragments appear because of DNA breaks, especially during cell division, or because of laggards being excluded from the nucleus (Ma, 1982). These micronuclei could be owing to the inhibition of DNA synthesis at the S-phase (Kumari et al., 2009).

Grant (1978) reported that binucleate cells rise as a consequence of the inhibition of cell-plate formation. Huang et al., (2009) reported that due to the disruption of the mitotic checkpoint, PLKI protein function controls the mitosis process, including cytokinesis, when exposed to TiO_2 NPs.

DNA content

Different treatments of NPs affect the DNA content. Kwon et al., (2014) suggest that small NPs cross the cellular membranes more easily and this can increase the potential for DNA damage. Within cells, many NPs end up in the lysosomes but some also appear in the cytoplasm and other cellular organelles, e.g., the Golgi body, the mitochondria, and the nucleus (Yuliang et al., 2010). The molecular mechanisms of NPs mostly depend on their chemical properties. Auffan et al., (2009) concluded that chemically stable metallic NPs have no significant cellular toxicity, while NPs that can be oxidized, reduced, or dissolved are cytotoxic and genotoxic for cellular organisms. Mehrian and Lima (2016) and Brunner et al., (2006) suggested three mechanisms involved in NPs toxicity. The first is the toxic substance from soluble NPs released into exposed media. These substances could contribute to DNA damage by their involvement in ROS generation (Fenton-type reaction) (Kruszewski et al., 2011). The second mechanism is the ROS generated through surface interactions with the media. The third mechanism is the direct physical interaction of NPs with biological targets such as cell membranes or DNA (Brunner et al., 2006). NPs can also interact with the mitochondria and other cell components and disrupt their functions. The ROS that result from the transfer of electrons' energy to oxygen are highly reactive and potentially harmful to living organisms (Wu et al., 2014). Van Breusegern and Dat (2006) reported that ROS as a result of NP interaction will interact with almost all cellular components, producing protein change, lipid peroxidation, and DNA damage.

In this study, the treatment of *A. sativum* with AlO_2 showed that a low concentration increases DNA content and a high concentration decreases it. Sjorgen and Larsen (2017) suggested that Al_2 inhibits the cells' entrance into the S-phase during the cell cycle, which will affect DNA content by decreasing the content frequency. On the other hand, the S-phase cells entered the G2/M phase, leading to an increase of DNA content frequency. Similar results were found by Silva et al., (2000) and Jaskowiak et al., (2018). Wu et al. (2014) demonstrate that the reductive dissolution of iron oxide NPs induced a

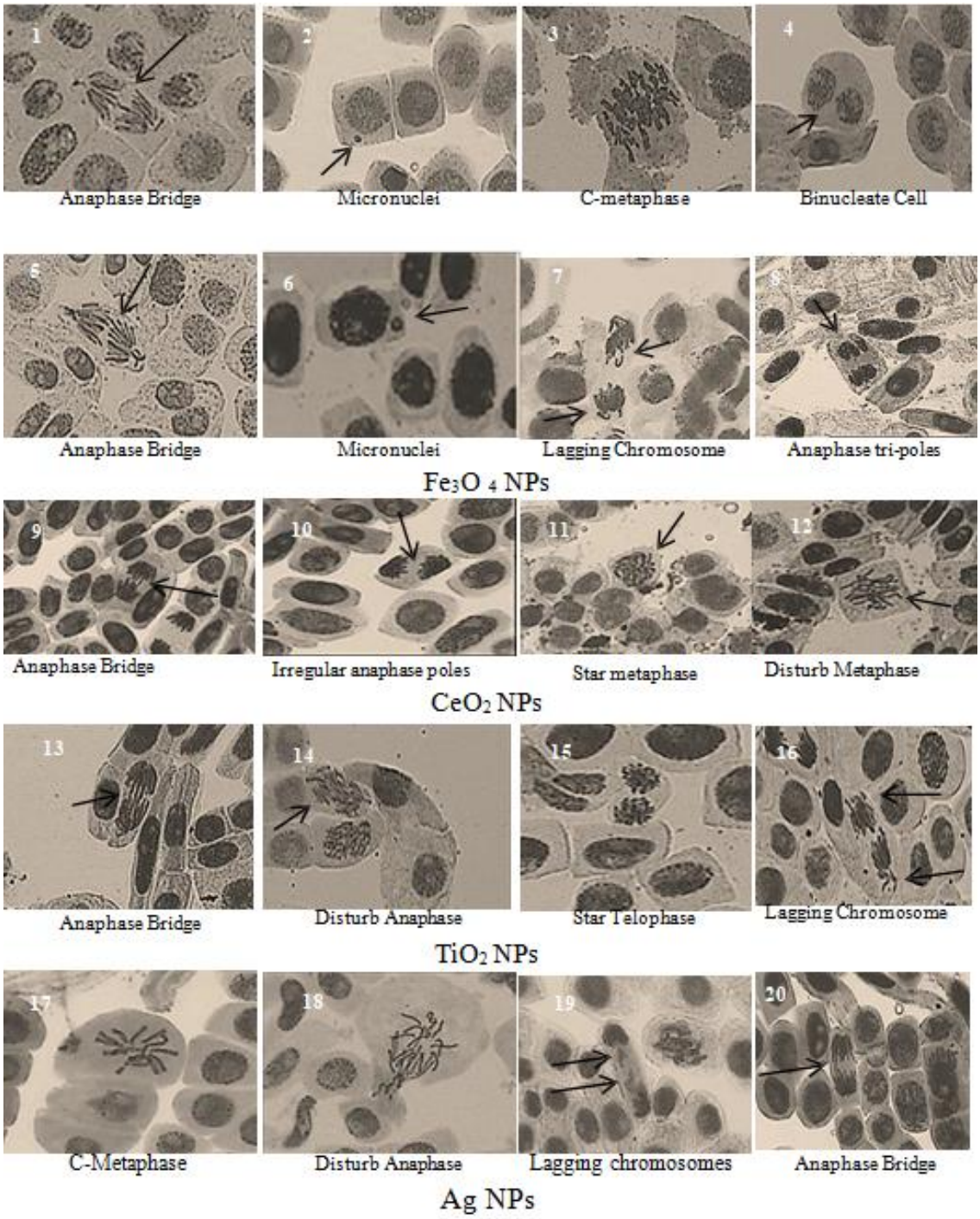

Figure 7. (1-4) Anaphase bridge, micronuclei, C-metaphase, binucleate cell. (5-8) Anaphase bridge, micronuclei, anaphase lagging chromosome, anaphase trip-polar. (9-12) Anaphase bridge, irregular anaphase polar, Star metaphase, disturb metaphase. (13-16) Anaphase bridge, disturb anaphase, Star telophase, anaphase lagging chromosome. (17-20) C-Metaphase, disturb anaphase, lagging chromosomes, anaphase bridge.

more homogeneous Fenton reaction, one that is more efficient in producing ROS. The availability of ROS inside the cell will affect cell components, and one of these components is DNA.

The CeO_2 NPs was the most harmful for DNA compared to other NPs and the control. This effect may be due to the instability of DNA caused by increasing oxidative stress, which leads to DNA damage that occurs due to the high presence of ROS (Mattiello et al., 2015). A high concentration of CeO_2 NPs effected DNA

content compared to the control but less than a low concentration, which has a greater impact on DNA content. This difference may be due to the superoxide dismutase (SOD) mimetic activity related to a high concentration of CeO_2 NPs, which causes the dismutation of superoxide anions into H_2O_2 (Mattiello et al., 2015). Vranová et al., (2002) suggested that the oxidative burst induced by the more harmful dose of CeO_2 NPs may be associated with the stimulation of cellular respiration that increases the signal requirement for energy. Mattiello et al., (2015) found that CeO_2 NPs affect the DNA by inducing visible modifications in the chromatin aggregation. A condensed chromatin is a part of the programmed cell death. A similar result was found by Liman et al., (2019) for the effect of CeO_2 NPs on the DNA content of *A. cepa*, by Benameur et al., (2015) for the effect on human dermal fibroblasts, and by Kumari et al., (2014) for the effect on Wistar rats. López-Moreno et al., (2010) found that CeO_2 NPs affect the integrity of DNA and genetic stability of soybean plants.

In this study, the TiO_2 NPs decreased the DNA content. As Rico et al., (2011) reported, this is due to the generation of superoxide radicals that cause lipid peroxidation in cells. Turkez and Geyikoglu (2007) reported that TiO_2 NPs could induce genotoxicity by inducing sister chromatid exchange and micronuclei in human white blood cells. Also, Ghosh et al., (2010) reported that the effect of TiO_2 NPs on DNA is due to the increased malondialdehyde (MDA) concentration that leads to lipid peroxidation, which leads to DNA damage. Pesnya (2013) concluded that TiO_2 NPs have a high potential to interact with DNA and cause primary DNA damage. The bio-uptake effect of TiO_2 NPs was explained by Pakrashi et al., (2014). They found a conjunction between the NPs uptake and the increase of ROS. An imbalance in intracellular ROS content caused by NPs exposure can induce DNA damages through oxidative stress owing to the oxidation of purine molecules (Afaq et al., 1998). Ghosh et al., (2012) found that treatment with TiO_2 NPs caused genotoxicity because of the generation of superoxide radicles. Schins and Knaapen (2007) suggested that the genotoxic effect of TiO_2 NPs might be due to oxidative stress and that the mechanism for this, as described by Donaldson et al., (1996) and Gilmour et al., (1997), is that TiO_2 NPs have hydroxyl radical activity. Similar results for TiO_2 NPs effect were found by Pakrashi et al., (2014).

Treatment of *A. sativum* with low and high concentrations of Ag NPs decreased the DNA content compared to the control. The Ag NPs induced toxicity due to their effect on ROS formation (Qian et al., 2013). Ma (1982) and Grant (1982) suggested that Ag NPs and their role in oxidative stress induced cellular death. Similar results were found by Sudhakar et al., (2001) and Babu et al., (2008). In higher plants, Saha and Gupta (2017) found that Ag NPs enter the plant cells and interfere with DNA repair, which leads to a blockage of DNA synthesis. Huijing et al., (2015) found that Ag NPs inhibit the new DNA synthesis in bacteria cells, which causes cell apoptosis.

This study showed that low concentrations of tested NPs had different effects on DNA. The Ag, TiO_2 and CeO_2 NPs decreased the DNA content, while AlO_2 and Fe_3O_4 NPs increased it. This difference may result from the ROS generation (Mcshan et al., 2014). Sharma et al. (2012) reported that ROS' destructive role depends on the equilibrium between ROS production and scavenging, that is, if a cell has developed a strong mechanism to control the ROS level by producing the enzymatic and non-enzymatic molecules needed to cope up with NPs-caused stress, it will decrease the effect of NPs on cell components including DNA.

The genotoxicity of NPs may result from their direct interaction with DNA or from indirect effects such as interacting with cells or tissues and releasing factors that cause harmful effects such as inflammation and oxidative stress (Singh et al., 2009; Magdolenova et al., 2014). Golbamaki et al., (2015) proposed that the genotoxic effects of NPs may be classified as primary genotoxicities or secondary genotoxicities. The second class may be due to the ROS generated during particle-induced inflammation, whereas the first class can be genotoxic without inflammation.

This study has revealed that different concentrations of the tested NPs affects the MI and that some treatments were significant at $p \leq 0.05$ particularly, Fe_3O_4 NPs after treatment with a low concentration for 24 h, TiO_2 NPs after treatment with low and high concentrations for 24 h, and Ag NPs after treatment with a high concentration for 16 h. This effect may be due to the free radicals generated by the interaction between NPs and cell components that raises the potential for cytotoxicity and decreases the MI. The tested NPs caused different types of chromosomal aberrations. Some of the scored types, e.g., micronuclei, lagging chromosomes, and chromosome bridges, indicated a genotoxic effect of NPs because these types of chromosome aberrations only occur if there is a direct effect on DNA. These NP effects may also be due to the time of interaction between the NPs and the cell cycle periods. It seems that NPs have greater effects during the S-phase of the cell cycle and wither this interaction starts during the beginning, middle, or end of the S-phase.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Afaq F, Abidi P, Matin R, Rahman Q (1998). Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed ultrafine titanium dioxide. Journal of Applied Toxicology 18(5):307-312.

Alarifi S, Ali D, ALkahtani S, Alhader MS (2014). Iron oxide nanoparticles induce oxidative stress, DNA damage, and caspase activation in the human breast cancer cell line. Biological Trace Element Research 159(1-3):416-424.

Auffan M, Rose J, Orsiere T, De Meo M, Thill A, Zeyons O, Proux O, Masion A, Chaurand P, Spalla O, Botta A, Wiesner MR, Bottero JY (2009). CeO2 nanoparticles induced DNA damage towards human dermal fibroblasts in vitro. Nanotoxicology 3(2):161-171.

Babu K, Deepa MA, Shankar SG, Rai S (2008). Effect of nano-Silver on Division and mitotic chromosomes: A prefatory Siren. Internet Journal of Nanotechnology 2(2):1-7.

Benameur L, Affuan M, Cassien M, Liu W, Culcasi M, Rahmouni H, Stocker P, Tassistro V,Bottero J, Rose J, Botta A, Pietri S (2015). DNA damage and oxidative stress induced by CeO2 nanoparticles in human dermal fibroblasts: Evidence of a clastogenic effect as a mechanism of genotoxicity. Nanotoxicology 9(6):696-705.

Brunner TJ, Wick P, Manser P, Spohn P, Grass RN, Limbach LK, Bruinink A, Stark WJ (2006). In vitro cytotoxicity of oxide nanoparticles: Comparison to asbestos, silica, and the effect of particle solubility. Environmental Science & Technology 40(14):4374-4381.

Buzea C, Pacheco BI, Robbie K (2007). Nanomaterials and nanoparticles: Sources and toxicity. Biointerphases 2(4):17-72.

Castiglione MR, Giorgetti L, Geri C, Cermonini R (2011). The effects of nano-TiO2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. Journal of Nanoparticle Research 13(6):2443-2449.

Çekiç FÖ, Ekinci S, Inal MS, Ünal D (2017). Silver nanoparticles induced genotoxicity and oxidative stress in tomato plants. Turkish Journal of Biology 41(5):700-707.

Chen M, von Mikecz A (2005). Formation on nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 Nanoparticles. Experimental Cell Research 305(1):51-62.

Debnath P, Mondal A, Hajra A, Das C, Mondal K (2018). Cytogenetic effects of silver and gold nanoparticles on *Allium cepa* roots. Journal of Genetic Engineering and Biotechnology 16(2):519-526.

Donaldson K, Beswick PH, Gilmour PS (1996). Free radical activity associated with the surface of particles: a unifying factor in determining biological activity. Toxicology Letters 88(1-3):293-298.

EL-Khodar S, Habib A, Haliem A (1990). Effect of the herbicides tribunnil on root mitosis of *Allium cepa*. Cytologia 55:209-215.

Faisa M, Saquib Q, Alatar A, Han MAM (2016). Cobalt oxide nanoparticles aggravate DNA damage and cell death in eggplant via mitochondrial swelling and NO signaling pathway. Biolgical Research 49:20.

Ghormade V, Deshpande MV, Pakmikar KM (2011). Perspectives for nano-biotechnology enabled protection and nutrition of plants. Biotechnology Advances 29(6):792-803.

Ghosh M, Bandyopadhyay M, Mukherjee A (2010). Genotoxicity of titanium dioxide (TiO2) nanoparticles at two trophies levels: Plant and human lymphocytes. Chemosphere 81(10):1253-62

Ghosh M, Manivannan J, Silnha S, Chakraborty A, Mallic SK, Bandyopadhyay M, Mukherjee A (2012). *In vitro* and *in vivo* genotoxicity of silver nanoparticles. Mutation Research 749(1-2):60-69.

Gilmour P, Brown DM, Beswick PH, Benton E, MacNee W, Donaldson K (1997). Surface free radical activity of PM10 and ultrafine titanium dioxide: a unifying factor in their toxicity? The Annals of Occupational Hygiene 41(1):32-38.

Golbamaki N, Rasulev B, Cassano A, Robinson RIM, Benfenati E, Leszczynski J, Cronin MTD (2015). Genotoxicity of metal oxide nanomaterials: Review of recent data and discussion of possible mechanisms. Nanoscale 7(6):2154-98.

Grant WF (1978). Chromosome Aberrations in Plants as a Monitoring System. Environmental Health Perspectives 27:37-43

Grant WF (1982). Chromosome aberration assay in Allium. A report of the US Environmental Protection Agency Gene-Tox Program. Mutation Research 99(3):273-291.

Grant WF (1999). Higher plant assays for the detection of chromosomal aberrations and gene mutations—a brief historical background on their use for screening and monitoring environmental chemicals. Mutation Research /Fundamental and Molecular Mechanisms of Mutagenesis 426(2):107-112.

Huang S, Chueh PJ, Lin YW, Shih TS, Chuang SM (2009). Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-Tio2 long-term exposure. Toxicology and Applied Pharmacology 241(2):182-94.

Huijing B, Xiaoxu Y, Xhen X, Zhaoyang L, Dianjun W, Yunde L (2015). New Toxicity Mechanism of Nanoparticles. Promoting Apoptosis and Inhibiting Proliferation. PLoS One 10(3):e0122535.

Hunt PR, Marquis BJ, Tyner KM, Conklin S, Olejnik N, Nelson BC, Sprand RL (2013). Nano silver suppresses growth and induces oxidative damage to DNA in *Caenorhabditis elegans*. Journal of Applied Toxicology 33(10):1131-1142.

Jaskowiak J, Tkaczyk O, Slota M, Kwasniewska J, Szarejko L (2018). Analysis of Aluminum toxicity in *Hordeum vulgare* roots with an emphasis on DNA integrity and cell cycle. PLoS One 13(2):e0193156

Jezek P, Hlavata I (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues and organism. The International Journal of Biochemistry and Cell Biology 37(12):2478-503.

Khodakovskaya MV, Silva K, Biris AS, Dervishi E, Villagarcia H (2012). Carbon Nanotubes Induce Growth Enhancement of Tobacco Cells. ACS Nano 6(3):2128-2135.

Klasterska I, Natarajan AT, Ramel C (1976). An interpretation of the origin of sub chromatid aberration and chromosome stickiness as a category of chromatid aberrations. Hereditas 83(2):153-162.

Klien K, Godnic J (2012). Genotoxicity of Metal Nanoparticles: Focus on *in vivo* Studies. Arh Hig Rada Toksikol 63(2):133-45.

Kovacic P, Somanathan R (2010). Biomechanisms of nanoparticles (toxicants, antioxidants and therapeutics): Electron transfer and reactive oxygen species. Journal of Nanoscience and Nanotechnology 10(12):7919-7930.

Kruszewski M, Brzoska K, brunborg G, Asare N, Dobrzynska M, Dusinska M, Marie Fjellsbo L, Georgantzopoulou A, Gromadzka-Ostrowska J, Gutleb AC, Lankoff A, Magdolenova Z, Pran ER, Rinna A, Instanes Ch Sandberg WJ, Schwarze PE, Maciej Stepkowski T, Wojewodzka M, Refsnes M (2011). Toxicity of silver nanomaterials in higher eukaryotes. Advances in Molecular Toxicology 5:179-218.

Kumari M, Kumari SI, Grover P (2014). Genotoxicity analysis of cerium oxide micro and nanoparticles in Wistar rats after 28 days of reported oral administration. Mutagenesis 29(6):467-479.

Kumari M, Mukherjee A, Chandrasekaran N (2009). Genotoxicity of Silver Nanoparticles on *Allium cepa*. Science of The Total Environment 407(19):5243-5246.

Kuriyama R, Sakai H (1974). Role of tubulin-SH group in polymerization to microtubules. Journal of Biochemistry 76(3):651-654.

Kwon JY, Koedrith P, Seo YR (2014). Current investigations into the genotoxicity of Zinc oxide and silica nanoparticles in mammalian models in vitro and in vivo. Carcinogenic/ genotoxic potential relevant mechanisms and biomarkers, artifacts, and limitations. International Journal of Nanomedicine 9(Suppl 2):271-86.

Landa P, Vankova R, Andrlova I, Hodek J, Marsik P, Storchova H, White JC, Vanek T (2012). Nanoparticle-specific changes in Arabidopsis thaliana gene expression after expouser to ZnO, TiO2, and gulleren soot. Journal of Hazardous Materials 241:55-62

Landsiedel R, Kapp MD, Schulz M, Wienc, K, Oesch F (2009). Genotoxicity investigations on nanomaterials: Methods, preparation and characterization of test material, potential artifacts and limitations- Many questions, some answers. Mutation Research 681(2-3):241-58.

Lee CW, Mahendra S, Zodrow K, Li DM, Tsai YC, Braam , Ivarez PJJ (2010). Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana* L. Environmental Toxicology and Chemistry 29(3):669-675.

Liman R, Acikbas Y, Hakki I (2019). Cytotoxicity and genotoxicity of cerium oxide micro and nanoparticles by Allium and comet tests. Ecotoxicology and Environmental Safety 168:408-414.

López-Moreno M, Guadalupe R, José ÁH, Hiram C, Cristina E, José

RP, Jorge LG (2010). Evidence of the differential biotransformation and genotoxicity of ZnO and CeO2 nanoparticles on soybean (*Glycine max*) plants. Environmental Science and Technology 44(19):7315-7320.
Ma C, Chhikara S, Xing B, Musante C, White JC, Dhankher OP (2013). Physiological and molecular response of Arabidopsis thaliana L. to nanoparticle cerium and indium oxide exposure. ACS Sustainable Chemistry and Engineering 1(7):768-778.
Ma TH (1982). Vicia cytogenetic tests for environmental mutagen. A report of the US Environmental Protection Agency Gene-Tox Program. Mutation Research 99(3):257-271.
Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, Dusinska M (2014). Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8(3):233-78.
Mattiello A, Filippi A, Poscic F, Musetti R, Salvtici MC, Giordano C, Vischi M, Bertolini A, Marchiol L (2015). Evidence of Phytotoxicity and Genotoxicity in *Horeum vulgare* L. Exposed to Ceo2 and TiO2 Nanoparticles. Forntiers in Plant Science 6:1043.
McShan D, Ray PC, Yu H (2014). Molecular toxicity mechanism of nano silver. Journal of Food and Drug Analysis 22(1):116-127.
Mehrian SK, De Lima R (2016). Nanoparticles cyto and genotoxicity in plants: Mechanisms and abnormalities. Environmental Nanotechnology, Monitoring and Management 6:184-193.
Mohandas T, Grant WF (1972). Cytogenetic effect of 2,4-D and amitol in relation to nuclear volume DNA content in some higher plants. Canadian Journal of Genetics and Cytology 14(4):773-783.
Mukherjee A, Peralta-Videa J, Gardea-Torresdey J (2016). Effects and Uptake of Nanoparticles in Plants. Engineered Nanoparticles and the Environment: Biophysicochemical Processes and Toxicity. https://doi.org/10.1002/9781119275855.ch20
Pakrashi S, Jerobin J, dalai S, Prathna TC (2014). In vivo Genotoxicity Assessment of Titanium Dioxide Nanoparticles by *Allium cepa* Root Tip Assay at High Exposure Concentration. PLoS One 9(2):e87789.
Park EJ, Choi J, Park YK, Park K (2008). Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. Toxicology 245(1-2):90-100.
Patil BC, Bhat GI (1992). A comparative study of MH and EMS in the induction of chromosomal aberration on lateral root meristem in *Clitoria ternate* L. International Journal of Cytology 57(2):259-264.
Patlolla AK, Berry A, May L, Tchounwou PB (2012). Genotoxicity of silver nanoparticles in *Vicia faba*: a pilot study on the environmental monitoring of nanoparticles. International Journal of Environmental Research and Public Health 9(5):1649–1662.
Pesnya DS (2013). Cytogenetic effects of chitosan-capped silver nanoparticles in the *Allium cepa* test. International Journal of Cytology, Cytosystematics and Cytogenetics 66(3):275-281.
Pulate PV, Ghurde MU, Deshmukh VR (2011). Cytological effects of the biological and chemical silver-nanoparticles in *Allium cepa* L. International Journal of Innovations in Biological and Chemical Sciences 1:32–35.
Qian H, Peng X, Han X, Ren J, Zhengwei F (2013). Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. Journal of Environmental Sciences 25(9):1947-1955.
Rajeshwari A, Kavitha S, Sruthi Ann A, Deepak K, Anita M, Natarajan C, Amitava M (2015). Cytotoxicity of Aluminum oxide nanoparticles on *Allium cepa* root tip- effects of oxidative stress generation and bio uptake. Environmental Science and Pollution Research 22(14):11057-66.
Rajiv S, Erobin J, Saranya V, Nainawat M, Sharma M, Makwana P, Gayathri C, Bharath L, Singh M, Kumar M, Mukherjee M, Chandrasekaran N (2015). Comparative cytotoxicity and genotoxicity of cobalt (II, III) oxide, iron (III) oxide, silicon dioxide, and aluminum oxide nanoparticles on human lymphocytes in vitro. Human and Experimental Toxicology 35(2):170-183
Rico CM, Duarte S, Garden M, Peralta-Videa JR, Gardea-Torrcsdcy JL (2011). Interaction of nanoparticles with edible plants and their possible implications in the food chain. Journal of Agricultural and Food Chemistry 59(8):3485-3498.
Saha N, Gupta SD (2017). A Glimpase on Silver Nanoparticles Genotoxicity in Higher Plants. Global Journal of Nanomedicine 2(2):1-2.
Salam HMH (2012). Effects of silver nanoparticles in some crop plants, common bean (*Phaseolus vulgaris* L.) and corn (*Zea mays* L.). International Research Journal of Biotechnology 3(10):190-197.
Schins RP, Knaapen AM (2007). Genotoxicity of poorly soluble particles. Inhalation Toxicology 19(Suppl 1):189-198.
Sendra M, Moreno-Garrido G, Yeste P, Gatica JM, Blasco J (2016). Behaviour of CeO2 nanoparticles and bulk and their toxicity in freshwater and seawater microalgae. Universidad de Alicante pp. 99-101.
Sharma P, Jha AB, Dubey RS, Pessarakli M (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany 217037:1-26.
Sheykhbaglou R, Sedghi M, Shishevan MT, Sharifi RS (2010). Effects of nano-iron oxide particles on agronomic traits of soybean. Notulae Scientia Biologicae 2(2):112-113.
Siddiqui MH, Mohamed HW, Mohammad F, Mutahhar YK (2015). Role of Nanoparticles in Plants. Nanotechnology and Plant Sciences 19-35
Silva JR, Smyth TJ, Moxley DF, Carter TE, Allen NS, Rufty TW (2000). Aluminum accumulating at nuclei of cell in the root tip. Fluorescence detection using lumogallion and confocal laser scanning microscopy. Plant Physiology 123(2):543-552.
Singh N, Manshian B, Jenkins GJ, Griffiths SM, Williams PM, Maffeis TG, Wright CJ, Doka SH (2009). The DNA damaging potential of engineered nanomaterials. Biomaterials 30(23-24):3891-3914.
Sjorgen CA, Larsen PB (2017). SUV2, which encode an ATR-related cell cycle checkpoint and putative plant ATRIP, is required for aluminum-dependant root growth inhibition in Arabidosis. Plant, Cell and Environment 40(9):1849-1860.
Song G, Gao Y, Wu H, Hou W, Zhang C, Ma H (2012). Physiological effect of anatase TiO2 nano-particles on *Lemna minor*. Environmental Toxicology and Chemistry 31(9):2147-2152.
Srinivas A, Rao PJ, Selam G, Murthy PB, Reddy PN (2011). Acute inhalation toxicity of cerium oxide nanoparticles in rats. Toxicology Letters 205(2):105-115.
Sudhakar R, Ninge Gowda KN, Govindappa V (2001). Mitotic Abnormalities Induced by Silk Dyeing Industry Effluents in the Cells of *Allium cepa*. Cytologia 66(3):235-239.
Tavares AM, Louro H, Antunes S, Quarre S, Simar S, De Temmerman PJ, Verleysen E, Mas J, Jense KA, Norppa H, Nesslany F, Silva MJ (2014). Genotoxicity evaluation on nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicology *in Vitro* 28(1):60-90.
Trouiller B, Reliene R, Westbrook A, Solaimani P, Schiestl RH (2009). Titanium Dioxide Nanoparticles induce DNA Damage and Genetic Instability in vivo in Mice. Cancer Research 69(22):8784-8789
Turkez H, Geyikoglu F (2007). An *in vivo* blood culture for evaluating the genotoxicity of titanium dioxide: the response of antioxidant enzymes. Toxicology and Industrial Health 23(1):19-23.
Van Breusegem F, Dat JF (2006). Reactive oxygen species in plant cell death. Plant Physiology 141:384-390.
Vranová E, Inzé D, Van Breusegem F(2002). Single transduction during oxidative stress. Journal of Experimental Botany 53(372):1227-1236.
Wu H, Yin J, Wamer WG, Zeng M (2014). Reactive oxygen species-related activities of nano-iron metal and nano-iron oxides. Journal of Food and Drug Analysis 22(1):86-94.
Yuliang Z, Bing W, Weiyue F, Chunli B (2010). Nanotoxicology: toxicological and biological activities of nanomaterials. In: Encyclopedia of Life support Systems (EOLSS): nanoscience and nanotechnologies. Paris: UNESCO-EOLSS Publisher. https://www.eolss.net/Sample-Chapters/C05/E6-152-35-00.pdf
Zheng L, Hong S, Lu C (2005). Effect of TiO2 on strength of naturally aged seeds and growth of spinach. Biological Trace Element Research 104(1):83-92.
Zhongwen Chen, Jun-Jie Yin, Yu-Ting Zhou, Zhang Y, Song L, Song M, Hu S, Gu N (2012). Dual enzyme-like activities of iron oxide nanoparticles and their implication for diminishing cytotoxicity. ACS Nano 6(5):4001-4012.
Zia-ur-Rehman M, Qayyum MF, Akmal F, Maqsood MA, Rizwan M, Waqar M, Azhar M (2018). Nanomaterials in Plants, Algae, and Microorganisms. Recent Progress of Nanotoxicology in Plants-Science Direct pp. 143-174.

Vol. 18(29), pp. 797-807, September, 2019
DOI: 10.5897/AJB2018.16591
Article Number: 6248B9661818
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Review

Biotechnological tools for detection, identification and management of plant diseases

Tibebu Belete* and Nuh Boyraz

Department of Plant Protection, Faculty of Agriculture, Selcuk University, Campus/Konya, Turkey.

Received 11 July, 2018; Accepted 10 September, 2018

Detection and identification of plant pathogens is one of the most important strategies for sustainable plant diseases management. For this reason, the availability of fast, sensitive and accurate methods for detection and identification of plant pathogens is increasingly necessary to improve disease control decision making process. In other words, new technologies and improved methods with reduced/fair cost and improved speed, throughput, multiplexing, accuracy and sensitivity have emerged as an essential strategy for the control of both fungal and bacterial diseases. The development of recombinant DNA technology is also possible to isolate individual genes and incorporate resistance genes into otherwise agronomically acceptable cultivars to develop genetically resistance variety for a particular disease. These advances have been complemented by the development of new nucleic acids extraction methods, increased automation, reliable internal controls, multiplexing assays, online information and on site molecular diagnostics. The different types of polymerase chain reaction (PCR) are the most common DNA amplification technology used for detecting various plant pathogens. With the applications of bioinformatics as a modern technology in plant pathology, identification of specific motifs, DNA sequences has become possible, which ultimately increase the accuracy of modern techniques in plant disease diagnosis. The newly emerged proteomic technology is also a promising tool for providing information about pathogenicity and virulence factors that will open up new possibilities for plant disease diagnosis and appropriate protection measures.

Key words: Biotechnology, molecular markers, marker assisted selection, quantitative trait loci (QTL), polymerase chain reaction (PCR), proteomics.

INTRODUCTION

Biotechnology is broadly defined as set of biological techniques developed through basic research and now applied to research and product development. In other words, it is the genetic manipulation and multiplication of any living organism through new technologies resulting in the production of improved and new organism and products can be used in a variety of ways (Agrios, 2005; Fagwalawa et al., 2013). It is an applied science in the field of agriculture and known as agricultural biotechnology. Based on knowledge of DNA, scientists have developed solutions to increase agricultural productivity (Agrios, 2005). New biotechnological tools enhance pathologists' ability to make improvements in crops regarding to their respective diseases. It can be

*Corresponding author. E-mail: tibebubelet@su.edu.et.

applied as genetic engineering, molecular markers, molecular diagnosis and tissue culture (James, 2003).

Chemical control of plant diseases is often non-specific in its effects, killing beneficial organisms as well as pathogens, and it may have undesirable health, safety, and environmental risks. Because of these factors, nowadays, control of plant disease is a subject of great interest for biotechnologists. Biotechnology will enhance our understanding of the mechanisms that control a plant ability to recognize and defend itself against disease causing organisms like fungi (Haggag, 2008) and the future of sustainable agriculture will increasingly rely on the integration of biotechnology with traditional agricultural practices.

Biotechnology is an important discipline for accurate diagnosis of plant disease. Enzyme-linked immunosorbent assays (ELISA) and nucleic acid techniques are used in the identification of fungal, viral and bacterial diseases. New techniques in plant genetic engineering strategies for the management of plant diseases are now available and the application of genetic engineering for plant disease resistance has been discussed and presented by many scholars. The different tools of biotechnology and genomic approaches have enhanced the process of identifying plant pathogens with greater accuracy. Plant genes and their products that interact with plant pathogens have been identified and either inserted into plants or used as specific molecular markers to improve plant breeding for disease resistance. Therefore, acquiring noble information from applied biotechnology to plant-pathogen interactions is providing new knowledge and new approaches to improve plant health, yield and quality of plants contributing to food (Fagwalawa et al., 2013).

Accurate identification and diagnosis of plant diseases are very important for sustainable food security as well as prevention of the spread of invasive pathogens (Balodi et al., 2017). In this regard, special interest should be taken in the early detection of pathogens in plant propagating materials (seeds, mother plants and vegetative propagative plant material) to avoid the introduction and further dispersal of new pathogens in a growing area where it is not present earlier. Therefore, rapid detection and identification of plant pathogens is strongly necessary by using fast, sensitive and accurate methods so as to come to last decision for appropriate control strategies. Some of the most important molecular methods for detection of plant pathogenic disease are the PCR isothermal amplification methods, fingerprinting [restriction fragment length polymorphism [RFLP], random-amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites], DNA hybridization technology and sequencing (McCartney et al., 2003; Barnes and Szabo, 2007; Kang et al., 2010). Successful management of plant diseases is primarily dependent on the accuracy and efficient detection of the pathogens, knowing the amount of genetic and pathogenic variability present in a pathogen population, development of disease resistant cultivars and quantifying disease resistance genes in different epidemiological regions. Beside conventional methods of pathogen detection and breeding resistant cultivars, recent development in molecular biology techniques particularly the advent of various DNA based markers have greatly influenced the plant protection methods. Therefore, the objective of this article is to review the major application of biotechnological tools to detect, identify and control of plant diseases as well as to give an overview of proteomics studies in bacterial diseases.

ADVANCED TOOLS FOR DETECTION AND IDENTIFICATION OF PATHOGENS

Detection protocols used for the diagnosis or quarantine measures should be reproducible, repeatable and should have minimum false results. All molecular detection methods should be sensitive to pathogen concentration, genetic variability within a target pathogen population, and similarities between the target and other organisms (Martin et al., 2016; Balodi et al., 2017).

Bacterial diseases

Detection of pathogenic bacteria in seed and other plant tissues (particularly in latent infections) is challenging because the target bacteria are often irregularly distributed and present as a small component of a much larger bacterial population. Moreover, it is often difficult to distinguish and identify pathogenic bacteria from all the soil-associated and other saprophytic bacteria normally present on plant surfaces. In addition to epiphytic and casual surface contaminants, non-detrimental or beneficial endophytic bacteria may also be present (Punja et al., 2008).

Traditional techniques to detect the presence of pathogenic bacteria involved in field inspection for symptoms and signs of disease as well as laboratory tests (Figure 1). Laboratory procedures for detection of the bacteria may involve grow-out assays, serological tests such as ELISA and immune-fluorescence microscopy. In addition, isolation of the bacteria on selective or semi-selective media is also done. Following isolation, strains need to be characterized by physiological, biochemical and pathogenicity tests. Use of traditional methods is reliable and efficient for some of the bacterial plant pathogens, but for many others they lack adequate sensitivity and specificity. Another major disadvantage is the long times required for grow-out assays, bacterial isolation and pathogenicity tests. Therefore, new and modern molecular techniques are the best option to diagnosis of bacterial pathogens (Punja et al., 2008).

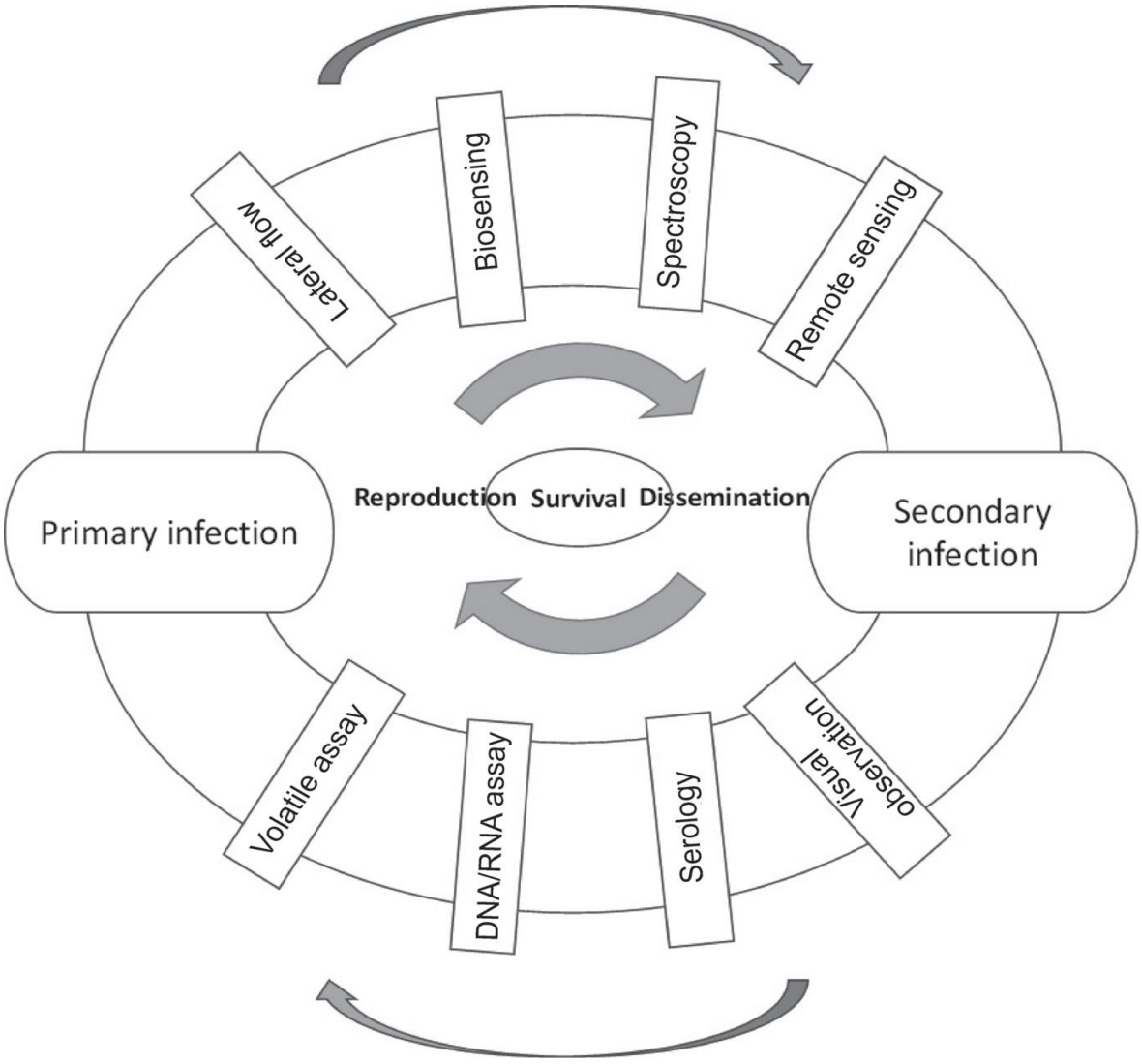

Figure 1. Detection methods of plant pathogens at various stages of disease development (adapted from Balodi et al., 2017).

Nucleic acid (DNA)-based techniques have been demonstrated to be generally more sensitive, specific and reliable for the detection, identification and quantification of bacterial plant pathogens than other methods. Among the nucleic acid-based diagnostic techniques, PCR assay or its variants have been used very widely for detection of bacterial pathogens in pure cultures or in single/multiple infections of plant hosts (Narayanasamy, 2011). For example, a PCR-based assay using the primers from DNA sequences of the phaseolotoxin gene was able to detect efficiently the pathogen *Pseudomonas savastanoi* pv. *phaseolicola*, causing bean halo blight disease, even in the presence of high populations of non-target bacteria. Similarly, *Xanthomonas axonopodis* pv. *manihotis* and *Ralstonia solanacearum* (from soil) can be detected by PCR, on amplifying an 898 and 288-bp fragment, respectively (Alvarez et al., 2008). In another study by Audy et al. (1994), *X. axonopodis* pv. *phaseoli* (*Xap*) was detected in the first time by employing primers from plasmid DNA, in PCR assay which had a detection limit of 10 to 100 fg of *Xcp* DNA (equivalent of 1 to 10 cfu).

To date, real-time PCR seed detection assays have been reported for *Acidovorax avenae* subsp. *citrulli* in watermelon seeds, *R. solanacearum* race 3, biovar 2, in asymptomatic potato tubers, *Xanthomonas arboricola* pv. *pruni* in *Prunus* species and *Xanthomonas oryzae* pv *oryzae* in rice by Real time Bio-PCR (Balodi et al., 2017).

Fungal diseases

Plant pathogenic fungi are the causal agents of the most detrimental diseases in plants, provoking considerable

yield losses worldwide (Aslam et al., 2017). Some of the basic methods used to detect fungal pathogens mostly rely on microscopic, morphological and cultural approaches that require extensive time, labor and classical taxonomy knowledge (Nilsson et al., 2011). Even though, these approaches are the cornerstone of fungal diagnostics, they can lead to the unreliable results due to the problems in weak identification. Therefore, advances in the development of molecular methods, like immunological methods, nucleic acid-based probe technology and PCR technology have provided diagnostic laboratories with powerful tools for detection and identification of phytopathogenic fungi (Aslam et al., 2017). These methods are much faster, more specific, sensitive, and accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise (Badali and Nabili, 2012). Additionally, these techniques allow the detection and identification of non-culturable microorganisms, and due to its high degree of specificity, molecular techniques can distinguish closely related organisms at different taxonomic levels (Capote et al., 2012). PCR technology includes multiplex PCR, nested PCR, real-time PCR and reverse transcriptase (RT)-PCR and DNA barcoding has been recently used as a molecular tool for detection and identification of fungal pathogens (Table 1).

PCR methods for identification of *Sclerotium rolfsii* and *Colletotrichum capsici* have been developed based on specific sequences of the internal transcribed spacer (ITS) region. Multiplex PCR technique has been used for the simultaneous detection and differentiation of fungal pathogens in different crops like in sunflower (*Podosphaera xanthii* and *Golovinomyces cichoracearum)*, in cedar trees and water samples (*Phytophthora lateralis).* It is also very important for determining the mating type of the pathogens *Tapesia yallundae* and *Tapesia acuformis*; for differentiating two pathotypes of *Verticillun alboatrum* infecting hop and for distinguishing among eleven taxons of wood decay fungi infecting hardwood trees (Guglielmo et al., 2007; Chen et al., 2008; Jeeva et al., 2010; Torres-Calzada et al., 2011; Capote et al., 2012). Padlock probes have been used for the simultaneous detection of *Phytophthora cactorum*, *Phytophthora nicotianae*, *Pythium ultimum*, *Pythium aphanidermatum*, *Pythium undulatum*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Fusarium solani*, *Myrothecium roridum*, *Myrothecium verrucaria*, *Verticillium dahliae* and *Verticillium alboatrum* in samples collected from horticultural water circulation systems in a single assay (Van Doorn et al., 2009; Capote et al., 2012). ISSR and SRAP markers were also used for molecular characterization of *Venturia inaequalis* isolates for the first time and they were more informative, easily applicable, reproducible, and specific (Kaymak et al., 2016).

A polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) detection tool based on the amplification of the ITS region has been recently applied to detect multiple *Phytophthora* species from plant material and environmental samples (Shamim et al., 2017). Real-time PCR is currently considered the gold standard method for detection of plant pathogens. This technique allows the monitoring of the reaction during the amplification process by the use of a fluorescent signal that increases proportionally to the number of amplicons generated and to the number of targets present in the sample (Alemu, 2014). Another advantage of real-time PCR is the capability to perform multiplex detection of two or more pathogens in the same reaction. *Microdochium nivale* in wheat seeds and *Fusarium circinatum* in pine seed were detected by real time-PCR assay (Balodi et al., 2017). SYBR Green real-time PCR assays can detect so many pathogenic fungi like *Botrytis cinerea, Fusarium oxysporum* f. sp. *vasinfectum, Colletotrichum acutatum, Phoma sclerotioides, Pythium irregular, Rhizoctonia solani, Verticillium dahliae,* etc. (Capote et al., 2012).

APPLICATION OF MOLECULAR MARKERS FOR RESISTANCE BREEDING

Disease resistance in crop plants is a major challenge in plant breeding. Conventional breeding for disease resistance is based on phenotypic identification and crossing with agronomically desirable but susceptible plants. It has made great progress in incorporating natural defense genes. This is performed based on a backcross program which takes more than 7 years to reach the final goal. However, the modern molecular techniques make it possible to use markers and probes to track the introgression of several resistance genes into a single cultivar from various sources during a crossing program. The advent of new biotechnology techniques such as marker-assisted selection provides new opportunities to enhance plant disease resistance (Torres, 2010; Torres-Calzada et al., 2011).

Different types of molecular markers (DNA based markers) have been developed and used for developing of disease resistance varieties. Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR), Microsatellites or Simple Sequence Repeat (SSR), Allele Specific Associated primers (ASAP), Expressed Sequence Tag (EST), Cleaved Amplified Polymorphic Sequence (CAPS), Diversity Arrays Technology (DArT), Sequence Characterized Amplified Regions (SCARs) and Single Nucleotide Polymorphism (SNP) are some of molecular markers and have been used in several crops (Table 2) (Doveri et al., 2008; Singh et al., 2013; Ragimekula et al., 2013). Application of molecular markers for breeding disease resistant varieties is especially interesting when breeding for

Table 1. The advantage and disadvantage of molecular detection and identification tools in fungi.

Molecular tools	Advantages	Disadvantages
Conventional PCR	Gives rapid and precise results when use the primer of specific species	Required much labor and cost
Nested PCR	Use of two sets of primer increase the yield and specificity of amplification of the target DNA	Risk of contamination, because of two cycles of amplification
Multiplex PCR	Time and money saver by using the several pairs of primers in a same reaction	Interference of primers and probes, reduce sensitivity
Reverse transcriptase (RT) PCR	Gives quantitative data about pathogens, more sensitive than conventional PCR	Formation of each assay is time consuming and requires the expensive equipment and the reagents
Real-time PCR (q PCR)	Automated and no need of post amplification analysis	Cost and complexity due to simultaneous thermal cycling and fluorescence detection
In situ hybridization	Maximum use of the short supply tissue	Difficulty in identifying targets that have low DNA and RNA copies
FISH	Can be used for non-dividing cells	Probe-preparing method is very difficult because it is necessary to tailor the probes to identify the particular sequences of DNA
Microarray	Easy to use because it does not require the large-scale DNA sequencing	Large amount of mRNA is required
LAMP	Rapid, sensitive and highly specific	Primer design is complex; recognizes only one specific pathogen; risk of sample contamination
NASBA	Expensive equipment is not needed. Better than RTPCR	Specificity of the reactions is dependent on thermolabile enzymes. Reaction temperature cannot be exceeded than 42°C without compromising it
RNA interference (RNAi)	Ability to simultaneously interrogate thousands of genes	Variability and incompleteness of knockdowns and the potential non specificity of reagents
Northern blotting	Detection of RNA size	Applied only on a small sample of the genes
SAGE	Prior knowledge of the subject's genome is not requisite	Specificity of tag sequence
RNA-Seq	Increased specificity and sensitivity	Needs expensive equipment. Bioinformatics knowledge requisite for data analysis

resistance traits that are difficult or expensive to assess phenotypically.

Resistance to *Turcicum* leaf blight in sorghum accession G-118 was found to segregate as a single dominant trait in a cross with susceptible cultivar HC-136. By using SSR markers coupled with bulk segregant analysis, a molecular marker linked to the locus for resistance to *Turcicum* leaf blight was identified (Mittal and Boora, 2005). In the same population, an SSR marker, Xtxp 309, produced amplification of a 450 bp band. This was found to be located at a distance of 3.12 cM away from the locus governing resistance to leaf blight which was considered to be closely linked and 7.95 cM away from the locus governing susceptibility to leaf blight (Mittal and Boora, 2005). By the RAPD technique with bulk-segregant analysis, it was possible to identify

Table 2. Some applications of molecular marker assisted selection (MAS) in four crops.

Character/Trait	Target genes	Types of marker used	References
Rice			
Bacterial blight resistance	*xa5, xa13 & Xa21*	CAPS for *xa5* (RG556+DraI), CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Sundaram et al. (2008)
Bacterial blight (BB) resistance + Grain quality	*xa13 & Xa21*	CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Gopalakrishnan et al. (2008)
Bacterial blight (BB) resistance	*Xa4, X17 & Xa21*	STS for *Xa4 & Xa7,* STS for *Xa21* (pTA248)	Perez et al. (2008)
Bacterial blight (BB) resistance	*xa5* and *xa13*	CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Sundaram et al. (2009)
Blast resistance	*Pi-9(t)*	*pB8*	Wen and Gao (2011)
Bacterial blight (BB) resistance	*Xa4, xa5, xa13 & Xa21*	STS for Xa4, CAPS for *xa5* (RG556+DraI), CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Shanti et al. (2010)
Bacterial blight (BB) resistance + Blast resistance	*xa5, xa13, Xa21 &Pi25*	CAPS for *xa5* (RG556+DraI), CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248) and STS for *Pi25* (SA7)	Zhan et al. (2012)
Bacterial blight (BB) resistance + Blast resistance + sheath blight (ShB)	*xa13, Xa21, Pi54 & qSBR11-1*	CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248), SSR for *Pi54* (RM206), SSR for *qSBR11-1* (flanking markers RM224 and RM7443)	Singh et al. (2012)
Bacterial blight (BB) resistance	*xa13 & Xa21*	CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Pandey et al. (2013)
Bacterial blight (BB) resistance + Blast resistance	*Xa21& Pi54*	STS for *Xa21* (pTA248), SSR for *Pi54* (RM206)	
Bacterial blight (BB) resistance	*Xa4, xa5, xa13 & Xa21*	STS for Xa4, CAPS for *xa5* (RG556+DraI), CAPS for *xa13* (RG136+HinfI), STS for *Xa21* (pTA248)	Dokku et al. (2013)
Barley			
Barley yellow mosaic virus I-III	-	RFLP	Okada et al. (2003)
Resistance to cereal cyst nematode	-	RFLP	Barr et al. (2000)
Barley stripe rust	-	RFLP, RAPD, AFLP	Hayes et al. (2003)
Barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2)	-	RAPD, SSR, STS	Werner et al. (2005)
Resistance to BYDV	-	CAPS, SSR, STS	Scholz et al. (2009)

Table 2. Contd.

Wheat			
Fusarium head blight (FHB) resistance	-	SSR	Miedaner et al. (2006)
Fusarium head blight QTL	-	SSR	Wilde et al. (2008)
Leaf rust (*Puccinia triticina*) resistance gene *Lr47*	-	SSR	Bainotti et al. (2009)
Powdery mildew (*Erysiphe graminis* f.sp. *tritici*) resistance genes	-	RFLP	Liu et al. (2000)
Soybean			
Soybean mosaic virus (SMV)	-	SSR	Shi et al. (2009)
SCN resistance	-	SSR	Arelli et al. (2007)
Resistance to frogeye leaf spot (Cercospora sojina)	-	SNP, SSR	Shannon et al. (2009)

PCR amplification products segregated with the response to Turcicum leaf blight (Boora et al., 1999). A three-gene pyramid line of rice was identified by MAS to possess broad-spectrum bacterial blight resistance and excellent grain quality (Sundaram et al., 2008). Pandey et al. (2013) improved the two traditional bacterial blight (BB) susceptible rice varieties through the strategy of limited marker-assisted backcrossing for introgression of two major BB resistance genes coupled with phenotype-based selection for improvement of their plant type and yield. Generally, many scholars identified new QTLs related to resistance genes, however, very few of them reported (shown in Table 2) have been used for MAS in breeding programs. Manulis et al. (1994) were able to identify the specific banding patterns that were subsequently used as probes to distinguish between the races of the carnation wilt fungal pathogen F. oxysporum f. sp. dianthi by using RAPDs. In another study, RAPD markers were used to infer the genetic relationships among the wheat bunt fungi. Globally, RAPD markers are also reported to be useful in diagnostic studies of many plant pathogens (Singh et al., 2013). Billard et al. (2012) also identified fungal pathogen using ASAP marker system. In addition, they also identified resistance gene by molecular markers, knowing the genetic variability and diversity of the disease causing agents are also imperative. Kaymak et al. (2016) used RAPD, ISSR, SSR and SRAP markers to evaluate the genetic divergences and the relationships of Venturia inaequalis isolates. They reported that SSR and SRAP markers were found to be more informative and consistent than other marker techniques during their study.

PROTEOMIC STUDIES AS A TOOL IN PATHOGENIC BACTERIA

Proteomics is the large-scale study of the whole set of proteins present in a cell, tissue or organism at a specific time point under specific conditions. In recent years, proteomics has played a key role in identifying changes in protein levels in plant hosts upon infection by pathogenic fungal and bacterial organisms and in characterizing cellular and extracellular virulence and pathogenicity factors produced by pathogens (Lodha et al., 2013). Mehta and Rosato (2001) reported the analysis of X. axonopodis pv. citri cultivated in the presence of the host Citrus sinensis leaf extract and recognized differentially expressed proteins, including a sulfate-binding protein, by NH2 terminal sequencing. The same authors suggested that the induction of this enzyme may have been caused by the amino acids or different sugars present in the leaf extract. Tahara et al. (2003) also analyzed the expressed proteins of X. axonopodis during interaction with the host *Passiflorae edulis* leaf extract, and identified an inorganic pyrophosphatase and an outer membrane protein up regulated in the presence of leaf extract, also by NH2 terminal sequencing. It was proposed that the protein which was identified in outer membrane may have an important role in pathogenicity (Tahara et al., 2003).

In a 2DE-mediated proteomic study of Xylella fastidiosa, the causal agent of citrus variegated chlorosis, showed that this pathogen did not produce significant changes in heat shock protein expression when compared with X. axonopodis pv. citri (Martins et al., 2007). However, it was found that X. fastidiosa constitutively expressed several stress-inducible proteins such as heat

shock protein A (HspA) and GroeS, which were induced in *X. citri* under stress conditions. The authors suggested that the constitutive expression of these proteins may help *X. fastidiosa* cope with sudden environmental changes and stresses.

With regard to plant defense responses, direct evidence of the involvement of target proteins has also been provided by proteomic studies (Table 3). Although few of the reports outlined clearly show the importance of proteomic approaches, which can aid significantly in the understanding of plant bacterium interactions. A detail understanding of plant defense response using successful combination of proteomic techniques is needed for practical application to secure and stabilize yield of many crop plants (Lodha et al., 2013). Jones et al. (2004) analyzed the proteomic and transcriptomic profiles of *Arabidopsis thaliana* leaves during early responses (1 to 6 h post-inoculation) to the challenge by *Pseudomonas syringae* pv. *tomato*. They compared the proteomic changes in *A. thaliana* in response to the *P. syringae* pv. *tomato* highly virulent strain DC3000, which results in successful parasitism, a DC3000 hrp mutant, which induces basal resistance, and a trans-conjugant of DC3000 expressing avrRpm1, which triggers a gene-for-gene-based resistance. As a follow-up study, Jones et al. (2006), examined the global proteomic profile in three sub-cellular fractions (soluble protein, chloroplast and mitochondria enriched) of *A. thaliana* responding to the same three *P. syringae* pv. *tomato* DC3000 strains. This was the first report to associate post-translational events (1 to 6 h post-inoculation) occurring before significant transcriptional re-programming. The results showed that several chloroplast systems are modified during all aspects of the defense response.

Many study was conducted between rice and bacterial association, some are pathogenic and cause severe damages to the crop, such as *X. oryzae* pv *oryzae*, *Burkholderia glumae, Burkholderia kururiensis* and *Pseudomonas fuscovaginae.* Among them, *B. kururiensis* is very often isolated from rice and has been studied recently for its potential beneficial effects on the plant and the mechanisms of interaction (Suarez-Moreno et al., 2012). The role of defense responsive proteins in the *X. oryzae* pv. o*ryzae* interaction was investigated through a proteomic approach (Mahmood et al., 2006). Cytosolic and membrane proteins were fractionated from the rice leaf blades 3 days post-inoculation with incompatible and compatible *X. oryzae* pv. *oryzae* races. From 366 proteins analyzed by 2DE, 20 were differentially expressed in response to bacterial inoculation. Analyses clearly revealed that the four defense related proteins [PR-5, probenazole-inducible protein (PBZ1), superoxide dismutase (SOD) and Prx] were induced for both compatible and incompatible *X. oryzae* pv. *oryzae* races, wherein PR-5 and PBZ1 were more rapid and showed higher induction in incompatible interactions and in the presence of jasmonic acid (JA). Study in the same rice *X. oryzae* pv. *oryzae* interaction, Chen et al. (2007) analyzed proteins from rice plasma membrane to investigate the early defense responses proteins involved in XA21-mediated resistance. XA21 is a rice receptor kinase, predicted to perceive the *X. oryzae* pv. *oryzae* signal at the cell surface, leading to the 'gene-for-gene' resistance response. At the same time, a total of 20 proteins were differentially regulated by pathogen challenge at 12 and 24 h post-inoculation and identified at least eight putative plasma membrane-associated and two non-plasma membrane-associated proteins with potential functions in rice defense were observed by the same authors.

By comparing two partially resistant lines and a susceptible line in a time course (72 and 144 h post-inoculation) experiment, proteins from the wild tomato species *Lycopersicon hirsutum* that are regulated in response to the causal agent of bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) were identified. Twenty six differentially regulated tomato proteins were identified by using 2DE and ESI-MS/MS, 12 of which were directly related to defense mechanisms.

Proteomic analysis was also used to detect the responses of the model legume *Medicago truncatula* to the pathogenic bacterium *Pseudomonas aeruginosa* in the presence of known bacterial quorum sensing signals, such as N-acyl homoserine lactone (AHL) (Mathesius et al., 2003). To make appropriate responses to the pathogen, the fast and reliable detection of bacterial AHL signals by plant hosts is essential. Therefore, *M. truncatula* is able to detect very low concentrations of AHL from *P. aeruginosa* and response in a global manner by significant changes in the accumulation of 154 proteins, 21 of which are related to defense and stress responses.

CONCLUSIONS

The science of plant disease**s** diagnostics and management has progressed in technological advancements from the visual inspection of signs and symptoms of diseases to molecular level detection of the pathogens. Currently, precise identification and diagnosis of plant pathogens to the species or strain level, information during their early stages of infection, and better understanding of pathogenicity factors are the crucial prerequisite for disease surveillance and development of novel disease control strategies. Therefore, the advancements in the field of plant pathology, coupled with biotechnology, bioinformatics and molecular biology have opened new avenues for development of specific and sensitive procedures of diagnosis. And currently more sensitive methods like Real Time PCR and Microarrays are being used. Genetic engineering is also one of the potential tools to provide an abundance of beneficial plant traits, particularly an

Table 3. Some examples of proteins expressed in plant-bacterial interactions and identified in plants using proteomic approaches (Jones et al., 2004, 2006; Mahmood et al., 2006; Chen et al*.,* 2007).

Protein	Studied organism	Pathogen
lutathione S-transferase	*A. thaliana*	*P. syringae*
Peroxiredoxin	*A. thaliana*	*P. syringae*
Peroxiredoxin, chloroplast	*O. sativa*	*X. oryzae pv. oryzae*
Glyceraldehyde 3-phosphate dehydrogenase	*O. sativa*	*X. oryzae pv. oryzae*
Triosephosphate isomerase, cytosolic (EC 5.3.1.1)	*O. sativa*	*X. oryzae pv. oryzae*
Thaumatin-like protein X	*O. sativa*	*X. oryzae pv. oryzae*
Superoxide dismutase	*O. sativa*	*X. oryzae pv. oryzae*
Alcohol dehydrogenase 1 a	*O. sativ*	*X. oryzae pv. oryzae*
Quinone reductase	*O. sativa*	*X. oryzae pv. oryzae*
Prohibitin	*O. sativa*	*X. oryzae pv. oryzae*
Ascorbate peroxidase	*O. sativa*	*X. oryzae pv. oryz**ae***
Remorin 1	*L. hirsutum*	*C. michiganensis* subsp *michiganensis*
Ascorbate peroxidase	*L. hirsutum*	*C. michiganensis* subsp. *michiganensis*
Glutathione S-transferase	*L. hirsutum*	*C. michiganensis* subsp *michiganensis*
Pathogenesis-related 3 (endochitinase precursor)	*L. hirsutum*	*C. michiganensis* subsp *michiganensis*

enhanced ability to withstand or resist attack by plant pathogens. In addition, developments in DNA marker technology together with the concept of marker-assisted selection provide new solutions for selecting and maintaining desirable genotypes. Marker assisted selection can be performed in early segregating populations and at early stages of plant development for pyramiding the resistance genes, with the ultimate goal of producing varieties with durable or multiple disease resistance. Generally, a timely detection of resistance levels in populations of phytopathogenic pathogens in a field would help the growers formulate proper decisions on resistance management programs to control plant diseases. Finally, future studies will be focused on the practical application of each traditional or innovative method, their cost and availability of instruments, specialization level, rapidity of analysis, and the stage of disease at which detection is possible.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to thank the staff members of Plant Protection Department, Selcuk University, Turkey for giving moral to develop this manuscript and helpful review.

REFERENCES

Agrios GN (2005). Plant Pathology. 5 th Ed., Elsevier Academic Press. New York pp. 213-223

Alemu K (2014). Real-time PCR and its application in plant disease diagnostics. Advances in Life Science and Technology 27:29-49.

Alvarez E, Llano G, Mejia J (2008). Applying biotechnology tools to improve control diseases of some tropical crops. Ftopatologia Colombiana 28(2):93-97.

Arelli PR, Pantalone VR, Allen FL, Mengistu A (2007). Registration of soybean germplasm JTN-5303. Journal Plant Registrations 1:69-70.

Aslam S, Tahir A, Aslam MF, Alam MW, Shedayi AA, Sadia S (2017). Recent advances in molecular techniques for the identification of phytopathogenic fungi - a mini review. Journal of Plant Interactions 12(1):493-504.

Audy P, Laroche A, Saindon G, Huang HC, Gilbertson RC (1994). Detection of bean common blight bacteria *Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var*. fuscans* using polymerase chain reaction. Phytopathology 84:1185-1192.

Badali H, Nabili M (2012). Molecular tools in medical mycology; where we are! Jundishapur Journal of Microbiology 6(1):1-3.

Bainotti C, Fraschina J, Salines JH, Nisi JE, Dubcovsky J, Lewis SM, Bullrich L, Vanzetti L, Cuniberti M, Campos P, Formica MB, Masiero B, Alberione E, Helguera M (2009). Registration of 'BIOINTA 2004 wheat. Journal of Plant Registrations 3:165-169.

Balodi R, Bisht S, Ghatak A, Rao KH (2017). Plant disease diagnosis: technological advancements and challenges. Indian Phytopathology 70(3):275-281.

Barnes CW, Szabo LJ (2007). Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. Phytopathology 97(6):717-727.

Barr AR, Jefferies SP, Warner P, Moody DB, Chalmers KJ, Langridge P (2000). Marker- assisted selection in theory and practice. Prodeedings of the 8th International Barley Genetics Symposium, Vol I., Adelaide, Australia pp. 167-178.

Billard A, Laval V, Fillinger S, Leroux P, Lachaise H, Beffa R, Debiue D (2012). The allele specific probe and primer amplification assay, a new real time PCR method for fine quantification of single nucleotide Polymorphism in pooled DNA. Applied and Environmental Microbiolgy 78(04):1063-1068.

Boora KS, Frederiksen RA, Magill CW (1999). A molecular marker that segregates with sorghum leaf blight resistance in one cross is maternally inherited in another. Molecular and General Genetics 261:317-322.

Capote N, Pastrana AM, Aguado A, Sánchez-Torres P (2012). Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In Cumagun CJ (Ed.), Plant Pathology, InTech, Rijeka, Croatia 362 p.

Chen RS, Chu C, Cheng CW, Chen WY, Tsay JG (2008). Differentiation of two powdery mildews of sunflower (*Helianthus annuus*) by a PCR-mediated method based on ITS sequences. European Journal of Plant Pathology 121(1):1-8.
Chen F, Yuan Y, Li Q, He Z (2007). Proteomic analysis of rice plasma membrane reveals proteins involved in early defense response to bacterial blight. Proteomics 7:1529-1539.
Dokku P, Das KM, Rao GJN (2013). Pyramiding of four resistance genes of bacterial blight in Tapaswini, an elite rice cultivar, through marker-assisted selection. Euphytica 192:87-96.
Doveri S, Maheswaran M, Powell W (2008). Molecular markers – history, features and applications. In: Kole C, Abbott AG (eds) Principles and Practices of Plant Genomics. Vol. 1: Genome Mapping. Science Publishers, Enfield, Jersey, Plymouth, England.
Fagwalawa LD, Kutama AS, Yakasai MT (2013). Current issues in plant disease control: Biotechnology and plant disease. Bayero Journal of Pure and Applied Sciences 6(2):121-12.
Gopalakrishnan S, Sharma RK, Rajkumar KA, Joseph M, Singh VP, Singh AK, Bhat KV, Singh NK, Mohapatra T (2008). Integrating marker assisted background analysis with foreground selection for identification of superior bacterial blight resistant recombinants in Basmati rice. Plant Breeding 127:131-139.
Guglielmo F, Bergemann SE, Gonthier P, Nicolotti G, Garbelotto M (2007). A multiplex PCR-based method for the detection and early identification of wood rotting fungi in standing trees. Journal of Applied Microbiology 103:1490-1507.
Haggag WM (2008). Biotechnological aspects of plant resistant for fungal diseases management. American-Eurasian Journal of Sustainable Agriculture 2(1):1-18.
Hayes PM, Corey AE, Mundt C, Toojinda T, Vivar H (2003). Registration of 'Tango' Barley. Crop Science 43:729-731.
James C (2003). Preview: Global status of commercialized transgenic crops: 2003. ISAAA Briefs No. 30. Ithaca, NY.
Jeeva ML, Mishra AK, Vidyadharan P, Misra RS, Hegde V (2010). A species-specific polymerase chain reaction assay for rapid and sensitive detection of *Sclerotium rolfsii*. Australasian Plant Pathology 39(6):517-523.
Jones AME, Thomas V, Truman B, Lilley K, Mansfield J, Grant M (2004). Specific changes in the Arabidopsis proteome in response to bacterial challenge: differentiating basal and R-gene mediated resistance. Phytochemistry 65:1805-1816.
Jones AME, Thomas V, Bennett MH, Mansfield J, Grant M (2006). Modifications to the Arabidopsis defense proteome occur prior to significant transcriptional change in response to inoculation with *Pseudomonas syringae*. Plant Physiology 142:1603-1620.
Kang S, Mansfield MA, Park B, Geiser DM, Ivors KL, Coffey MD, Grünwald NJ, Martin FN, Lévesque CA, Blair JE (2010). The promise and pitfalls of sequence-based identification of plant-pathogenic fungi and oomycetes. Phytopathology 100(8):732-737.
Kaymak S, Boyraz N, Daniels J (2016). Molecular markers to evaluate genetic diversity among *Venturia inaequalis* isolates obtained from apple plantations in Isparta Province. Turkish Journal of Agriculture and Forestry 40:489-498.
Liu J, Liu D, Tao W, Li W, Wang CP, Cheng S, Gao D (2000). Molecular marker-facilitated pyramiding of different genes for powdery mildew resistance in wheat. Plant Breeding 119:21-24.
Lodha TD, Hembram P, Tep N, Basak J (2013). Proteomics: A successful approach to understand the molecular mechanism of plant-pathogen interaction. American Journal of Plant Sciences 4:1212-1226.
Mahmood T, Jan A, Kakishima M, Komatsu S (2006). Proteomic analysis of bacterial-blight defenceresponsive proteins in rice leaf blades. Proteomics 6:6053-6065.
Manulis S, Kogan N, Reuven M, Yephet YB (1994). Use of the RAPD technique for identification of *Fusarium oxysporum* f. sp. *dianthi* from carnation. Phytopathol 84:98-101.
Martin RR, Constable F, Tzanetakis IE (2016). Quarantine regulations and the impact of modern detection methods. Annual Review of Phytopathology 54:189-205.
Martins D, Astua-Monge G, Coletta-Filho HD, Winck FV, Baldasso PA, de Oliveira BM, Marangoni S, Machado MA, Novello JC, Smolka MB (2007). Absence of classical heat shock response in the citrus pathogen Xylella fastidiosa. Current Microbiology 54:119-123.
Mathesius U, Mulders S, Gao M, Teplitski M, Caetano-Anolles G, Rolfe BG, Bauer WD (2003). Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proceedings of the National Academy of Sciences USA 100:1444-1449.
McCartney HA, Foster SJ, Fraaije BA, Ward E (2003). Molecular diagnostics for fungal plant pathogens. Pest Management Science 59(2):129-142.
Mehta A, Rosato YB (2001). Differentially expressed proteins in the interaction of *Xanthomonas axonopodis pv. citri* with leaf extract of the host plant. Proteomics 1:1111-1118.
Miedaner T, Wilde F, Steiner B, Buerstmayr H, Korzun V, Ebmeyer E (2006). Stacking quantitative trait loci (QTL) for *fusarium* head blight resistance from non-adapted sources in a European elite spring wheat background and assessing their effects on deoxynivalenol (DON content and disease severity. Theoretical and Applied Genetics 112:562-569.
Mittal M, Boora KS (2005). Molecular tagging of gene conferring leaf blight resistance using microsatellites in sorghum {*Sorghum bicolour* (L.) Moench}. Indian Journal of Experimental Biology 43(5):462-466.
Narayanasamy P (2011). Microbial plant pathogens-detection and disease diagnosis, Springer Dordrecht Heidelberg London New York, 2:280.
Nilsson RH, Abarenkov K, Larsson KH, Kõljalg U (2011). Molecular identification of fungi: rationale, philosophical concerns, and the UNITE database. The Open Applied Informatics Journal 5:81-86.
Okada Y, Kanatani R, Arai S, Asakura T, Ito K (2003). Production of a novel virus-resistant barley line introgression to the rym1 locus with high malting quality using DNA marker assisted selection. Journal of the Institute of Brewing 109:99-102.
Pandey MK, Shobha Rani N, Sundaram RM, Laha GS, Madhav MS, Srinivasa Rao K, Injey Sudharshan, Yadla Hari, Varaprasad GS, Subba Rao LV, Kota Suneetha, Sivaranjani AKP, Viraktamath BC (2013). Improvement of two traditional Basmati rice varieties for bacterial blight resistance and plant stature through morphological and marker-assisted selection. Molecular Breeding 31:239-246.
Perez DR, Lopez GE, Rivera J, Ferreira A, Fontan G (2008). Naturally occurring Bruton's tyrosine kinase mutations have no dominant negative effect in an Xlinked agammaglobulinaemia cellular model. Clinical and Experimental Immunology 152:33-38.
Punja ZK, De Boer SH, Sanfaçon H (2008). Biotechnology and plant disease management. CAB International, Canada 590 p.
Ragimekula N, Varadarajula NN, Mallapuram SP, Gangimeni G, Reddy RK, Kondreddy HR (2013). Marker assisted selection in disease resistance breeding. Journal of Plant Breeding and Genetics 1(2):90-109.
Scholz M, Ruge-Wehling B, Habekub A, Schrader O, Pendinen G, Fischer K, Wehling P (2009). Ryd4Hb: A novel resistance gene introgressed from *Hordeum bulbosum* into barley and conferring complete and dominant resistance to the barley yellow dwarf virus. Theoretical and Applied Genetics 119:837-849.
Shamim M, Kumar P, Kumar RR, Kumar M, Kumar RR, Singh KN (2017). Assessing fungal biodiversity using molecular markers. In: Singh B., Gupta V. (eds) Molecular markers in mycology. Fungal biology. Springer, Cham, Switzerland.
Shannon JG, Lee JD, Wrather JA, Sleper DA, Mian MAR, Bond JP, Robbins RT (2009). Registration of S99-2281 soybean germplasm line with resistance to frogeye leaf spot and three nematode species. Journal of Plant Registrations 3:94-98.
Shanti ML, Shenoy VV, Lalitha Devi G, Mohan Kumar V, Premalatha P, Naveen Kumar G, Shashidhar HE, Zehr UB, Freeman WH (2010). Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivar and parental lines of hybrid rice. Journal of Plant Pathology 92(2):495-501.
Shi A, Chen P, Li D, Zheng C, Zhang B, Hou A (2009). Pyramiding multiple genes for resistance to soybean. Mosaic virus using molecular markers. Molecular Breeding 23:113-124.
Singh A, Singh KV, Singh SP, Pandian RTP, Ranjith EK, Singh D, Bhowmick KP, Gopala Krishnan S, Nagarajan M, Vinod KK, Singh UD, Prabhu KV, Sharma TR, Mohapatra T, Singh AK (2012). Molecular breeding for the development of multiple disease resistance in Basmati rice. www.aobplants.oxfordjournals.org.

Singh Y, Singh J, d Pandey AK (2013). Molecular markers in diagnosis and management of fungal pathogens: A Review. International Journal of Advanced Biotechnology and Research 4(2):180-188.

Sundaram RM, Vishnupriya MR, Biradar SK, Laha GS, Reddy GA, Shoba Rani N, Sarma NP, Sonti RV (2008). Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. Euphytica 160:411-422.

Sundaram RM, Priya MRV, Laha GS, Shobha Rani N, Srinivasa Rao P, Balachandran SM, Ashok Reddy G, Sarma NP, Sonti RV (2009). Introduction of bacterial blight resistance into Triguna, a high yielding, mid-early duration rice variety by molecular marker assisted breeding. Biotechnology Journal 4:400-407.

Suarez -Moreno ZR, Caballero-Mellado J, Coutinho BG, Mendonça-Previato L, James EK, Venturi V (2012). Common features of environmental and potentially beneficial plant-associated Burkholderia. Microbial Ecology 63:249-66.

Tahara ST, Mehta A, Rosato YB (2003). Proteins induced by *Xanthomonas axonopodis* pv. *passiflorae* with leaf extract of the host plant (*Passiflorae edulis*). Proteomics 3:95-102.

Torres AM (2010). Application of molecular markers for breeding disease resistant varieties in crop plants. In Molecular techniques in crop improvement. Springer, Dordrecht pp. 185-205.

Torres-Calzada C, Tapia-Tussell R, Quijano-Ramayo A, Martin-Mex R, Rojas-Herrera R, Higuera-Ciapara I, Perez-Brito D (2011). A species-specific polymerase chain reaction assay for rapid and sensitive detection of *Colletotrichum capsici*. Molecular Biotechnology 49(1):48-55.

Van Doorn R, Slawiak M, Szemes M, Dullemans AM, Bonants P, Kowalchuk GA, Schoen CD (2009). Robust detection and identification of multiple oomycetes and fungi in environmental samples by using a novel cleavable padlock probe-based ligation detection assay. Applied and Environmental Microbiology 75(12):4185-4193.

Wen S, Gao B (2011). Introgressing blast resistant gene *Pi-9(t)* into elite rice restorer luhui17 by marker-assisted selection. Rice Genomics and Genetics 2(4):31-36.

Werner K, Friedt W, Ordon F (2005). Strategies for pyramiding resistance genes against the barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2). Molecular Breeding 16:45-55.

Wilde F, Schon C, Korzun V, Ebmeyer E, Schmolke M, Hartl L, Miedaner T (2008). Marker-based introduction of three quantitative-trait loci conferring resistance to *fusarium* head blight into an independent elite winter wheat breeding population. Theoretical and Applied Genetics 117:29-35.

Zhan XD, Zhou HP, Chai RY, Zhuang JY, Cheng SH, Cao LY (2012). Breeding of R8012, a rice restorer line resistant to blast and bacterial blight through marker-assisted selection. Rice Science 19 (1):29-35.

Vol. 18(29), pp. 808-818, September, 2019
DOI: 10.5897/AJB2019.16888
Article Number: 19D047E61831
ISSN: 1684-5315

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Biopreservation of meat using the essential oil from *Hyptis suaveolens* Poit. (Lamiaceae) in Burkina Faso

Henriette B. MIHIN[1], Marius K. SOMDA[1,2], Donatien KABORE[3], Souleymane SANON[4], Agbémébia Y. AKAKPO[1,5], Zenabou SEMDE[1,3], Alfred S.TRAORE[2] and Aboubakar S. OUATTARA[1*]

[1]Laboratoire de Microbiologie et Biotechnologie Microbienne, Centre de Recherche en Sciences Biologiques, Alimentaires et Nutritionnelles (CRSBAN), Département de Biochimie Microbiologie, Université Joseph KI-ZERBO. 03BP:7021, Burkina Faso.
[2]Laboratoire de Technologies Alimentaires, Département de Biochimie Microbiologie, Université Joseph KI-ZERBO. 03BP:7021, Burkina Faso.
[3]Departement de Technologie Alimentaire, Institut de Recherche en Sciences Appliquées et Technologies. 03BP:7047, Ouagadougou, Burkina Faso.
[4]Centre National de Recherche et de Formation sur le Paludisme. 01 BP 2208 Ouagadougou, Burkina Faso.
[5]Laboratoire de Contrôle Qualité et Normalisation (LCQN), Institut Togolais de Recherche Agronomique (ITRA). BP: 1163, Lomé-Togo.

Received 12 June, 2019;Accepted 23 August, 2019

Essential oils are natural substances which can be used as natural conservators for foods. The present work aimed to evaluate biological properties of essential oil of *Hyptis suaveolens* and its effect on shelf-life of beef. The extraction was carried out by hydro-distillation. Antioxidant activity was determined by method of DPPH and FRAP. Antimicrobial activity was determined by the microdilution and agar diffusion methods. The sensitivity of eleven pathogen microbial strains was tested. The conservation test of ground meat using essential oil was carried out by monitoring the evolution of the microbial groups. Result showed that extraction yield of essential oil was 0.21% (m/v). The DPPH assay gave IC_{50} of 16.367 ± 0.0739 µL and was confirmed by the FRAP assay. The essential oil from *H. suaveolens* showed inhibitory activity on microbial strains. Biopreservation assay revealed a significant decrease of microbial charge during conservation time. Thus essential oil was successful *in vitro* and its activity on prolonging shelf-life of beef was found to be 7 days. The results of the present study demonstrated the possibility of essential oil from *H. suaveolens* to be used as natural conservator for food industry.

Key words: *Hyptis suaveolens*, essential oil, biopreservation, biological properties, meat.

INTRODUCTION

The sanitary quality of a food is one of essential bases of its ability to satisfy the safety of the consumers. A food

*Corresponding author. E-mail: mhenrietteb@gmail.com. Tel: (+226) 75100566.

exposed to microbial spoilage could lose its organoleptic, nutritional and sanitary qualities (Guiraud, 2003). Despite the improved techniques for preserving food, the kind of preservatives remains as one of the most important issues for public health (Burt, 2004). One of the main problems in food industry is to ensure safe food preservation for consumers (Nessrien and Mohamed, 2007). To cope with the problems of oxidation and contamination of foodstuffs, new chemical compounds have often been used to prevent the deterioration of food (Nakahara et al., 2003a). Preservatives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-Butylhydroquinone (TBHQ) used in food preservation have been limited in several countries due to their undesirable toxicological and carcinogenic effects at short or long time (Rachid et al., 2010). Also, the current trend of consumers to seek a more natural diet has increased. The demand by customers for natural preservative has directed researchers' interest to develop protective methods for reliable foods having better nutritional and organoleptic properties and with high microbial quality (Goni et al., 2009). Aromatic plants have interesting properties due to metabolic substances during their secondary metabolism (Rashid et al., 2010). These substances (the essential oils) are getting more interest for industries and scientific research due to their antioxidant, antibacterial and antifungal activities (Dung et al., 2008). They are also useful as natural preservatives in agro-food industries (Rasooli et al., 2008). In the developing countries where the demographic growth rate is raised, the meat consumption progresses quickly. It is in the case in sub-Saharan Africa, where the beef represent the main of the additional consumption of the area. If the majority of the beef consumed in sub-Saharan Africa is produced in the area, half of additional consumption will come from the importation. The beef consumption will increase gradually during ten next years. It should increase approximately 17% in the areas under development (OCDE/FAO, 2017).

Essential oils are a source of natural preservatives for perishable dairy products in Sub-Saharan Africa where the capacity of food preservation by cold chain is very limited. Among commodities, meat products are very susceptible to deterioration. Poorly preserved meat can lead to economic losses and health consequences in most of developing countries. In Burkina Faso, beef is a food of choice because of its importance as a source of protein. However, rapid deterioration can be observed due to microbial contamination. Some microorganisms can be pathogenic and harmful for consumers' health. To overcome or stop meat spoiling, it is important to use natural preservatives such as essential oils. Although the antimicrobial activity of essential oils derived from plants has been proved by in-vitro tests, more research is required in order to investigate their activities on food. The present study aimed to investigate the biological properties of essential oils from *H. suaveolens* as well as it capacity to prolong shelf-life of meat.

MATERIALS AND METHODS

Plant material

The leaves from *H. suaveolens* were collected in different areas of Ouagadougou. The identification of the plant (*H. suaveolens*) was carried out by the Laboratory of Plant Ecology Biology in University Joseph Ki-Zerbo. Fresh plant material was dried about 25°C for 72 h.

Indicator microorganisms

The antimicrobial activity study focused on eleven (11) microbial strains used as indicator microorganisms:

1. Nine (09) bacterial strains including 5 Gram-negative (*Escherichia coli* ATCC8739, *Salmonella typhi*, *Salmonella paratyphi*, *Shigella dysenteria*, and *Pseudomonas aeruginosa* ATCC9027) and 4 Gram-positive strains (*Staphylococcus aureus* ATCC25923, *Listeria monocytogenes*, *Clostridium perfringens* and *Bacillus cereus*), maintained as stock cultures at -80°C in Mueller-Hinton broth medium (Liofilchem, Italy) with 20% (v/v) glycerol as cryoprotectant.
2. Two (02) fungal strains (*Candida albicans* and *Aspergillus niger*), maintained as stock cultures at -80°C in Sabouraud broth medium (Liofilchem, Italy) with 20 % (v/v) glycerol as cryoprotectant.

Food matrix: Ground beef

Ground beef was purchased at Zogona market (Ouagadougou). The meat samples were placed in icebox to maintain refrigerated conditions and transported to the laboratory.

Essential oil extraction

The essential oil was extracted from the plant material by hydro-distillation according to the method described by Baser and Buchbauer (2010). 500 g of dry matter was immersed in a Clevenger type apparatus containing about 5 L of distilled water. The mixture was boiled for three (03) hours. The obtained essential oil was collected in a sterile amber glass bottle for preservation. Yield, expressed as a percentage (% weight/weight), and was calculated using the following equation:

$$\text{Yield} = (\text{Mass of essential oil (g)}) / (\text{Mass of dry matter (g)}) \times 100$$

Analysis of the organoleptic properties of essential oil

Organoleptic properties such as color, appearance and odor have been determined directly by the sense organs. The essential oil was first transferred to a transparent bottle and analysis by using of standard procedures.

Determination of biological activities of essential oil

Antioxidant activity

DPPH radical scavenging assay: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was performed according to the method described by Joshi et al., (2010). Different volumes of essential oil (5, 10, 15, 20 and 25 µL) were mixed with 5 mL of ethanolic solution of DPPH (0.004 % weight/volume). The mixture obtained was incubated in dark space for 30 min and absorbance was read at

517 nm using a spectrophotometer (Jasco, Japan). Butylhydroxytoluene (BHT) (0.05 M), ascorbic acid (0.005 M) and quercetin (0.05 M) (5, 10, 15, 20 and 25 µL) were used as standard antioxidants and a negative control was also prepared in the same conditions like the samples. The tests were carried out in triplicate.

The percentage of inhibition (% I) was calculated according to the following equation:

% Inhibition = $[(A_{blank}–A_{sample}) / A_{blank}] \times 100$; where A_{blank} is the absorbance of the negative control and A_{sample} is the absorbance of the essential oil.

The antioxidant activity of the essential oil was expressed by the 50% inhibitory concentration (IC50) which is the amount of essential oil needed to reduce the initial concentration of DPPH by 50%. The 50% inhibitory concentration was calculated using a regression line (% inhibition = f (concentrations)). The tests were carried out in triplicate.

Ferric reduction antioxidant power (FRAP): The reducing power of essential oil of *H. suaveolens* was determined according to the method of Singh et al. (2006). Different volumes of essential oil (5, 10, 15, 20 and 25 µL) were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of potassium ferricyanide ($K_3Fe(CN)_6$) at 1%. The mixture was incubated at 50°C for 30 min then 2.5 mL of trichloroacetic acid (10%) were added to the mixture followed by centrifugation at 650 G for 10 min. The supernatant was collected (5 mL) and mixed with 5 mL of distilled water and 1 mL of 0.1% iron ($FeCl_3$) chloride. Absorbance was then measured at 700 nm using a spectrophotometer. Ascorbic acid was used as a standard at 5 mg/Ml. A negative control (blank) was also included in each test. The standard and blank were prepared according to the process of essential oil. An increase in absorbance indicates an increase in reducing power. A standard curve of essential oil absorbance was developed. The tests were carried out in triplicate.

Screening of essential oil for antimicrobial activity

Preparation of the inocula of bacterial and fungal strains

The inocula of bacterial and fungal strains used as indicators were prepared before inoculating during the assays.

A suspension of each bacterial strain was prepared in 10 mL of Mueller-Hinton Broth for 18 to 24 h at 37°C. Using the sterile diluent (physiological saline) (Liofilchem, Italy), the concentration was adjusted in each tube to about 1.0×10^8 CFU/mL comparable to that of the McFarland 0.5 standard according to Lennette et al. (1987). For the fungal suspension, each strain was prepared in Sabouraud broth (Liofilchem, Italy) for 18 to 48 h at 30°C. With a sterile diluent, the concentration was adjusted to about 1.0×10^8 CFU/mL comparable to that of the McFarland 0.5 standard (Lennette et al., 1987).

Agar diffusion method

Petri dishes containing Sabouraud Chloramphenicol medium (for fungal strains) and Mueller-Hinton agar (for bacterial strains) were inoculated aseptically with the inocula. Seeding was done by flooding the Petri dish and the excess was aspirated. After drying the dishes, wells were cut with a sterile cork borer (diameter: 6 mm) in the agar and 10 µL of *H. suaveolens* essential oil was added into the different wells. The dishes were exposed at room temperature for one (1) hour before incubation to promote diffusion of essential oil on agar plate. The dishes were incubated at 37 °C for 24 hours for bacteria and at 30°C for 48 h for fungal strains. The presence of a clear zone around the well indicates the inhibition. The results were read by measuring the diameters of inhibition zones in mm (Rhayour, 2002).

The activity of essential oil was assessed according to the diameter of inhibition values as described by Negreiros et al., (2016). The microbial strains were classified as non-sensitive when a diameter is less than 8 mm, sensitive from 9 to 14 mm, highly sensitive from 15 to 19 mm and extremely sensitive for more than 20 mm.

Determination of minimum inhibitory concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) was performed in a sterile 96-well microplate.

For bacterial strains, 190 µL of Muller-Hinton broth supplemented with tween 80 (0.5%) were introduced into the wells of line 1 and 100 µL into the other wells of the microplate from line 2 to line 12, then 10 µL of essential oil were added to the wells of line 1. The contents of the wells of line 1 were well mixed. 100 µL were collected from these wells (line 1) for cascade dilutions in the other wells (line 1) to the 0.004 % concentration.

The inoculum density was adjusted with sterile saline solution (NaCl 0.9 % w/v) to McFarland 0.5 corresponding to 10^8 CFU/mL. 100 µL of bacterial inoculum were added to all wells except the wells in line 11 which contained only the essential oil and Muller-Hinton broth. Line 11 (without inoculum) served as a negative control. Line 12 containing Muller-Hinton broth and bacterial suspension served as a positive control. The microplates were closed and incubated for 18 to 24 h at 37°C (Yu et al., 2004; Obame, 2009).

For fungal strains, Sabouraud broth supplemented with tween 80 (0.5%) was used. The same procedure as described previously was performed for experiment. The microplates were incubated at 30°C for 48 to 72 h.

Microbial growth is indicated by reading the optical density of culture (Yu et al., 2004). The lowest essential oil concentration inhibiting the microbial growth after incubation period was identified as minimum inhibitory concentration (MIC). The treatments were repeated three times and mean values calculated.

Determination of minimum bactericidal concentration (MBC)

One hundred (100) microliter were taken from wells without detectable growth after 24 h of incubation at 37°C and seeded by spreading on Mueller-Hinton agar (Ouedraogo, 2012). The lowest concentration at which 99.99% of bacteria cells were inhibited after 24 h of incubation was identified as minimum bactericidal concentration (MBC). Experiments were done in triplicate.

Determination of minimum fungicidal concentration (MFC)

A volume of one hundred (100) µL was collected from wells that did not present detectable growth after incubation at 30°C for 72 h. It was seeded on Sabouraud Chloramphenicol agar (Ouedraogo, 2012). The lowest concentration at which 99.99% of fungal cells were inhibited after 72 h of incubation was identified as minimum fungicidal concentration (MFC). Experiments were done in triplicate.

Determination of ratio MBC/MIC and MFC/MIC

The ratio MBC/MIC and MFC/MIC allowed the determination of the bactericidal or bacteriostatic and fungicidal or fungistatic capacities of essential oil on the strains tested according to Canillac and Mourey (2001) and Derwich et al., (2010):

Table 1. Organoleptic characteristics of the essential oil of *Hyptis suaveolens.*

Color	Light yellow
Appearance	Fluid, limpid, very transparent, very bright
Odor	Very strong, aromatic, minty, characteristic of the plant

Essential oil is bacteriostatic when MBC/MIC>4;
Essential oil has bactericidal property when MBC/MIC≤4;
Essential oil has fungicidal effect when MFC/MIC<4;
Essential oil has fungistatic effect when MFC/MIC≥4.

Preservation of ground meat by essential oil

The essential oil of *H. suaveolens* (20 µL) was incorporated in a Stomacher bag containing 50 g of fresh ground meat; a second bag containing only ground meat was used as control. The samples were stored in the refrigerator at 4°C for 7 days. Microbiological analysis was carried out on the 1st, 4th and 7th day on ground meat treated with the essential oil and on the control. Enumeration included total mesophilic aerobic bacteria (TMAB), total coliforms (TC) and thermotolerant coliforms (TTC).

For all the determinations, 10 g of the samples were homogenized in a stomacher (Stomacher 400) with 90 mL of sterile peptone buffered water (Liofilchem, Italy). Tenfold serial dilution was prepared and spread-plated for microorganisms count. 1 mL of each dilution was used for spreading.

Aerobic mesophilic bacteria (AMB) were enumerated on pour plates of Plate Count Agar (Liofilchem, Italy) incubated at 30°C for 72 h (ISO 4833, 2003). Coliforms were enumerated on Eosin Blue Methylene Agar (Liofilchem, Italy), incubated at 37°C (Total coliforms) or 44°C (thermotolerant coliforms) for 24 h according to ISO 4832 (2006) and AFNOR NF V 08-060 (2009) respectively.

Statistical analysis

The data were collected on Excel and analyzed using XLSTAT software version 7.5.2. Variance analysis (ANOVA) was used and Fisher's Least Significant Difference (LSD) was used for comparisons of means in case of significant difference. The difference between means was significant when p value < 0.05. The values were estimated with a 95% confidence interval.

RESULTS AND DISCUSSION

Characteristics of essential oil

Yield and organoleptic characteristics of essential oil from H. suaveolens

The essential oil yield from *Hyptis suaveolens* leaves was 0.215 ± 0.002%. This yield corroborates with that obtained by Ngom et al., (2014) in Senegal which was 0.22%. In Benin, Adjou and Soumanou (2013) obtained similar results (0.23 ± 0.02%). On the other hand, the yield obtained is lower than those obtained by Ilboudo (2009) and Goly et al., (2015) which were respectively 0.23 and 0.34 ± 0.02%. Differences in yield could be explained by several factors such as geographical origin, ecological climatic factors such as temperature and humidity, soil type, plant organ, stage of growth, period of harvesting, conservation of plant material, extraction time and drying time (Marzoukia et al., 2009, Aprotosoaie et al., 2010).

The organoleptic characteristics of the essential oil have been presented in Table 1. The essential oil of *Hyptis suaveolens* was light yellow, its appearance was fluid, limpid, very transparent and very bright with a very strong, aromatic, minty and characteristic smell of the plant. The color of essential oil is different of that obtained by Ngom et al., (2014) which was light green.

Biological properties of essential oil from *H. suaveolens*

Antioxidant activity

Capacity of inhibition of the radical DPPH: Antioxidant activity was determined and compared to that of the reference antioxidants (ascorbic acid, BHT and quercetin). Figure 1 shows the variation of the percentage of inhibition as a function of the quantity of essential oil and standards.

The increase in the percentage of inhibition of the free radical was directly proportional to the increase in the quantity of essential oil and standards. Statistical analysis showed a significant difference ($p < 0.0001$) between percent inhibition of essential oil and standards. For each of the essential oil and the standards, the concentration needed to reduce the DPPH free radical by 50% was determined from the linear regression equations. The 50% inhibitory concentrations (IC_{50}) were presented in Table 2.

For the essential oil from *H. suaveolens*, its antioxidant activity was higher than that of ascorbic acid (0.005 M), but was lower than that of BHT (0.05 M) and quercetin (0.05 M). Indeed, the IC_{50} of *H. suaveolens* was 16.367 ± 0.074 µL whereas with ascorbic acid, BHT and quercetin, the IC_{50} was respectively 22.098 ± 0.128, 13.013 ± 0.053 and 12.142 ± 0.234 µL.

Reducing power of essential oil of *H. suaveolens:* The reducing power of essential oils was evaluated using the FRAP method. The diagram showing the variation of the reducing power as a function of the quantity of essential oils and the standard (Ascorbic acid) are recorded in Figure 2.

The results obtained by the FRAP test show that the reduction of ferric ions was related to the amount of

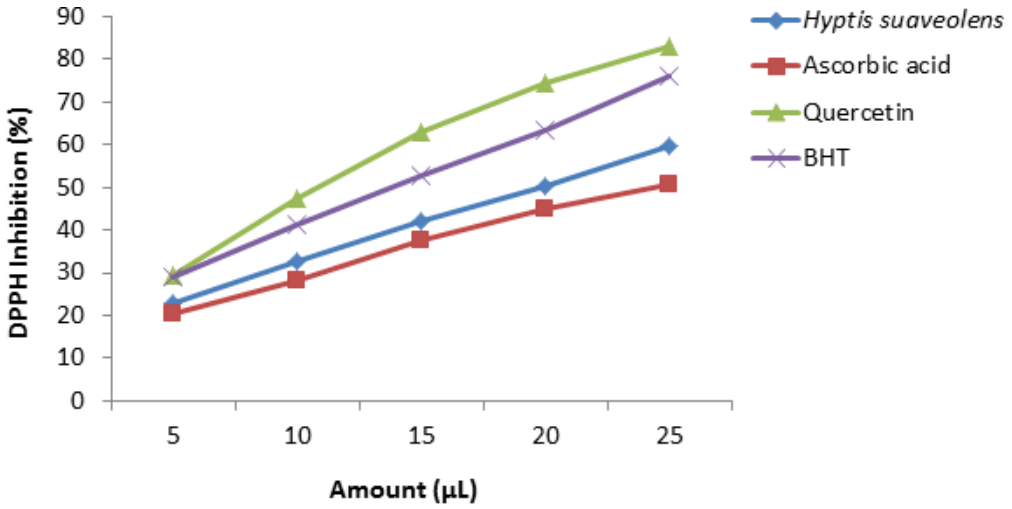

Figure 1. DPPH radical scavenging power.

Table 2. Inhibitory concentrations at 50% of the essential oil of *H. suaveolens.*

Essential oil and standards	Regression equation	R^2	IC_{50} (µL)
H. suaveolens	Y = 2.2789x + 12.698	0.9894	16.367 ± 0.074
Ascorbic acid (0.005 M)	Y = 1.7179x + 12.080	0.9894	22.098 ± 0.128
Quercetin (0.05 M)	Y = 2.9372x + 14.316	0.9967	12.142 ± 0.234
BHT (0.05 M)	Y = 2.083x + 22.891	0.9908	13.013 ± 0.053

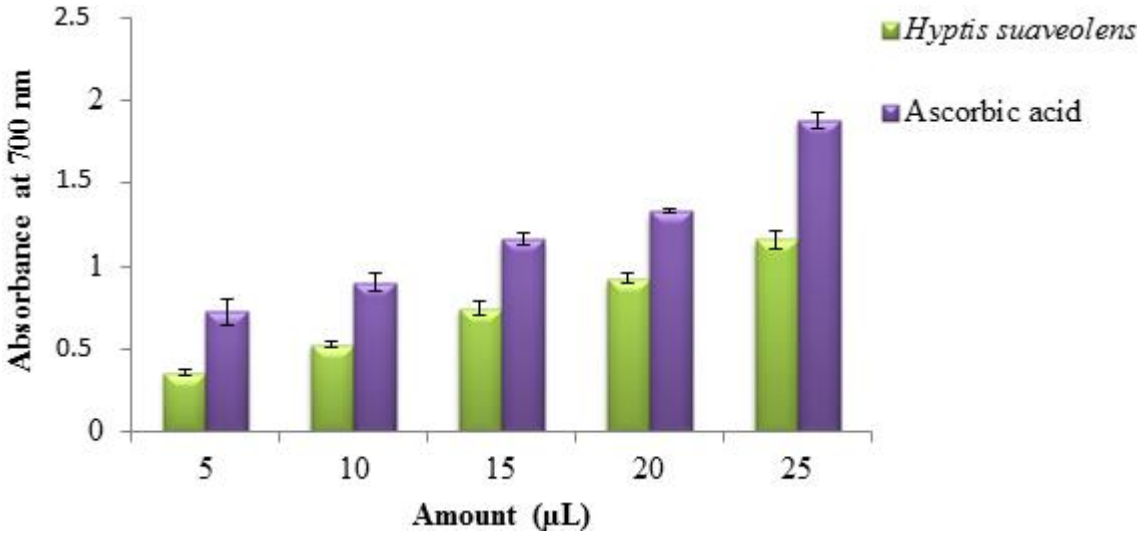

Figure 2. Reducing power of essential oil and ascorbic acid.

essential oils and that the ability of *H. suaveolens* essential oils to reduce iron was lower than that of ascorbic acid.

The evaluation of the antioxidant activity by the DPPH technique and that of FRAP revealed that the essential oil of *H. suaveolens* has antioxidant properties. Previous studies have shown that oxygenated monoterpenes such as thymol, carvacrol and α-terpineol are mainly responsible for the antioxidant potential of their essential oils (Bicas et al., 2011). Monoterpenes such as β-

Table 3. Average diameters of inhibition of the essential oil of *H. suaveolens.*

Bacterial strain	Diameters of inhibition (mm)	Sensitivity of strains
Shigella dysenteria	43.667 ± 1.732[a]	Extremely sensitive
Staphylococcus aureus ATCC25923	36.167 ± 2.055[b]	Extremely sensitive
Salmonella paratyphi	18.833 ± 1.434[c]	Very sensitive
Listeria monocytogenes	18.167 ± 2.718[cd]	Very sensitive
Bacillus cereus	18.000 ± 1.780[cde]	Very sensitive
Escherichia coli ATCC8739	14.333 ± 1.247[def]	Sensitive
Clostridium perfringens	14.167 ± 1.027[def]	Sensitive
Salmonella typhi	13.500 ± 2.273[f]	Sensitive
Pseudomonas aeruginosa ATCC9027	12.833 ± 1.546[f]	Sensitive
Fungal strains		
Aspergillus niger	13.667 ± 1.434[ef]	Sensitive
Candida albicans	10.667 ± 0.471[f]	Sensitive

Values with the same superscript letters are not significantly different ($p<0.05$).

caryophyllene, also has free radical scavenging activity using the DPPH test (Dar et al., 2011). Oxygen-rich essential oils have a stronger anti-radical activity compared to hydrocarbon terpenes. This could explain the moderate antioxidant activity of the essential oil of *H. suaveolens* (Miladi et al., 2013).

Antimicrobial activity

Inhibiting capacity of essential oil of *H. suaveolens*: The well diffusion method highlights the antimicrobial capacity of the essential oils against nine bacterial strains and two fungal strains. The average inhibition diameters obtained are mentioned in Table 3.

Table 3 shows that the essential oils studied have antimicrobial activity. The importance of the action of each essential oil varied according to the microorganism tested.

The essential oil of *H. suaveolens* showed antimicrobial activity on the strains tested. The largest diameter was obtained with *Shigella dysenteria* (43.667 mm) followed by *Staphylococcus aureus* (36.167 mm) and the smallest diameter with *Candida albicans* (10.667 mm). The sensitivity of *H. suaveolens* essential oil was not related to bacteria nature. *S. aureus* (Gram positive) and *S. dysenteria* (Gram negative) were more sensitive to the essential oil. This activity of the essential oil of *H. suaveolens* could be explained by its chemical advantage (Ngom et al., 2014).

The antimicrobial activity could be explained by the presence of phenolic hydroxyl compounds by forming hydrogen bonds with the active sites of the targeted cell enzymes (Derwich et al., 2010). The factors determining the activity of essential oils are the chemical composition, the functional groups present in active components and their synergistic interactions (Chouhan et al., 2015).

Minimum inhibitory concentrations of essential oil of *H. suaveolens:* The results of the determination of minimum inhibitory (MIC), bactericidal (MBC) and fungicidal (MFC) concentrations are shown in Table 4.

Minimum inhibitory concentrations (MIC) ranged from 0.203 to 2.083%. The highest MIC was obtained with *E. coli* and the lowest MIC with *S. paratyphi*. Minimum bactericidal concentrations (MBCs) ranged from 0.625 to 5%. The largest MBC was obtained with *Staphylococcus aureus* (5.000±0.049%) and *C. albicans* (5.000±0.045 %) and the lowest with *C. perfringens* (0.625±0.021%).

Ratio MBC/MIC and MFC/MIC of essential oil from *H. suaveolens*

The results of the MBC/MIC and MFC/MIC report are presented in Table 5.The essential oil of *Hyptis suaveolens* had bactericidal activity on *E. coli*, *S. typhi*, *S. dysenteria*, *B. cereus*, *P. aeruginosa*, *L. monocytogenes* and *C. perfringens*. It had Bacteriostatic on *S. paratyphi* and *S. aureus*. Its effect was fungicidal on *A. niger* and fungistatic on *C. albicans*.

Effect of essential oil on prolonging shelf-life of food matrix

Initial microbial load of ground meat

Prior to the incorporation of the essential oil (EO) on ground meat (GM) for conservation testing, the initial microbial load of this meat was assessed by counting the total aerobic mesophilic flora, total and thermotolerant

Table 4. MIC, MBC and MFC of the essential oil of *H. suaveolens.*

Bacterial strains	MIC	MBC
Escherichia coli ATCC8739	2.083±0.189^{a}	2.167 ±0.302^{b}
Salmonella paratyphi	0.203±0.074^{e}	2.500 ±0.224^{b}
Salmonella typhi	0.625±0.000^{cde}	2.500 ± 0.072 ^{b}
Shigella dysenteria	0.521±0.048^{de}	1.250±0.044^{cd}
Bacillus cereus	1.042±0.012^{bc}	4.167±0.039^{a}
Pseudomona aeruginosa ATCC9027	0.625±0.189^{cde}	1.042±0.081^{cd}
Staphylococcus aureus ATCC25923	0.833±0.121^{bcd}	5.000±0.049^{a}
Listeria monocytogenes	0.521±0.140^{de}	1.250±0.053^{cd}
Clostridium perfringens	0.625±0.032^{cde}	0.625±0.021^{d}
Fungal strains	**MIC**	**MFC**
Candida albicans	1.250±0.000^{b}	5.000±0.045^{a}
Aspergillus niger	1.042±0.113^{bc}	1.667±0.062^{bc}

Values in the same column with the same superscript letters are not significantly different ($p < 0.05$).

Table 5. MBC/MIC and CMF/MIC ratio of essential oil *H. suaveolens.*

Bacterial strains	MBC/MIC	Sensitivity of strains
Escherichia coli ATCC8739	1	Bactericidal
Salmonella paratyphi	12	Bacteriostatic
Salmonella typhi	4	Bactericidal
Shigella dysenteria	2	Bactericidal
Pseudomonas aeruginosa ATCC9027	1	Bactericidal
Bacillus cereus	4	Bactericidal
Staphylococcus aureus ATCC25923	6	Bacteriostatic
Listeria monocytogenes	2	Bactericidal
Clostridium perfringens	1	Bactericidal
Fungal strains	**MFC/MIC**	
Candida albicans	6	fungistatic
Aspergillus niger	1	Fungicidal

coliforms. Figure 3 shows the initial microbial flora of minced meat before storage.

The Initial microbial load of ground meat was found to be 7.0×10^{4} CFU/g for total aerobic mesophilic bacteria, 1.0×10^{4} CFU/g for total coliforms and 5.0×10^{2} CFU/g for thermotolerant coliforms.

The high number of total initial aerobic mesophilic bacteria (TMAB) indicates that the meat was of poor microbiological quality, which could influence its shelf life. The presence of coliforms indicates recent fecal contamination that may be explained by the lack of good hygiene practices throughout the meat production chain.

Effect of essential oil on total mesophilic aerobic bacteria

The evolution of total mesophilic aerobic bacteria (TMAB) was followed in untreated minced meat (control) as well as in that incorporated with essential oils. The results obtained were recorded in Figure 4.

Figure 4 shows the evolution of TMAB during conservation. Thus, on Day 1, the microbial load was 8.3×10^{4} CFU/g for untreated ground meat, 3×10^{4} CFU/g for the ground meat (GM) + essential oil (EO) of *H. suaveolens*. On day 7, the microbial load was 2×10^{5} CFU/g for untreated ground meat and 7.3×10^{4}UFC/g for the GM + EO of *H. suaveolens*. Statistical analysis showed a significant difference with p value<0.0001. An increase in microbial load was noted during storage. This increase was much greater in untreated minced meat than in that incorporated with the essential oil. The reduction of microorganisms concentration (42%) in ground meat containing the essential oil could be explained by the inhibitory effect of this essential oil. The increase of microbial load in meat incorporated with

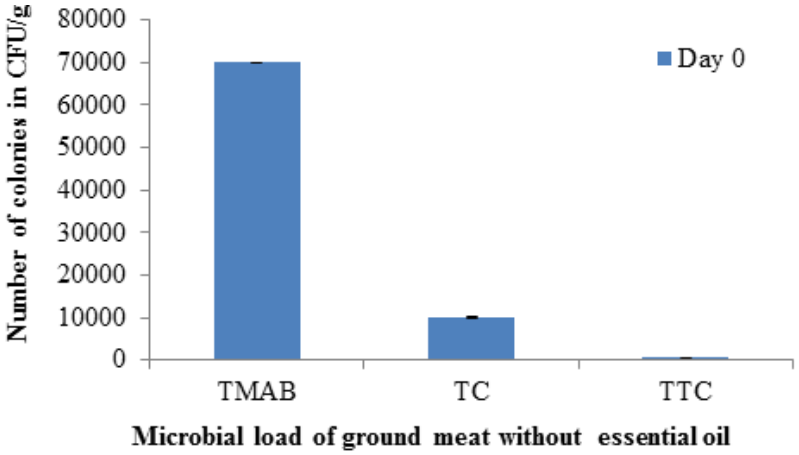

Figure 3. Initial microbial flora of bovine ground meat. TMAB: Total mesophilic aerobic bacteria; TC: Total coliforms; TTC: Thermotolerant coliforms.

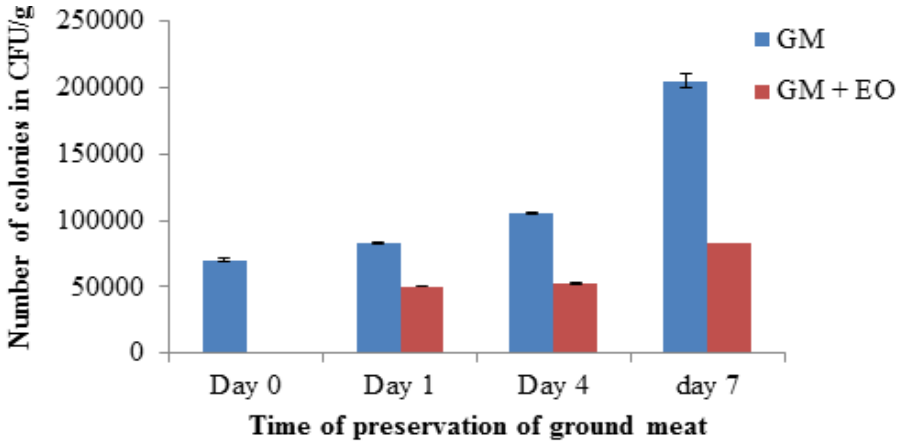

Figure 4. Evolution of TMAB loads during conservation. GM: Ground meat; GM+EO: Ground meat + essential oil.

essential oils could be related to the bacteriostatic effect of these essential oils on certain bacterial strains and the loss of bioactive molecules properties during storage. Meat and meat products can have a high fat content and rich source of protein, minerals, and vitamins. This structure reduces the effect of essential oils against bacteria (Hernandez et al., 2011; Seda and Nukhet, 2016).

Effect of essential oil on total coliform

The evolution of total coliforms concentration in untreated ground meat and in that incorporated with this essential oil is recorded in Figure 5.

The total coliform count at Day1 was 1.5×10^4UFC/g for untreated minced meat and 1.3×10^4 CFU/g for the GM + EO of *H. suaveolens*. After seven days of storage (Day 7), the numbers were 7.4×10^4 CFU/g for untreated minced meat and 8.0×10^3 CFU/g for GM + EO from *H. suaveolens*. Statistical analysis showed a significant difference ($p < 0.0001$).

An increase in total coliforms during storage was observed in untreated ground meat. In the meat supplemented with essential oil, there was a regression (80%) of the bacterial load, which reflects an important antibacterial activity of the essential oil of *H. suaveolens*.

Effect of the essential oil on the evolution of the thermotolerant coliforms load

The evolution of the number of thermotolerant coliforms during refrigeration is presented in Figure 6.

For the evolution of fecal coliforms, the microbial count varied from 7.3×10^3 CFU/g (Day 1) to 1.24×10^4 CFU/g

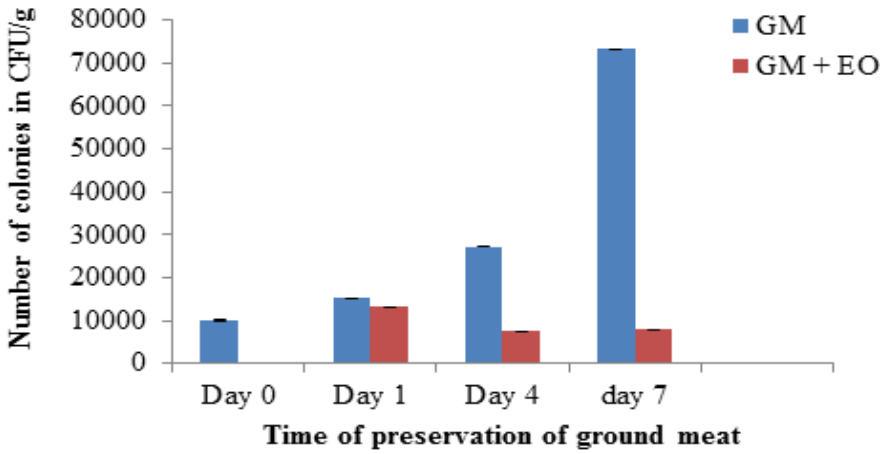

Figure 5. Evolution of total coliform loads during conservation. GM: Ground meat; GM+EO: Ground meat + essential oil.

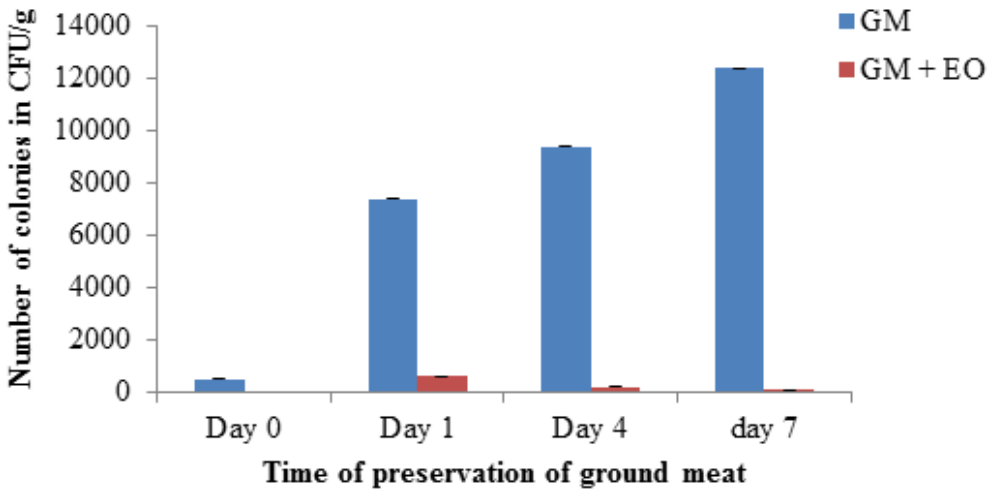

Figure 6. Evolution of thermo tolerant coliform loads during conservation. GM: Ground meat; GM+EO: Ground meat + essential oil.

(Day 7) for untreated ground meat. This number varied from 6.0×10^2UFC/g (Day 1) to 1.0×10^2 CFU/g (Day 7) for the GM+ EO of *H. suaveolens*. Statistical analysis showed a significant difference with p value < 0.0001.

Unlike untreated minced meat, there was a considerable reduction (84%) in the number of thermotolerant coliforms in minced meat incorporated with essential oils during storage. The almost complete inhibition (98%) of the number of thermotolerant coliforms could be explained by the bactericidal effect of these essential oils on *E. coli*. The presence of germs in the meat incorporated with essential oil could be explained by the presence of total coliforms. The results of the conservation tests confirm those of the antimicrobial activity observed previously by this essential oil. The results corroborate with several works that have dealt with the application of essential oils to foods in order to reduce the microbial count as compared to control (Smaoui et al., 2016). Thus, the studies of Caillet and Lacroix (2007) showed that the incorporation of the essential oil in ground beef contributed to the improvement of microbiological quality and the reduction of fat oxidation beyond its normal storage life.

Conclusion

The present work aimed to evaluate biological properties of essential oil of *H. suaveolens* and its effect on shelf-life of bovine ground meat. The essential oil from *H. suaveolens* showed inhibitory activity on microbial strains. Biopreservation assay revealed a significant

decrease of microbial charge during conservation time.

The results obtained in the present study show that the essential oil from *H. suaveolens* could be used as a promising bioconservative agent for the food industry that can prevent oxidation and reduce microbial spoilage.

CONFLICT OF INTERESTS

Authors have declared no conflict of interest.

REFERENCES

Adjou ES, Soumanou MM (2013). Efficacité des extraits de plantes dans la lutte contre les moisissures toxinogènes isolées de l'arachide en post-récolte au Bénin. Journal of Applied Biosciences 70:5555-5566. DOI: 10.4314/jab.v70i1.98755. ISSN 1997–5902.

Aprotosoaie AC, Spac AD, Hancianu M, Miron A, Tanasescu VF, Dorneanu V, Stanescu U (2010). The chemical profile of essential oils obtained from fennel fruits (*Foeniculum vulgare* Mill.). FARMACIA 58(1):46-54. Reference ID: 1433431.

Baser KHC, Buchbauer G (2010). Handbook of essential oils: Science, Technology, and Applications. Ed. Taylor and Francis Group, LLC. United States of America 994 p.

Bicas JL, Neri-Numa IA, Ruiz ALTG, De Carvalho JE, Pastore GM (2011). Evaluation of the antioxidant and antiproliferative potential of bioflavors. Food and Chemical Toxicology 49:1610-1615. https://doi.org/10.1016/j.fct.2011.04.012.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods. *A Review.* International Journal of Food Microbiology 94:223-253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Caillet S, Lacroix M (2007). Les huiles essentielles: leurs propriétés antimicrobienne et leurs applications potentielles en alimentaires laboratoire de recherche en science appliquée à l'alimentation. INRS-Institues Armand- Frappier, (RESALA), 8p. https://e-dition.net/des-huiles-essentielles-aux-proprietes-antimicrobiennes.

Canillac N, Mourey A (2001). Antibacterial activity of the essential oil of *Picea excelsa* on *Listeria*, *Staphylococcus aureus* and coliform bacteria. Journal of Food Microbiology 18:261-268. https://doi.org/10.1006/fmic.2000.0397.

Chouhan S, Sharma K, Guleria L (2017). Antimicrobial Activity of Some Essential OilS-Present Status and Future Perspectives. Medicines 4(58):1-21. DOI:10.3390/medicines4030058.

Dar MY, Shah WA, Rather MA, Qurishi Y, Hamid A, Qurishi MCA (2011). Chemical composition, *in vitro* cytotoxic and antioxidant activities of the essential oil and major constituents of *Cymbopogon jawarancusa* (Kashmir). Food Chemistry 129(4):1606-1611. DOI: 10.1016/j.foodchem.2011.06.016.

Derwich E, Benziane Z, Boukir A (2010). GC/MS Analysis and antibacterial activity of the essential oil of *Mentha pulegium* grown in Morocco. Reseach Journal of Agriculture and Biological Sciences 6(3):191-198. Record Number: 20103179519.

Dung NT, Kim JM, Kang SC (2008). Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of *Cleistocalyx operculatus* (Roxb.) Merr and Perry buds. Food and Chemical Toxicology 46(12):3632-3639. DOI:10.1016/j.fct.2008.09.013.

Hernandez Ochoa L, Aguirre Prieto YB, Nevarez Moorilon GV, Gutierrez Mendez N, Salas Munoz E (2011). Use of essential oils and extracts from spices in meat protection. Journal of Food Science Technology 51(5):957-963. DOI 10.1007/s13197-011-0598-3.

Goly KRC, Soro Y, Dadié A, Kassi ABB, Dje M (2015). Antibacterial activity of essential oils and extracts from the leaves of *Hyptis suaveolens* and *lippia multiflora* on multi-resistant bacteria. Rasayan Journal of Chemistry 8(4):396-403. ISSN: 0974-1496. http://www.rasayanjournal.com.

Goni P, Lopez P, Sanchez-Jarabo C, Nerin C (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chemistry 116(4):982-989. DOI: 10.1016/j.foodchem.2009.03.058.

Guiraud JP (2003). Microbiologie alimentaire. Ed. DUNOD, Paris.110 - 112.https://www.librairiedialogues.fr/livre/107946-microbiologie-alimentaire-joseph-pierre-guiraud-dunod

Ilboudo Z (2009). Activité Biologique de quatre huiles essentielles contre *Callosobruchus maculatus* Fab. *(Coleoptera : Bruchidae),* insecte ravageur des stocks de niébé au Burkina Faso. Thèse de doctorat. Université de Ouagadougou 150 p.

Joshi L, Gupta B, Prakash R (2010). Chemical synthesis of poly(5-carboxyindole) and poly (5- carboxyindole)/carboxylated multiwall carbon nanotube nanocomposite. Thin Solid Films 519(1):218-222. DOI: 10.1016/j.tsf.2010.07.123.

Lennette HE, Bellows A, Hausler JW, shadomy HJ (1987). Manual of Clinical microbiology, 4th Ed. pp. 336-359. Number ID: 971432431. ISBN-13: 978-0914826699.

Marzoukia H, Elaissib A, Khaldic A, Bouzidd S, Falconierie D, Marongiu B, Pirasa A, Porcedda S (2009). Seasonal and geographical variation of *Laurus nobilis* L. essential oil from Tunisia. The Open Natural Products Journal 8(2):86–91. DOI: 10.2174/1874848100902010086.

Miladi H, Ben Slama R, Mili D, Zouari S, Bakhrouf A, AE (2013). Chemical composition and cytotoxic and antioxidant activities of S*atureja montana* L. essential oil and its antibacterial potential against *Salmonella Spp.* strains. Journal of Chemistry 9–18. https://www.hindawi.com/journals/jchem/2013/275698/

Nakahara K, Alzoreky NS, Yoshihashi T, Nguyen HTT (2003a). Chemical composition and antifungal activity of essential oil from *Cymbopogon nardus* (Citronella Grass). Japan Agricultural Research Quarterly 37(4):249-252. Record ID:JP2005000711.

Negreiros MO, Pawlowski A, Soares GLG, Motta AS, Frazzon AP (2016). *In vitro* antimicrobial activity of essential oils from Heterothalamus Less. (*Asteraceae*) against clinically relevant bacterial and fungal species. Brazilian Journal of Biosciences 14(1):26-31. PUBMED : 27590861.

Nessrien MNY, Mohamed AT (2007). Antioxidant and antimicrobial effects of marjoram and thyme in coated refrigerated semi fried mullet fish fillets. World Journal Dairy and Food Science 2(1):01-09. ISSN 1817-308X.

Ngom S, Diop M, Mbengue M, Faye F, Kornprobst JM, Samb A (2014). Composition chimique et propriétés antibactériennes des huiles essentielles d'*Ocimum basilicum* et d'*Hyptis suaveolens* (L.) Poit récoltés dans la région de Dakar au Sénégal. Afrique Science 10(4):109-117.

Norme AFNOR NF V 08-060 (2009). Numeration of fecal coliforms by cell counting at 44°C, 10 p.

Norme Internationale ISO 4832 (2006). Food Microbiology-Flat Method horizontale for coliforms counting 6 p.

Norme Internationale ISO 4833 (2003). Food Microbiology-Flat Method horizontale for microorganisms counting. 6 p. Technic of colonies counting at 30°C. 9 p.

Obame ELC (2009). Study of phytochemical antimicrobial and activities of some medicinales aromatiques African plants. Thesis of PhD. University of Ouagadougou. 227p. http://www.beep.ird.fr/collect/uouaga/index/assoc/M11006.dir/M11006.pdf.

OCDE/FAO (2017). OCDE/FAO (2017). «Perspectives agricoles de l'Organisation de Coopération et de Développement Économiques (OCDE) et de l'Organisation des Nations Unies pour l'alimentation et l'agriculture (FAO)». Statistiques agricoles. http://dx.doi.org/10.1787/agr-outl-data-fr.

Ouedraogo I (2012). Anti-oxydant et antimicrobial activité of essentials oils of *Cymbopogon citratus* (DC) Stapf, *Citrus sinensis* (L.) Obsbeck and *Zingiber officinale* Roscoe for food preservation. Master Degree. University of Ouagadougou 94 p.

Rashid A, Qureshi MZ, Raza SA, William J, Arshad M (2010). Quantitative determination of antioxidant potential of *Artemisia persica*. Bucuresti–Chimie (serie Noua) 19(1):23-30. search.ebscohost.com.

Rhayour K (2002). Etude du mécanisme de l'action bactéricide des huiles essentielles sur Esherichia coli, Bacillus subtilis et sur Mycobacterium phlei et Mycobacterium fortuitum. Thèse de doctorat.

Université Sidi Mohamed Ben Abdellah, Maroc. 170p
Seda O, Nukhet NDZ (2016). Evaluation of usage of essential oils instead of spices in meat ball formulation for controlling *Salmonella* spp. Food Science and Technology International 22(2):93-101. DOI: 10.1177/1082013215571118.
Smaoui S, Hsouna AB, Lahmar A, Ennouri K, Mtibaa-Chakchouk A, Sellem I (2016). Bio-preservative effect of the essential oil of the endemic *Mentha piperita* used alone and in combination with BacTN635 in stored minced beef meat. Meat Science 117:196-204. DOI: 10.1016/j.meatsci.2016.03.006.
Yu J, Lei J, Yu H, Cai X, Zou G (2004). Chemical composition and antimicrobial activity of the essential oil of *Scutellaria barbata*. Phytochemistry 65(7):881-884. DOI:10.1016/j.phytochem.2004.02.005.

Vol. 18(29), pp. 819-830, September, 2019
DOI: 10.5897/AJB2019.16776
Article Number: 661655E61833
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

Recombination fraction and genetic linkage among key disease resistance genes (*Co-4^2/Phg-2 and Co-5*/"P.ult") in common bean

Dennis Okii[1*], Arfang Badji [1], Thomas Odong[1], Herbert Talwana[1], Phinehas Tukamuhabwa[1], Allan Male[2], Clare Mukankusi[2] and Paul Gepts[3]

[1]Department of Agricultural Production, Makerere University, P. O. Box 7062, Kampala, Uganda.
[2]International Centre for Tropical Agriculture (CIAT)/Pan African Bean Research Alliance (PABRA), P. O. Box 6247, Kampala, Uganda.
[3]Section of Crop and Ecosystem Sciences, Department of Plant Sciences/MS1, University of California, 1 Shields Avenue, Davis, CA 95616-8780, USA.

Received 19 February, 2019; Accepted 26 June, 2019.

Anthracnose (*Colletotrichum lindemuthianum*), Angular leaf spot (*Pseudocercospora griseola*) and *Pythium* root rot are important pathogens affecting common bean production in the tropics. A promising strategy to manage these diseases consists of combining several resistance (R) genes into one cultivar. The aim of the study was to determine genetic linkage between gene pairs, *Co-4^2/Phg-2*, on bean-chromosome Pv08 and *Co-5*/"P.ult" on-chromosome Pv07, to increase the efficiency of dual selection of resistance genes for major bean diseases, with molecular markers. The level of recombination was determined by tracking molecular markers for both BC3F6 and F2 generations. Recombination fraction r, among gene pairs, the likelihood of linkage, L(r), and logarithm of odds (LOD) scores were computed using the statistical relationship of likelihood which assumes a binomial distribution. The SCAR marker pair SAB3/PYAA19 for the gene pair *Co-5*/"P.ult" exhibited moderate linkage (r = 32 cM with a high LOD score of 9.2) for BC_3F_6 population, but relatively stronger linkage for the F_2 population (r = 21 cM with a high LOD score of 18.7). However, the linkage among SCAR marker pair SH18/SN02, for the gene pair *Co-4^2/Phg-2* was incomplete for BC_3F_6 population (r = 47 cM with a low LOD score of 0.16) as well as F_2 population (r = 44 cM with a low LOD score of 0.7). Generally, the weak or incomplete genetic linkage between marker pairs studied showed that all the four genes mentioned earlier have to be tagged with a corresponding linked marker during selection. The approaches used in this study will contribute to two loci linkage mapping techniques in segregating plant populations.

Key words: Genetic linkage, recombination, likelihood, logarithm of odds (LOD) score, sequence characterized amplified regions (SCAR) markers, genes.

INTRODUCTION

Diseases are critical production constraints for common beans in tropical countries, causing total crop failure when several pathogens attack susceptible bean genotypes under favorable conditions (Schwartz and Galvez, 1980; Mahuku et al., 2011). Management of diseases at farm level in short and long term is practical, through genetic control by incorporating resistant alleles for different pathogens into selected cultivars through

marker assisted gene pyramiding techniques (Young and Kelly, 1996; Malav et al., 2016).

Marker assisted breeding strategies for pyramiding genes requires information on marker co-transmissions (Mahuku et al., 2011; http://www.extension.org/pages/32465/gene-pyramiding-using-moleculermarkers#.ViTJovlViko). Molecular markers and genetic maps accelerate identification of desirable homozygotes without need for progeny tests (Pathania et al., 2014).

Genetic maps and markers of major disease of beans are reported for bean breeding (Kelly et al., 2003; Oblessuc et al., 2013; Perseguini et al., 2016) and in the Phaseolus Genes database (http://phaseolusgenes.bioinformatics.ucdavis.edu/; Miller et al., 2018) and may be utilised in gene pyramiding.

Generating breeding information on linkages of sequence characterized amplified region (SCAR) marker pairs and their co-segregations could help to reduce sample sizes and time during marker assisted pyramiding (stacking together) of major genes in breeding programs for managing multiple diseases of common bean.

Practically, a polymorphic marker must co-segregate with the gene of interest and so be present in the resistant progeny lines but absent in susceptible ones (Miller et al., 2018). Once linkage is established between markers, the chromosomal region flanking the marker can then be analyzed for alternative markers.

Genetic linkage is the tendency of genes that are located proximally to each other on a chromosome to be inherited together during meiosis and can therefore be used as a tool for estimating the genetic distance between two loci (Ott et al., 2015). Two statistical approaches, termed parametric and nonparametric linkage analysis have been used to test, linkage analysis or observed recombination between two loci and is detailed by Bailey-Wilson and Wilson (2011).

Parametric linkage analysis was applied in this study and its test statistic is called the logarithm of odds (LOD) score (Balding et al., 2007; Strachan and Read, 2011). A LOD score higher than 3.0 is generally accepted as the evidence supporting linkage, whereas a LOD score lower than -2.0 is considered evidence against linkage (Ott et al., 2015).

LOD score analysis is a simple way to determine the linkage between Mendelian traits (or between a trait and a marker, or two markers). On the other hand, the nonparametric linkage analysis is a model-free approach that studies the probability of an allele being identical by descent. Balding et al. (2007) and Strachan and Read (2011) describe the LOD score method in greater detail. Briefly, it works as follows: estimates of recombinant and non-recombinant fraction is made, the overall likelihood, given linkage and the likelihood, given no linkage and a LOD score is calculated for each estimate of recombination fraction.

The recombination fraction estimate with the highest LOD score is considered the best estimate. The two-point LOD score between two loci, that is, a trait and a marker or marker-marker loci in this study was typically calculated over several recombination fractions between 0 and 1/2, and the recombination fraction that maximizes the likelihood (the maximum LOD score) is considered to be the best estimate of the recombination fraction (Bailey-Wilson and Wilson, 2011).

For most occurrences of crossing-over, genes located at close physical postion are co-inherited due to linkage, while genes far apart tend to segregate independently (Ott et al., 2015). Genetic recombination through chromosomal cross-over after hybridization produces new haplotypes during meiosis through inter-chromosomal genetic material exchange and plays a critical role in the evolution of organisms (Coop and Przeworski, 2007).

Estimates of recombination rates are traditionally obtained by directly counting the number of such events during meiosis (Kaplan and Hudson, 1985). This approach is however, limited by the extremely low fraction of recombinations between tightly linked genes (Gao et al., 2016). Two loci that are far apart on the chromosome have a high probability of recombination in any meiosis, such that they assort independently to offspring (Bailey-Wilson and Wilson, 2011). While, loci that are very far apart experience recombination about 50% of the time, and thus appear to be assorting independently, just as loci on different chromosomes do.
Probability refers to knowing parameters (measurable characteristic of a system) and being able to predict their outcomes, while Likelihood is a synonym for probability where observed data is used to estimate parameters (Edwards, 1972). The likelihood ratio test that is the basis of modern parametric likelihood ratio tests for linkage (Bailey-Wilson and Wilson, 2011) likelihood ratio test that is the basis of modern parametric likelihood ratio tests for linkage (Bailey-Wilson and Wilson, 2011). In this study, the likelihood hypothesing linkage was compared to a hypothesis of no linkage with some specific recombination fraction ($r < 1/2$).

At data analysis levels, observations are already completed, the data is fixed and there is no probabilistic part of the data anymore (Edwards, 1972). Likelihood of the model parameters that underlie the fixed data would then be of most interest. Maximum likelihood estimation (MLE) thus aims to find the parameter value(s) that makes the observed data most likely (Staub et al., 1996; Toomet and Henningsen, 2009).

*Corresponding author. E-mail: dokii@caes.mak.ac.ug or dennis.okii@gmail.com.

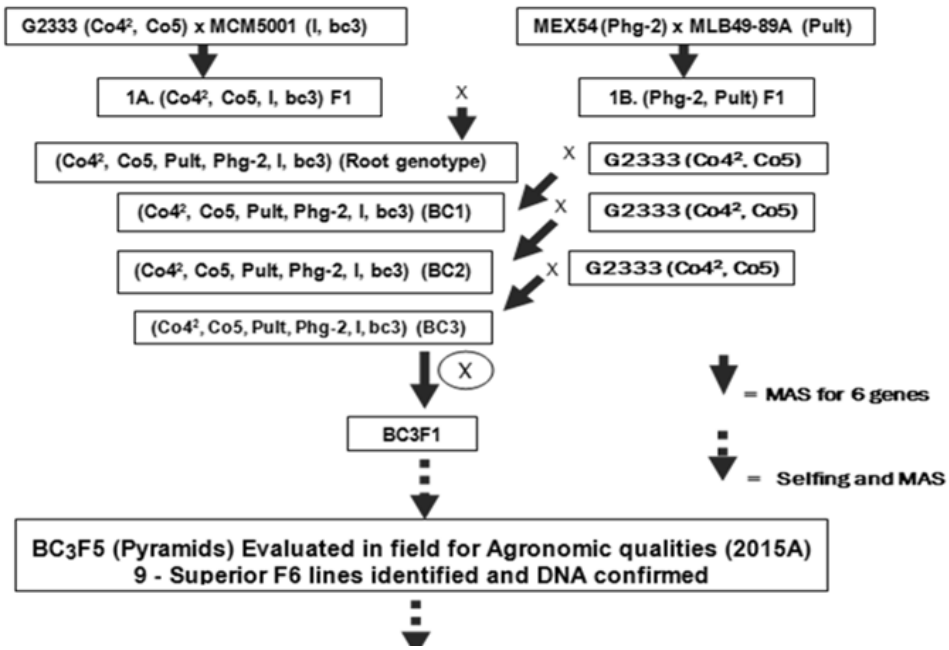

Figure 1. Simultaneous gene pyramiding scheme developed and used via MAS.

This study aimed at estimating recombination frequencies and genetic linkage between gene pairs, *Co-4²/Phg-2* on bean chromosome Pv08 and *Co-5*/"P.ult" on chromosome Pv07.

In the study, the gene symbol "P.ult" is for *Pythium ultimum* root rot resistance gene linked to (SCAR) marker PYAA19 developed by Mahuku et al. (2007) for selecting bean lines resistant to several species of *Pythium* root rot in common bean. According to the developers of the SCAR marker, the linked gene symbol is *Pyult1*. The gene symbol for root rot disease used in this study is "P.ult", shortened from the targeted pathogen's genus (*Pythium)* and species (*ultimum)* names. The gene symbol ("P.ult") was thus put between quotation marks within the text and not italicised, unlike the other genes in this study (that is, *Co-4²* , *Phg*-2 and *Co-5*) because it is not an official genetic symbol in common beans.

The findings will contribute to the efficiency of marker assisted pyramiding of disease resistance genes in common bean leading to simultaneous expression of more than one gene in a variety to develop durable resistance expression (Malav et al., 2016).

MATERIALS AND METHODS

Population development

Two segregating bean populations: BC_3F_6 and F_2 were developed for this study and are described as follows. In the start, a BC_3F_5 population previously developed at CIAT of Kawanda (Uganda) to combine six disease resistance genes of common bean, namely *Co-4², Co-5, Phg-2,* "P.ult"*, I* and *bc-3* (Figure 1) formed the genetic material for this study.

The BC_3F_5 population was planted in the fields at CIAT, Kawanda in 2015 to advance it to BC_3F_6 population from which DNA of 345 plants was collected and genotyped with SCAR markers in the molecular laboratory facility at CIAT, Kawanda as detailed subsequently. Bean plants with single dorminant genes (specifically, *Co-4², Co-5, Phg-2,* "P.ult"*)* were identified among the 345 plants of BC_3F_6 population using genotypic electrophoresis gel profiles, harvested and seed used as parents to develop new crosses and populations (Figure 2) in the screen house facility at CIAT, Kawanda.

Five representative plants from progenies of the BC_3F_6 population with single genes were planted in 5 litre's plastic pots with sterile soils, labelled and monitored with daily watering until flowering and crosses made through hand pollinations to generate two F1 populations between respective plants with targeted single genes (*Co-4² x Phg-2 – population 1* and *Co-5 x* "P.ult" – *population 2*). The F1 seeds from the two populations were harvested separatelty dried and planted in the screen house in the second season in 2015 to generate two populations of F2 plants which were monitored until maturity and harvested. The F2 seeds were planted in the screen house in sterile soils in raised wooden trays measuring 75 cm long × 45 cm wide and height of 13 cm during the third season to generate plants for DNA extraction.

DNA extraction

The genomic DNA from 345 plants from BC_3F_6 population in the field was collected from young leaves before flowering in eppendorf tubes, clearly labelled and transferred to the laboratory for extraction. DNA of each plant was isolated in the molecular laboratory facility at CIAT, Uganda, using the CTAB method according to Mahuku (2004), and kept in Eppendorf tubes at -20°C for further analyses.

The two F2 populations having 219 plants targeting genes; *Co-4² x Phg-2* – population 1 and 236 plants targeting *Co-5 x* "P.ult" - population 2 were planted in trays in the screen house and DNA was extracted from seedlings at the second trifoliate stage. The

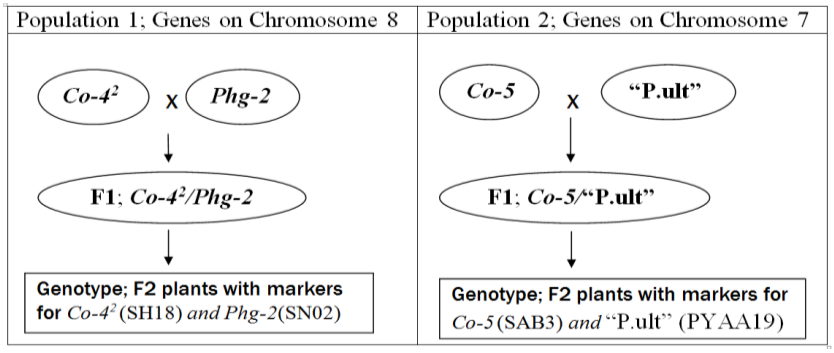

Figure 2. Scheme used to develop populations for testing linkage among genes physically located on bean chromosomes eight and seven.

DNA was extracted using the Whatman FTA card technology according to Chilagane et al., (2013). The leaf sample was placed over the marked area of the FTA Plant Saver card and the leaf was overlaid with parafilm. The leaf was pounded using a pestle, making sure that the leaf material was transferred to the paper by checking the back of the FTA card.

The samples were left to air dry and later transferred to the laboratory. The Harris 2 mm unicore punch, was used to cut the samples from the FTA cards with the assistance of the cutting mat and placed into the PCR tube and then washed twice using 200 ml of FTA purification reagent followed by 200 ml of 1X TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) and the sample left to incubate at room temperature for about 10 min in each wash, then the leaf disks were left to dry and ready for PCR reaction.

Polymerase chain reaction (PCR) and electrophoresis

DNA samples for amplification through PCR were diluted to a factor of 1 in 30 µl solution and sorted out according to the field plan. The PCR reaction mix contained 5 µl of the *Accu-Power PCR* premix composed of DNA polymerase, dNTPs, reaction buffer, blue tracking dye and patented stabiliser. One microlitre of DNA for plants from BC_3F_6 population and leaf disk for the two F2 populations, 0.3 µl of forward and reverse primer, and 3.4 µl water was added to the premix to make a total reaction volume of 10 µl. The test sample tubes were placed in a thermocycler (MyGenie, Daejeon) for the PCR reaction cycles. Forward (F) and Reverse (R) primers of SCAR marker SBB14 (F-GTGGGACCTGTT-CAAGAATAATAC, R-GTGGGACCTGGGTAGTGTAGAAAT) was used to tag common bean lines with the *Co-4²* gene, SN02 (F-ACCAGGGGCATTATGAACAG, R-ACCAGGGGCAACATACTATG) for the *Phg-2* gene, PYAA19 (F-TTAGGCATGTTAATT-CACGTTGG, R-TGAGGCGTGTAAGGTCAGAG) for "P.ult" and SAB3 (F-TGGCGCACACATAAGTTCTCACGG, R-TGGCGCACACCATCAAAAAAGGTT) for *Co-5*. The PCR products of markers used were separated on 1.2% agarose gel in 1X TBE at 140V for 30 min. The gel was then stained in 0.5ug/mL ethidium bromide for 20 min and the image was captured using the Syngene G: BOX gel documentation system (Syngene, Fredrick, MD).

Data analysis

Marker scoring and establishing number of recombinants

The SCAR marker frequencies (SH18 for *Co-4²*, SN02 for *Phg-2*, SAB3 for *Co-5* and PYAA19 for "P.ult") were computed for BC_3F_6 and F2 bean populations to determine the number of recombinants and for further linkage analysis. This involved scoring polymorphic bands observed on electrophoresis gel pictures for each genotype using one for presence and zero for absence of bands. Recombinant genotypes were identified by counting band patterns in gel pictures in both BC_3F_6 and F_2 populations.

Recombinants, LOD score and likelihood ratio analysis

The methods for estimating LOD score, likelihoods and recombination value were previously described by Geffroy et al., (2008). Recombination fraction, r, among gene pairs, the likelihood L(r) and LOD scores were generated using the following statistical relationship, assuming a binomial distribution of data in MS office Excel.

$$Likelihood,\ L(r) = \binom{N}{R} r^R (1-r)^{N-R}$$

where N = total plants genotyped and R = number of recombinants. The likelihood, L(r) of obtaining the aforenetioned data set for recombination rates was computed using the following relationships, for example using data from BC_3F_6 population.

$$Likelihood,\ L(r) = \binom{N}{R} r^R (1-r)^{N-R}$$

$$Constant\ , \binom{N}{R} = \frac{N!}{R!(N-R)!}$$

$$\text{Constant } for\ \text{Co4}^2/\text{Phg - 2} = \frac{345!}{162!(183)!}$$

$$\text{Constant } for\ \text{Co5/P.ult} = \frac{345!}{112!(233)!}$$

The LOD (log-odds) score is often used to assess the evidence for linkage and according to Balding et al. (2007) is defined by the formula:

$$LOD = log_{10}\left[\frac{L(data \mid r)}{L(data \mid r = \frac{1}{2})}\right]$$

Table 1. Likelihoods (r) and LOD scores for testing linkage of *Co-4²*/*Phg-2* and *Co-5*/"P.ult" from BC_3F_6.

Loci	r	r^R	1-r	$(1-r)^{N-R}$	L_(r)	Lr/L(r=1/2)	LOD score [Log10(Lr/L(r=1/2))]
Co-4²/Phg-2		**(R=162)**		**(N-R=183)**			
	0.05	1.71E-211	0.95	8.38E-05	2.33E-113	1.03E-111	-111.00
	0.10	1.00E-162	0.90	4.23E-09	6.88E-69	3.03E-67	-66.52
	0.15	3.36E-134	0.85	1.21E-13	6.63E-45	2.92E-43	-42.53
	0.20	5.85E-114	0.80	1.84E-18	1.75E-29	7.72E-28	-27.11
	0.25	2.93E-98	0.75	1.37E-23	6.51E-19	2.87E-17	-16.54
	0.30	1.97E-85	0.70	4.50E-29	1.44E-11	6.33E-10	-9.20
	0.35	1.38E-74	0.65	5.80E-35	1.30E-06	5.72E-05	-4.24
	0.40	3.42E-65	0.60	2.52E-41	0.0014	0.062	-1.21
	0.45	6.61E-57	0.55	3.06E-48	**0.033**	1.45	0.16
	0.50	1.71E-49	0.50	8.16E-56	**0.023**	1.00	-1.24E-10
***Co-5*/"P.ult"**		**(R=112)**		**(N-R=233)**			
	0.05	1.93E-146	0.95	6.45E-06	1.57E-58	8.90E-48	-47.05
	0.10	1.00E-112	0.90	2.18E-11	2.76E-30	1.56E-19	-18.81
	0.15	5.28E-93	0.85	3.60E-17	2.40E-16	1.36E-05	-4.868
	0.20	5.19E-79	0.80	2.63E-23	1.73E-08	9.79E+02	3.00
	0.25	3.71E-68	0.75	7.75E-30	0.0004	2.06E+07	7.31
	0.30	2.74E-59	0.70	8.10E-37	0.02	1.59E+09	9.20
	0.35	8.62E-52	0.65	2.56E-44	0.02	1.58E+09	9.20
	0.40	2.70E-45	0.60	2.04E-52	0.0007	3.94E+07	7.60
	0.45	1.44E-39	0.55	3.20E-61	5.84E-07	3.31E+04	4.52
	0.50	1.93E-34	0.50	7.24E-71	1.76E-11	1.00E+00	2.08E-07

where r = recombination fraction. *L*(*data* | r) = likelihood value at maximum estimate (MLE), while *L*(*data* | r = ½) = likelihood value at maximum recombination fraction of 0.5.

The two-point LOD score between two loci, that is, marker-marker loci was calculated over several recombination fractions between 0 and 1/2, and the recombination fraction that maximizes the likelihood (the maximum LOD score) was considered to be the best estimate of the recombination fraction (Bailey-Wilson and Wilson, 2011).

RESULTS

Recombinants, likelihood and LOD scores in BC_3F_6 population

The results of likelihoods (r) and LOD scores for testing linkage among gene pairs, *Co-4²*/*Phg-2* and *Co-5*/"P.ult" from bean populations at BC_3F_6 generation are shown in Table 1. For gene pairs, *Co-4²*/*Phg-2*, 162 genotypes were recombinants (having two genes, *Co-4²*/*Phg-2*) and the rest of the genotypes (n= 183) were non-recombinants (Table 1). Similarly, for gene pairs, *Co5*/"P.ult"*, 112* genotypes were recombinants and 233 non-recombinants.

Graphics of likelihood, recombination rates and LOD score in BC_3F_6 population

The graphical presentation of likelihood (r) and maximum recombination rates at 0.47 (the maximum likelihood estimate (MLE)) extracted from Table 1 for gene pairs *Co-4²*/*Phg-2* are as shown in Figure 3 and 4. Similarly, the graph of the plot between LOD score from Table 1 and recombination rates ranging from 0 to 0.5 had the same pattern (Figure 4), suggesting that the recombination fraction between *Co-4²*/*Phg-2* was 0.47 showing weak linkage. However, the graph of the plot of likelihood (r) and recombination rates was maximum at 0.32 for gene pairs *Co-5* and "P.ult" (Figure 5). Similarly, the graph of LOD score and recombination rates (Figure 6) had the same pattern, suggesting that the recombination fraction between *Co-5* and "P.ult" was 0.32 showing a stronger linkage.

Recombinants, likelihood and LOD scores in F_2 populations

The results of likelihoods (r) and LOD scores for testing linkage among gene pairs, *Co-4²*/*Phg-2* and *Co-5*/"P.ult"

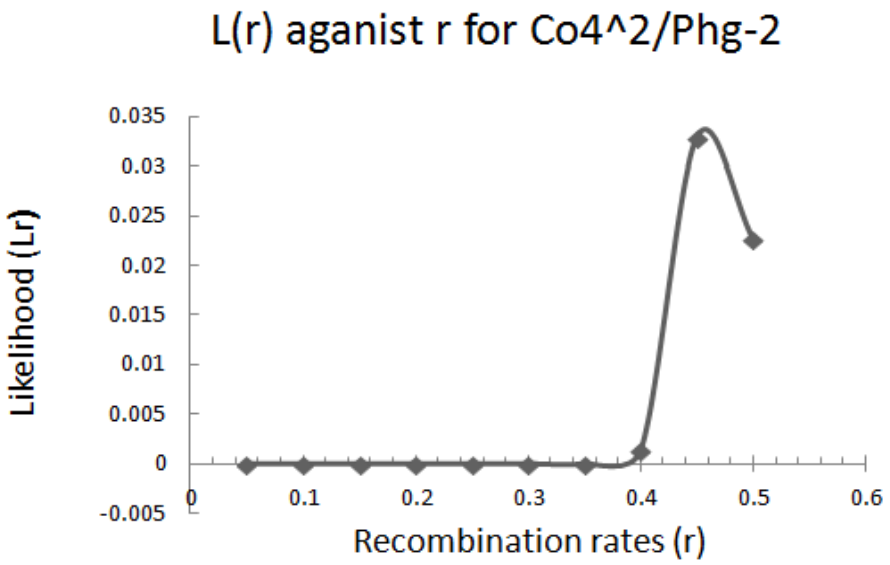

Figure 3. Plot of Likelihood (r) against Recombination rates, showing maximum likelihood estimate (MLE) of recombination fraction at 0.47, so that is the best estimate of linkage for gene pairs *Co-4^2/Phg-2* on bean chromosome Pv08, using BC_3F_6 population.

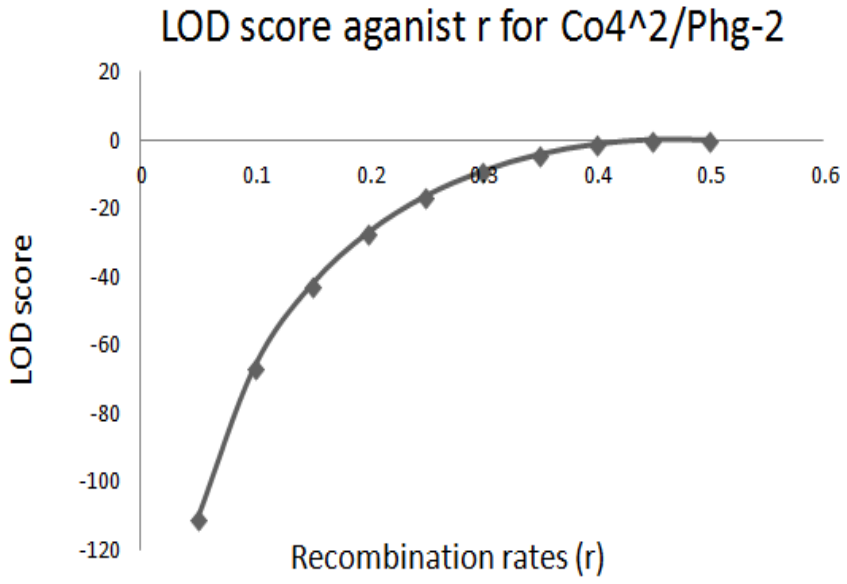

Figure 4. Plot of LOD scores against Recombination rates, LOD is maximum around, r = 0.47, so that is the best estimate of linkage for gene pairs *Co-4^2/Phg-2* on bean chromosome Pv08, using BC_3F_6 population.

from bean populations at F_2 generation are shown in Table 2. For gene pairs, *Co-4^2/Phg-2*, 96 genotypes were recombinants (having two genes, *Co-4^2/Phg-2*) and the rest of the genotypes (n= 123) were non-recombinants (Table 2). Similarly, for gene pairs, *Co-5*/"P.ult", *49* genotypes were recombinants and 187 non-recombinants.

Graphics of likelihood, recombination rates and LOD score in F_2 populations

The graphical presentation of likelihood (r) and maximum recombination rates of 0.44 (the maximum likelihood estimate (MLE)) from Table 2, for gene pairs *Co-4^2/Phg-2*

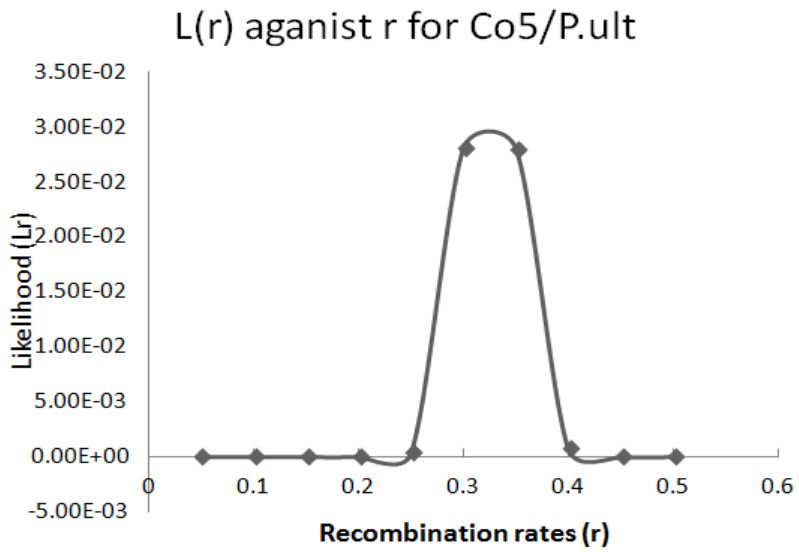

Figure 5. Plot of Likelihood (r) against Recombination rates, showing maximum likelihood estimate (MLE) of recombination fraction at 0.32, so that is the best estimate of linkage for gene pairs *Co-5*/ "P.ult" on bean chromosome Pv07, using BC_3F_6 population.

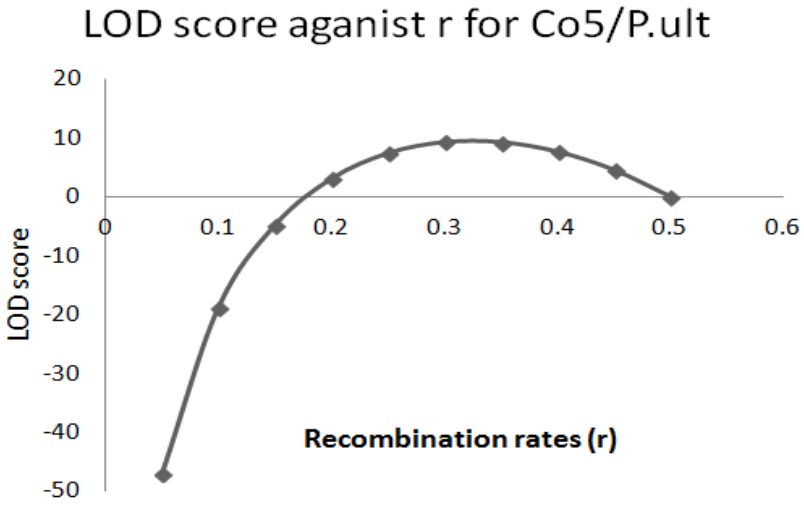

Figure 6. Plot of LOD scores against Recombination rates, LOD is maximum around, r = 0.32, so that is the best estimate of linkage for gene pairs *Co-5*/ "P.ult" on bean chromosome Pv07, using BC_3F_6 population.

is as shown in Figures 7 and 8. Similarly, the graph of the plot between LOD score from Table 2 and recombination rates ranging from 0 to 0.5 had the same pattern (Figure 8), suggesting that the recombination fraction between *Co-4*^{2}*/Phg-2* was 0.44, showing weak linkage. However, the graph of the plot of likelihood (r) and recombination rates was maximum at 0.21 for gene pairs *Co-5* and "P.ult" (Figure 9). Similarly, the graph of LOD score and recombination rates (Figure 10) had the same pattern, suggesting that the recombination fraction between *Co-5* and "P.ult" was 0.21 showing a stronger linkage.

Testing linkage using log-likelihood statistic

The summary of recombination fraction and likelihood data is shown in Table 3 and was used to test the significance of linkages through comparison of log-

Table 2. Likelihoods (r) and LOD scores for testing linkage of *Co-4²/Phg-2* and *Co-5*/"P.ult" from F_2 populations.

Loci	r	r^R	1-r	$(1-r)^{N-R}$	L_(r)	Lr/L(r=1/2)	LOD score [Log10(Lr/L(r=1/2))]
Co-4²/Phg-2		**(R=96)**		**(N-R=123)**			
	0.05	1.26E-125	0.95	1.82E-03	1.98E-64	1.94E-62	-61.71
	0.10	1.00E-96	0.90	2.35E-06	2.03E-38	1.98E-36	-35.70
	0.15	8.03E-80	0.85	2.08E-09	1.44E-24	1.41E-22	-21.85
	0.20	7.92E-68	0.80	1.20E-12	8.21E-16	8.03E-14	-13.10
	0.25	1.59E-58	0.75	4.29E-16	5.89E-10	5.76E-08	-7.24
	0.30	6.36E-51	0.70	8.85E-20	4.85E-06	4.75E-04	-3.32
	0.35	1.70E-44	0.65	9.74E-24	1.43E-03	1.39E-01	-0.86
	0.40	6.28E-39	0.60	5.16E-28	0.02790	2.72856	0.44
	0.45	5.11E-34	0.55	1.16E-32	0.05108	4.99553	0.69
	0.50	1.26E-29	0.50	9.40E-38	0.01022	0.99999	-2.05E-08
***Co-5*/"P.ult"**		**(R=49)**		**(N-R=187)**			
	0.05	1.78E-64	0.95	6.83E-05	1.75E-17	1.34E+03	3.13
	0.10	1.00E-49	0.90	2.78E-09	4.00E-07	3.06E+13	13.49
	0.15	4.25E-41	0.85	6.33E-14	3.88E-03	2.97E+17	17.47
	0.20	5.63E-35	0.80	7.55E-19	6.12E-02	4.69E+18	18.67
	0.25	3.16E-30	0.75	4.33E-24	0.019687051	1.51E+18	18.18
	0.30	2.39E-26	0.70	1.08E-29	0.000372339	2.85E+16	16.46
	0.35	4.56E-23	0.65	1.03E-35	6.80449E-07	5.21E+13	13.72
	0.40	3.17E-20	0.60	3.27E-42	1.49241E-10	1.14E+10	10.06
	0.45	1.02E-17	0.55	2.80E-49	4.11E-15	3.15E+05	5.49
	0.50	1.78E-15	0.50	5.10E-57	1.30E-20	1.00E+00	1.12E-06

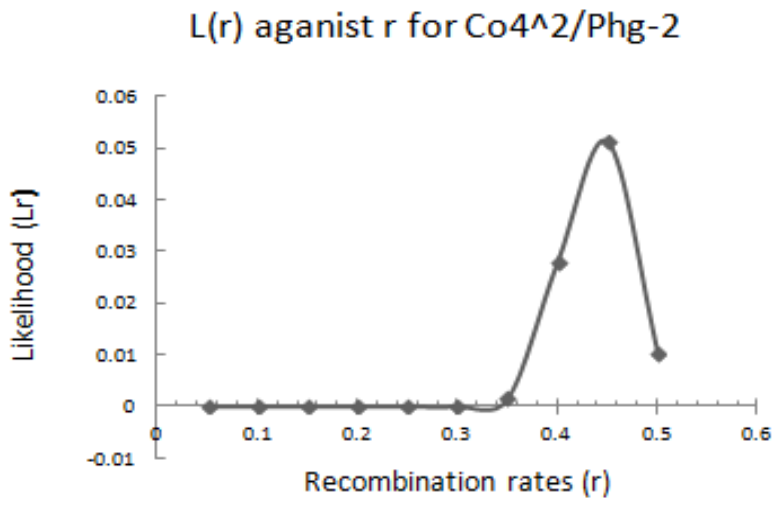

Figure 7. Plot of Likelihood (r) against Recombination rates, showing maximum likelihood estimate (MLE) of recombination fraction at 0.44, so that is the best estimate of linkage for gene pairs *Co-4²/Phg-2* on bean chromosome Pv08, using F_2 population.

likelihood under null and alternate hypotheses (where $\chi^2_{tabulated} = 3.84$) for a given gene pair in both BC_3F_6 and F_2 populations. For gene pair, Co-4²/Phg-2 the computed chi value of 0.32 and 1.4 in BC_3F_6 and F_2 populations, respectively (Table 3) shows no significant difference between the null hypothesis (r = 0.5, no linkage) and the

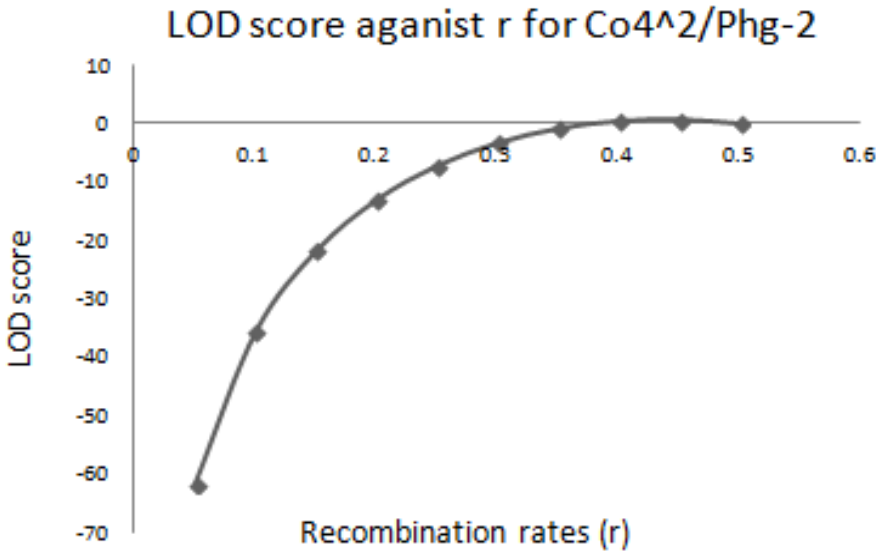

Figure 8. Plot of LOD scores against Recombination rates, LOD is maximum around, r = 0.44, so that is the best estimate of linkage for gene pairs *Co-4²/Phg-2* on bean chromosome Pv08, using F_2 population.

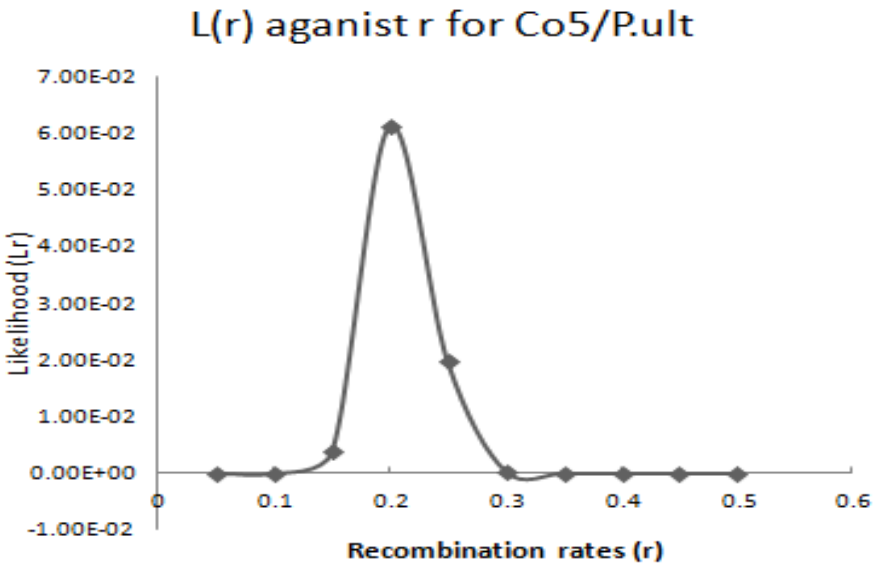

Figure 9. Plot of Likelihood (r) against Recombination rates, showing maximum likelihood estimate (MLE) of recombination fraction at 0.21, so that is the best estimate of linkage for gene pairs *Co-5*/"P.ult" on bean chromosome Pv07, using F_2 population.

alternative hypothesis (r = 0.47, suggesting linkage). The marker loci for gene pairs *Co-4²/Phg-2* are thus not linked in BC_3F_6 and F2 bean populations studied. In contrast, for gene pairs, *Co-5*/"P.ult", the computed chi value of 18.4 and 37.18 in BC_3F_6 and F_2 populations, respectively (Table 3) shows significant difference between the null hypothesis and the alternative hypothesis. The marker loci for gene pairs *Co-5*/"P.ult" are thus linked in BC_3F_6 and F2 bean populations studied.

DISCUSSION

The objective of this study was to estimate recombination

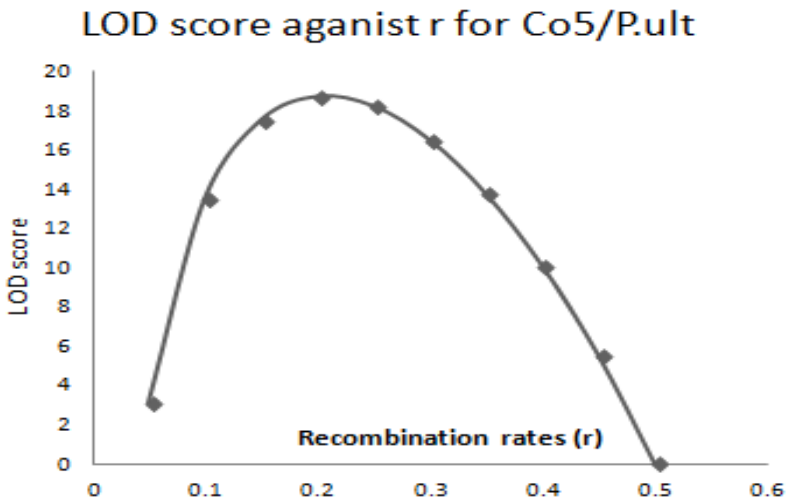

Figure 10. Plot of LOD scores against Recombination rates, LOD is maximum around, r = 0.21, so that is the best estimate of linkage for gene pairs Co5/"P.ult" on bean chromosome Pv07, using F_2 population.

Table 3. Comparison of log-likelihood under alternate and the null hypothesis for Co-4^2/Phg-2 and Co-5/"P.ult" gene pairs in BC_3F_6 and F_2 populations

Gene pair	Hypotheses	BC_3F_6			F2		
		Recombination fraction (r)	Likelihood of r, L(r)	Log of Likelihood	r	L(r)	Log of Likelihood
Co4^2/Phg-2	H_A	0.47	0.033	-1.48	0.44	0.051	-1.29
	H_0	0.5	0.022	-1.64	0.5	0.010	-1.99
	$2(LH_A - LH_0)$	-	-	0.32	-	-	1.4
Co-5/"P.ult"	H_A	0.32	0.028	-1.55	0.21	6.12E-02	-1.21
	H_0	0.5	1.77E-11	-10.75	0.5	1.30E-20	-19.8
	$2(LH_A - LH_0)$	-	-	18.4*	-	-	37.18*

Alternative hypothesis (H_A) is when r is estimated at its MLE and the null hypothesis (H_0) is when r is fixed at 0.5. $2(LH_A-LH_0)$ shows that the difference between the log-likelihood multiplied by a factor of 2 for technical reasons, so that this quantity will be distributed as the familiar chi (χ^2) statistic. Critical value for test-statistics at 1% level of significance is *3.84. Asteric (*) of log-likelihood shows significant difference between recombination fraction (r) values at H_A versus H_o suggesting linkage between corresponding gene pairs.*

fractions and genetic linkage between gene pairs, *Co-4^2/Phg-2* on bean chromosome Pv08 and *Co-5*/"P.ult" on chromosome Pv07. A strong genetic linkage among a pair of molecular markers located less than five centi Morgans (cM) apart on common bean chromosome implies that their two linked genes could be selected with only one marker to reduce genotyping costs.

The results show incomplete genetic linkage between gene pairs, *Co-4^2/Phg-2* and *Co-5*/"P.ult" on common bean chromosomes Pv08 and Pv07, respectively.The recombination fraction summarized in Table 3 are indicators of the degree of linkage and was higher in BC_3F_6 than F_2 populations for gene pairs, *Co-4^2/Phg-2* (47cM vs 44 cM) and *Co-5*/"P.ult" (32 cM vs. 21 cM). This difference was attributed to the two generation studied with significant differences in levels of genetic variations.

As reported under population development under the materials and methods, the F_2 populations derived their parents from from progenies of the BC_3F_6 population with single genes targerted. At BC_3F_6 the bean populations had possibly accumulated more recombinations than in $F_{2s.}$ Secondly, the progeny lines in BC_3F_6 genotyped were derived from a four way cross comprising four parents used to develop the genetic pyramids (Figure 2) with a high genetic diversity and population structure (Okii et al., 2017).

The hypothesis that physically linked genes for bean diseases, for example anthracnose (*Co-4^2*) and angular leaf spot resistance (*Phg-2*) located on bean chromosome Pv08 co-segregate in bean populations due to genetic

linkages was tested in this study. However, the weak genetic linkage between marker pairs studied shows that each of the four genes mentioned earlier have to be tagged with a corresponding linked markers during MAS. The study aimed to suggest strategies for reducing population size during gene pyramiding by finding a single marker locus (position of the chromosome) between genes or markers for simultaneous selection of resistance genes on the same bean chromosome(s), with linkages in coupling (Staub et al., 1996). Theoretical investigations that probe the potential of MAS are, however, of practical importance (Staub et al., 1996).

In other studies on common bean, maximum linkage with no recombinants (0.0 cM) was reported for gene pairs, *Co-1*^{4} and *Phg-1* for anthracnose and angular leaf spot diseases in bean cultivar AND277 (Gonçalves-Vidigal et al., 2011) and suggested overlap or very tight linkage of *Co-1*^{4} and *Phg-1* loci in the bean genome. The large genetic separation of SCAR markers SBB14 for *Co-4*^{2} and SN02 for *Phg-2* corroborates their physical positions in the Andean bean reference cultivar G19833 (Schmutz et al., 2014), with SBB14 situated at 2,758,731 base pairs (Burt et al., 2015) and SN02 at 58,535,517-58,536,216 on Pv08 (http://phaseolusgenes.bioinformatics.ucdavis.edu/).

To define whether two markers are in linkage is to test whether the recombination fraction between these two markers is less than 0.5 (Balding et al., 2007). This hypothesis testing problem can be carried out using the likelihood ratio test Ott et al, 2015; Balding et al., 2007). Similarly, Sun et al. (2012) proposed recombination frequencies of 15 cM as the threshold for strong linkages among loci, while LOD scores above 3 indicate strong genetic linkages (Ott et al., 2015). The threshold genetic distance of 5 cM was recommended as strong indicator of linkage between molecular markers and resistance genes during MAS (Collard and Mackill, 2008).

Therefore, the approaches used in this study contributed to two loci linkage mapping techniques in segregating plant populations through genotyping with markers. However, in situations where computer programs are used to integrate phenotypic and genotypic data sets, estimated LOD scores and likelihood values provide a threshold value for testing genetic linkage (Churchill and Doerge, 1994).

Key statistical methods used in the study are reported by Geffroy et al., (2008) to show possible ways of reducing the number of laboratory samples screened with markers to reduce genotyping costs while still improving the efficiency of MAS for traits influenced by few genes such as diseases in common bean. The results should complement other useful genetic maps developed earlier for improving common bean for combining resistance to several diseases and quantitative trait loci (QTL) of economic importance (Kelly et al., 2003).

This study was based on dominant SCAR markers, which cannot differentiate homozygotes progenies from heterozygotes, we therefore recommend use of co-dominant markers to estimate linkage among gene pairs; *Co-4*^{2}*/Phg-2, Co-5/*"P.ult" in early generations such as F_2s and F_3s using a moderate population size (of 50 individuals) proposed by Sun et al., (2012). The following are other recommendations: 1) sequencing SCAR markers which are strongly linked to targeted resistance genes in parental cultivars, then annotate chromosomal regions flanking the markers to find alternative markers and potential candidate genes; 2) Phenotyping the bean populations with pathogens in addition to genotyping and establish the correlations; and 3) Reciprocal crosses can be used to test the effect of maternal effects on linkage estimates among gene pairs or markers.

Conclusion

There was weak linkage among gene pair, *Co-4*^{2}*/Phg-2* on bean chromosome eight. The linkage was however relatively stronger among gene pair, *Co-5/*"P.ult". There was difference in the value of recombination fraction between the BC_3F_6 and F_2 population. This implies that selection for each of the resistance genes, *Co-4*^{2}*, Phg-2, Co-5* and "P.ult" requires to be selected with their own SCAR marker due to lack of strong genetic linkages among these genes during marker assisted gene pyramiding targerting all the four genes in the same background.

ACKNOWLEDGEMENTS

This research was funded by African Development Bank (AfDB) - Higher Education, Science and Technology (HEST) project through CIAT, Uganda. The National Agricultural Research Laboratories/NARO, Kawanda and Kirkhouse Trust (UK) made significant support to this work and are acknowledged. The authors appreciate assistance from research assistants at CIAT, Uganda.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Bailey-Wilson JE, Wilson AF (2011). *Linkage analysis in the next-generation sequencing era.* Human Heredity 72(4): 228-236.

Balding DJ, Bishop M, Cannings C (2007). Third Edition. Handbook of Statistical Genetics. John Wiley & Sons, Ltd, West Sussex, England. 1-1390.

Burt AJ, William HM, Perry G, Khanal R, Pauls KP, Kelly JD, Navabi A (2015). Candidate gene identification with SNP marker-based fine mapping of anthracnose resistance gene Co-4 in common bean. PLoS One 10(10):e0139450.

Chilagane LA, Tryphone GM, Protas D, Kweka E, Kusolwa PM, Nchimbi-Msolla M (2013). Incorporation of resistance to angular leaf spot and bean common mosaic necrosis virus diseases into adapted common bean (*Phaseolus vulgaris* L.) genotype in Tanzania. African Journal of Biotechnology 12(27):4343-4350.

Churchill G, Doerge R (1994). Empirical threshold values for quantitative trait mapping. Genetics 138(3):963-971.
Coop G, Przeworski M (2007). An evolutionary view of human recombination. Nature Reviews Genetics 8(1):23-34.
Collard BCY, Mackill DJ (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philosophical Transactions of the Royal Society B: Biological Sciences 363(1491):557-572.
Edwards AWF (1972). Likelihood. Cambridge University Press.
Gao F, Ming C, Hu W, Li H (2016). New software for the fast estimation of population recombination rates (FastEPRR) in the genomic era. Genetics 6(6):1563-1571.
Geffroy V, Sévignac M, Billant P, Dron M, Langin T (2008). Resistance to *Colletotrichum lindemuthianum* in *Phaseolus vulgaris*: A case study for mapping two independent genes. Theoretical and Applied Genetics 116(3):407-415.
Gonçalves-Vidigal MC, Cruz AS, Garcia A, Kami J, Vidigal-Filho PS, Sousa LL, McClean P, Gepts P, Pastor-Corrales MA (2011). Linkage mapping of the Phg-1 and Co-14 genes for resistance to angular leaf spot and anthracnose in the common bean cultivar AND 277. Theoretical and Applied Genetics 122(5):893–903.
Kaplan N, Hudson RR (1985). The use of sample genealogies for studying a selectively neutral m-loci model with recombination. Theoretical Population Biology 28(3):382-396.
Kelly JD, Gepts P, Miklas PN, Coyne DP (2003). Tagging and mapping of genes and QTL and molecular-marker assisted selection for traits of economic importance in bean and cowpea. Field Crops Research 82(2-3):135-154.
Mahuku GS (2004). A simple extraction method suitable for PCR-based analysis of plant, fungal, and bacterial DNA. Plant Molecular Biology Reporter 22(1):71-81.
Mahuku G, Navia M, Buruchara R, Matta A, Otsyula R (2007). Development of PCR markers tightly linked to Pyult1, a gene that confers Pythium root rot resistance in the common bean genotype AND 1062. Abstract. Annual Meeting, July 28–August 1. San Diego, California. American Phytopathological Society.
Mahuku GS, Henriquez MA, Montoya C, Jara C, Teran H, Beebe S (2011). Inheritance and development of molecular markers linked to angular leaf spot resistance genes in the common bean accession G10909. Molecular Breeding 28(1):57-71.
Malav AK, Indu, Chandrawat KS (2016). Gene pyramiding: An overview. International Journal of Current Research in Biosciences and Plant Biology 3(1):22-28.
Miller T, Gepts P, Kimmo S, Arunga E, Chilagane LA, Nchimbi-Msolla S, Namusoke A, Namayanja A, Tedla YR (2018). Alternative markers linked to the Phg-2 angular leaf spot resistance locus in common bean using the *Phaseolus* genes marker database. African Journal of Biotechnology 17(26):818-828.
Oblessuc PR, Perseguini J, Baroni RM, Chiorato AF, Carbonell SAM, Mondego JMC, Vidal RO, Camargo LEA, Benchimol-Reis LL (2013). Increasing the density of markers around a major QTL controlling resistance to angular leaf spot in common bean. Theoretical and Applied Genetics 126(10):2451-2465.
Okii D, Mukankusi C, Sebuliba S, Tukamuhabwa P, Tusiime G, Talwana H, Odong T, Namayanja A, Paparu P, Nkalubo S, Otim M, Ugen M, Buah S, Gepts P (2017). Genetic variation, heritability estimates and GXE effects on yield traits of Mesoamerican common bean (*Phaseolus vulgaris* L) germplasm in Uganda. Plant Genetic Resources 16(3):237-248.
Ott J, Wang J, Leal SM (2015). Genetic linkage analysis in the age of whole-genome sequencing. Nature Reviews Genetics 16(5):275-284.
Pathania A, Sharma SK, Sharma PN (2014). Common bean. In: Singh et al. (eds.), Broadening the Genetic Base of Grain Legumes. Springer, India pp. 11-50.
Perseguini JMKC, Oblessuc PR, Rosa JRBF, Gomes KA, Chiorato AF, Carbonell SAM (2016). Genome-wide association studies of anthracnose and angular leaf spot resistance in common bean (*Phaseolus vulgaris* L.). PLoS One 11(3):e0150506.
Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimwood J (2014). A reference genome for common bean and genome-wide analysis of dual domestications. Nature Genetics 46(7):707-713.
Schwartz HF, Galvez GE (1980). Eds. Bean Production Problems: Diseases, Insect, Soil and Climatic constraits of phaseolus vulgaris. CIAT, Cali, Colombia.
Staub EJ, Serquen FC, Gupta M (1996). Genetic markers, map construction, and their application in plant breeding. Horticultural Science 31(5):729-739.
Strachan T, Read AP (2011). Human Molecular Genetics. 4th edition. Garland Science, Taylor and Francis Group, New York.
Sun Z, Li H, Zhang L (2012). Estimation of recombination frequency in bi-parental genetic populations. Genetics Research 94(3):163-177.
Toomet O, Henningsen A (2009). maxLik: Tools for Maximum Likelihood Estimation. R. package version 0.5. http://CRAN.R-project.org.
Young RA, Kelly JD (1996). Characterization of the genetic resistance to *Colletotrichum lindemuthianum* in common bean differential cultivars. Plant Disease 80(6):650-654.

Vol. 18(29), pp. 831-844, September, 2019
DOI: 10.5897/AJB2019.16854
Article Number: 929AE5F61835
ISSN: 1684-5315

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Aqueous Extracts of *Pericopsis angolensis* and *Swartzia madagascariensis* with High Antimicrobial Activities against *Escherichia coli* O157, Shigella spp. and *Salmonella enterica* subsp. enterica (*Serovar typhi*)

Chingwaru Constance[1], Tanja Bagar[2] and Chingwaru Walter[1,2]*

[1]Biological Sciences Department, Faculty of Science and Engineering, Bindura University of Science Education, Zimbabwe.
[2]ICANNA – International Institute for Cannabinoids. Kodeljevo Castle, Ulica Carla Benza 16. SI-1000 Ljubljana, Slovenia.

Received 11 May, 2019; Accepted 29 July, 2019

This study determined the potential antidiarrhoeal potential of *Pericopsis angolensis* and *Swartzia madagascariensis* extracts against *Escherichia coli* O157, *Shigella* species and *Salmonella* Typhi. Extracts were obtained using the following methods: (i) hot water extraction (90°C) (LHWE), (ii) cold water extraction (CWED) and (iii) ethanolic extraction (EED). Antimicrobial effects of the extracts were determined using the well diffusion assay. Phytochemical analysis was performed using standard biochemical methods. The LHWE extracts exhibited significantly greater inhibition than CWED and EED extracts as follows: (i) *P. angolensis* bark extract at 0.8 mg/ml against *Shigella* spp. and (ii) *P. angolensis* bark extract at 1.6 mg/ml and *S. madagascariensis* bark extract at 1.6 mg/ml against *S.* Typhi. The aqueous methods largely resulted in *P. angolensis* and *S. madagascariensis* extracts rich in flavonoids, saponins and tannins. The aqueous extraction methods (CWED and LHWE) are therefore suitable to obtain extracts with high antimicrobial effects against *E. coli* O157, *Shigella* species and *S.* Typhi.

Key words: Antidiarrhoeal, phytochemicals, extraction, *Pericopsis angolensis*, *Swartzia madagascariensis*.

INTRODUCTION

The use of plants or their products in traditional medicines has, since historic times, remained significant in the treatment of various medical ailments such as diarrhoea (Maroyi, 2016). Notably, there is a renewed public interest in the use of traditional medicines owing to the high costs of orthodox medicines and the associated side effects, especially antimicrobial resistance (Patwardhan et al., 2005). In African countries, approximately 80% of the population reportedly rely on traditional medicines owing to their low cost and ease of

*Corresponding author. E-mail: wchingwaru@yahoo.co.uk or wchingwaru@buse.ac.zw. Tel: +263-7777 66606.

access, as well as for cultural reasons (Maroyi, 2016).

Diarrhoeal diseases have remained a global burden and a leading public health threat characterised by high morbidity and mortality especially among children under the age of 5 years (Maroyi, 2016). Diarrhoea is thought to account for between 1 and 9 million deaths among children aged 5 years or younger globally, with the highest rates occurring in low income countries (LIC) especially those in sub Saharan Africa and Asia (Njume et al., 2011).

Plants have maintained their place in traditional medicine owing to their rich composition of healthful bioactive chemical compounds/metabolites (Smith, 2007). Notably, most communities in LIC tend to rely of phytomedicines to manage various forms of diarrhoea including cholera, typhoid and various forms of gastroenteritis. Bioactive compounds are accumulated plant tissues as secondary metabolites (Smith, 2007). These metabolites are often accessed through the use of extraction techniques, with some plant materials being consumed whole to achieve the desired therapeutic or prophylactic effect (Semenya and Maroyi, 2013). The composition and bioactivity of an extract depends on its inherent chemical composition, the solvent used as well as the protocol followed during the extraction process (Muhamad et al., 2017). Traditional medicine has often relied on organic solvents including the use of cold and hot water (steeping) as extractants (Ngarivhume et al., 2015; Palombo, 2011; Wachtel-Galor, 2004). However, the utilisation of organic solvents is often limited by a low extraction yield (Wachtel-Galor, 2004). To enhance the extraction processes, scientists have adopted protocols that use a combination of organic solvents (Pilon et al., 2016). The traditional extraction methods, relying primarily on hot or cold water, utilise fresh or dried plant materials, with the obtained extracts being administered within hours from the time of plant material collection (De Wet and Ngubane, 2014; Odunmbaku et al., 2018). Different extractive strategies reportedly yield products with different clinical efficacies (Odunmbaku et al., 2018). With the availability of more modern extraction methods including maceration, percolation, reflux extraction, super critical fluid extraction (SFC), pressurised liquid extraction (PLE) and microwave assisted extraction (MAE)-with advantages that include enhanced extraction efficiency and improved extract bioactivities (Zhang et al., 2018), their utility compared to that of the traditional hot and cold water extraction methods have remained unappraised.

The current study compared the antidiarrhoeal potential of hot water extraction and cold water extraction methods (LHWE and CWED, respectively) to that of ethanolic extraction method (EED). Specifically, the current study sought to assess the validity and utility of the aqueous extraction methods, which are considered methods of choice in the preparation of traditional antidiarrhoeal medicines. Two plant species with a history of use in the traditional management of diarrhoea in Southern Africa, namely *Pericopsis angolensis* (Baker) Meeuwen and *Swartzia madagascariensis* (Desv.) J.H. Kirkbr. & Wiersama (Table 1), were used for the study. The extracts were tested for antimicrobial activities against selected diarrhoeagenic *Escherichia coli* O157, *Shigella* species and presumptive *Salmonella* Typhi. Additionally, the phytochemical composition of the extracts was determined. This study was considered important as it informs both the traditional and orthodox medicinal practices on the relative utilities of these extraction methods.

MATERIALS AND METHODS

Collection and processing of plant materials

Fresh samples of *P. angolensis* bark, *S. madagascariensis* bark and *S. madagascariensis* leaves were collected in Chiraswa Village in Murehwa, Mashonaland East Province of Zimbabwe (-17°.69′71.55″S, 31°.96′48.90″E) during the months of October-December 2018. Species identification was done by qualified botanists at the National Herbarium and Botanic Garden in Harare (Zimbabwe). Voucher specimens were deposited in the Biological Sciences laboratory for future reference.

The collected plant materials were washed to remove debri and then separated into two batches. Half of each fresh sample was frozen in airtight plastic bags for future use. The other half of each plant sample was air-dried for 72 h, then ground into fine powders with an electric grinder and stored in air tight containers in the dark at room temperature.

Extraction

Cold water (*CWED*) *and ethanolic extraction* (*EED*)

The powdered samples were extracted into cold distilled water (150 ml) and 70% ethanol (150 ml). The plant-solvent mixtures were continuously swirled at 150 rpm on a rotary shaker for 72 h. The extracts were filtered through Whatman No. 41 filter paper (pore size 20-25 µm) and the collected filtrates were evaporated at room temperature. Each dried extract was resuspended into between 1 and 2 ml of sterile Ringers solution. Concentrations of each stock solution were stored at -20°C until further analysis.

Hot water extraction (*THWE*)

Fresh plant samples (10 g) were added to 100 ml of hot boiled water (90°C) and steeped for 30 min. The samples were filtered through Whatman No. 41 filter paper (pore size 20-25 µm) and were stored at -20°C for further tests.

Phytochemical analyses

Qualitative chemical analyses of the extracts were conducted using the following biochemical tests. Ringers solution was used as a negative control for all phytochemical tests.

Test for tannins (*Ferric chloride test*)

A few drops of 0.1 ferric chloride were added to 2 ml of aqueous extracts (CWED, EED and THWE). A blue coloration indicated the

Table 1. Description of selected plant species used in the management of diarrhoea and other ailments.

Plant species	Common name	Distribution in Zimbabwe	Parts of plants used	Extraction method	Bioactive compounds
Pericopsis angolensis (Baker) Meeuwen	Afrormosia (English), Muwanga (Shona), Ubanga (Ndebele)	North, Central and Eastern parts [14]	Roots: abortifacient, aphrodisiac and a tonic, decoctions blood circulation stimulant, diarrhoea, bronchial and chest pains, nausea and eye problems. Dried and powdered root: relieve pain, treat oedema and tumours. Bark: diarrhoea, sore throat, toothache, eye bath. Leaves: vapour for headaches, anthelmintic (http//www.prota.org)	No information found	No information found
Swartzia madagascariensis (Desv.) J.H. Kirkbr. & Wiersama	Snake bean (English), Mucherekese (Shona) [14]	East [14]	Bark: ear treatments, laxative, venereal diseases. Leaves: astringents, mammal and bird poisons, rodenticides. Root: diarrhoea, dysentery, vermifuges, abortifacients, ecbolics, antidotes. Pod: insecticides, arachnicides, leprosy (Royal botanical gardens.	Solvent extraction (ethanol and hexane and ethyl acetate) using Soxhlet apparatus [15]	Hexane extracts (steroids and triterpenes present) [15], Ethanol extracts (flavonoids, saponins, triterpenes, alkaloids. Fruits: glycosides, saponins, steroids. Leaf, root and seeds: tannins

presence of tannins [16].

Test for flavonoids

Dilute ammonia (5 ml) solution was added to 1 ml of each plant extract. Concentrated sulphuric acid (5 ml) was added and a yellow coloration in each plant extract indicated the presence of flavonoids (Zohra et al., 2012).

Test for alkaloids

Aqueous 1% hydrochloric acid (0.2 ml) was added to 2 ml each extract. Each solution was heated in a steam bath for 10 min. The aqueous extract solution was treated with 6 to 10 drops of Dragendoff's reagent. A creamish precipitate indicated the presence of alkaloids (Zohra et al., 2012).

Test for saponins

Aqueous extracts (2 ml) were mixed with distilled water (5 ml) and shaken vigorously for stable persistence froth. The froth was mixed with 3 drops of olive oil and was shaken vigorously. Emulsion indicated the presence of saponins (Zohra et al., 2012).

***Test for reducing sugars* (*Benedict's test*)**

To 1 ml of the plant extract, a few drops of Benedict's reagent (alkaline solution containing cupric citrate solution) were added and boiled in a water bath. A reddish brown precipitate indicated the presence of reducing sugars (Avinash and Waman, 2014).

Bacterial strains

The microorganisms used in determination of the antibacterial activity of the plant extracts' were as follows: presumptive *E. coli* O157, *Shigella* spp. and *S.* Typhi. All bacterial strains were obtained from our in-laboratory stock of environmental isolates. The isolated strains were maintained on Nutrient agar. The bacterial cultures were prepared by transferring a colony of the bacteria into a universal bottle containing 10 ml of nutrient broth and incubated overnight at 37°C. The concentration of the bacterial cultures was standardised to a concentration of 1×10^8 colony forming units per millilitre (CFU/ml) (internal protocol), which is equivalent to an optical density of 0.2 using a Biobase EL 10B Microplate Reader (Jinan, China) at optical density 620 nm. Ringer's solution was used as a negative control for all antimicrobial tests.

Antibacterial screening

Antibacterial tests were performed using standard agar well diffusion assay as described by Soman and Ray (2016). Briefly, agar plates were prepared using sterile HiCrome O157: H7 agar (Sigma-Aldrich, Saint Quentin Fallavier, France) and XLD agar (Sigma-Aldrich, Saint Quentin Fallavier, France) for *E. coli* O157, *Shigella* spp. and *S.* Typhi, respectively. The standardised cultures were evenly spread onto the surface of the agar plates using sterile swabs. Wells were made in each plate with a sterile auger (10 mm diameter). 40 µl of ethanol and aqueous extracts (100 mg/ml) were added in each well, with streptomycin (300 µg, Mast Diagnostics, UK) being used as positive control. The plates were incubated at 37°C for 24 h. Each extract was tested in triplicate.

Antibacterial activity was tested by observing bacterial growth and was indicated as the presence of clear zones around the well

(zones of inhibition). The absence of the zone of inhibition around the wells was interpreted as the absence of activity. The zones of inhibition were measured in millimetres. Only extracts that showed antimicrobial activities were used to determine the minimum inhibition and minimum bactericidal concentration of each preparation.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration of the plant extracts/control was determined using the well diffusion assay (Soman and Ray, 2016). Agar plates were prepared using sterile HiCrome 0157: H7 media for *E. coli* 0157 and XLD media for *Salmonella* and *Shigella* spp. The standardised cultures were evenly spread on the surface of the agar plates using sterile swabs under sterile conditions. Wells were made in each plate with a sterile auger (10 mm diameter). 40 µl of plants extracts (two fold concentrations ranging from 0.781 to 100 mg/ml) were added in each triplicate wells. Streptomycin (300 µg, Mast Diagnostics, UK) was used as the positive control. The diffusion of the extracts was allowed at room temperature for 1 h in a sterile laminar flow cabinet and the plates were incubated at 37°C for 24 h. The plates were observed for antimicrobial activity and the zones of inhibition (mm) indicated the minimum concentration at which the extracts inhibited the growth of the test microorganisms. The concentration at which there was no zones of inhibition were recorded as the minimum inhibition concentration.

Determination of minimal bactericidal concentration (MBC)

A modified assay to that described by as modified from the Soman and Ray (2016) method was used to determine the MBC of each extract. Briefly, using the agar plates from the MIC assay, a sterile inoculating loop was used to touch the zone of inhibition of different concentrations of extracts where there was invisible growth. The loops were used to streak labelled and prepared agar plates. The plates were incubated for 24 h and observed for growth at different concentration.

RESULTS

Extracts from plant materials listed in Table 1 were exposed to different solvents and conditions. Briefly, plant materials were exposed to the following: (i) hot water (steeping) for 1 h (LHWE), a method simulating the traditional extraction method; (ii) cold water for 72 h (CWED) followed by evaporation at room temperature and (iii) ethanol for 72 h followed by evaporation at room temperature (EED). Yields per extract (mg) were obtained by weighing the dried samples and subtracting the weight of the containers (Petri dishes). Table 2 provides details of the amount of material used and the yield of extract (mg).

Standard phytochemical analyses were conducted on extracts described in Table 3. Briefly, extracts were exposed to various chemicals in accordance with standard biochemical protocols. Colour and other changes in the extracts were used to show the presence of the target compounds. Relative phytochemical concentration was determined relying on intensities of the extracts. Table 3 provides information on chemical composition of each extract used.

The traditional hot water extraction method (LHWE) yielded extracts that contained the following phytochemicals [flavonoids (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts, saponins (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts) and reducing sugars (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts)] (Table 3). The cold water extraction method (with evaporation) (CWED) yielded extracts that contained the following phytochemicals [flavonoids (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts, saponins (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts), tannins (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts) and reducing sugars (*P. angolensis* bark extract and *S. madagascariensis* bark and leaf extracts)] (Table 3). The ethanolic extraction method (EED) yielded extracts with greater concentrations of reducing sugars (*P. angolensis* bark extract)] (Table 3). All extracts showed no presence of alkaloids.

The traditional hot water extraction method (LHWE) yielded the highest antimicrobial activities in terms of the attainment of the lowest minimum inhibition concentration (MIC)/highest zone of inhibition (ZOI) against *Shigella* spp. in comparison with the other two extraction methods (CWED and EED) as follows: (i) *P. angolensis* bark extract (ZOI = 21 mm) (Figure 3) and (ii) *S. madagascariensis* bark extract (1.56 mg/ml) (Figure 1). The LHWE extract was shown to yield significantly greater ZOI at 100 mg/ml than CWED and EED against *Shigella* spp. (p = 0.001 and p = 0.0003, respectively) (Table A1). Additionally, LHWE extracts of the following extracts attained significantly greater antimicrobial activity (ZOI) against the strain of *Shigella* spp. (at 100 mg/ml) than Streptomycin (300 µg/ml) (ZOI = 20 mm) with the following: (i) *S. madagascariensis* leaf extract (ZOI = 23 mm) (Figure 2) and (ii) *P. angolensis* bark extract (ZOI = 21 mm) (Figure 1). Additionally, the LHWE extract retained the highest activity against *Shigella* spp. compared to the other two (CWED and EED) across all concentrations tested (Figures 1, 2 and 3).

The LHWE extraction method was shown to yield extracts with significantly greater ZOI against *Shigella* spp. than those from the other methods with the following: (i) *P. angolensis* bark extract (LHWE > CWED - p = 0.001) and (ii) *P. angolensis* bark extract (LHWE > EED – p = 0.0003) (Table A1). Additionally, LHWE extracts were shown to have significantly greater ZOI at 100 mg/ml than other against the *S.* Typhi as follows: (i) *S. madagascariensis* bark extract (LHWE > CWED – p < 0.0001); (ii) *S. madagascariensis* bark extract (EED > LHWE – p = 0.002); (iii) *S. madagascariensis* leaf extract (LHWE > CWED – p = 0.0001); (iv) *S. madagascariensis* leaf extract (LHWE > EED – p = 0.0003); (v) *P.*

Table 2. Extract yield per unit weight of plant material used and extraction method (traditional hot water extraction - LHWE, cold water extraction with concentration - CWED and ethanolic extraction with concentration - EED).

Plant species and extract		Weight of plant material used (g)	Yield (mg)
P. angolensis bark	CWED	45.18	510
	EED	45.18	465
	LHWE	10	N.D
S. madagascariensis bark	CWED	45.17	320
	EED	45.17	1 275
	LHWE	10	N.D
S. madagascariensis leaf	CWED	45.17	320
	EED	45.17	720
	LHWE	10	N.D

N.D: Not determined. Extract was used without evaporation; hence its dry weight was not determined. CWED: Cold water extract with desiccation; EED: ethanol extract with desiccation; LHWE: aqueous extract obtained by the traditional extraction method.

Table 3. Qualitative phytochemical composition of extracts obtained using traditional hot water extraction (LHWE), cold water extraction with concentration (CWED) and ethanolic extraction with concentration (EED).

Plant species and extract		Saponins	Alkaloids	Flavonoids	Tannins	Reducing sugars
P. angolensis bark	CWED	+++	-	+++	+++	+
	EED	++	-	+	-	+++
	LHWE	++	-	+	-	+
S. madagascariensis bark	CWED	+++	-	+	-	+
	EED	-	-	+	++	-
	LHWE	+++	-	+	+++	++
S. madagascariensis leaf	CWED	++	-	+	-	+++
	EED	-	-	+	++	-
	LHWE	++	-	+++	-	+++

CWED: Cold water extract with desiccation; EED: ethanol extract with desiccation; LHWE: aqueous extract obtained by the traditional extraction method; + = presence of phytochemical in trace amounts; -: absence of phytochemical; ++: moderate amount of phytochemical; +++: appreciable amounts of phytochemicals.

angolensis bark extract (LHWE > CWED – p = 0.002) and (vi) *P. angolensis* bark extract (LHWE > EED – p = 0.0004) (Table A1, Figure 4 and 5).

The cold water extraction method with desiccation (CWED) yielded the highest antimicrobial activities in terms of the attainment of the lowest minimum inhibition concentration (MIC) / highest zone of inhibition (ZOI) against *S.* Typhi in comparison with the other two extraction methods (LHWE and EED) as follows: (i) *S. madagascariensis* bark extract (MIC = 0.78mg/ml) (Figure 1d) and *S. madagascariensis* leaf extract (MIC = 1.56mg/ml) (Figure 1e). Additionally, the CWED extract retained the highest activity against *S. typhi* compared to the other two (LHWE and EED) across all concentrations of *S. madagascariensis* bark extract (Figure 1d). Additionally, the CWED extract from *P. angolensis* bark was shown to have significantly greater ZOI at 100mg/ml than that of EED extract against the *S. typhi* (CWED > EED – p = 0.02) (Figure 6).

The hot water extraction method (LHWE) yielded extracts that had no antimicrobial activity against *E. coli.* The cold water extraction method with desiccation (CWED) of *S. madagascariensis* leaf extract (ZOI = 21 mm) yielded the higher antimicrobial activities in terms of the attainment of the highest zone of inhibition (ZOI) against *E. coli* than that of extracts from the other two extraction methods (LHWE and EED) (Figure 8). Additionally, CWED extracts attained greater antimicrobial activity against the strain of *E. coli* (at 100 mg/ml) than streptomycin (300 µg/ml) (ZOI = 23 mm) as follows:

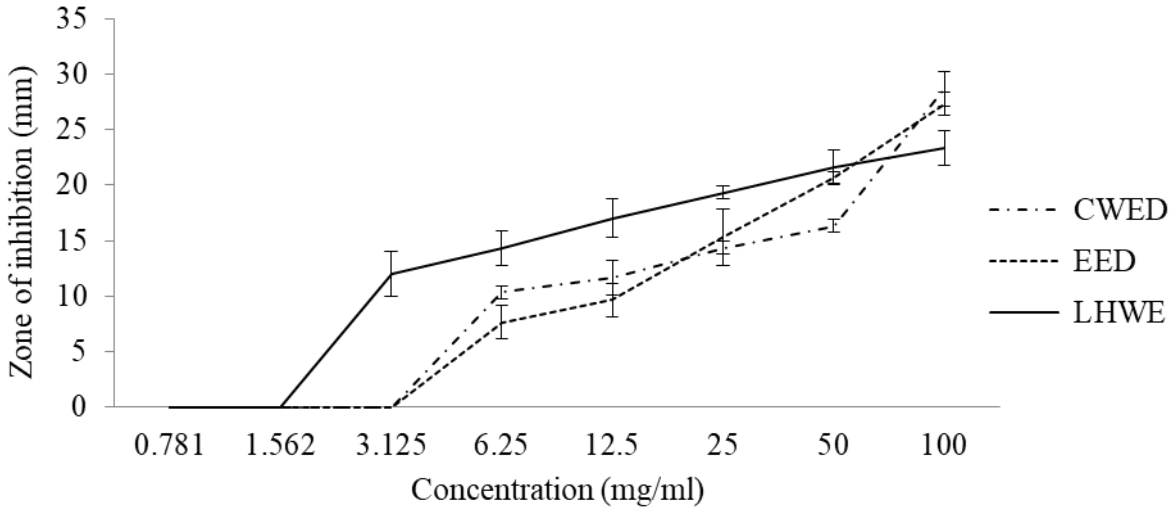

Figure 1. Inhibition of *Shigella* spp. isolate by varying concentrations of *S. madagascariensis* leaf extract.

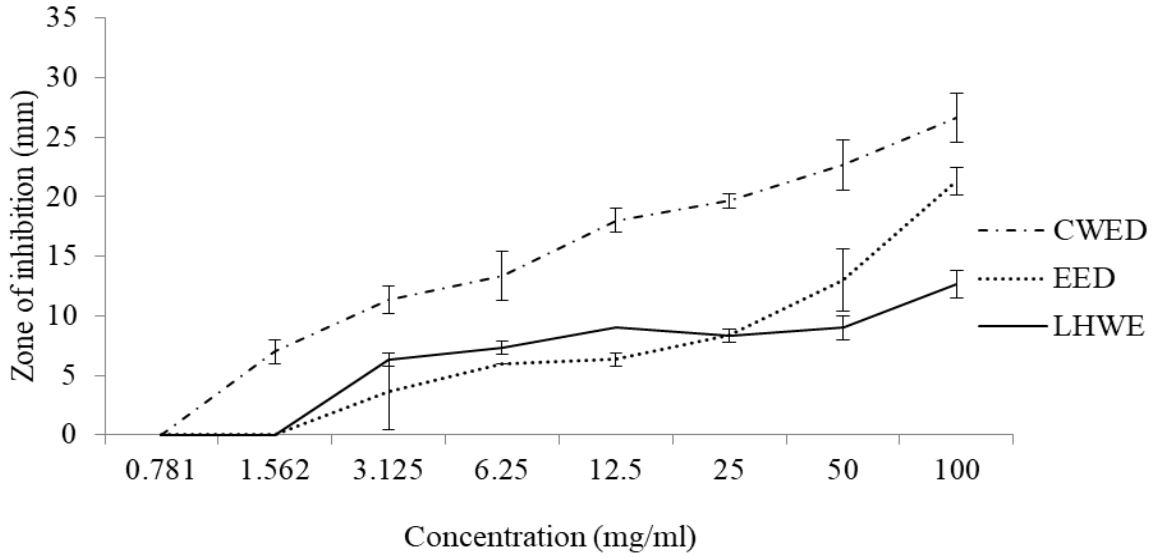

Figure 2. Inhibition of *Shigella* spp. isolate by varying concentrations of *S. madagascariensis* bark extract.

(i) *S. madagascariensis* bark extract (ZOI = 26 mm) (Figure 7), (ii) *S. madagascariensis* leaf extract (ZOI = 34 mm) (Figure 8) and (iii) *P. angolensis* bark extract (ZOI = 30 mm) (Figure 9). Additionally, the CWED extract retained the highest activity against *S. typhi* compared to the other two (LHWE and EED) across all concentrations of *S. madagascariensis* leaf (Figure 8).

The following extracts were shown to have significantly greater ZOI at 100 mg/ml than the other against the *E. coli*: (i) *S. madagascariensis* bark EED extract > CWED extract: $p = 0.02$ and *S. madagascariensis* leaf CWED extract > EED extract: $p < 0.0001$ (Table A1).

The ethanolic extraction with desiccation method yielded extracts with greater antimicrobial activities against *E. coli* O157 as follows: (i) *S. madagascariensis* bark extract (MIC = 0.39 mg/ml / ZOI = 29 mm) (Figure 7) and (ii) *P. angolensis* bark extract (ZOI = 31 mm) (Figure 9). The following extracts achieved greater ZOI than streptomycin against *E. coli* (300 µg/ml) (ZOI = 23 mm): (i) CWED and EED of *S. madagascariensis* bark extracts (26 and 29 mm) (Figure 7), (ii) CWED of *S. madagascariensis* leaf extract (34 mm) (Figure 8) and (iii) CWED and EED of *P. angolensis* bark extracts (30 and 31 mm) (Figure 9).

Overall, the aqueous extraction methods (CWED and LHWE) yielded extracts with greater antimicrobial

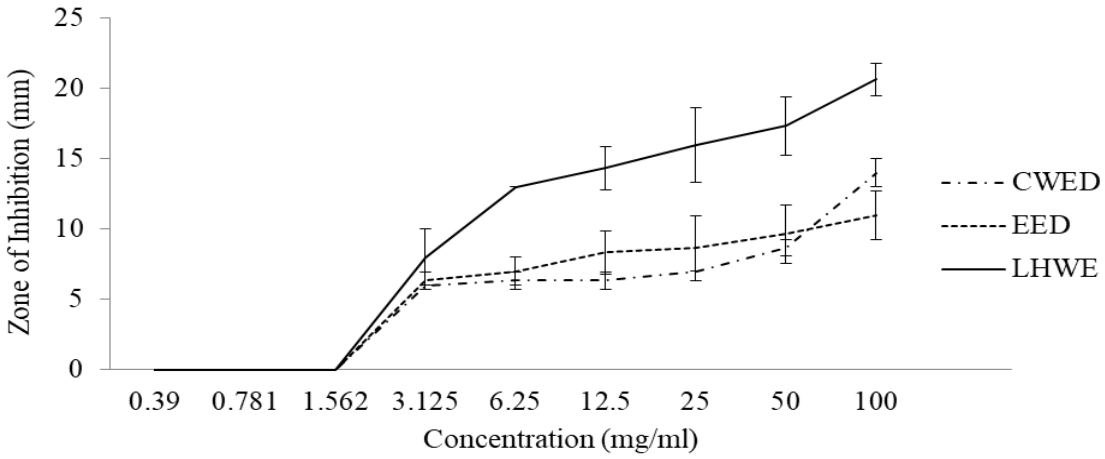

Figure 3. Inhibition of *Shigella* spp. isolate by varying concentrations of *P. angolensis* bark extract.

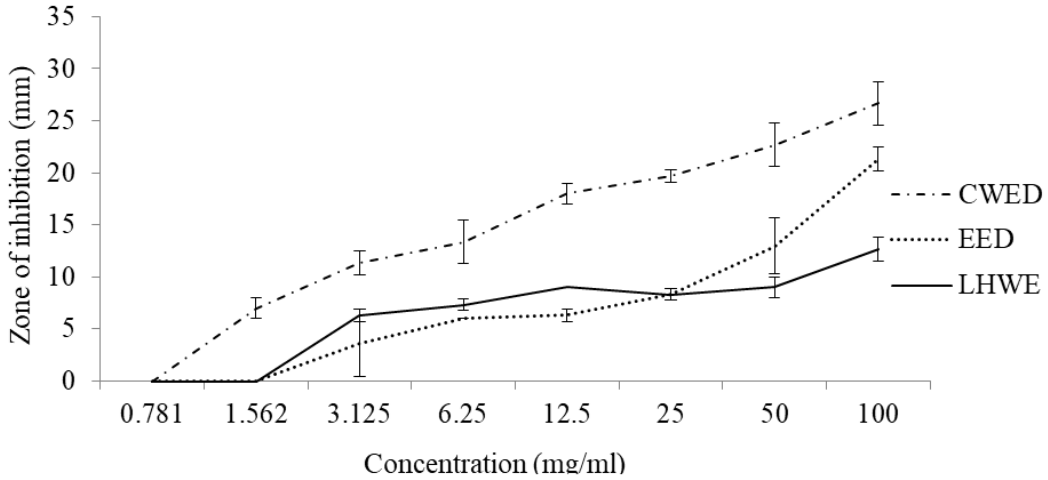

Figure 4. Inhibition of *S.* Typhi isolate by varying concentration of *S. madagascariensis* bark extract.

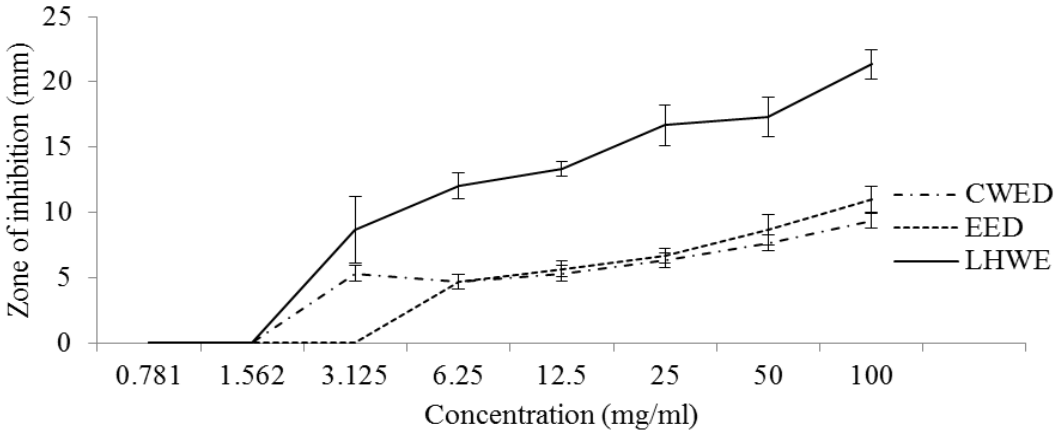

Figure 5. Inhibition of *S.* Typhi isolate by varying concentration of *S. madagascariensis* leaf extract.

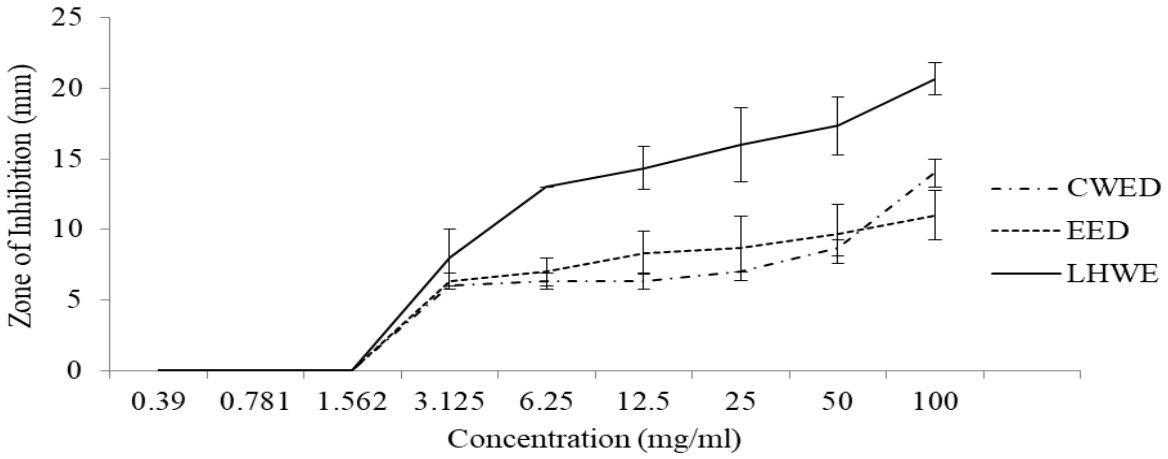

Figure 6. Inhibition of *S.* Typhi isolate by varying concentration of *P. angolensis* bark extract.

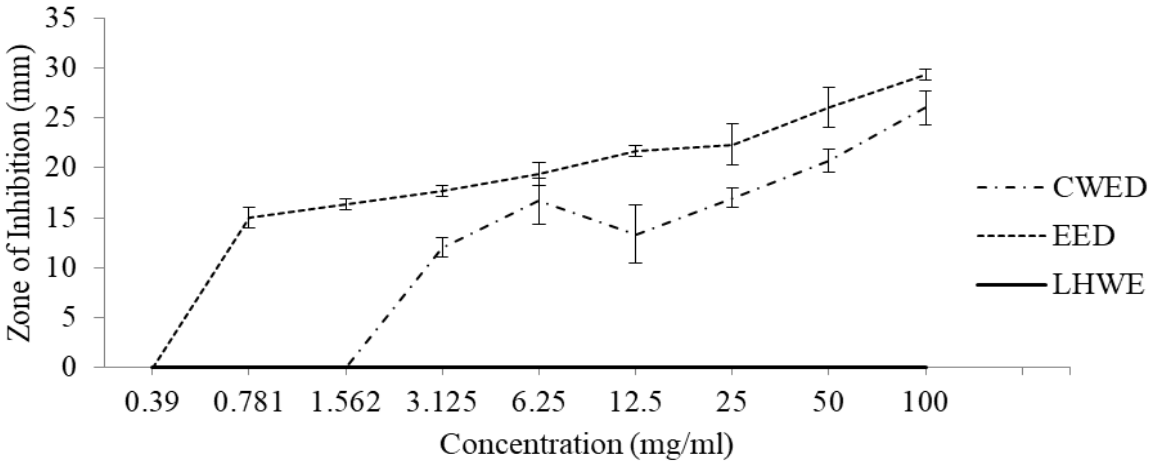

Figure 7. Inhibition of *E. coli* O157:H7 isolate by varying concentration of *S. madagascariensis* bark extract.

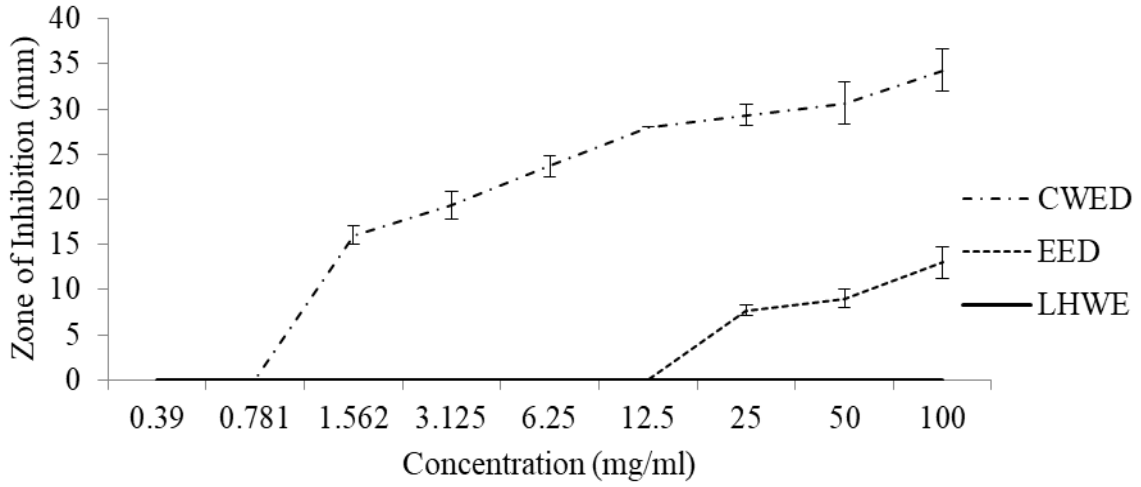

Figure 8. Inhibition of *E. coli* O157:H7 isolate by varying concentration of *S. madagascariensis* leaf extract.

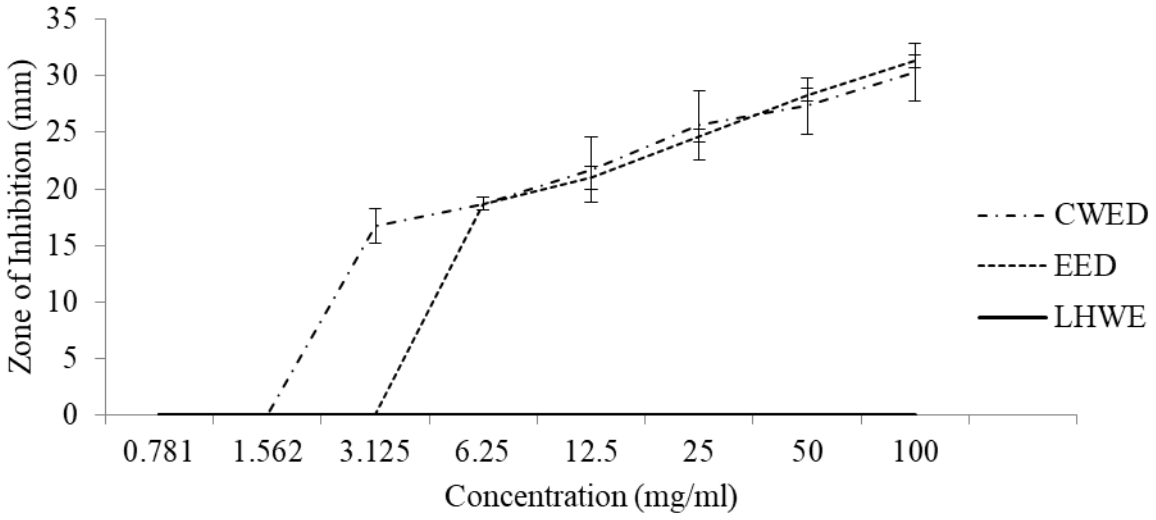

Figure 9. Inhibition of *E. coli* O157:H7 isolate by varying concentration of *P. angolensis* leaf extract.

activities than the other two methods in terms of the attainment of the lowest MIC values and higher ZOI per concentration used depending on plant species or plant part used. Generally, the LHWE method was largely better than the other two methods in (lowest MIC values and higher ZOI against *Shigella* spp. and presumptive *S.* Typhi per concentration used) with different plants. However, the LHWE technique yielded extracts with no antimicrobial activities against *E. coli.* The CWED and EED methods yielded greater antimicrobial activities against the bacteria as follows: (i) CWED with *S. madagascariensis* leaf extract (Figure 8) and (ii) EED with *P. angolensis* bark (Figure 9).

Table 4 shows ZOI of each extract (at the highest concentrations) against *Shigella* spp. that were greater than that for streptomycin (300 µg, Mast Diagnostics, UK) (20 mm). The extract from the traditional hot water extraction method (LHWE) had greater inhibitory activities than that for streptomycin (300 µg) against the following microorganisms with the following extracts (*Shigella* spp.: *S. madagascariensis* bark and leaf extracts and *P. angolensis* bark extract) (Table 4). The cold water extract of *S. madagascariensis* bark showed greater ZOI compared to that for streptomycin against *E. coli* and *S.* Typhi. The cold water extract of *S. madagascariensis* leaf showed greater ZOI compared to that of streptomycin against *E. coli*. Whereas the ethanolic extraction method yielded an extract of *S. madagascariensis* bark and *P. angolensis* bark with greater ZOI than streptomycin.

The traditional hot water extraction method (LHWE), cold water extraction with desiccation (CWED) and the ethanol with desiccation extraction (EED) methods yielded equal or similar MIC values, as seen with *P. angolensis* bark extract (0.78 mg/ml) (Figure 1). The CWED method yielded equal but higher MIC values than LHWE and EED method when used to obtain *S. madagascariensis* bark extract (CWED - 0.2 mg/ml vs. LHWE and EED - 0.78 mg/ml) (Table 5). The LHWE method yielded greater antimicrobial activities (ZOI) at concentrations higher that the respective MIC against presumptive *S. typhi* for *P. angolensis* bark extract and *S. madagascariensis* bark extract (Table 5). Overall, the LHWE method was largely better than the other two methods in (lowest MIC values and higher ZOI per concentration used) for *P. angolensis* bark extract and *S. madagascariensis* bark extract, whereas EED proved inferior (Figure 1).

The traditional hot water extraction method (LHWE) yielded extracts had no antimicrobial effects against the strain of *E. coli* O157 used (results not shown). The CWED method yielded higher MIC values than EED method and LHWE when used to obtain the following: *P. angolensis* (MIC = 0.78 mg/ml, greater activity between 0.78 and 1.56 mg/ml and *S. madagascariensis* (MIC = 0.196 mg/ml, greater activity between 0.196 and 6.25 mg/ml (Figure 1). The EED method yielded higher MIC values than CWED method and LHWE when used to obtain *S. madagascariensis* bark extract (MIC: EED = 0.098 mg/ml, CWED = 0.781 mg/ml).

Minimum inhibition concentration (MIC) of extracts obtained using traditional hot water extraction (LHWE), cold water extraction with concentration (CWED) and ethanolic extraction with concentration (EED) were obtained following the well diffusion protocol described earlier. Table 5 shows MIC values for each extract against each of the microorganisms tested.

The ethanolic extraction (with concentration) method (EED) yielded extracts that had dually greater antimicrobial effect (MIC) against the strains and extracts (than the other two methods): *S.* Typhi and *E. coli* with *P. angolensis* bark extract. The cold water aqueous

Table 4. Extracts with ZOI (at 100 mg/ml) greater than that of Streptomycin against *E. coli, Shigella* spp. and *S.* Typhi.

Microorganism	Plant/Part	Extract	ZOI (100 mg/ml)
***Shigella* spp.**		**Streptomycin**	**20**
	S. madagascariensis bark	LHWE	24
	S. madagascariensis leaf	LHWE	24
	P. angolensis bark	LHWE	24
***S.* Typhi**		**Streptomycin**	**25**
	S. madagascariensis bark	CWED	26
E. coli		**Streptomycin**	**23**
	S. madagascariensis bark	CWED	26
		EED	29
	S. madagascariensis leaf	CWED	34
	P. angolensis bark	EED	31
		CWED	31

CWED: Cold water extract with desiccation; EED: ethanol extract with desiccation; LHWE: aqueous extract obtained by the traditional extraction method.

extraction (with concentration) method (CWED) yielded extracts that had dually greater antimicrobial effect (MIC) against the strains and extracts (than the other two methods): *S.* Typhi and *E. coli* with *S. madagascariensis* bark.

DISCUSSION

In this study, extracts obtained from the extraction LHWE, CWED and EED were analysed for antimicrobial activity against *presumptive E. coli* O157, *Shigella* spp. and *S.* Typhi and were characterised for phytochemical composition. The extraction methods gave yields (dry mass of desiccated extracts) that lied between 711 and 2833 mg (0.07 and 0.25% respectively) per 100 g of plant material used. This means for one to obtain 1 kg of desiccated product, between 35 and 140 kg of dried plant material. Should the plants not be domesticated, harvest for widespread use in the management of diseases would not be sustainable. We recommend the domestication or replanting of such medicinal plants.

Qualitative phytochemical analysis revealed the presence of saponins, flavonoids, tannins and reducing sugars in plant extracts obtained from different extraction methods.

The traditional hot water extraction method (LHWE) yielded greater antimicrobial activities (significantly greater ZOI than the other extracts against *S.* Typhi: (i) *S. madagascariensis* bark extract (LHWE > CWED – $p < 0.0001$), (ii) *S. madagascariensis* bark extract (EED > LHWE – $p = 0.002$), (iii) *S. madagascariensis* leaf extract (LHWE > CWED – $p = 0.0001$), *S. madagascariensis* leaf extract (LHWE > EED – $p = 0.0003$), (v) *P. angolensis* bark extract (LHWE > CWED – $p = 0.002$) and (vi) *P. angolensis* bark extract (LHWE > EED – $p = 0.0004$). Similar dominance of LHWE was shown against *Shigella* spp. as follows: (i) *P. angolensis* bark extract (LHWE > CWED - $p = 0.001$) and (ii) *P. angolensis* bark extract (LHWE > EED – $p = 0.0003$). *Swartzia madagascariensis* has a history of being used in concoctions (mixed with *Isoberlinia doka*, *Annona senegalensis*, *Gardenia ternifolia*, *Terminalia glaucescens* and *Erythrina senegalensis*) that have shown significant antibacterial activities against *Bacillus cereus*, *Mycobacterium fortuitum*, *Staphylococcus aureus*, or *Candida albicans* (Magassouba et al., 2007). No evidence of use of *S. madagascariensis* or *P. angolensis* as sole antimicrobials in the traditional management of diseases was found.

The barks of *P. angolensis* and *S. madagascariensis* were shown to contain a number of phenolic compounds (pterocarpins) (Harper et al., 1969) which could account for the high antimicrobial activities of LHWE against *S.* Typhi and *Shigella* spp. The observed antimicrobial activities in the selected plants may be attributed to high composition of flavonoids and tannins in *S. madagascariensis* leaf extract or pterocarpins in *P. angolensis* (Harper et al., 1969). Flavonoids have been shown to harbour antimicrobial activities against *Salmonella* spp. (Dzoyem et al., 2017), for example quercetin (Wang et al., 2017), rutin (Arima et al., 2002) and others. Generally, no other studies reporting chemical composition of *P. angolensis* were found. Interestingly, all LHWE extracts did not yield antimicrobial

Table 5. Minimum inhibition concentration (MIC) (mg/ml) of extracts obtained using traditional hot water extraction (LHWE), cold water extraction with concentration (CWED) and ethanolic extraction with concentration (EED).

Species	Plant part	Extract	Microbial species	MIC (mg/ml)
P. angolensis	Bark	CWED	*Shigella* spp.	1.562
			S. Typhi	0.781
			E. coli	0.781
		EED	*Shigella* spp.	12.5
			S. Typhi	0.196
			E. coli	0.098
		LHWE	*Shigella* spp.	0.0915
			S. typhi	0.781
			E. coli	0
S. madagascariensis	Bark	CWED	*Shigella* spp.	0
			S. Typhi	0.195
			E. coli	0.098
		EED	*Shigella* spp.	12.5
			S. Typhi	0.781
			E. coli	0.781
		LHWE	*Shigella* spp.	1.562
			S. Typhi	0.781
			E. coli	0
	Leaves	CWED	*Shigella* spp.	1.562
			S. Typhi	0.781
			E. coli	0.196
		EED	*Shigella* spp.	1.562
			S. Typhi	0.781
			E. coli	6.25
		LHWE	*Shigella* spp.	0.781
			S. Typhi	0.781
			E. coli	0

CWED: Cold water extract with desiccation; EED: ethanol extract with desiccation; LHWE: aqueous extract obtained by the traditional extraction method.

effects against *E. coli.*

Ahmed et al. (2014), in a study on the effect of hot versus cold water extraction of *Hibiscus sabdariffa* calyxes revealed greater accumulation of total phenolics, total flavonoids and tannins with short time high temperature extraction process, as well as high antioxidant activity (DPPH assay) than with the cold water extraction method. Yung et al. (2010) demonstrated an increase in phenolics content and antioxidant activities of Pegaga (*Centella asiatica*) extracts with boiling temperature (90°C). The observed high accumulation of the phenolic substances (flavonoids and tannins) as well as saponins may be due to the increased dissolution of these substances with the hot water extraction method in the present study. Saponins are glycosidic secondary metabolites that exert a wide range of pharmacological properties (Podolak et al., 2010).

The LHWE could have attained greater antimicrobial activities due to the short processing time (30 min) that could have prevented antioxidative deterioration of phytochemicals within. Whereas extraction with the EED and CWED methods was done over a period of 72h, plus

a desiccation step that took at least 48 h. The length of exposure to agents of the atmosphere and the time taken could have had deleterious effects on the chemicals.

The aqueous extraction method (with desiccation) (CWED) generally yielded extracts with higher antimicrobial activities against *E. coli* than against *S.* Typhi and *Shigella* spp. where the zones of inhibition were as follows (respectively): *P. angolensis* bark extract (31 mm for *E. coli*). CWED extracts were also shown to have greater concentrations of the following: flavonoids (*P. angolensis* bark extract), tannins (*P. angolensis* bark extract) and saponins (*P. angolensis* bark extract). The cold water extract of *S. madagascariensis* leaves (CWED) was shown to have significantly greater antimicrobial activity against *E. coli* than the ethanolic counterpart (EED) ($p < 0.0001$). The cold water extraction method (with evaporation) (CWED) yielded extracts of *S. madagascariensis* were shown to be rich in the following phytochemicals: flavonoids, saponins and tannins. These components are thought to account for the high antimicrobial activities of the *S. madagascariensis* extracts.

Conclusion

The aqueous extraction methods (CWED and LHWE) were shown to yield extracts with greater antimicrobial activities than the ethanolic extraction method (EED) (significantly lower MIC values or significantly higher ZOI against *Shigella* spp. and *S.* Typhi per concentration used) with the three selected plants. However, the LHWE technique yielded extracts with no antimicrobial activities against *E. coli.* The high antimicrobial activities of CWED and LHWE could be because of the presence of bioactive compounds that exert antimicrobial properties such as flavonoids, saponins, alkaloids and tannins. The hot water extraction method was shown to be an extraction method of choice as it resulted in significantly greater antimicrobial activities against the three diarrhoeagenic microorganisms with the three plant species. The novelty of the hot water extracted preparations is thought to lie with the freshness of such extracts (used within hours from extraction) – meaning reduced oxidative degradation of their phytochemistry. The current study therefore validates the widespread use of aqueous extraction methods in traditional medicinal practices.

ACKNOWLEDGEMENT

The authors thank Mrs. A. Chingwaru for her sterling work in sourcing and identifying the plants used.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ahmed ZS, Abozed SS, Abd El-Kader AE (2014). Extraction Optimization and Quality Characterization of Traditionally Prepared *Hibiscus sabdariffa* Beverage Using Response Surface Methodology. World Journal of Dairy and Food Sciences 9:154-165.

Arima H, Ashida H, Danno GI (2002). Rutin-enhanced antibacterial activities of flavonoids against *Bacillus cereus* and *Salmonella enteritidis*. Bioscience, Biotechnology, and Biochemistry 66(5):1009-1014.

Avinash DK, Waman SN (2014). Phytochemical Constituents of Leaves of *Celastrus paniculatus* Wild: Endangered Medicinal Plant. International Journal of Pharmacognosy and Phytochemical Research 6:792-794.

De Wet H, Ngubane SC (2014). Traditional herbal remedies used by women in a rural community in northern Maputaland (South Africa) for the treatment of gynaecology and obstetric complaints. S. South African Journal of Botany 94:29-139.

Dzoyem JP, Melong R, Tsamo AT, Tchinda AT, Kapche DG, Ngadjui BT, McGaw LJ, Eloff JN (2017). Cytotoxicity, antimicrobial and antioxidant activity of eight compounds isolated from *Entada abyssinica* (Fabaceae). BMC Research Notes 10(1):118.

Harper SH, Kemp AD, Underwood WGE, Campbell RVM (1969). Pterocarpanoid constituents of the heartwoods of *Pericopsis angolensis* and *Swartzia madagascariensis*. Journal of the Chemical Society 8:1109-1116.

Magassouba FB, Diallo A, Kouyate M, Mara F, Mara O, Bangoura O, Camara A, Traore S, Diallo AK, Zaoro M, Lamah K (2007). Ethnobotanical survey and antibacterial activity of some plants used in Guinean traditional medicine. Journal of Ethnopharmacology 114(1):44-53.

Maroyi A (2016). *Ximenia caffra* Sond. (Ximeniaceae) in sub-Saharan Africa: A synthesis and review of its medicinal potential. Journal of Ethnopharmacology 184:81-100.

Muhamad II, Hassan ND, Mamat SN, Nawi NM, Rashid WA, Tan NA (2017). Extraction technologies and solvents of phytocompounds from plant materials: Physicochemical characterization and identification of ingredients and bioactive compounds from plant extract using various instrumentations. In Ingredients Extraction by Physicochemical Methods in Food pp. 523-560.

Ngarivhume T, van Klooster CI, de Jong JT, Van der Westhuizen JH (2015). Medicinal plants used by traditional healers for the treatment of malaria in the Chipinge district in Zimbabwe. Journal of Ethnopharmacology 159:224-237

Njume C, Jide AA, Ndip RN (2011). Aqueous and organic solvent-extracts of selected South African medicinal plants possess antimicrobial activity against drug-resistant strains of *Helicobacter pylori*: inhibitory and bactericidal potential. International Journal of Molecular Sciences 12(9):5652-5665.

Odunmbaku LA, Sobowale SS, Adenekan MK, Oloyede T, Adebiyi JA, Adebo OA (2018). Influence of steeping duration, drying temperature, and duration on the chemical composition of sorghum starch. Food Science and Nutrition 6(2):348-355.

Palombo EA (2011).Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. ECAM.

Patwardhan B, Warude D, Pushpangadan P, Bhatt N (2005). Ayurveda and traditional Chinese medicine: a comparative overview. ECAM. 2(4):465-473.

Pilon AC, Carnevale Neto F, Freire RT, Cardoso P, Carneiro RL, Da Silva Bolzani V, Castro-Gamboa I (2016). Partial least squares model and design of experiments toward the analysis of the metabolome of Jatropha gossypifolia leaves: extraction and chromatographic fingerprint optimization. Journal of separation science 39(6):1023-1030.

Podolak I, Galanty A, Sobolewska D (2010). Saponins as cytotoxic agents: a review. Phytochemistry Reviews 9(3):425-74.

Semenya SS, Maroyi A (2013). Medicinal plants used for the treatment of tuberculosis by Bapedi traditional healers in three districts of the Limpopo Province, South Africa. African Journal of Traditional, Complementary and Alternative Medicines 10(2):316-323.

Smith E (2007). Plant secondary metabolites: occurrence, structure and

role in the human diet. Phytotherapy Research 21(9):904-904.
Soman S, Ray JG (2016). Silver nanoparticles synthesized using aqueous leaf extract of *Ziziphus oenoplia* (L.) Mill: characterization and assessment of antibacterial activity. Journal of Photochemistry and Photobiology 163:391-402.
Wachtel-Galor S (2004). Herbal medicine. In Herbal and Traditional Medicine. CRC Press pp. 78-89.
Wang S, Yao J, Zhou B, Yang J, Chaudry MT, Wang M, Xiao F, Li Y, Yin W (2017). Bacteriostatic effect of Quercetin as an antibiotic alternative *in vivo* and its antibacterial mechanism *in vitro.* Journal of Food Protection 81(1):68-78.
Yung OH, Maskat MY, Mustapha WAW (2010). Effect of Extraction on Polyphenol Content, Antioxidant Activity and pH in Pegaga (*Centella asiatica*) Extract. Sains Malaysiana 39(5):747-752.
Zhang QW, Lin LG, Ye WC (2018). Techniques for extraction and isolation of natural products: A comprehensive review. Chinese Medicine 13(1):20.
Zohra SF, Meriem B, Samira S, Muneer MA (2012). Phytochemical screening and identification of some compounds from mallow. Journal of Natural Product and Plant Resources 2(4):512-516.

APPENDIX A

Analysis of variance: ZOI at highest concentration of extracts used (*100 mg/ml*)

The inhibitory activities of the extracts obtained using the different extraction methods (traditional African hot water extraction - LHWE, cold water extraction with concentration - CWED and ethanolic extraction with concentration - EED) were analysed using One Way Analysis of Variance (ANOVA) statistical tool. Table A1 shows that there was no significant difference in zones of inhibition at 100mg/ml of each extract ($p > 0.005$).However, the LHWE method was shown to yield extracts with greater antimicrobial activities than the other two methods in (lowest MIC values and higher ZOI against *Shigella* spp. and *S. typhi* per concentration used) with different plants. Notably, the LHWE technique yielded extracts with no antimicrobial activities against *E. coli.* The CWED and EED methods yielded greater antimicrobial activities against the bacteria as follows: (i) CWED with *P. angolensis* and *S. madagascariensis* bark extracts, and (ii) EED with: *S. madagascariensis* bark extract.

Table A1. Analysis of Variance of antimicrobial activities (zone of inhibition) of extracts from extraction methods (traditional African hot water extraction - LHWE, cold water extraction with concentration - CWED and ethanolic extraction with concentration - EED) against *E. coli, S. typhi* and *Shigella* spp.

Microorganism	Pair of extracts compared	F value	P value	Comment
Shigella spp.	*S. madagascariensis* bark (CWED vs LHWE)	294	< 0.0001	Significance (CWED > LHWE)
	S. madagascariensis bark (CWED vs EED)	54	0.002	Significance (CWED > EED)
	S. madagascariensis bark (EED vs LHWE)	96	0.0006	Significance (EED >LHWE)
	S. madagascariensis leaf (CWED vs LHWE)	37.5	0.004	Significance (CWED > LHWE)
	S. madagascariensis leaf (CWED vs EED)	1.5	0.30	No significance (CWED vs EED)
	S. madagascariensis leaf (LHWE vs EED)	24	0.008	Significance (EED > LHWE)
	P. angolensis bark (CWED vs LHWE)	73.5	0.001	Significance (LHWE > CWED)
	P. angolensis bark (CWED vs EED)	13.5	0.02	Significance (CWED > EED)
	P. angolensis bark (LHWE vs EED)	150	0.0003	Significance (LHWE > CWED)
S. typhi	*S. madagascariensis* bark (CWED vs LHWE)	294	< 0.0001	Significance (LHWE > CWED)
	S. madagascariensis bark (CWED vs EED)	96	0.0006	Significance (EED > CWED)
	S. madagascariensis bark (EED vs LHWE)	54	0.002	Significance (LHWE > EED)
	S. madagascariensis leaf (CWED vs LHWE)	216	0.0001	Significance (LHWE > CWED)
	S. madagascariensis leaf (CWED vs EED)	6	0.07	No significance (CWED vs EED)
	S. madagascariensis leaf (LHWE vs EED)	150	0.0003	Significance (LHWE > EED)
	P. angolensis bark (CWED vs LHWE)	54	0.002	Significance (LHWE > CWED)
	P. angolensis bark (CWED vs EED)	13.5	0.02	Significance (CWED > EED)
	P. angolensis bark (LHWE vs EED)	121.5	0.0004	Significance (LHWE > EED)
E. coli	*S. madagascariensis* bark (CWED vs EED)	13.5	0.02	Significance (EED > CWED)
	S. madagascariensis leaf (CWED vs EED)	726	< 0.0001	Significance (CWED > EED)
	P. angolensis bark (CWED vs EED)	1.5	0.290	No significance (CWED vs EED)

Vol. 18(29), pp. 845-856, September, 2019
DOI: 10.5897/AJB2019.16856
Article Number: C196E6461893
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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Extreme resistance to late blight disease by transferring 3 *R* genes from wild relatives into African farmer-preferred potato varieties

Ethel N. Webi[1,2], Kariuki Daniel[2], Kinyua Johnson[2], Njoroge Anne[1], Ghislain Marc[1] and Magembe Eric[1*]

[1]International Potato Center, Biosciences East and Central Africa (BecA-ILRI hub), P. O. Box 25171-00603 Nairobi, Kenya.
[2]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200 Nairobi, Kenya.

Received 13 May 2019; Accepted 29 August 2019

Three late blight resistance (*R*) genes from wild potato species were transferred as a stack into the farmer-preferred varieties 'Tigoni' and 'Shangi'. Transgenic events were tested by detached-leaf assays (DLA) and whole-plant assays (WPA) with isolates of *Phytophthora infestans* using 20,000 sporangia / mL inoculum. For the first DLA, 9 out of 13 'Tigoni' and 10 out of 12 'Shangi' transgenic events had scores below 3% of leaf affected area. 17 of the 19 transgenic events with low scores were subjected to a second DLA using two different *P. infestans* isolates. 8 of them exhibited extreme LB resistance while the remaining 9 transgenic events showed hypersensitive response (HR). 6 transgenic events (2 'Tigoni' and 4 'Shangi') with extreme resistance by DLA were subjected to WPA and they all exhibited extreme resistance to LB. Hence, this study demonstrates that a simple DLA predicts high level of resistance to late blight. *R* gene expression analysis in 18 transgenic events showed different transgenic events exhibiting different levels of expression in the three genes. However, that pathogen induction and / or high *R* gene expression are necessary for extreme resistance when transgenic events bear a stack of three *R* genes was not observed.

Key words: Detached-leaf assay, late blight, *Phytophthora infestans*, resistance.

INTRODUCTION

Agriculture is the mainstay of African nations where 48% of the total African population and almost 70% in East Africa rely on agriculture for their livelihood (NEPAD, 2013). Therefore, the African Union (AU) chose to make agriculture one of the pillars of the New Partnership for African Development (NEPAD). It is geared towards contribution to development goals such as poverty eradication through improved crop production, job creation and boosting Africa trade and investments. Potato (*Solanum tuberosum* L.), being the world's fourth

*Corresponding author. E-mail: e.magembe@cgiar.org.

largest crop after maize, wheat and rice is universally significant (IYP, 2008). The crop is cultivated in tropical and subtropical regions of the world and can grow from sea level up to over 4,000 m above sea level (Wurster, 1977). Production and consumption have increased significantly in recent years mainly through expansion of cultivated areas (Devaux et al., 2014).

Processing of potatoes into food products has become increasingly important in African urban areas. Therefore, potato is a significant crop in Africa and its year-round production a common feature in many African countries which gives it a great potential for providing food security. At present, developing countries produce more potatoes than developed countries. In Sub-Saharan Africa, India and China, production continues to rise with the total production being close to 250 million tons up from 35 million tons in 1960 (Haverkort and Struik, 2015). In Kenya, potato is the second most important food and cash crop mainly grown by small-scale farmers (Laititi, 2014; Muthoni et al., 2013). It provides nutritional benefits such as vitamin C and provides employment for more than 2.5 million people (Kaguongo et al., 2013). Varieties available in Kenya include 'Shangi', 'Tigoni', 'Asante', 'Ambition', 'Rudolf', 'Kenya Mavuno' among others. The first two are some of the most preferred varieties in Kenya, due to their, taste, versatile use, short dormancy period and fast maturity of approximately three months (NPCK, 2015).

Late blight (LB) caused by the oomycete *Phytophthora infestans*, is the most devastating disease of potato in the world. It is a major biotic constraint to production and has been observed by farmers as a problem that causes heavy economic losses that can be as high as 90% if the disease strikes early in the growing season (Ssengooba and Hakiza, 1999). *P. infestans* is an economically important pathogen that was responsible for the Irish potato famine in 1840 which led to the death of 1 million people and the migration of 1.5 million others (Schumann and D'Arcy, 2005). Loss of potatoes due to LB in the world has been estimated at 5.2 billion euros per annum (Haverkort et al., 2009). Susceptible potato varieties require fungicide application as the only option to control the disease. However, the cost of fungicide, machinery and labor are unaffordable to resource poor farmers. Furthermore, fungicide resistance is on the rise and the population structure of *P. infestans* in East Africa is changing (Njoroge et al., 2016).

LB infection can be delayed by the use of certified seeds and through crop rotation (Hannukkala et al., 2007); but the most cost-effective way to control any plant disease is by host resistance since this offers prevention from the disease. Thus, conventional breeding has long been pursued for the development of host resistance. However, it takes several decades to obtain resistant cultivars from the first cross with resistant wild species and these varieties still suffer from linkage drag of undesirable alleles from wild relatives (Haverkort et al., 2009). Resistant cultivars often use single *R* gene-based resistance which is rapidly overcome by virulent *P. infestans* strains that have a compatible interaction with the resistant plants (Vleeshouwers et al., 2000).

Several *R* genes against *P. infestans* originating from a wide variety of wild species have been isolated in the last decade such as *RB* (same as *Rpi-blb1*) and *Rpi-blb2* genes from *Solanum bulbocastanum*, a Mexican wild species, and *Rpi-vnt1.1* from *S. venturii*, an Argentinean wild species. The latter has three similar allelic variants; *Rpi-vnt1.1, Rpi-vnt1.2* and *Rpi-vnt1.3*. These *R* genes confer extreme resistance to *P. infestans* but have not been extensively used in potato breeding due to the time lag to produce acceptable varieties and the short-lasting resistance mediated by single *R* genes (Vleeshouwers et al., 2011). Consequently, no virulent strains of *P. infestans* have been selected which make these genes likely to confer a longer lasting resistance than those previously introduced into potato varieties such as those from *S. demissum*.

Numerous *R* genes have been cloned and can be stacked in existing potato varieties which would result in new LB resistant varieties, in a much shorter time with longer lasting resistance and no linkage drag. This strategy has been explored and achieved extreme resistance to late blight disease (Zhu et al., 2012; Ghislain et al., 2018). Interestingly, Haesaert et al*.,* (2015) showed that by stacking several *R* genes (*Rpi-sto1, Rpi-vnt1.1* and *Rpi-blb3*), complete resistance to *P. infestans* was achieved in the field whereas 'Desiree' transgenic events transformed with single *R* genes only achieved partial resistance. Though their study covered only 2 to 3 years, plants with stacked *R* genes appeared more stable than the single *R* gene plants which got infected with virulent isolates during their field experiments.

Each of the three *R* genes, belonging to the nucleotide-binding site (NBS) and leucine rich repeats (LRR) domains, expresses proteins that recognize *P. infestans* AVR-BLB1, AVR-BLB2 and AVR-VNT1 effectors, activating the extreme resistance known as effector-triggered immunity (ETI; Oh et al., 2009). The ETI is characterized by a hypersensitive response (HR) and associated disease-resistance responses (Oh et al., 2014). The 2A_1 clonal lineage of *P. infestans* is the dominant genotype in Kenya that has completely displaced the old US-1 lineage on potato (Njoroge et al., 2016). 2A_1 is also rapidly spreading in the other east African countries and was also the only clonal lineage used for bioassays in this study. This *P. infestans* lineage expresses all the three corresponding AVR-BLB1, AVR-BLB2 and AVR-VNT1 effectors in the field and therefore will not be able to overcome quickly these 3*R* transgenic varieties (Ghislain et al., 2018).

In this study, 3 *R* genes was introduced as a stack made of genes from wild relatives of *S. tuberosum* (*RB* and *Rpi-blb2* from *S. bulbocastanum* and *Rpi-vnt1.1* from

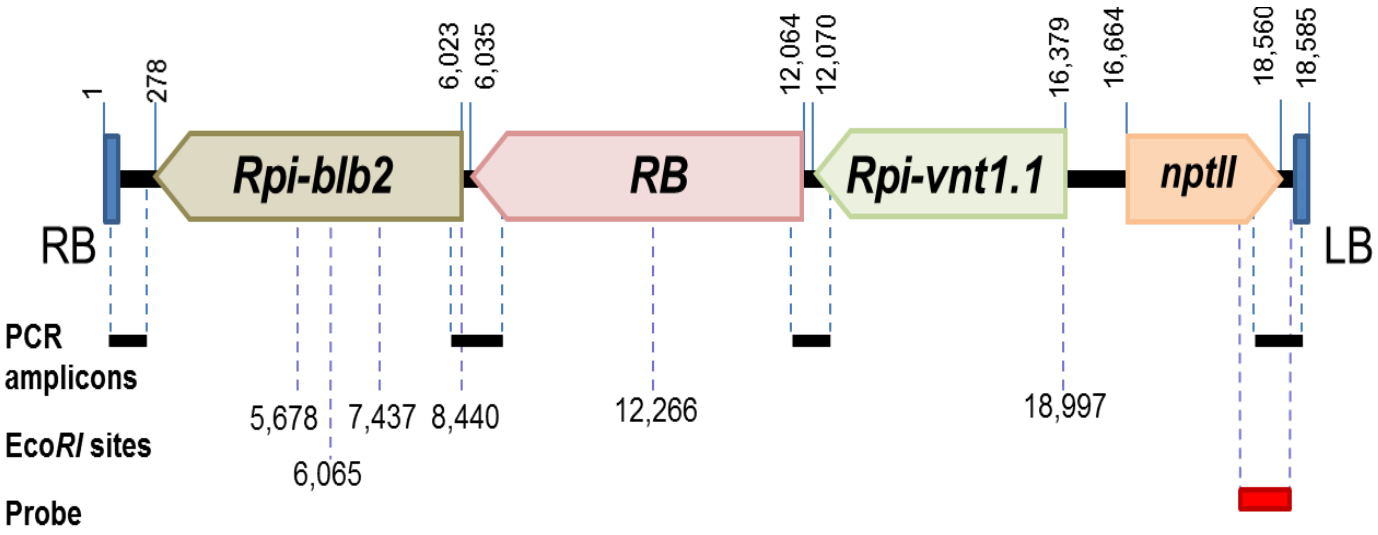

Figure 1. T-DNA carrying the 3 *R* genes (*Rpi-blb2*, *RB*, and *Rpi-vnt1.1*) and the selectable marker gene (*nptII*) used for transformation of potato. The T-DNA is a 18,585 bp long insert of the potato binary vector pCIP99 (24,819bp). PCR amplicons used to determine completeness of the inserted T-DNA are indicated by black lines. Positions of the *Eco*RI sites are indicated by dotted lines and numbers written below. The *nptII* probe which has been used for Southern blotting is indicated in red.

S. venturii) into African farmer-preferred potato varieties 'Tigoni' and 'Shangi'. Detached-leaf assay versus whole-plant leaf assay was tested to predict the level of resistance to late blight. Finally, the gene expression of each of the three *R* genes in transgenic events, from both varieties was analyzed.

MATERIALS AND METHODS

Construction of the 3*R* potato gene construct

The pCIP99 gene construct is described elsewhere (Ghislain et al., 2018). Briefly, the backbone vector is the pCAMBIA2300 plant transformation binary vector (vector provided by CAMBIA Australia) in which from the left border to the right border, the *nptII* gene in transcription orientation was inserted toward the left border and the three *R* genes in transcription orientation towards the right border (*Rpi-vnt1.1*, *RB* and then *Rpi-blb2*). The full size of the T-DNA is 18,585 bp whereas the resulting plant transformation vector pCIP99 is 24,819 bp long (Figure 1). The vector pCIP99 was then transferred by electroporation into the *Agrobacterium tumefaciens* hypervirulent strain EHA105 (Hood et al., 1993).

Agrobacterium-mediated transformation of 'Tigoni' and 'Shangi' varieties

Internodal explants from 4-week-old plantlets were used for transformation using previously published protocol with minor modifications (Cuellar et al., 2006). The *Agrobacterium tumefaciens* EHA105 strain bearing pCIP99 was incubated for 15 h at 28°C in LB semi-solid medium containing 100 mg/L kanamycin. A single colony was taken with a scalpel and then used to cut internodal explants of potato plantlets of the 'Tigoni' and 'Shangi' varieties. The infected internodes were placed on co-culture medium and subsequently transferred to regeneration medium (Cuellar et al., 2006) containing 50 mg/L kanamycin and 200 mg/L carbenicillin. To avoid false kanamycin resistant regenerants, calli were induced from leaf segments of the putative transgenic plantlets and cultured for 4 weeks on a highly selective medium (4.3 g/L salt Murashige and Skoog, 20 g/L sucrose, 20 g/L mannitol, 0.5 g/L 2-(*N*-Morpholino) ethanesulfonic acid, 0.5 g/L polyvinylpyrrolidone, 0.2 g/L glutamine, 0.04 g/L adenine sulfate, 1 ml/L of vitamins GAP, 1 mg/L naphthalene acetic acid, 0.1 mg/L 6-Bencyl-aminopurine, 200 mg/L kanamycin, and 2 g/L Gelrite, pH 5.6) under *in vitro* propagation conditions. Plantlets that did not form calli were deemed false kanamycin resistant regenerants and discarded.

Molecular characterization by PCR

Genomic DNA was extracted from putative transformed plants using a phenol-chloroform extraction method (Gawel and Jarret, 1991). To confirm the presence of the T-DNA in putative 'Tigoni' and 'Shangi' events, primers overlapping the region between *RB* - *Rpi-blb2* and *RB* - *Rpi-vnt1.1,* and primers overlapping the left and right border regions flanking the T-DNA insert, were designed and used for PCR amplification (Table 1). Conventional PCR was done in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) under the following conditions: 94˚C for 5 min followed by 40 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 1 min then 72˚C for 10 min and 10˚C hold.

Copy number analysis by Southern blot

Genomic DNA was extracted using a modified protocol of Gawel and Jarret (1991). 40 µg of the DNA was digested using *Eco*RI enzyme (20,000 U/ml *Eco*RI-HF™ NEB#R3101S) with 14 µL CutSmart Buffer 10X (NEB #B7204S) and 80 µL nuclease-free water to a final reaction volume of 200 µL. The DNA was digested overnight at 37°C and then separated in 0.8% agarose for 4 h at 55 V/20 cm. DNA was transferred to a positively charged nylon membrane (Roche) overnight by capillary method. Non-transgenic genomic DNA restricted by EcoRI was used as negative control and a transgenic event (Vic.1) by Ghislain et al. (2018) previously

Table 1. Primer sequences for molecular characterization of transgenic events.

Target region	Primer name	Primer sequence	Tm (°C)	Expected size (bp)
RB-Rpi-Vnt1.1 Overlap	RB-Vnt1F	AAAAAGAAGATTTCATGCGC	55	363
	RB-Vnt1R	CATTTTGGTTCATGAGTTCA	55	
RB-Rpi-Blb2 Overlap	RB-blb2F	GCTGCGTTAATTATTTACAT	55	587
	RB-blb2R	GTTGGTTGATTACTTGAACT	55	
nptII gene	35SNPTIIF	TCCTGTCATCTCACCTTGCTCC	60	400
	35SNPTIIR	ACGATTCCGAAGCCCAAACC	60	
Left Border	GSPc_LB	TCTCCATAATAATGTGTGAGTAGTTCCC	67	250
	LB_R	CAGCTTAGTTGCCGTTCTTCCG	67	
Right Border	GSPb_RB	ACCGATCGCCCTTCCCAACAGTTG	67	250
	RB_R	CACTATAGCAGCGGAGGGGTTG	67	
RB gene transcript	qRT-RB-F	CACGAGTGCCCTTTTCTGAC	60	132
	qRT-RB-R	ACAATTGAATTTTTAGACTT	60	
Rpi-blb2 gene transcript	qRT-Rpi-blb2-F	TTCAAAACCCCAAATAAGTTTCAAC	60	214
	qRT-Rpi-blb2-R	CCATGCTTGCTGTACTTTGCA	60	
Rpi-vnt1.1 gene transcript	qRT-Rpi-vnt1.1-F	GGTAAGGTATTGGCTCTG	60	81
	qRT-Rpi-vnt1.1-R	CTTCTCAGCAATCCACATA	60	
β-*tubulin* gene transcript	β-tubulin F	ATGTTCAGGCGCAAGGCTT	60	104
	β-tubulin R	TCTGCAACCGGGTCATTCAT	60	

proven to be single copy was used as a positive control. A Digoxigenin (Roche) labelled probe was made according to the manufacturer's instructions.

P. infestans isolate collection and purification

P. infestans isolates used in this study were collected from farmers' fields in major potato growing regions in Kenya including Kiambu (Limuru and Muguga), Molo (Elburgon, Turi and Kapsita) and Meru (Timau, and Kibirichia). Single lesion leaflets from different farms were collected during the potato-growing season of June to July 2016. The leaflets were taken to the laboratory at BecA/ILRI hub in Kenya for pathogen isolation. The diseased leaflets were washed under running tap water, tapped dry on paper towels and then incubated overnight at 18˚C in humid chambers (Petri dishes with moist paper) to induce sporulation of *P. infestans.* To obtain axenic cultures of the pathogen, fresh potato tubers of the susceptible variety Asante were cleaned under running tap water and disinfected in 10% bleach (commercially referred to as Jik; Reckitt Benckiser®) for ten minutes. A sterilized pocket knife was used to cut potato tuber slices of ~1 cm thickness in a laminar flow hood. Approximately 10 mm of the sporulating leaflets were cut and placed beneath the potato tuber slices in clean petri dishes. The petri dishes were sealed with Parafilm and kept at 18°C until mycelia grew through the tuber slices. Mycelia were then transferred to modified V8 agar media made as follows. 120 g of ripened tomatoes, 200 g of fresh carrots and 4 g of clean and young potato leaves were cut to small pieces and blended in 1 L distilled water. The mixture was sieved to obtain the filtrate that was measured to 200 mL in a glass measuring cylinder. The 200-mL filtrate was then mixed with 15 g of technical agar (Oxoid Agar No. 3™ LP0013), 3 g of calcium carbonate (Uni-Chem Calcium Carbonate powder) and 50 mg of β-sitosterol (Sigma). The mixture was topped up to 1 L and autoclaved at 121˚C for 15 min. After cooling, the media was dispensed into petri dishes and *P. infestans* isolates were maintained in culture. The cultures were observed under a microscope at a total magnification of 40X (4X * 10X) to confirm that the isolates were *P. infestans* based on morphological descriptors. This was done by observing the presence of the characteristic lemon shaped sporangium that is found in *P. infestans* and the aseptate tubular hyphae that are found in oomycetes. All the isolates selected for detached-leaf assay had the *P. infestans* characteristics.

Inoculum preparation

Inoculum was made by adding 5 mL of distilled water to the culture plates and gently scraping off mycelia using sterile blades. The mycelial fragments were then transferred to clean Falcon® tubes containing 15 mL distilled water, vortexed for about 5 min and then sieved through four layers of cheesecloth. The resulting sporangia suspension was quantified using a haemocytometer and afterwards standardized to a concentration of 20,000 sporangia/mL. The sporangia suspensions were incubated at 4˚C for 3 to 4 h to induce the release of zoospores. Presence of viable zoospores was confirmed under the microscope before the inoculations were performed.

Detached-leaf assays

Detached-leaf assays were done using the method described by Knapova and Gisi (2002). Leaf materials of uniform size from 'Tigoni' and 'Shangi' transgenic events and non-transgenic varieties were obtained from glasshouse grown plants at 45 days after planting. These were cleaned with distilled water and placed abaxial side up in 9 cm petri dishes containing moistened paper towels. Three isolates from Kiambu, Molo and Meru labelled, isolate 1 (Latitude 1˚10'52.08949" S; Longitude 36˚38'39.49569" E Elevation 2,142 m), isolate 2 (S 00'15'22.3", E 035'42'58.1" Elevation 2,533 m) and isolate 3 (S 00'05'11.3", E 037'35'43.6" Elevation 1,904 m) respectively were selected for the initial detached-leaf assay (DLA). For each isolate, five Petri dishes containing a single leaflet per potato event were used. Four leaflets

per event were inoculated at the midrib with 50 µL droplet of the 20,000 sporangia/mL suspension, while the fifth leaflet was inoculated with 50 µL droplet of sterile distilled water as a check leaflet. After inoculation, each set of five Petri dishes per isolate for each event was placed in transparent plastic bags and sealed to maintain humidity. The Petri dishes were then incubated at 18˚C for 7 days with a 12 h photoperiod and 80% relative humidity. The leaf affected area (LAA) was assessed visually on the 7th day and was defined as the necrotic plus green area covered with sporangia. The size of the affected area was measured using a Vernier digital caliper. This experiment was repeated once for events that exhibited low or no infections. The repetition was done to verify that indeed the resistant events from the first assay would hold their resistance when inoculated with different isolates. The second assay was done using isolates from Kiambu and Meru labelled isolate 4 (Latitude 1˚10'52.08949" S; Longitude 36˚38'39.49569" E Elevation 2,142 m) and 5 (S 00'05'11.3", E 037'35'43.6" Elevation 1,904 m) respectively. The length and width of the LAA of individual leaflets was measured in millimeters and used to calculate the average percentage of the leaf affected area using the formula (π*a*b) which calculates the area of an ellipsoid. π is the mathematical constant '*pi*', 'a' is the radius of the length and 'b' is the radius of the width. Analysis of variance (ANOVA) was performed to measure the variation in resistance of the potato events to *P. infestans* isolates. The resistance scale of 0 to 9 (0 being the most resistant and 9 being the most susceptible) of Yuen and Forbes, 2009, was used as follows:

*Standard scale= (Severity of test plant/Severity of control plant) *9*

The average of the two experiments was used to determine the resistance scale values since the experiment was repeated once.

Whole-plant assays

These assays were done according to the method adapted from Sharma et al*.,* (2013). Transgenic events were grown from first generation tuber seeds in the glasshouse with three replicates for each event and a non-transgenic control for each. At six weeks, the plants were moved to a controlled environment (CONVIRON, Argus controls) chamber prior to inoculation. The inoculum was made and quantified as previously described but consisted of mixed sporangia of five isolates from Muguga and Meru at a final concentration of 20,000 sporangia/mL. Inoculations were performed by spraying the test plants with the sporangial suspensions using a hand-held sprayer until all the leaves were wet. The plants were covered with a transparent plastic bag until 24 h after inoculation to maintain high humidity and enhance the infection process. The Conviron chamber was maintained at a relative humidity of between 80 and 100% with a 12 h photoperiod and temperatures of 18°C for 10 days. Plants were misted once daily with sterile distilled water and were monitored every day until the experiment was terminated. The severity of infection was recorded as a percentage of foliage area that was affected 10 days post inoculation (dpi). The same resistance scale used for the DLA was used to assign resistant score values in this WPA.

R gene expression

Transcript level of the *Rpi-vnt1.1*, *RB*, and *Rpi-blb2* genes in the transgenic events was done from infected plants by RTqPCR. The analysis was done at two-time points (1 day before inoculation referred to 'Day 0' and 3 days after inoculation referred to 'Day 3'). RNA was extracted using the RNeasy plant minikit (Qiagen) from the infected leaves which had been frozen with liquid nitrogen and stored at -80°C. Three repetitions of samples and RNA extractions (biological replicates) per treatment were done. The extracted RNA concentration was estimated by spectrophotometry using a NanoDrop Micro Photometer (Thermo Scientific). cDNA was synthesized using the *AccuPower*® CycleScript RT kit (Bioneer) following the manufacturer´s protocol. To assess the relative expression values of each of the 3*R* genes, qPCR reactions were set up using Luna® Universal Probe qPCR Master Mix with gene specific primer sets (Table 1) and qRT-Rpi-vnt1.1-F and qRT-Rpi-vnt1.1-R for *Rpi-vnt1.1* gene (Roman et al., 2017), qRT-RB-F and qRT-RB-R for *RB* gene (Kramer et al., 2009), qRT-Rpi-blb2- and qRT-Rpi-blb2-R for *Rpi-blb2* gene (EPA 2006). The RTqPCR was normalized to β-tubulin housekeeping gene with primer β-tubulin F and β-tubulin R (Kramer et al., 2009) (Table 1). All reactions were prepared in a total volume of 10 µl using Luna® Universal qPCR Master Mix (New England Biolabs) as follows: 5 µl of Luna® qPCR master mix, 0.5 µl of each primer (2.5 µM), 1 µL of cDNA and 3 µl of nuclease free water. The RTqPCR was done as follows; initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C or 15 s and 60°C for 1 min. The melting curve was run at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s. Data was analyzed using DataAssist^{TM} Software (Applied Biosystems).

RESULTS

Agrobacterium-mediated genetic transformation

3,000 internodes from both 'Tigoni' and 'Shangi' varieties were *agro*-infected. 282 putative transformed shoots was regenerated for 'Tigoni', of which 76 were confirmed to be highly resistant to kanamycin which represents a regeneration efficiency of 9.4%. In the case of 'Shangi', we regenerated 328 putative transformed shoots, of which 108 were confirmed to be highly resistant to kanamycin representing a regeneration efficiency of 10.9%. PCR was performed on 76 'Tigoni' and 108 'Shangi' putative events. 32 'Tigoni'' and 31 'Shangi' events were confirmed to be positive for the *nptII* gene which represents a transformation efficiency of 1.1% for 'Tigoni' and 1% for 'Shangi'. This transformation efficiency was lower than that reported by Orbegozo et al., (2016) of approximately 6% and Roman et al., (2017) at 7.5% for the Desiree variety transformed with *Rpi-blb2* and *Rpi-vnt1.1* single gene construct. However, the efficiency was comparable to that reported by Ghislain et al. (2018) at 1.5% for Victoria variety transformed with the same 3*R*-gene construct.

Molecular characterization of transgenic plants

PCR with overlapping primers to detect all the 3 *R* genes resulted in 22 'Tigoni' and 13 'Shangi' positive events. These transgenic events were further screened for the absence of vector backbone sequences by PCR. 13 'Tigoni' and 12 'Shangi' events did not have vector backbone sequences. Southern blot analysis was done on these 25 transgenic events to determine the transgene copy numbers using a 35S-*nptII* gene specific probe. 5 'Tigoni' and 5 'Shangi' (40%) events had single copies of the T-DNA while the rest had multiple copies (Figure 2).

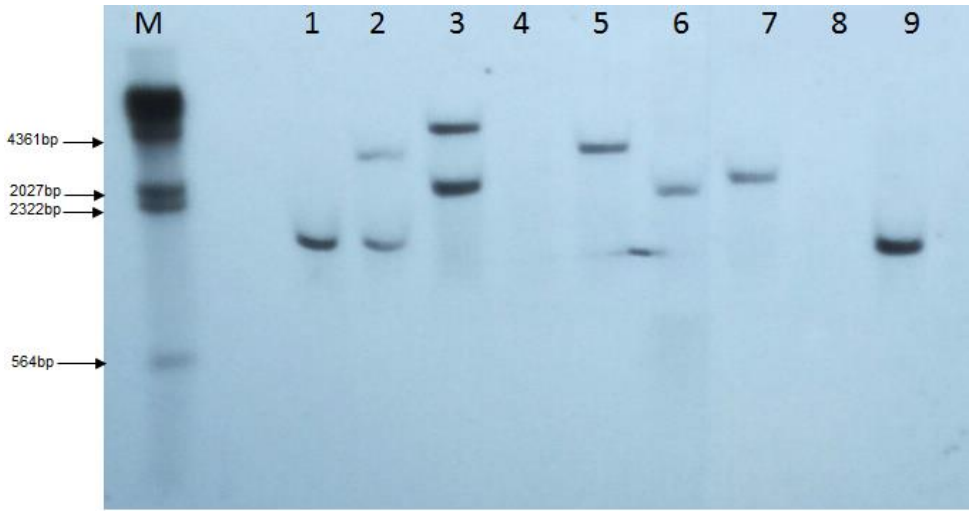

Figure 2. Southern blot analysis of DNA restricted by *Eco*RI using *nptII* gene as a probe from transgenic events from the variety 'Shangi' (sample 1 to 5) and 'Tigoni' (sample 6 to 8), non-transgenic 'Tigoni' variety (sample 8) and Vic.1 (sample 9) transgenic event containing a single copy as positive control. 'M' is the DIG labeled molecular size marker.

Table 2. ANOVA analysis at a *P*-value of 0.05 comparing the variation of individual transgenic events to isolates 1, 2 and 3.

ANOVA: Single factor						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between events[a]	42144.38	23	1832.36	21.75	3.87071E-18	1.76
Within events[b]	4044.33	48	84.26			
Total	46188.71	71				

[a]Differences amongst events as affected by different isolates 1,2 and 3; [b]Differences between events as affected by individual isolates; *P* values less than the alpha value (α=0.05) indicate significant variation while values greater than the alpha value do not.

This result was comparable to the result obtained by Ghislain et al. (2018) where 50% of the events had single T-DNA copies.

Detached-leaf assay to rapidly identify the late blight resistant transgenic events

The 13 'Tigoni' and 12 'Shangi' transgenic events that were positive for the 3*R*-gene stack were evaluated for resistance to *P. infestans* together with their non-transgenic equivalent varieties. Three *P. infestans* isolates were used for the first DLA and two isolates for the second DLA all being from the dominant 2A_1 clonal lineage in Kenya as reported by Njoroge et al., (2016). ANOVA results in Table 2 show how individual transgenic events exhibited resistance to inoculation by *P. infestans* isolates 1, 2 and 3 in the first assay. The output of the results showed highly significant differences ($P \leq 0.05$) amongst the events in the first DLA. Out of 25 events, 19 (76%) exhibited resistance ranging from 0 to 3% of the average leaf affected area by LB at 7 days after inoculation. This number of resistant events was comparable to what was reported by Ghislain et al. (2018) where 75% of transgenic events transformed with this *3R* gene construct were highly resistant to *P. infestans*. The non-transgenic 'Tigoni' and 'Shangi' varieties were completely susceptible. Transgenic events that showed susceptibility with a resistance scale values of 6-9 (Tig.8, Tig.16, Tig.141, Tig.225, and Sha.6) had multiple lesions with heavy sporulation. These events with susceptibility to late blight based on the first DLA results were not included in the second DLA. Results of the first and second assay are represented in Figure 3 where a few events exhibited susceptibility to LB in the first DLA.

The average of the resistance values in the first and second assay was used to calculate resistance values of the events. Of the 25 events tested, 19 exhibited high resistance (Table 3). The remaining five events were susceptible with visible lesions and sporulation. As expected, the two non-transgenic controls were highly

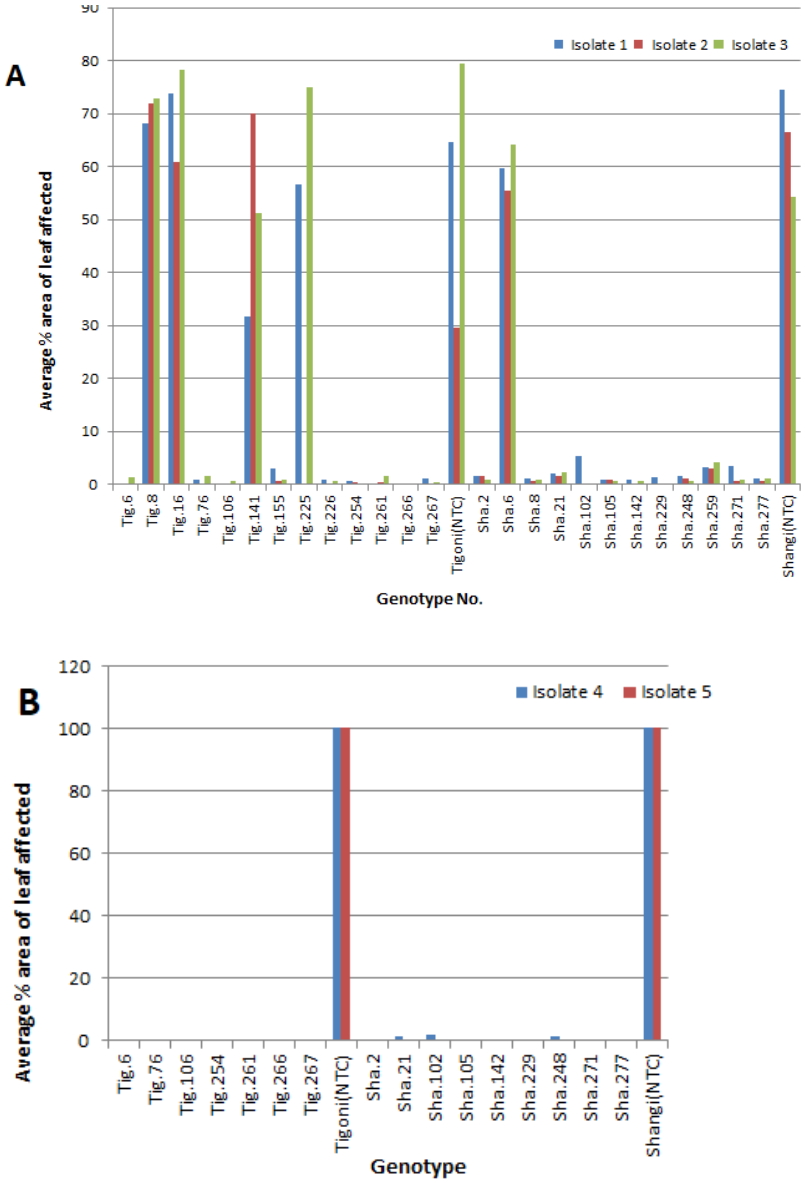

Figure 3. Detached-leaf assay of the high and low lesion transgenic events tested in the first (A) and second (B) assay showing the average percent of leaf affected area by *Phytophthora infestans* isolates 1, 2 and 3 (first DLA) and 4 and 5 (second DLA) for each genotype of 'Tigoni' (Tig), 'Shangi' (Sha) transgenic events and non-transformed controls (NTC). The average percent of leaf affected area was calculated based on the area of the lesions.

susceptible and high sporulation was evident.

Whole-plant assay on DLA late blight resistant transgenic events

Results of the whole-plant assay were comparable to those of the DLA. Six transgenic events (Tig.155, Tig.267, Sha.259, Sha.2, Sha.105, and Sha.248) randomly chosen from the 20 DLA-based resistant transgenic events, were tested in a single WPA and all exhibited complete resistance to LB with no infection (Figure 4). The non-transgenic 'Tigoni' and 'Shangi' varieties developed lesions at 3 days post infection (dpi) and at the end of the assay (10 dpi) the plant foliage had well over 80% infection. These results were like those observed by Ghislain et al. (2018) where transgenic events were all resistant to LB in WPA. This observation extended to the field where transgenic events that exhibited resistance in WPA maintained their resistance and non-transgenic varieties got LB (Ghislain et al., 2018). The percentage of damaged area varied much less among repetitions for

Table 3. Resistance scores of 'Tigoni' and 'Shangi' transgenic events infected by *Phytophthora infestans* isolates in detached-leaf assays on a resistance scale of (0-9); 0 being highly resistant and 9 being highly susceptible.

Genotype[a]	Average % of leaf affected area[b]	Standard scale values[c]	Genotype[a]	Average % of leaf affected area[b]	Standard scale values[c]
	TIGONI			**SHANGI**	
Tig.6	0.29	0.04	Sha.2	0.79	0.09
Tig.8	70.96	8.54	Sha.6	59.73	6.80
Tig.16	70.96	8.54	Sha.8	1.46	0.17
Tig.76	0.48	0.06	Sha.21	1.52	0.17
Tig.106	0.11	0.01	Sha.102	1.94	0.22
Tig.141	51.03	6.14	Sha.105	0.51	0.06
Tig.155	1.45	0.17	Sha.142	0.38	0.04
Tig.225	65.84	7.92	Sha.229	0.39	0.04
Tig.226	0.57	0.07	Sha.248	0.99	0.11
Tig.254	0.28	0.03	Sha.259	3.51	0.40
Tig.261	0.38	0.05	Sha.271	1.14	0.13
Tig.266	0.07	0.01	Sha.277	0.63	0.07
Tig.267	0.30	0.04	Shangi **(NTC)**	79.08	9.00
Tigoni (**NTC**)	74.77	9.00			

[a]Potato genotypes inoculated with *P. infestans;* [b]Average leaf affected area that formed lesion after the 1st and 2nd detached-leaf assays; [c]Values indicating the level of susceptibility/resistance of transgenic potato genotypes to *P. infestans;* Tig - Tigoni, Sha – Shangi and NTC - non-transgenic control.

the WPA than for the DLA and between replications for the non-transgenic varieties. Therefore, the WPA as a more reliable screening method for late blight resistance was considered.

R gene expression of transgenic events

Expression study of *Rpi-vnt1.1*, *RB* and *Rpi-blb2* genes was done by RT-qPCR on 17 transgenic events (9 for 'Tigoni' and 8 for 'Shangi') that were available for RNA extraction and had been tested as highly resistant by detached-leaf assays. β-Tubulin housekeeping gene demonstrated stability in expression and was therefore used for relative comparison to the target genes expression. Different transgenic events showed different patterns of expression at day 0 and day 3. The *Rpi-vnt1.1* gene showed a lower basal expression level compared to β-tubulin at day 0 in 15 out of 18 transgenic events and was always the lowest of the 3 *R* genes. The *RB* gene showed a higher basal level of expression in 13 out of 18 transgenic events compared to β-tubulin and presented for all of them a higher expression 3 days after infection. The *Rpi-blb2* gene showed a higher basal level of expression compared to β-tubulin at day 0 in all transgenic events and was overall the *R* gene with slightly higher expression but with the lowest induced expression than the other 2 *R* genes. Twelve transgenic events showed an increase in expression after inoculation for all the three *R* genes. Five events (Tig.266, Sha.102, Sha.142 and Sha.229) indicated a lower *R* gene expression in comparison to the reference gene expression for at least one of the three *R* genes after inoculation. There was insignificant change in expression levels of the *RB* gene for two transgenic events (Tig.76 and Sha.229). Different levels of expression of the three *R* genes were observed in different transgenic events. In some cases, expression levels were enhanced after inoculation and in other instances expression levels were diminished in one or all the three *R* genes after inoculation (Figure 5).

DISCUSSION

In this study, the ability of a 3*R*-gene stack containing *Rpi-vnt1.1*, *RB*, and *Rpi-blb2* genes was assessed to provide extreme resistance against *P. infestans* in farmer-preferred potato varieties 'Tigoni' and 'Shangi'. These varieties are popular in Kenya but are also grown in neighboring east African countries. The researchers decided to use the 3*R*-gene stack that had been shown to confer extreme resistance to *P. infestans* on the transgenic events from another farmer preferred variety 'Victoria' in Uganda as described by Ghislain et al. (2018). The resistance of 3*R* transgenic events of 'Tigoni' and 'Shangi' was characterized by a total absence of symptoms of LB after detached-leaf and whole-plant assays indicating a successful defense response. It was observed that the detached-leaf assay predicts very well the resistance in the whole-plant assay. In addition, the study demonstrated that the resistant transgenic events

Figure 4. Damaged Shangi non-transgenic control (NTC) and two healthy Shangi transgenic plants (3R) 10 days-post-inoculation with *P. infestans.*

display an increase in expression of at least one of the three *R* genes.

In the *Agrobacterium*-mediated genetic transformation, a transformation efficiency of 1.1% was obtained for 'Tigoni' and 1% for 'Shangi' which was lower compared to those reported previously at 3.4, 2.8, 6 and 7.5% for the variety Desiree (Ghislain et al., 2018; Ahmad et al., 2012; Orbegozo et al., 2016; Roman et al., 2017). However, this was similar to that reported for the variety 'Victoria' at 1.5% (Ghislain et al., 2018). This observation confirms that transformation efficiency is genotype dependent and lower for larger T-DNA (Heeres et al., 2002). A regeneration efficiency of 9.4% was obtained for 'Tigoni' and 10.9% for 'Shangi' which is within the range of those reported previously by Orbegozo et al. (2016) at 9.5% and Roman et al., (2017) at 13% for the 'Desiree' variety transformed with *Rpi-blb2* and *Rpi-vnt1.1* single gene constructs. This does not seem to indicate a significant difference in the regeneration efficiency of these two varieties.

The DLA technique was used for the first time in Kenya to evaluate resistance of potato to *P. infestans* infection in addition to WPA that had previously been used by Ghislain et al. (2018). It is demonstrated herein that detached-leaf bioassays can be used to quickly determine the level of LB resistance in transgenic potato events. For the first DLA, all the events that showed resistance were equally resistant to all three isolates. The resistant transgenic events were tested for the second time using 2 isolates and they similarly showed equal resistance. DLA facilitates handling of many plant genotypes and allows individual genotypes to be challenged with several pathogen isolates. Noteworthy, the first and second assays gave slightly different results in that, the transgenic events exhibited a higher resistance in the second assay. Eight of the transgenic events had no infection (0% of leaf affected area) in the second assay while they exhibited a low but visible % of leaf affected area in the first assay. This difference could be attributed to the decrease in pathogen aggressiveness during the lapse of time between the first and the second assay. While the first assay was performed when the isolates were freshly isolated from the field, the second assay was performed one month after the first DLA raising the possibility that the isolates had reduced aggressiveness. Aggressiveness of isolates is usually reduced when an isolate is frequently cultured in the laboratory, additionally, different isolates differ in aggressiveness, but the differences tend to be smaller in genetically similar populations (Cooke et al., 2006). A slight difference was observed between the WPA and the DLA where the events that had exhibited hypersensitive response in DLA, exhibited extreme resistance in the WPA. This could be attributed to the different physiological states of the plants. It is expected that all the transgenic events that exhibited resistance in the WPA will exhibit extreme resistance in the field since Sharma et al., (2013) and Ghislain et al. (2018) observed that transgenic events that exhibited partial resistance in the glasshouse were completely resistant in the field. This was anticipated due to a lower natural inoculum pressure in the field compared to that used for DLA or WPA at 20,000 sporangia/ml. Additionally, field conditions cannot be controlled where sub-optimal conditions

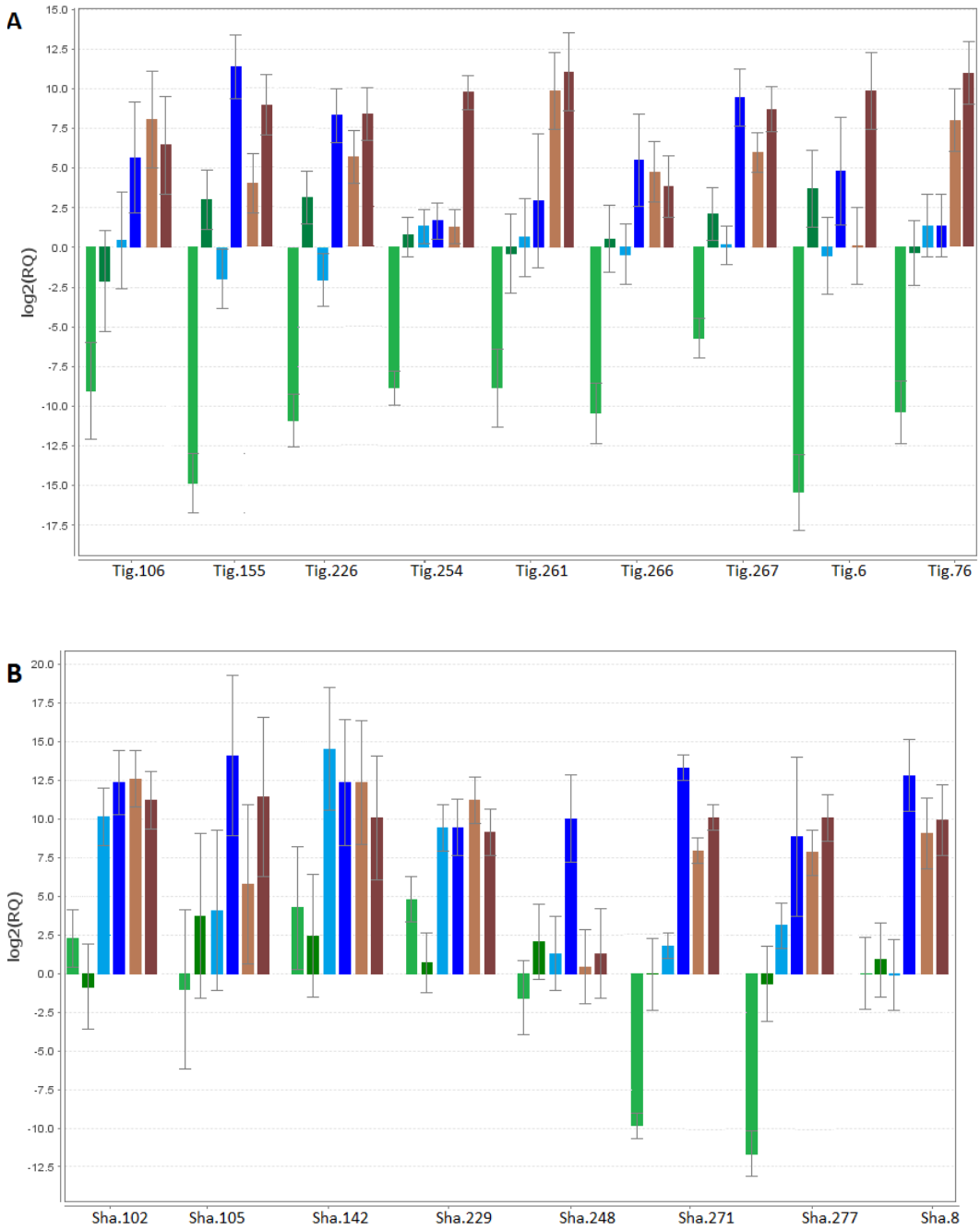

Figure 5. Relative quantification (RQ) plot of expression profiles of each of the *R* genes before *P. infestans* inoculation at day 0 and day 3 after inoculation for highly resistant transgenic events from 'Tigoni' and 'Shangi'. Figure A contains 'Tigoni' transgenic events and B has 'Shangi' transgenic events. Green bars represent *Rpi-vnt1.1* gene, blue bars represent *RB* gene and brown bars represent *Rpi-blb2* gene. Bars of the lighter shade represent day 0 and the darker shade represent day 3.

lead to a lower infection potential. It is therefore evident from this work that DLA is a reliable method to rapidly identify transgenic events that have extreme resistance to *P. infestans*. This reduces the amount of time and resources needed to evaluate resistance of transgenic events.

R gene expression was shown to be induced after inoculation with *P. infestans,* slowly decreasing over the subsequent three days (Kramer et al., 2009; Vleeshouwers et al., 2011; Orbegozo et al., 2016; Roman et al., 2017, Ghislain et al., 2018). In this study, there was significant variation in the expression levels of the three *R*

genes before and after inoculation. Ghislain et al. (2018) reported that there were insignificant differences of gene expression at different time points for the unique highly resistant transgenic event studied. Here, *R* gene expression was assayed on 17 highly resistant transgenic events. Results showed enhanced expression after inoculation in two third of the transgenic events while it reduces slightly in others. This observation appears to be consistent with that observed by Millet et al., (2009) in nwhich *RB* gene transcript abundance varied in different transgenic lines which was attributed to the location in which the *R* gene was introduced.

Five transgenic events that expressed a lower expression than that of the reference gene for at least one of their *R* genes still exhibited late blight resistance after inoculation. Millet et al., (2009, 2015) showed that disease resistance was affected by plant age and cultivar; even though *R* gene expression varied in young plants, they exhibited higher resistance in comparison to older plants and vice versa. In future analysis, screening of the transgenic events at more than two time points could provide more information on expression of the three *R* genes. Additionally, it would be important to do more experiments on *R* gene expression at different physiological stages of transgenic events. The pathogen induction of *R* gene expression behavior has been observed in many other plant species where expression of *R* genes has been enhanced after pathogen challenge (Kramer et al., 2009; Alice and Joy, 2016). However, due to the differences observed in the expression of the *R* genes before and after inoculation, the results do not support that an induction or high level of expression are necessary to obtain highly resistant transgenic events when using a 3*R*-gene stack.

In conclusion, it was demonstrated here that the three *R* genes (*Rpi-vnt1.1, RB* and *Rpi-blb2*) from the wild species *S. bulbocastanum* and *S. venturii* can confer extreme, possibly stable and durable, resistance to LB in the potato varieties 'Tigoni' and 'Shangi' cultivated in east Africa. Out of 13 'Tigoni' and 12 'Shangi' transgenic events, at least 9 transgenic events from each variety that are highly resistant were identified. These events have single T-DNA copy insertions and do not have vector backbone sequence inserted which is also desirable for releasing these transgenic events as varieties. Field evaluations under natural infection by *P. infestans* are needed to confirm the extreme resistance to the local *Pi* population since host-pathogen interactions is always influenced by environmental factors. These extremely late blight resistant transgenic events have the potential to be eventually commercialized for food security and be used in breeding programs to develop other late blight resistance varieties. However, it is yet to be demonstrated that at least one of them has conserved the integrity of all three *R* genes and has not interrupted an allele of an essential gene of the potato. A LB resistant variety will be important to smallholder farmers because farmers will realize increased yields and save on the costs of fungicides while reducing exposure of fungicides, thereby generating more revenue and improving their livelihoods. Genetic control of late blight in potatoes not only provides a strategy to improve food security but also boost economic development in Sub-Saharan Africa where agriculture employs more than half of the total manpower and provides a livelihood for numerous small-scale producers (IMF, 2012). However, social acceptance of biotech or genetically engineered crops in Africa will need to improve, especially in countries where transgenic crops have not yet been introduced and where the regulatory environment is still under development (Chambers et al., 2014).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This research was undertaken as part of the CGIAR Research Program on Roots, Tubers and Bananas (RTB). Funding support for this work was provided by the United States Agency for International Development (USAID), the 2Blades foundation, and the Japan International Cooperation Agency (JICA who funded the field sampling and isolation of *P. infestans*).

REFERENCES

Ahmad ZM, Hussain I, Muhammad A, Ali S, Ali MG, Roomi S, Zia AM, Ijaz A (2012). Factor affecting *Agrobacterium*-mediated transformation of rice chitinase gene in *Solanum tuberosum L*. African Journal of Biotechnology 11(41):9716-9723.

Alice MQ, Joy B (2016). Modulation of *R*-gene expression across environments. Journal of Experimental Botany 67(7): 2093-2105

Chambers JA, Zambrano P, Falck-Zepeda JB, Gruère GP, Sengupta D, Hokanson K (2014). GM agricultural technologies for Africa: a state of affairs. International Food Policy Research Institute.

Cooke D, Lees A, Shaw D, Ruairidh B, Cooke L (2006). *Variation in aggressiveness in Phytophthora infestans* . British potato council. doi: Available at: http://blightmaps.ahdb.org/secure_downloader.php?index_id=91&sec doc_id=410. Accessed March 16, 2017.

Cuellar W, Gaudin A, Solórzano D, Casas A, Ñopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M (2006). Self-excision of the antibiotic resistance gene *nptII* using a heat inducible Cre-loxP system from transgenic potato. Plant molecular biology 62(1-2):71-82.

Devaux A, Kromann P, Ortiz O (2014). Potatoes for sustainable global food security. Potato Research 57(3-4):185-199.

EPA Ireland (2006). Response from BASF to request further information, publicly available download at: https://www.epa.ie/pubs/reports/other/gmo/field/RESPONSE%20to%20Request%20for%20further%20information4thand%205th%20combined_CW.pdf

Gawel NJ, Jarret RI (1991). A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea.* Plant Molecular Biology Reporter 9(3):262-266.

Ghislain M, Arinaitwe AB, Magembe E, Njoroge A, Rivera C, Román

ML, Tovar JC, Gamboa S, Forbes GA, Kreuze JF, Barekye A, Kiggundu A (2018). Stacking three late blight resistance genes from wild species directly into African highland potato varieties confers complete field resistance to local blight races. Plant Biotechnology Journal 17(6):1119-1129 doi: 10.1111/pbi.13042.

Haesaert G, Vossen HJ, Custers R, Loose M, Haverkort A, Heremans B, Hutten R, Kessel G, Landschoot S, Droogenbroeck B, Visser R, Gheysen G (2015). Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions. Crop protection 77:163-175.

Hannukkala AO, Kaukoranta T, Lehtinen A, Rahkonen A (2007). Late blight epidemics on potato in Finland 1933-2002; increased and earlier occurrence of epidemics associated with climate change and lack of rotation. Plant Pathology 56(1):167-176.

Haverkort A, Struik P (2015). Yield levels of potato crops: recent achievements and future prospects. Field Crops Research 182:76-85

Haverkort AJ, Struik PC, Visser RGF, Jacobsen E (2009). Applied Biotechnology to Combat Late Blight in Potato Caused by *Phytophthora Infestans.* Potato Research 52(3):249-264

Heeres P, Schippers-Rozenboom M, Jacobsen E, Visser RG (2002). Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124(1):13-22.

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993). New *Agrobacterium* helper plasmids for gene transfer to plants (EHA105). Transgenic Research 2(4):208-218.

International Monetary Fund (IMF) (2012). International Jobs Report, Economist Intelligence Unit, Washington, DC.

International Year of the Potato (IYP) (2008). The Potato United Nations Food and Agricultural Organization Retrieved 6 August 2015.

Kaguongo W, Nyangweso A, Mutunga J, Nderitu J (2013). A policymakers' Guide to Crop Diversification: The Case of the Potato in Kenya, FAO, Rome, http://www.fao.org/docrep/018/i3329e/i3329e.pdf

Knapova G, Gisi U (2002). Phenotypic and genotypic structure of *Phytophthora infestans* populations on potato and tomato in France and Switzerland. Plant Pathology 51(5):641-653 https://doi.org/10.1046/j.1365-3059.2002.00750.x

Kramer LC, Choudoir MJ, Wielgus SM, Bhaskar PB, Jiang J (2009). Correlation between transcript abundance of the *RB* gene and the level of the *RB*-mediated late blight resistance in potato. Molecular Plant-Microbe Interactions 22(4):447-455.

Laititi M (2014). Potato market survey in Kenya: An agricultural product value chain approach. International Journal of Business and Management Review 2(6):59-87.

Millet BP, Mollov DS, Lorrizo M, Carputo D, Bradeen JM (2009). Changes in disease resistance phenotypes associated with plant physiological age are not caused by variation in *R* gene transcript abundance. Molecular Plant-Microbe Interactions 22(3):362-368.

Millet BP, Gao L, Lorrizo M, Carputo D, Bradeen JM (2015). Potato tuber blight resistance phenotypes correlate with *RB* transgene transcript levels in an age-dependent manner. Phytopathology 105(8):1131-1136.

Muthoni J, Shimelis H, Melis R (2013). Potato production in Kenya: Farming systems and production constraints. Journal of Agricultural Science 5(5):182-197.

National Potato Council of Kenya (NPCK) (2015). Potato Variety Catalogue Retrieved from the National Potato Council of Kenya Website http://www.npck.org/index.php

New Partnership for Africa's Development (NEPAD) (2013). Agriculture in Africa: Transformation and Outlook: Prepared by Roger Blein, Martin Bwalya, Sloans Chimatiro, Benoît Faivre-Dupaigre, Simon Kisira, Henri Leturque and Augustin Wambo-Yamdjeu 15 p.

Njoroge A, Tusiime G, Forbes G, Yuen J (2016). Displacement of US-1 clonal lineage by a new lineage of *Phytophthora infestans* on potato in Kenya and Uganda. Plant Pathology 65(4):587-592.

Oh S-K, Kim H, Choi D (2014). *Rpi-blb2*-mediated late blight resistance in *Nicotiana benthamiana* requires *SGT1* and salicylic acid-mediated signaling but not *RAR1* or *HSP90*. FEBS Letters 588(7):1109-1115.

Oh S-K, Young C, Lee M, Oliva R, Bozkurt TO, Cano LM, Win J, Bos JI, Liu HY, van Damme M, Morgan W, Choi D, van der Vossen EA, Vleeshouwers VG, Kamoun S (2009). In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. The Plant Cell 21:2928-2947.

Orbegozo J, Roman ML, Rivera C, Gamboa S, Tovar JC, Forbes GA, Lindqvist-Kreuze H, Kreuze JF, Ghislain M (2016). *Rpi-blb2* gene from *Solanum bulbocastanum* confers extreme resistance to late blight disease in potato. Plant Cell, Tissue and Organ Culture 125(2):69–281.

Roman ML, Izarra M, Lindqvist-Kreuze H, Rivera C, Gamboa S, Tovar JC, Kreuze JF, Forbes GA, Ghislain M (2017). *R*/*Avr* gene expression study of *Rpi-vnt1.1* transgenic potato resistant to the *Phytophthora infestans* clonal lineage EC-1. Plant Cell, Tissue and Organ Culture 131(2):259-268.

Schumann GL, D'Arcy CJ (2005). Late blight of potato and tomato. The Plant Health Instructor 724-733 DOI: 10.1094/PHI-I-2000-0724-01

Sharma BP, Forbes AG, Manandhar KH, Shrestha SM, Thapa RB (2013). Determination of Resistance to *Phytophthora infestans* on Potato Plants in Field, Laboratory and Greenhouse Conditions. Journal of Agricultural Science 5(5):9752-9760 ISSN 1916-9752E-ISSN 1916-9760

Ssengooba T, Hakiza JJ (1999). The current status of late blight caused by *Phytophthora infestans* in Africa, with emphasis on eastern and southern Africa. Proceedings of Global Initiative on Late Blight Conference, March 16-19 Quito, Ecuador pp. 25-28.

Vleeshouwers VGA, Dooijeweert W, Govers F, Kamoun S, Colon TL (2000). The hypersensitive response is associated with host and non-host resistance to *Phytophthora infestans*. Planta 210(6):853-864

Vleeshouwers VG, Raffaele S, Vossen JH, Champouret N, Oliva R, Segretin ME, Pel MA (2011). Understanding and exploiting late blight resistance in the age of effectors. Annual Review of Phytopathology 49:507-531.

Wurster R (1977). Social science research and training: report of the planning conference on social science research and training held at CIP-Lima, Peru, August 8-12, 1977. Lima: Centro Internacional de la papa.

Yuen JE, Forbes GA (2009). Estimating the level of susceptibility to *Phytophthora infestans* in potato genotypes. Phytopatholog 99(6):782-786.

Zhu S, Li Y, Vossen JH, Visser GRF, Jacobsen E (2012). Functional stacking of three resistance genes against *Phytophthora infestans* in potato. Transgenic Research 21(1):89-99.

Vol. 18(29), pp. 857-864, September, 2019
DOI: 10.5897/AJB2019.16895
Article Number: 4B00BE661895
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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Characterization of products from sawdust biodegradation using selected microbial culture isolated from it

Lennox, Josiah A., Asitok, A., John, Godwin E. and Etim, Blessing T.

Food and Industrial Microbiology Unit, Department of Microbiology, Faculty of Biological Sciences, University of Calabar, Calabar, Cross River State, Nigeria.

Received 18 June, 2019; Accepted 8 August, 2019.

Characterization of products from sawdust degradation using selected microbial culture isolated from it was carried out. A composite sawdust waste sample was collected from MCC timber market in Calabar. The sample was diluted using serial dilution method and inoculated on Nutrient agar and Sabauraud dextrose agar using pour plate technique and incubated at 370C and 270C respectively. Five bacteria species and 3 species of fungi were identified which included Bacillus sp, Serracia mercescens, Micrococcus sp, Pseudomonas aeruginosa, Streptococcus sp, Aspergillus niger, Rhizopus sp and Penicillumsp. The bacteria and fungi species were individually and in combination inoculated into sterile sawdust containing sterile water. These were kept at 270C for 21 days. Protein, glucose contents and chemical components in the degraded sawdust samples were determined using GC-MS chromatography. It was observed that Aspergillus niger degradation had protein content 7.87g/l, Serracia mercescens and Aspergillus niger 6.56g/l, and Serracia mercescens 5.75g/l. These organisms used lignocellulolytic enzymes and similar enzymes in the degradation process. In the case of glucose, the combined degradation produced 2.24g/l, Aspergillus niger 2.05g/l and Serracia mercescens 1.94g/l. It was also observed that sawdust inoculated with Serratia marcescens produced 14 compounds while the combined species of Aspergillus niger and Serratia marcescens produced only 12 compounds. The following compounds Methylene cyclo propane carboxylic acid, Glycine N-Cyclopropylcarbonyl-methyl ester, acetic acid, propanoic acid, benzaldehyde, thiophene, tetrahydro, furfural and propanoic acid 2,2-Dimethyl-2-ethylhexyl ester were detected from the combination of Serratia marcescens and Aspergillus niger though in different concentrations. The percent concentrations of the compounds from Serratia marcescens range from n-Hexadecanoic acid 3.59 to Methylene cyclo propane carboxylic acid with 11.56 while Serratia marcescens and Aspergillus niger range from Butanoic acid, 4-chloro 3.59 to Benzaldehyde with 11.56. Microbial degradation of sawdust produced economically important products and therefore can contribute to the economy of this country at the same time reduce pollution caused by it which is a novel.

Key words: Sawdust, biodegradation, bioactive, extracts, microbial culture, lignocellulosic.

INTRODUCTION

Microbial biodegradation is the use of bioremediation and biotransformation methods to harness the naturally occurring ability of microbial xenobiotic metabolism to degrade, transform or accumulate environmental

pollutants including hydrocarbons (example oil).

Biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms or their enzymes (Godliving and Yoshitoshi, 2002). These organic substances can either be degraded aerobically with oxygen, or anaerobically without oxygen. Interest in the microbial biodegradation of agricultural wastes has intensified in recent years (Koukkou and Anna-Irini, 2011).

Sawdust is a by-product of woodworking operations such as sawing, milling, planning, routing, drilling and sanding. These operations shatter lignified wood cells and break out whole cells and groups of cells. Shattering of wood cells creates dust known as sawdust. This dust causes a lot of health hazards (Baran and Teul, 2007; Zhang, 2004). It is composed of fine particles of wood made up of 3 major components, cellulose, hemicelluloses and lignin (Green, 2006; Alexander, 1997; Erikson et al., 1990). It is generally used for blotting material, hand cleaner, wood filler, compost, packing and for writing notes (Berendeohu, 2018). The general recalcitrance of cellulose, lignin and hemicelluloses and the importance of their biodegradation in the environment have received much attention for several years (Erikson et al., 1990). Raw sawdust takes an average of 180 days to decompose in the soil due to deficiency of nitrogen (Olayinka and Adebayo, 1989). Microbial degradation of sawdust has been very difficult due to the presence of lignin, a highly recalcitrant component that does not degrade easily (Erkson et al., 1990). Despite this, Lennox et al. (2010) were able to degrade sawdust at certain degrees using microorganisms. Shide et al., (2004) reported that wood sawdust was degraded by *Lentinus squarrosolus* (Mont) singer, a basidiomycete also known as a white rot fungi to form protein, glucose and ethanol. Cellulose fibrils have high tensile strength which is used in the textile industry, paper and miscellaneous materials like vulcanized fibre, plastic filters, filtering media and surgical cotton; other uses include adhesives, explosives, thickening agents, coated paper, cellophane, artificial leather, films and foils (Hitchner and Leatherwood, 1982). This is to say that numerous products can be gotten from biodegradation of sawdust. Fungi of the classes hyphomycetes, zygomycetes, pyrenomycetes, hymenomycetes and the actinomycetes and bacteria of the groups *Cytophage, Erwinia, Pseudomonas, Sporolytophaga, Xanthamonas and Streptomonas* species degrade hemicelluloses (Lennox et al., 2010; Wuyep et al., 2012).

This research is therefore aimed at characterization and identification of products of biodegradation of lignocellulosic material, sawdust using microbial culture isolated from it.

MATERIALS AND METHODS

Samples collection

Fresh and dry undecomposed sawdust composite samples were collected from MCC timber market, Calabar, Cross River State, Nigeria in sterile polythene bags and transported to the laboratory for analysis.

Isolation and identification of indigenous bacteria and fungi involved in sawdust degradation

Bacteria were isolated and identified by carrying out a ten-fold dilution of the wet decomposing sawdust as described by Lennox et al. (2010). Antifungal agent, nystatin was incorporated into nutrient agar. One milliliter of the diluted solution of the sawdust was plated and incubated at 37°C for 18 to 24 h. The discrete colonies were sub-cultured and stock cultures were prepared from the pure cultures and stored at 4°C until needed. The method of Anderson et al., (1973) was used in the isolation of fungi. One milliter from the dilution above was inoculated on potato dextrose agar and incubated at 27°C for 7 days. The discrete colonies were sub-cultured and the pure cultures were stocked and stored until needed. The isolated colonies were characterized and identified.

Purification and maintenance of isolates

Each discrete colony on a Petri dish was transferred using a sterile inoculating loop into plates containing freshly prepared nutrient agar and were incubated at 37°C for 24 to 48 h, respectively. After incubation, the cultural characteristics of the isolates were recorded and compared with descriptive features contained in Bergey's Manual for Determinative Bacteriology (Holt et al., 1994). The isolates were then preserved on nutrient agar slants stored in the refrigerator at 4°C for biochemical characterization and identification.

Biochemical characterization and identification of isolates

The method of Oranusi et al., (2004) as modified was employed for the identification of the bacteria isolates while the fungal isolates were identified based on the taxonomic schemes described by Fawole and Oso (1988). Biochemical and sugar fermentation tests were used for the identification of the bacteria isolates. The biochemical tests carried out were: Catalase, methyl-red, oxidase, citrate utilization, coagulase, indole, coagulase and oxidase tests. The identities of the bacteria were confirmed using the identification aid outlined in Bergey's Manual for Determinative Bacteriology (Holt et al., 1994) as well as that of known taxa as described by Cheesbrough (2003). The fungal isolates were characterized based on colonial morphological features and microscopic examination using the Lactophenol cotton blue stained slide cultures and the results compared with fungal atlas.

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

Table 1. Cultural and morphological characterization of fungal isolates.

Isolate code	Colony morphology	Microscopic morphology	Organism
PF_1	Growth begins as yellow colonies that soon develop a black, dotted surface as conidia are produce within 26 days the colony becomes jet black and powdery and the reverse remains cream color	Exhibits septate hyphae long conidiophores that support spherical vesicle that give rise to mutulae and phalides from which conidia are produce	*A. niger*
PF_2	Black fluffy coloration with powdery appearance.	Non-septate hyphae with sporangiophores	*Rhizopus sp.*
PF_3	Green colonies, surface of colonies becomes powdery due to presence of conidia	Hyphae are septate and produce brush like conidiophores, conidiophores produce metulae from which phalides producing chains of conidia arise	*Penicillium sp.*
PF_4	Growth begins as yellow colonies that soon develop a black, dotted surface as conidia are produce within 26 days the colony becomes jet black and powdery and the reverse remains cream color	Exhibits septate hyphae long conidiophores that support spherical vesicle that give rise to mutulae and phalides from which conidia are produce	*A. niger*
PF_5	Green colonies, surface of colonies becomes powdery due to presence of conidia	Hyphae are septate and produce brush like conidiophores, conidiophores produce metulae from which phalides producing chains of conidia arise	*Penicillium sp.*

Preparation of bacterial and fungal inoculum

The identified bacteria and fungi strains isolated from sawdust sample were transferred from stock agar slant cultures to nutrient agar broths and incubated until a sizable inoculum size of 10^7 cfu/ml was obtained at 35°C.

Sawdust inoculation and degradation

The collected sawdust samples were first autoclaved to kill all indigenous bacteria and fungi present in it. Three sterile conical flasks were obtained and 25 g of sawdust was introduced into each and properly labeled; bacteria, fungi and combined (bacteria + fungi). 150 ml of distilled water was added into each of the conical flasks followed by the addition of the inoculums size of 10% v/v (17.5 ml) into each of the sawdust solutions. The conical flasks were then incubated at room temperature (26 to 28°C) for 21 days. At the end of the 21 days, the supernatants in the conical flasks were carefully filtered out and the residue sawdust was analysed.

Estimation of protein and glucose contents of fermented sawdust

Protein and glucose contents of the fermented sawdust samples were determined using ASTM standard method (1974) as reported by Bakulin et al., (2007).

Gas chromatography mass spectrometry (GC-MS) analysis

The analysis for chemical components present in sawdust degradation extracts was carried out using GC-MS analyzer (BRUKER SCION 436-GC SQ). Extracts were dissolved in methanol (high-performance liquid chromatography grade) and filtered through Whattman TM FILTER DEVICE (0.2 µm). Helium (99.99%) was used as carrier gas, at a flow rate of 1 ml/min in split mode. RESTEK Rtx®-5 (Crossbond® 5% diphenyl/95% dimethyl polysiloxane) with 30 m length, 0.25 µmdf, and 0.25 mm ID column was used for separation of chemical components. 2 µL of sample was injected into the column. The injector temperature was 320°C. The temperature of oven starts at 70°C and hold for 2 min, and then, it was raised at a rate of 7°C per minute up to 320°C, hold for 1 min. Temperature of ion sources was maintained at 250°C. The mass spectrum was obtained by electron ionization at 70eV and detector operates in scan mode 30 to 500 Da atomic units. Total running time was 38.71 min including 3 min solvent delay. The spectrum of the unknown component was compared with the spectrum of the known components using computer searches on a NARICT Ver.2.1 MS data library. The name, molecular weight, retention time and structure of the components of the test materials were ascertained and results were recorded.

RESULTS

Cultural and morphological characterization of fungal isolates

The cultural and morphological characteristics of the fungal isolates results are shown in Table 1. Based on the colony and microscopic morphology, three species of fungi were identified.

Morphological and biochemical characterization and identification of the bacteria isolates

Table 2 shows the results of morphological and biochemical characterization and identification of the

Table 2. Biochemical characterization and identification of the isolates from sawdust sample.

Isolate code	Gram reaction	Shape	Catalase	Motility	Oxidase	Indole	Citrate	MR	VP	Pigment	Glucose	Lactose	Mannose	Sucrose	Organism
P_1	+	Rods	+	+	-	-	+	-	+	-	+	±	+	+	*Bacillus sp*
P_2	+	Cocci	-	-	-	+	+	-	-	-	+	+	+	+	*Streptococcus sp*
P_3	-	Rods	+	+	+	-	+	-	-	+	-	-	-	-	*P. aeruginosa*
P_4	+	Cocci	+	-	+	±	-	+	-	+	±	-	+	-	*Micrococcus sp*
P_5	-	Rods	+	+	-	-	+	-	+	+	+	-	+	+	*S. marcescens*

Table 3. Estimation of protein and glucose contents of degraded sawdust sample.

S/N	Name of sample		Protein content (g/l)	Glucose content **(g/l)**
1	*S. marcescens*	Initial	5.25	3.74
		Degraded	5.75	1.94
2	*A. niger*	Initial	5.25	3.74
		Degraded	7.87	2.05
3	*S. marcescens* *A. niger*	Initial	5.25	3.74
		Degraded	6.56	2.24

isolates from the sawdust sample analyzed. Five bacteria species were identified.

Estimation of protein and glucose contents

The available protein and glucose contents in the degraded sawdust sample were estimated as presented in Table 3. From the result, it was observed that the fungus degradation had higher protein content available followed by the combined (bacteria and fungi) with the bacterium degradation having the least available protein. However, in the case of glucose, the combined degradation recorded the highest glucose content while the least was seen in the bacterium degradation.

Sawdust degradation (bacteria) components identified by GC-MS analysis

The results of GC-MS analysis of sawdust sample degraded by inoculated bacteria isolate identified fourteen bioactive chemical constituents present in the extracts. The gas chromatogram shows the relative concentrations of various compounds getting eluted as a function of retention time as presented in Table 4 and Figure 1 respectively. The heights of the peak indicate the relative concentrations of the compounds of the components present in the plant extracts. The mass spectrometer analyzed the compounds eluted different times to identify the natures and structure of the compounds. The compounds which were identified by GC-MS analysis were 12 in total (Table 5). Identification of the bioactive compounds was carried out by comparison of their mass spectra and retention time with those of reference standard and published data in NIST MS library (Figure 2).

DISCUSSION

A total of 8 microbial species were isolated from the sawdust; 5 bacteria and 3 fungi. Microbial degradability of sawdust through the isolation of autochthonous bacterial and fungal species utilizing sawdust as their carbon and energy sources and the observation of differences in sawdust before and after degradation have been demonstrated by Lennox et al. (2010). This corroborates the findings of Godliving and Yoshitoshi (2002) and Zhang et al., (2018) that bacteria and fungi degrade wood sawdust by producing enzymes such as carboxymethyl cellulose, lignin peroxidase and xylanase. Beguin and Aubert (1994) also reported the biodegradability of cellulose. This agrees with the finding of Dosoretz et al*.,* (1990), when they reported the reduction in carbon content of sawdust when subjected to microbial degradation. Hitchner and Leatherwood (1982) reported the ability of cellulase enzyme in the degradation of

Table 4. Sawdust degradation components identified by GC-MS analysis using *S. marcescens.*

Temperature	RT (min)	Name of compound	Concentration (%)
320°C	24.57	Methylene cyclo propane carboxylic acid	11.56
	30.46	Glycine, N-cyclopropylcarbonyl-methyl ester	7.58
	32.12	Acetic acid	4.64
	16.53	Propanoic acid	3.59
	16.76	2(5H)-furanone	5.84
	24.15	Benzaldehyde	5.91
	36.06	1H-pyrazole, 3,4-dimethyl(C)	4.83
	12.56	Pentanoic acid 2-ethylhexyl ester	10.6
	28.97	Thiophene, tetrahydro	9.38
	28.98	Furfural	4.63
	44.2	Thiophene, tetrahydro	3.98
	37.84	Propanoic acid, 2,2-dimethyl-,2-ethylhexyl ester	4.2
	33.86	4H-Pyran-4-one	6.09
	44.19	n-Hexadecanoic acid	3.59

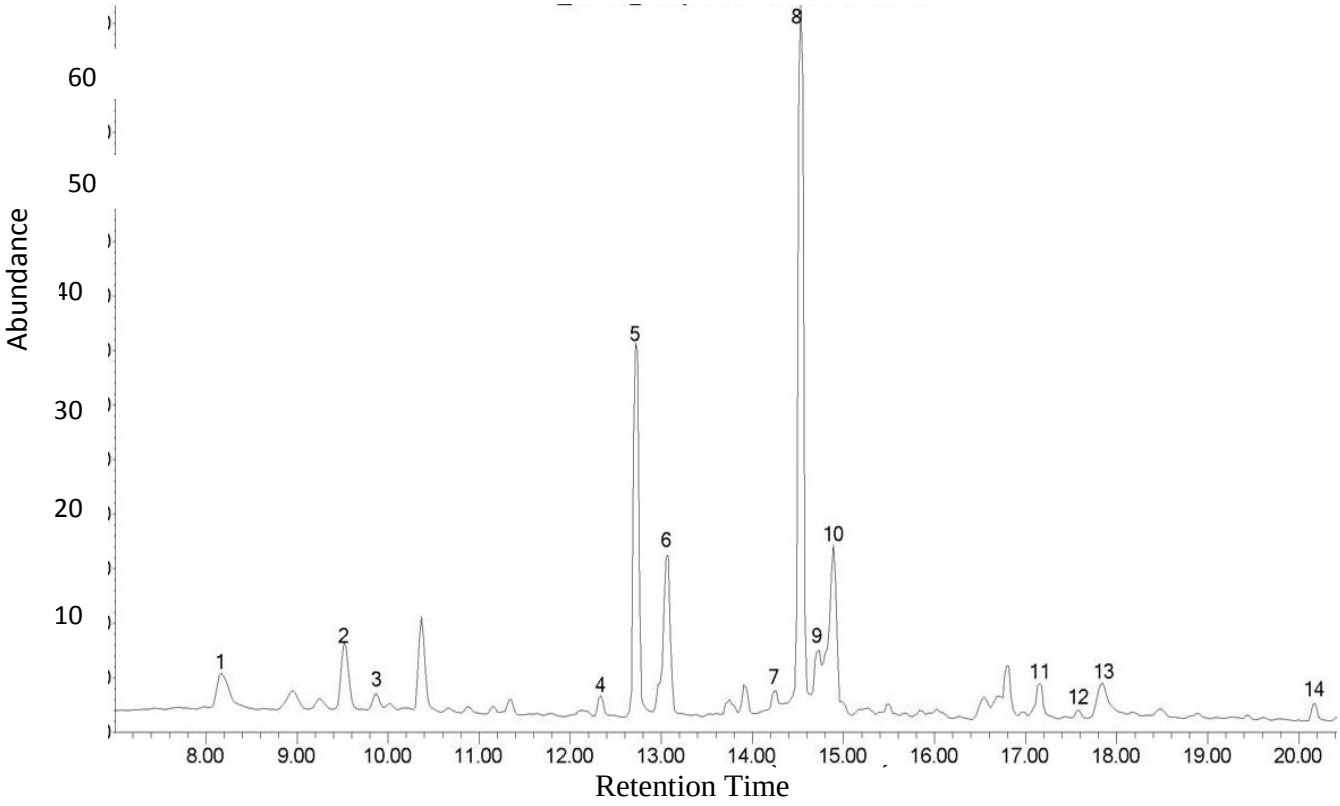

Figure 1. GC-MS chromatogram obtained from *sawdust* degraded extract by *Serratia marcescens.*

cellulose. Microbial species have different capabilities of degradation of sawdust (Lennox et al., 2010). This indicates that only few of the isolates are capable of utilizing sawdust as its source of carbon and energy for growth. The microbial isolates, *Bacillus* sp*, Streptococcus* sp.*, P. aeruginosa, Micrococcus* sp., *S. marcescens, A. niger, Rhizopus* sp. and *Penicillium* sp. in this study are similar to those isolated by *Lennox* et al. (2010) except *Cellulomonas* sp., *Cytophaga* sp., *Flavobacterium* sp., *Xanthomonas* sp., for bacteria, but only 2 fungi species, *Rhizopus* sp. and *Penicillium* sp. agree with the findings of Lennox et al. (2010). Two out of the eight microbial

Table 5. Sawdust degradation components identified by GC-MS analysis using **S**. *marcescens* and *A. niger*.

Temperature	RT (min)	Name of compound	Concentration (%)
320°C	24.57	Benzaldehyde	11.56
	30.46	Acetic acid	7.58
	32.12	Furfural	4.64
	16.53	Butanoic acid, 4-chloro	3.59
	16.76	Propanoic acid, 2,2-dimethyl-,2-ethylhexyl ester	5.84
	24.15	4H-Pyran-4-one	5.91
	36.06	n-Hexadecanoic acid	4.83
	12.56	thiocyanic acid, methylene ester	10.6
	28.97	Methylene cyclo propane carboxylic acid	9.38
	28.98	Propanoic acid	4.63
	44.2	Thiophene, tetrahydro	3.98
	37.84	Glycine, N-cyclopropylcarbonyl-methyl ester	4.2

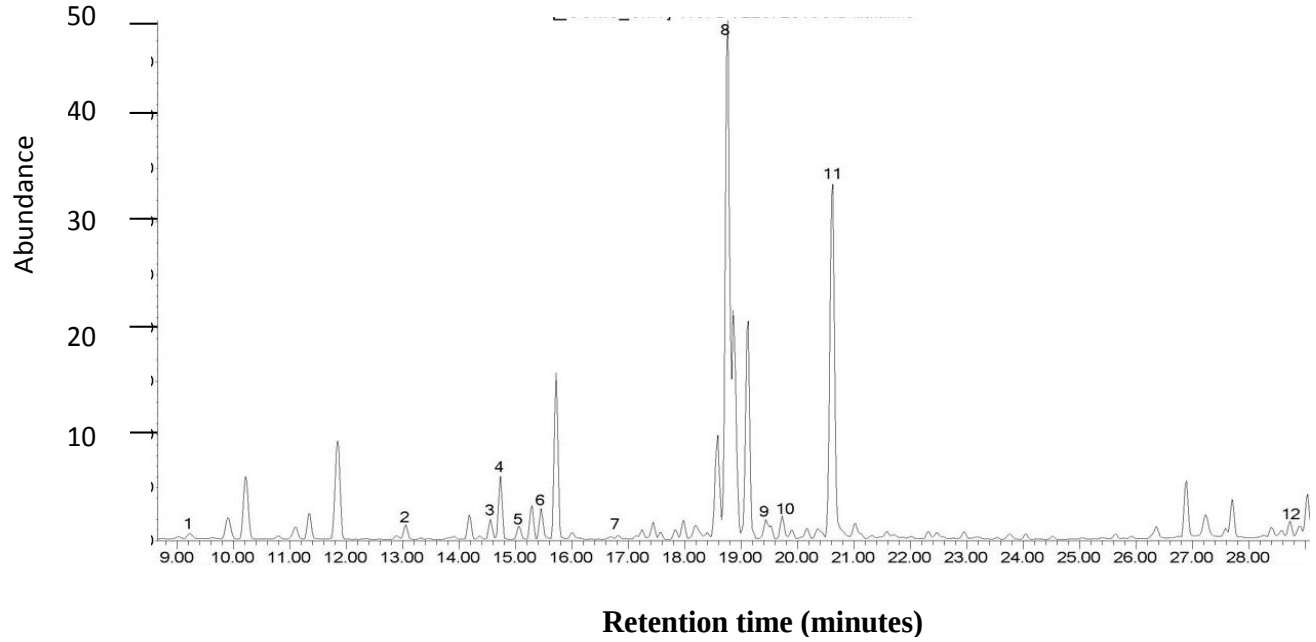

Figure 2. GC-MS chromatogram obtained from *sawdust* degraded extract by *S. marcescens* and *A. niger*.

species isolated from the sawdust were used for this research. *S. marcescens* and *A. niger* used in the biodegradation showed their capability to utilize saw dust to produce important products. The efficacy of fungi in cellulose degradation has also been reported (Deeble and Lee, 1985; Kelsey and Shafizadeh, 1980). These reports have provided insight into the possibility of degradation of sawdust using indigenous microorganisms, thereby paving way for enhanced natural attenuation of sawdust polluted sites (Lennox et al., 2010). The saw dust inoculated with *S. marcescens* produced 14 compounds while the combined species of *A. niger* and *S. marcescens* produced only 12 compounds. The following compounds, Methylene cyclo propane carboxylic acid, Glycine N-Cyclopropylcarbonyl-methyl ester, acetic acid, propanoic acid, benzaldehyde, thiophene tetrahydro, furfural and propanoic acid 2,2-Dimethyl-2-ethylhexyl ester were detected in both the *S. marcescens and A.niger/S. marcescens* inoculated saw dust samples though in different concentrations. Methylene cyclo propane carboxylic acid with 11.56% was the highest produced by *S. marcescens* but only 9.38% from the combined. This could be as a result of some antagonistic activities of the organisms by producing several hydrolytic enzymes like chitinase, protease, lipase and cellulase (Purkayastha et al., 2018).

S. marcescens produced 10.6% of pentanoic acid 2-ethylhexyl ester which was absent in the combined. This also could be some antagonistic or use of the compound by *A. niger.* Benzaldehyde concentration was 11.56% from the combined but only 5.91% from *S. marcescens*. Thiocyanic acid, methylene ester 10.6% produced by the combination was not produced by *S. marcescens*. The results here show that selective utilization of these organisms in the degradation of saw dust can lead to the production of economically important products. These compounds produced by these microorganisms are the first of its kind in scientific world and therefore a major contribution to knowledge.

The protein and glucose analysis revealed an increase in protein content by 9.5, 49.9 and 25% using *Sarratia marcescens, Aspergillus niger* and combination of *S. marcescens* and *A. niger* respectively. For the glucose, there were decreases of 48.6, 45.2 and 40% using *S. marcescens, A. niger* and combination of *S. marcescens* and *A. niger* respectively. This is to say that these microorganisms were able to utilize saw dust to increase the protein content while at the same time utilized the glucose contained in the saw dust. This is contrary to the findings of Bohdan and Yaser (2011), Reddy and Yang (2009), Van Wyk (2001) and Ndukwe et al., (2012) who detected an increase in glucose levels, though the saw dust samples were pretreated. This is pretreatment of the lignocelluloses wastes to a greater extent must have removed lignin from the different saw dust materials. This research has shown that saw dust waste can be bio converted to useful products without pretreatment as has always been believed. Most of the products produced by the fermentation of the sawdust are very useful. Glycine, N-Cyclopropylcarbonyl-methyl ester produced by *S.marcescens* is used as additive in pet food and animal feed, in foods and pharmaceuticals as a sweetener/taste enhancer, or as a component of food supplements and protein drinks. It is also used in cosmetics. Benzaldehyde, 11.56% produced by *S. marcescens* and *A. niger* is also an important compound used chiefly in the organic synthesis of dyes, perfumes, and flavors, and as a solvent. Methylene cyclo propane carboxylic acid (11.56%) and Pentanoic acid 2-Ethylhexyl ester (10.6%) also produced from the degradation of sawdust by *S. marcescens* have been found to be very useful as an intermediates in the production of pesticides, pharmaceuticals and other agrochemicals. Acetic acid (7.58%) produced by *S. marcescens* and *A. niger* has several health benefits which include reducing inflamation, lowering blood pressure, and keeping blood sugars spikes at bay. Acetic acid is also used as a condiment and in the pickling of raw vegetables and other foods

The direct fermentation of sawdust without pretreatment with selected indigenous microbial flora is a novel and the amounts of economically important products resulting from the fermentation are also novels. The use of sawdust for the production of these compounds will lead to reduction of pollution caused by sawdust.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Alexander M (1997). Soft rot bacteria. Introduction to Soil Microbiology 6th Edition, John Wiley and Sons, New York pp. 148-162.

Anderson NA, Wang SS, Schwandt JW (1973). The pleurotus ostreatus sapidus species complex. Mycologia 6:28-35.

ASTM (1974). Standard Test Methods for Moisture, Ash, and Organic Matter ofPeat and Other Organic Soils, ASTM International, West Conshohocken, PA; 2007.

Bakulin MK, Grudtsyna AS, Pletneva A (2007). Biological fixation of nitrogen and growth of bacteria of the genus *Azotobacter* in liquid media in the presence of Perfluoro carbons. Applied Biochemistry and Microbiology 4:399-402.

Baran S, Teul I (2007). Wood dust: occupational hazard which increases risk of respiratory disease. Journal of Physiology and Pharmacology 58 Suppl 5(Pt 1):43-50.

Beguin P, Aubert P (1994). The biological degradation of cellulose. FEMS Microbiology Reviews 13(1):25-58.

Berendeohu R (2018). Six things you can do with sawdust and wood shavings. Popular Mechanics 28(7):14-20. www.popularmechanics.com

Bohdan V, Yaser D (2011). Assessment of pretreatments and enzymatic hydrolysis of wheat straw as a sugar source for bioprocess industry. International Journal of Energy and Environment 2(3):427-448.

Cheesbrough M (2003). Microbiological tests. Medical laboratory manual for tropical countries. Cambridge University Press, New Delhi, India pp. 22-36

Deeble MF, Lee JM (1985). Enzymatic hydrolysis of cellulosic substances in an attrition bioreactor. Biotechnology Bioengineer Symposium 15(2):277-293.

Dosoretz GG, Chenn HC, Greth HE (1990). Effect of Environmental conditions of extracellular protease activity in lignolytic cultures of *Phanerochaete chrusosporium*. Applied Environmental Microbiology 56(24):395-400.

Erikson KEL, Blanchette RA, Ander P (1990). Microbial and Enzymatic Degradation of Wood and Wood Components. *Wood decomposition*. Springerverlag, New York pp. 41-62.

Fawole MO, Oso BA (1988). Laboratory manual of Microbiology. Spectrum Books Ltd, Nigeria pp. 26-31.

Godliving M, Yoshitoshi P (2002). Continuous production of lignin degrading enzymes by Bjer Kandora adusta immobilized on polyurethane foam. Biotechnology 24:173-1747.

Green H (2006). Wood, Craft, Culture, History. Penguin Books, New York. P 403.

Hitchner E, Leatherwood J (1982). Use of cellulose depressed mutant of cellulomonas in the production of single-cell protein from cellulose. Applied Environmental Microbiology 41:465-583.

Holt JG, Krieg NR, Sneath PH, Staley JT, Williams ST (1994). Bergeys manual of determinative bacteriology. Lippincott Wilkins. A Wolters Kluwer Company. Philadelphia.

Kelsey R, Shafizadeh F (1980). Enhancement of cellulose accessibility and enzymatic hydrolysis by simultaneous wet milling. Biotechnology and Bioengineering 22(5):1025-1036.

Koukkou I, Anna-Irini E (2011). Microbial Bioremediation of Non-metals: Current Research. Caister Academic Press pp. 312-320.

Lennox JA, Abriba C, Alabi BN, Akubuenyi FC (2010). Comparative degradation of sawdust by microorganisms isolated from it. African Journal of Microbiology Research 4(13):1352-1355.

Ndukwe NA, Okiel WO, Alo BI, van Wyke JPH, Mamabolo TM, Igwe CC (2012). Saccharification of delignified saw dust from 20 different trees in the Lagos area of Nigeria. African Journal of Biotechnology 11(100):16625-16629.

Oranusi SU, Oguoma OI, Agusi E (2004). Microbiological quality assessment of foods sold in student's cafeterias. Global Research Journal of Microbiology 3(1):1-7.

Olayinka A, Adebayo A (1989). Effect of pre-incubated sawdust based

cow dung on the growth and nutrient uptake of *Zea mys* L. and on soil chemical properties. Biology and Fertility of Soils 7(2):176-179.
Purkayastha GD, Mangar P, Saha D (2018). Evaluation of the biocontrol efficacy of a *Serratia marcescens* strains indigenous to tea rhizosphere for the management of root rot disease in tea. PLOS One 13(2):1-15.
Reddy N, Yang Y (2009). Natural cellulose fibers from soybean straw. Bioresearch and Technology 100(14):3593-3598.
Shide EG, Wuyep PA, Nok AJ (2004). Studies on degradation of wood sawdust by *Lentinus squarrosulu* (Mont.) singer. African Journal of Biotechnology 3(8):395-398.
Van Wyk JPH (2001). A Review: Biotechnology and the utilization of organic waste as a resource for bioproduct development. Trend Biotechnology 19(5):172-177.
Wuyep PA, Odunola MB, Balogun NA (2012). Purification and partial characterization of laccase from *Lachnocladium* sp. International Journal of Biological Chemistry 6(2):612-620.
Zhang H, Krafft T, Gao D, Zheng G, Cai L (2018). Lignocellulose biodegradation in the biodrying process of sewage sludge and sawdust. Drying Technology, an International Journal 36(3):316-324.
Zhang Y (2004). Indoor air quality. Engineering. Boca Raton, CRC Press, Florida P 18.

Vol. 18(29), pp. 865-874, September, 2019
DOI: 10.5897/AJB2019.16757
Article Number: 8CF852C61944
ISSN: 1684-5315

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Phenotypic characterization of selected Kenyan Khat (*Catha edulis)* cultivars based on morphological traits

Loise N. Ngari[1,2*], Shadrack N. Njagi, James K. Kamau[1], Rose C. Lagat[1], Amos M. Musyoki[1], Beryl O. Akoth[2], Festus Kioko[1], Zedekiah-A Okwany[2] and Mathew P. Ngugi[1]

[1]Department of Biochemistry and Biotechnology, Kenyatta University, P. O. Box 43844-00100, Nairobi, Kenya.
[2]National Museums of Kenya, Nairobi, Kenya.

Received 30 January, 2019; Accepted 29 March, 2019

Khat (*Catha edulis* Vahl) is an evergreen shrub habitually ingested for its euphoric and stimulatory effects. The crop is grown in the Middle East, Somalia, East Africa and Ethiopia. It is referred to as *Miraa* in Kenya, while in Yemen it has several names like *Qat, Kat, Kath, Gat, Chat* and *Tschat*. It belongs to the sub-order Rosidae, family Celastraceae and characterized by astringent taste. Despite the daily use and consumption of khat by millions of people in Kenya, little is known about its phenotypic. Phenotypic characterization is an essential approach for assessment of khat diversity; however, it is limited by morphological plasticity and multiple lineage evolution. The study aimed at evaluating the phenotypic diversity of selected khat cultivars grown in Embu and Meru Counties. Ninety samples from 18 cultivars were collected for phenotypic characterization. MINITAB 17 Software was used for description of principal component and construction of dendrogram using the Euclidean distance tool where 58.7% variability was observed among 13 traits studied in 90 samples of khat. Phenotypes grouped into 2 clusters phenotypic diversity showed considerable variability based on 13 khat traits. This will be useful in breeding and characterization programmes of khat cultivars.

Key words: Phenotypic, khat, diversity, cultivar.

INTRODUCTION

Khat (*Catha edulis* Vahl) is ever green and an edible plant (Ngari et al., 2018). It is classified in the kingdom Plantae, class Magnoliopsida, order Celastrales, family Celastraceae, genus *Catha,* and species *edulis* (Sikiru, 2012). The plant was first described a by Swedish botanist called Peter Forskal. He encountered the plant as he travelled to Yemen through Egypt in an expedition that was paid for by King of Denmark Friederick, who wanted all the natural collections (Al Motarreb et al., 2002). Countries and communities have different names for the plant such as *Qat* and *Chat* in Yemen and Ethiopia, *Jaad* and *Qaad* in Somalia, *Muguka* and *Miraa* in Kenya, and *Jimma* in the Oromo language. In most western countries, it is recognized as khat (Ngari et al., 2018).

Khat is said to have originated from Ethiopia and then spread to East Africa and Yemen (El-Menyar et al., 2015). In Kenya, khat is grown in Meru and Embu Counties for commercial purposes. In Meru County, it is grown around Nyambene hills in Meru North, 320 km North East of Nairobi and the main outlet is Chyulu Maua Town (Nyongesa and Onyango, 2010). In Embu county, khat is mainly found in lowland area of Mbeere region

*Corresponding author. E-mail: loisengithi@gmail.com. Tel: +254723544164.

which are usually dry (Kiunga et al., 2016).

Khat also flourishes in arid and semi-arid environments where temperatures range from 5 to 35°C with free draining soil (New Agriculturalist, 2007; Nyongesa and Onyango, 2010). Khat leaves are harvested in the morning then covered with fresh banana leaves and polythene bags to maintain freshness (Nyongesa and Onyango, 2010). Khat is more potent during the dry and sunny season of the year (Ng'ethe, 2012). Harvesting is done throughout the year and planting is spread over a period of time to obtain a continuous supply. Khat is usually consumed by chewing the leaves while fresh, although occasionally, leaves are dried, then consumed as a stimulating juice (Wabe, 2011).

Mainly, khat chewing is a male habit though it has gained popularity among women (Mwenda et al., 2003; Kiunga et al., 2016). More than 10 million people worldwide use khat for psychostimulating effect (Gitonga et al., 2017). In many countries, khat is chewed for social and psychological reasons (Al-Kholani, 2010). Current trends indicate that khat is used by all societal groups regardless of age, gender, affluence, class, education and occupation (Gesese, 2013).

Various khat cultivars are identified based on the communities that consume them. In Yemen, local cultivars of khat are described according to geographical location, growth habit and physical appearance (colour of the leaf, stem sizes and potency) (Al-Thobhani et al., 2008). It is documented that different kinds of khat vary in the extent of their pharmacological activity. Farmers in Yemen have four cultivars, namely, *Abyadh, Azraq, Aswad* and *Ahma*r categorized according to the colour of shoots and growing twig (Ngari et al., 2018). Also forty kinds of khat cultivar were recognized according to geographical origin. In Ethiopia, two major cultivars have been described as *Dimma* (red) or *Ahde* (white). Getahun and Krikorian, classified khat into three types, namely 'madness-causing, intoxicating-like spirit and insomnia-causing based on their effects (Al-Thobhani et al., 2008; Ayana and Mekonen, 2004). Little is known of how *C. edulis* differs morphologically, therefore, in this study the plant was characterized morphologically.

Morphological traits have been used for classification of variety duplicates, determination of genetic diversity and correlation with characteristics of agronomic significance (Zeng, 2015). Morphological characterization of khat cultivars in Embu and Meru counties was done by assessing variations in 13 khat traits. This has been traditionally used for classification of khat cultivars. It is clear that most users' knowledge is limited to only the narcotic effects of the plant (Al-Thobhani et al., 2008).

Khat plant cultivars are notable by the level of cathionone (or the narcotic effect of the plant) present in the plant material and also their morphological differences. The varying morphological features and cathionone levels within khat may also be as a result of genetic variations. Morphologic studies have not been conducted for this crop in Mt. Kenya region where it has become a major income earner. As a traditional method, morphological traits are used to assess genetic divergence and classify existing germplasm materials. In addition, this technique is easier, cost effective, and easy to score and requires less time and finally it does not need any technical knowledge (Malek et al., 2014).

MATERIALS AND METHODS

Collection sites

The germplasm was collected from 12 major khat producing wards (Figures 1 and 2). The wards included: Maua, Kianjai, Gaiti, Kangeta, Muthaara in Meru County and Kaaga South, Kithimu, Kaaga North, Mbeti south, Mbeti North, Mavuria, and Muminji in Embu County. Meru County is found in eastern region of Kenya approximately 225 km northeast of Nairobi, it covers an area of 6,936 km^2. The area receives about 1366 mm per annum. The climate of Meru is described as cool and warm with temperature ranging between 16°C during cold season and 23°C during hot warm season. Embu County is located approximately at 120 km northeast of Nairobi towards Mt. Kenya. It covers an area of 2,818 km^2 and lies between latitude 0° 8" and 0° 35" South and longitude 37° 40" East.

Collection of plant materials

Khat fruits, flowers and fully grown leaves were harvested from selected khat plants for morphological characterization. A total of 90 samples were collected from locally available cultivars and three replicates picked randomly from each of the sample giving a total number of 90 khat samples where different local khat names were given.

For morphological characterization, a small branch of the khat plant was cut aseptically and each packed in between a newspaper and later transported to National Museums Herbarium Department, where identification and morphological measurements were carried out. All the information on these plants was recorded based on local names given by Meru and Embu communities as well as geographic distribution (Appendix 1). The local names given by farmers included: *Kira Kieru*-1*, Kira Kieru*-2*, Kira Kieru*-3*, Kira Kiiru*-1*, Kira Kiiru*-2*, Kira Gitune*-1*, Kira Gitune*-2*, Muchuri, Kithara, Mutimutiri, Mugiza*-1*, Mugiza*-2*, Mugumo*-1*, Mugumo*-2*, Mugumo*-3*, Mugumo*-4*, Mugumo*-5*, Muguka*-1*, Muguka*-2*, Muguka*-3*, Muguka*-4*, Muguka*-5, *Mugukawakarimi, Gitu,Mutamucii*, *Mukurukuru*, *Muruti*, *Muceke*, *Mitune*, and *Mumbu.*

A total of 13 morphologic traits of 90 khat samples were evaluated according to guidelines provided by Robson (1994). The traits studied were leaf length, leaf width, leaf margin, petiole length, inflorescence length, peduncle length, sepal length, petal length, and diameter of the stamen, diameter of the filament, ovary length, capsule length and the length of the wing (Appendix 2). For each of the khat sample, 3 leaf samples were randomly selected and their measurement taken using a digital vernier caliper. Flowers and fruits measurements were taken using wild MSA Switzerland dissecting microscope at magnification of ×10.

Data management and statistical analysis

Raw data was entered into Microsoft excel spreadsheet which was then imported to Minitab software version 17.0 (State College Pennsylvania-USA) software. Data was analyzed statistically for the

Figure 1. A map showing Meru County.
Source: Survey of Kenya (2011).

Figure 2. A map showing Embu County.
Source: Survey of Kenya (2011).

differences in means for 13 khat traits, through one-way ANOVA followed by Tukey's post hoc test. Statistical significant differences were set at p≤0.05. Cluster analysis yielded a dendrogram that was used to examine the morphological relatedness among the 90 khat samples while Principal Component Analysis (PCA) was used to assess the underlying source of variation in morphology.

RESULTS

The measurements of the 13 phenotypic traits studied were found to vary across the 90 khat samples, *Muguka*-4 had the highest mean leaf length of 99 mm, while *Mutimutitiri* had the lowest mean leaf length of 53.67 mm. The mean leaf length of the 90 khat samples had significant differences. Regarding leaf width, *Muguka*-4 had the highest mean value of 45.67 mm, while *Kira Kieru*-1 had the lowest mean leaf length of 19.33 mm (Appendix 2).

The mean sepal length of the studied samples did not show significant differences among themselves with the highest mean length of 1.47 mm being recorded in *Mugumo*-1, *Mugiza*-1, and *Kithara*. The lowest mean sepal length of 1.03 mm was observed in *Mugumo*-3 and *Mugumo*-4, *Mugukawakarimi*, *Mukurukuru*, *Mumbu*, *Muruti, Mutamucii* and *Mutimutiri*. The highest mean petal length of 1.67 mm was observed in *Kira Gitune*-1, *Kira Kieru*-1 and *Kira Kiiru*-1 cultivar from Meru, while the lowest mean petal length of 1.00 mm was observed in *Muguka*-2. The mean petal length of the 90 khat samples did not show significant differences among themselves. The mean stamen diameter of the studied khat samples did not show significant differences with the highest diameter of 2.37 mm being shown in *Kira Gitune*-1 and the lowest mean diameter being 1.07 mm in *Kira Kieru*-2 (Appendix 2).

The mean filament diameter of all samples ranged from 1.53 to 1.07 mm. The highest mean filament diameter was observed in *Muchuri* and *Kira Kiiru*-1, while the lowest filament diameter was shown in *Gitu*, *Kira Kieru*-2, *Muguka*-4, *Muguka*-5, *Muguka*-1, *Muguka*-2*, Mugukawakarimi*, *Mugumo*-3, *Mukurukuru*, *Muruti* and *Mutimutiri* cultivars. There were no significant differences in ovary length mean among all cultivar with *Mugiza*-1 showing the highest length of 1.57 mm, while *Kira Kieru*-3 and *Kira Kiiru*-2 showed the lowest mean ovary length of 1.10 mm. There were significant differences in mean capsule length among the 90 khat samples with the highest capsule length of 8.43 mm being observed in *Gitu* and lowest mean capsule length of 5.1 mm in *Kira Gitune*-1, and *Kira Kieru*-3. The mean wing length varied significantly among various cultivar with the highest mean wing length of 3.5 mm in *Muguka*-1 and lowest wing length of 2.07 mm in *Kira Gitune*-1.

The leaf margin had wide variations ranging from a mean of 107.33 mm (*Muguka*-4) to a mean of 55.00 mm (*Mutimutiri*). The mean leaf margin of *Mutimutiri* cultivar was significantly lower than the other cultivars (p≤0.05). Besides, the mean petiole length did not show significant differences among the cultivar with the highest mean petiole length being 5.67 mm (*Muguka*-1 and *Muguka*-2) and the minimum mean petiole length being 2.33 mm observed in *Mugiza*-1 and *Muguka*-3 cultivars (p>0.05). The mean inflorescence length of 90 khat samples did not show significant difference among the 90 samples. *Kira Kieru*-1 had the highest mean inflorescence length of 21.00 mm, while *Mutimutiri* had the lowest inflorescence length of 14.33 mm. The mean peduncle length of 30 samples was not significantly different with *Kira Gitune*-1 having the highest mean of 7.33 mm, and *Mugumo*-1, *Mugumo*-2 and *Mugiza*-1 having lowest mean peduncle length of 4.00 mm (p>0.05).

A dendrogram constructed from data set of mean values of the 13 khat traits showed 2 super clusters namely I and II. Super cluster I had two sub clusters Ia and Ib. The sub cluster Ia comprised *Mugukawakarimi* and *Kira kieru*-3 cultivars from Embu and Meru counties, respectively, while sub cluster Ib had *Muguka*-4 cultivars from Embu county, clustering independently. The sub cluster II was more diverse and clustered into two sub clusters IIa and IIb. The sub cluster IIa was divided into IIai and IIaii sub clusters. The sub cluster IIai had three groups where the first group comprised *Gitu* and M*uguka*-3 cultivars from Embu County while the second group comprised *Kira kiiru*-2, *Kira kieru*-2 and *Kiithara* cultivars from Meru County. The third group comprised *Kira gitune*-2 and *Kira kieru*-1 cultivars from Meru county and *Mugumo*-4 cultivar from Embu county*.* The sub cluster llaii had one group which comprised *Muguka* -5 and *Mugumo*-1 genotypes both from Embu County.

The sub cluster IIbi had four groups. The first group comprised of *Gitune*, *Muruti, Mukurukuru Mugumo*-5, *Mugiza*-2 and *Muguka*-1 cultivar from Embu County and *Kira kiiru*-1 cultivar from Meru County. The second group comprised *Muceke* and *Mugiza*-1 from Embu County. The third group comprised *Mugumo*-2, *Muguka*-2, *Mugumo*-3 and *Mumbu* from Embu County, while the fourth group comprised *Kira gitune*-1 and *Muchuri* cultivar from Meru county, and *Mutamucii* from Embu county. The sub cluster IIbii had *Mutimutiri* cultivar which originated from Embu County (Figure 3).

Principal component analysis (PCA)

The PCA was performed for all the 13 traits in the 90 khat samples as indicated in Table 1. Out of the 13 traits, three principal components (pc1, pc2 and pc3) exhibited more than one Eigen value and showed about 58.7% variability among the 13 studied traits. The three principal components: pc1, pc2 and pc3 had 25.0, 18.4 and 15.3% variability, respectively among the cultivars for the traits under study. The pc1, pc2 and pc3 had Eigen values of 3.247, 2.386, and 1.995, respectively.

The pc1 was positively correlated to leaf margin, petiole

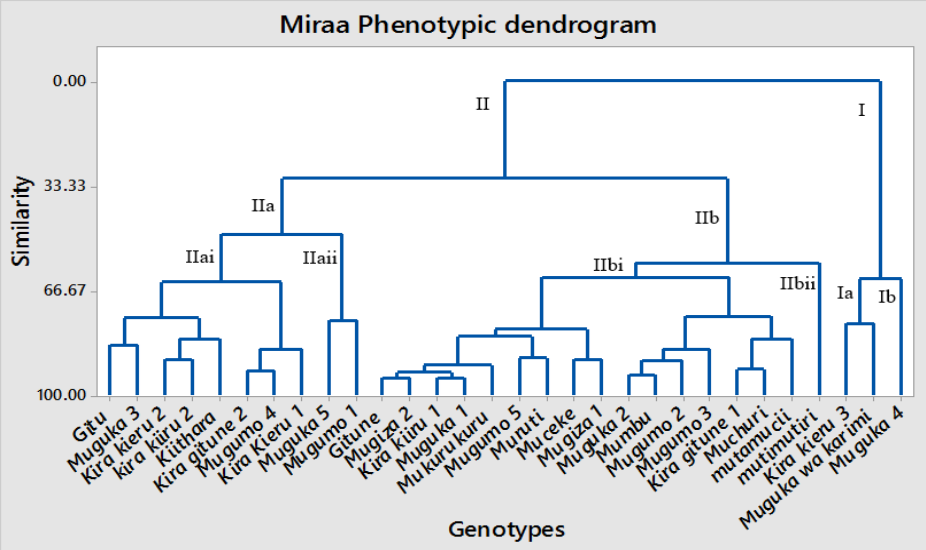

Figure 3. Euclidean distance based dendrogram developed from mean values of the 13 traits of 90 khat samples.

Table 1. Principal Component Analysis among the 30 khat cultivars.

Parameter	PC1	PC2	PC3
Eigen value	3.247	2.386	1.995
% Total variance	25.0	18.4	15.3
% Cumulative	25.0	43.3	58.7
Trait	**Eigen vectors**		
Leaf length (mm)	-0.062	0.310	-0.546
Leaf width (mm)	-0.323	0.030	-0.305
Leaf margin (mm)	0.107	0.488	-0.359
Petiole length (mm)	0.191	0.163	0.247
Inflorescence length (mm)	-0.120	0.037	-0.167
Peduncle length (mm)	0.466	0.159	0.090
Sepal length (mm)	0.124	-0.279	-0.541
Petal length (mm)	0.402	-0.148	-0.004
Diameter of stamen	0.386	-0.143	-0.111
Diameter of filament	0.224	-0.391	-0.147
Ovary length	0.074	-0.485	-0.214
Capsule length	-0.416	-0.314	0.053
Wing length	-0.241	-0.077	0.087

length, peduncle length, sepal length, petal length, stamen diameter, filament diameter and ovary length. However, it was negatively correlated to leaf length, leaf width, inflorescence length, capsule length and wing length. The pc2 showed a highly positive correlation to leaf length, leaf margin, petiole length, inflorescence length and peduncle length. However, it was negatively correlated to sepal length, petal length, stamen diameter, filament diameter, ovary length and wing length. The pc3 showed a positive correlation to petiole length, capsule length, peduncle length and wing length but negatively correlated to leaf length, leaf width, leaf margin, inflorescence length, sepal length, petal length, stamen diameter, filament diameter and ovary length (Table 1).

The scatter plot of the 30 khat samples based on the 13 studied traits was also drawn in this study. It was observed that the first quadrant comprised of *Muguka*-1, *Muguka*-2, *Muguka*-3 and *Mugiza*-2, *Muguka wa karimi, muguka*-4, *Muguka*-5 and *Gitu* cultivars, which were from Embu county. The second quadrant had six cultivars,

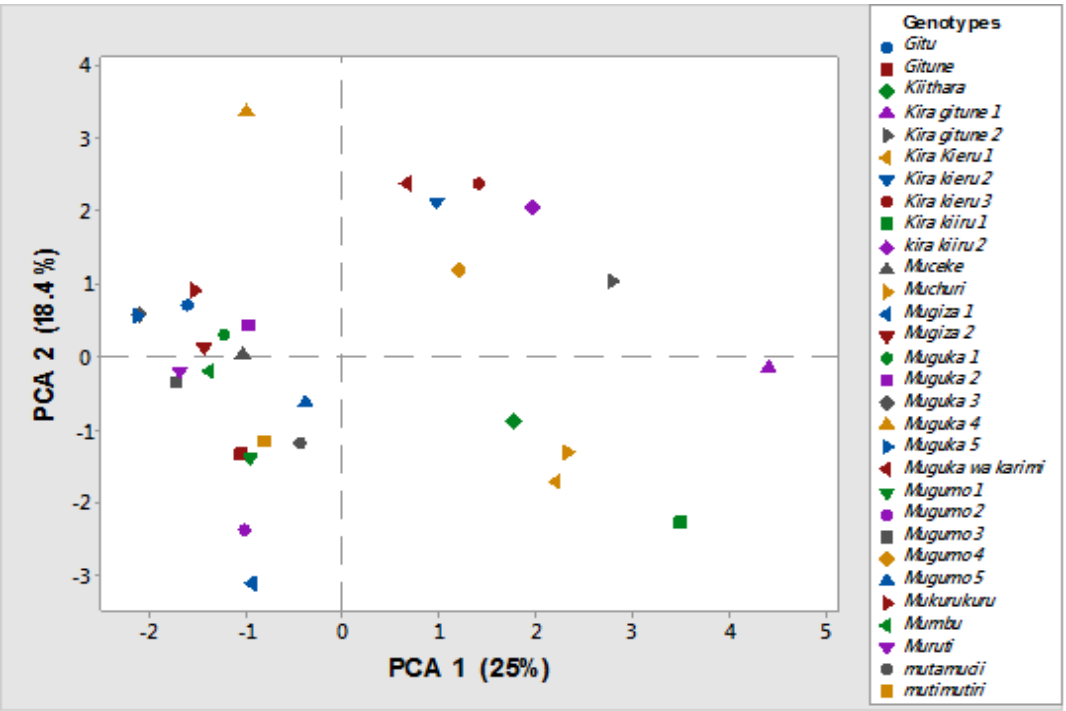

Figure 4. The scatter plots of 30 khat cultivars based on 13 traits

comprising *Kira kieru*-2, *Kira kieru*-3, *Kira kiiru*-2, *Kira gitune*-2, from Meru County, as well as *Mugumo*-4 and *Mukurukuru* cultivar from Embu County. The third quadrant comprised *Mugumo*-3, *Mugiza*-1, *Mugumo*-1, *Mugumo*-2, *Gitune*, *Mutimutiri, Muruti*, *Mumbu, Muceke* and *Mugumo*-5 cultivar from Embu County. The fourth quadrant comprised five cultivar, which originated from Meru county and they included *Kira gitune*-1, *Kiithara, Muchuri*, *Kira kieru*-1 and *Kira kiiru*-1 (Figure 4).

DISCUSSION

The mean leaf length of different cultivars indicated significant differences, whereby *Muguka*-4 cultivar recorded the highest mean leaf length while *Mutimutiri* cultivar showed the lowest mean leaf length. Both cultivars were collected from the same geographical location and environmental conditions. The lowest leaf length of *Mutimutiri* cultivar could be due to the ability of the cultivar to adapt to relatively drier and saline environment. Plants growing in such conditions usually have reduced leaf area in order to minimise loss of water during evaporation (Deblonde and Ledent, 2001).

The mean leaf width of different cultivar was significantly different, *Muguka*-4 cultivar recorded the highest mean leaf width while *Kira Kieru*-1 recorded the lowest leaf width. The lowest leaf width of *Kira Kieru*-1 could be as a result of adaptations to conserve water. *Kira Kieru*-1 had narrow leaves and less total surface area than *Muguka*-4 and, therefore, loses more water compared to *Kira Kieru*-1. Studies have shown that broad leaves heat up more than narrow leaves of the same length, hence narrow leaf plants are well adapted to dry and hot environments. The cultivar with the highest mean leaf width (*Muguka*-4) could have been in moist and shady environments, which enhanced their ability to absorb sunlight. This was confirmed in tropical vines (Tardieu, 2013).

Cultivars with long inflorescence produce more flowers, fruits and seeds compared to cultivars with short inflorescence (Rahman et al., 2009). This suggests that both the number of inflorescences and the number of female flowers are the main factors in determining yield. On the other hand, determining the number of inflorescences per plant and the number of female flowers both depend on environmental factors (Sangoi, 2001; Domiciano et al., 2014). Flowering and determination of the flower type are influenced by the occurrence of low temperatures and high rainfall (Inouye, 2008; Prasad et al., 2001). Therefore, the highest inflorescence length in *Kira Kieru*-1 could have resulted from high rainfall and low temperature, while the lowest inflorescence length in *Mutimutiri* could be due to low rainfall and high temperature.

The mean peduncle length showed significant differences among different cultivars. Drought and stress cause reduction in peduncle length. From this study *Kira Gitune*-1, which had the highest peduncle length was not drought stressed while *Mugumo*-1, *Mugumo*-2 and

Mugiza-1 which had the lowest peduncle length were drought stressed. This was confirmed by Amiri et al. (2013) on 80 bread wheat genotypes.

The mean sepal length of different genotypes showed significant differences among different cultivars. The difference in sepal and petal length could be related to availability of water (Kwak et al., 2007). Therefore, the longer the petal and sepal the higher the water availability. Hence cultivars *Mugumo*-1, *Mugiza*-1, *Kiithara*, *Kira Gitune*-1, *Kira Kieru*-1 and *Kira Kiiru*-1 were in place with enough water. The mean stamen length of many cultivars showed significant differences with the highest diameter of 2.37 mm being recorded in *Kira gitune*-1 cultivar from Meru and the lowest mean diameter of 1.07 mm was recorded in *Kira kieru*-2 cultivar from Meru. Availability of water results to increased flowers size and stamen length. Consequently, the traits usually related to floral attractiveness are increased (Natalia et al., 2015). However, fecundity potential is not increased. These results suggest that population differentiation in floral characters could be a caused by random genetic drift that occur in relatively small or isolated populations. It may also be attributed to restricted gene flow (Medrano et al., 2005).

The mean filament diameter showed significant difference among the cultivar with mean filament diameter ranging between 1.53 and 1.07 mm. *Kira Kiiru*-4 had the highest, while *Gitu*, *Muguka*-4 and *Muguka*-5 had the least 1.07. The floral parts of a plant are highly affected by the environment and this could be reasons as to why significant difference was recorded in filament diameter. The mean ovary length of all cultivars indicated no significant difference. This could be due to similar ancestry of all the cultivars studied. The mean capsule length showed significant difference among 30 khat samples, such could be used to discriminate cultivars. *Gitu* had the highest capsule length while *Kira gitune*-1 and *Kira kieru*-3 cultivar had the lowest. The difference in position of the inflorescence or the fruit could account for the variation in the capsule length (Buide, 2008). From the results it indicates that *Gitu* fruit was far from the stalk, while *Kira Gitune*-1 and *Kira Kieru*-1 fruit was near the stalk.

The mean length of the wing was significantly different among the various cultivars. *Muguka*-1 cultivar from Embu had the highest and *Kira Gitune*-1 had the lowest. This could be explained by the possibility that the age of the plant could have had an impact on the length of the seed wing (Georg-kraemer et al., 2004). The one with high mean wing length could be older compared to the one with short wing length. From the results this could mean *Muguka*-1 cultivar was older than *Kira Gitune*-1 cultivar.

Principal Component Analysis (PCA) is a method that summarizes the data without much loss of information based on the similarities and the differences of the data. The PCA results of 13 traits among 30 khat samples indicated that the first principal component had a higher contribution to the total variation compared to the second and third principal components. The traits which contributed the highest variability value in PC1 included leaf margin, petiole length, peduncle length, sepal length, petal length, diameter of stamen, diameter of filament and ovary length (Table 1). This showed that these traits were responsible for the most of the diversity exhibited in the first principal component. The cumulative value of the first three principal components (58.7%) was slightly above the first principal component obtained by Mawia et al. (2015) (53.8%) in 13 rice genotypes. The principal component two showed variability of six traits while principal component three showed variability of four traits (Table 1). Leaf margin, petiole length and peduncle length contributed to variation across all the three principal components, indicating that they were most important agronomic traits in *C. edulis*. The first Eigen value (3.247) was close to that found by Asudi et al. (2010) who reported a value of 3.147 on phenotypic features of Kenyan papaya. The second Eigen value of 2.386 was lower than that obtained by Mawia et al. (2015) in 13 rice genotypes. Conversely, the third Eigen value of 1.995 was higher than that obtained by Mawia et al. (2015) (0.38) in 13 rice genotypes.

The distribution of the cultivars on the scatter plot indicated the existence of wide variations among the studied cultivars. Cultivars that were close to each other on the same scatter plot showed phenotypic relatedness, while cultivars that were far away from each other were regarded as phenotypically distant. For example, the graphical closeness of *Mugumo*-5 and *Muguka*-3 indicated morphological relatedness. Most of the cultivars, from the same quadrant were collected from the same geographical region. For example *Mugumo*-5, *Mugumo*-2, *Mugiza*-2 and *Muguka*-3 were found in the same quadrant and were collected from the same geographical region. *Gitune*, *Muceke* and *Mumbu* were from the same county. However, cultivars from different geographical regions were found clustering together for instance *Kirakiiru* from Meru clustered together with *Muguka*-1*, Mutimutiri and Muguka*-4 which were cultivars from Embu county, while *Mugumo*-4,*Gitu,Mugumo*-1,*Muruti, MugukawaKarimi* clustered together with *Kira Gitune*-2, *Kira Kieru*-1 which were cultivars from Meru county.

The dendrogram generated from 13 phenotypic traits showed two main clusters (clusters I and II), which indicated an existing variation in the cultivars. Cluster II showed high phenotypic variation, indicating the enormous phylogenetic divergence among cultivars in this cluster. Cluster II had two sub-clusters, IIa and IIb each of these sub-cluster, clustered into two subcluster again, IIai, IIaii and IIbi and IIbii. Cultivars in these cluster I included *Kira Kieru*-3, *Mugukawakarimi* and *Muguka*-4 cultivars where 2 cultivars were from Embu and 1 from Meru County. Most of the cultivars clustered in cluster II, some of the cultivars included *Kira Kiiru*-2 and *Kira Kieru*-2 which

were collected from Meru in cluster IIai, *Muguka*-2*, Mumbu, Mugumo*-2*,* and *Mugumo*-3 cultivars from Embu was found in IIbi. In these findings, those cultivars which clustered together were collected from the different geographical location. For the scatter plot, for example *Kira Kieru*-2 and *Kira Kiiru*-2 cultivars which were on the same scatter plot showed little variation in dendrogram clustering. Further studies are recommended due to the limitations associated with morphological traits as a means of determining variations, the diversity of these khat cultivars should be validated using superior traits such molecular markers.

Conclusions

From this study, it was concluded that phenotypic diversity showed considerable variability; clustering of the phenotypes was not based on the geographical origin of the plant.

CONFLICT OF INTERESTS

The authors have not declares any conflict of interests in phenotypic variations in *Catha edulis* along different growth intervals should be determined.

REFERENCES

Al Motarreb A, Baker K, Broadley KJ (2002). Khat: Pharmacological and medical aspects and its social use in Yemen. Journal of Phytotherapy Research 16(5):403-413.

Al-Kholani AI (2010). Influence of Khat Chewing on Periodontal Tissues and Oral Hygiene Status among Yemenis. Dental Research Journal 7(1):1-6.

Al-Thobhani MAH, Sathyanarayana BN, Simon L, Sondur SN (2008). First comparative genotypic study on khat (Catha edulis Forsk) genotypes from Yemen.Middle East. Russian Journal of Plant Science and Biotechnology 2:1-8.

Amiri R, Bahraminejad S, Jalali-Honarmand S (2013). Effect of terminal drought stress on grain yield and some morphological traits in 80 bread wheat genotypes. International Journal of Agriculture and Crop Science 5(10):1145-1153.

Asudi GO, Ombwara FK, Rimberia FK, Nyende AB, Ateka EM, Wamocho LS, Shitanda D, Onyango A (2010). Morphological diversity of Kenyan papaya germplasm. African Journal of Biotechnology 9(51):8754-8762.

Ayana AM, Mekonen Z (2004). Khat (Catha edulis Forsk). Chewing, socio-demographic description and its effects on academic performance, Jimma University Students.

Buide ML (2008). Disentangling the causes of intrainflorescence variation in floral traits and fecundity in the hermaphrodite Silene acutifolia. American Journal of Botany 95(4):490-497.

Deblonde PMK, Ledent JF (2001). Effects of moderate drought conditions on green leaf number, stem height, leaf length and tuber yield of potato cultivars. European Journal of Agronomy 14(1):31-41

Domiciano GP, Alves AA, Laviola BG, Albrecht JC (2014). Analysis of morpho-agronomic and climatic variables in successive agricultural years provides novel information regarding the phenological cycle of Jatropha in conditions of the Brazilian cerrado. Anais da Academia Brasileira de Ciências Journal 86(4):1985-1998.

El-Menyar A, Mekkodathil A, Al-Thani H, Al-Motarreb A (2015). Khat use: history and heart failure. Oman Medical Journal 30(2):77-82.

Georg-kraemer JE, Caierão E, Minella E, Barbosa-Neto JF, Cavalli SS (2004). The glutamates in malting barley: enzyme survival and genetic and environmental effects. Journal of the Institute of Brewing 110:303-308.

Gesese D (2013). Is khat a social ill? Ethical arguments about 'stimulant' among learned Ethiopians, ASC Working Paper 108.

Gitonga GM, Ngeranwa J, Machocho AK, Muthee DG, Kimutai R, Gitonga AW(2017). Nephrotoxicity effects of Khat (Catha edulis) on mice when administered orally. Journal of Phytopharmacology 6(1):27-33.

Inouye DW (2008). Effects of climate change on phenology, frost damage, and floral abundance of montane wildflowers. Journal of Ecology 89 (2):353-362.

Kiunga JL, Dossaji C, Yenesew AS (2016). A Survey of Traditional Medicinal Uses of Cathaedulis (Celastraceae) in Meru and Embu Counties of Kenya. International Journal of Ethnobiology and Ethnomedicine 3(1):1-12.

Kwak MS, Min SR, Lee SM, Kim KN, Liu JR, Paek KH, Shin JS, Bae JM (2007). A sepal-expressed ADP-glucose pyrophosphorylase gene (NtAGP) is required for petal expansion growth in 'Xanthi' tobacco. Plant Physiology 145(1):277-89.

Malek MA, Rafii MY, Afroz SS, Nath UK, Mondal M (2014). Morphological characterization and assessment of genetic variability, character association, and divergence in soybean mutants. The Scientific World Journal pp. 1-12.

Mawia MA, Wambua KF, Daniel A, Nyamai WD, Felix M (2015). Phenotypic Diversity Studies on Selected Kenyan and Tanzanian rice (*Oryza sativa* L): Genotypes Based on Grain and Kernel Traits. Journal of Rice Research 3(4):150-161.

Medrano M, Herrera CM, Barrett SCH (2005). Herkogamy and mating patterns in the self compatible daffodil (*Narcissus longispathus*). Annals of Botany Journal 95:1105-1111.

Mwenda JM, Arimi MM, Kyama MC, Langat DK (2003). Effects of khat (Catha edulis) consumption on reproductive functions: a review. East African Medical Journal 80:318-323.

Natalia SL, Jamie O, David P, Rachel C, Adams, Katarina K, Frederick V (2015). Training response inhibition to food is associated with weight loss and reduced energy intake, Journal of Appetite 95:17-28.

New Agriculturalist (2007). Growing demand for Kenyan khat,Wren Media, Suffolk, UK.

Ng'ethe JW (2012). Factors influencing the consumption and control of Khat among the youth in Igembe South District, Meru County, Kenya (Doctoral dissertation, University of Nairobi, Kenya).

Ngari LN, Njagi SN, Kamau JK, Lagat RC, Musyoki AM (2018). Genotypic Characterization of Catha edulis in Mt. Kenya Region, Kenya. Journal of Phylogenetics Evolutionary Biology 6:203.

Nyongesa AW, Onyango DW (2010). Khat (Catha edulis Forsk): A bane or boon to humanity. Journal of Ethnomedicine 28:187-209.

Prasad PVV, Craufurd PQ, Kakani VG, Wheeler TR, Boote KJ (2001). Influence of high temperature during pre-and post-anthesis stages of floral development on fruit-set and pollen germination in peanut. Functional Plant Biology Journal 28(3):233-240.

Rahman I, Ahmad H, Sirajuddin I, Ahmad I, Abbasi FM, Islam M, Ghafoor S (2009).Valuation of rapeseed genotypes for yield and oil quality under rainfed conditions of district Mansehra. African Journal of Biotechnology 8(24):6844-6849.

Robson NKB (1994). Flora of Tropical East Africa-Celastraceae.CRC Press.

Sangoi L (2001). Understanding plant density effects on maize growth and development: an important issue to maximize grain yield. Ciência Rural Journal 31(1):159-168.

Sikiru L (2012). Flower of paradise (Khat: Catha edulis): Psychosocial, health and sports perspectives. African Journal of Health Sciences 22:61-175.

Tardieu F (2013). Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit. Frontiers in physiology 4:17. doi: 10.3389/fphys.2013.00017

Wabe NT (2011). Chemistry, pharmacology, and toxicology of khat (catha edulis forsk): a review. Addiction and Health 3(3-4):137-49.

Zeng X (2015). Genetic variability in agronomic traits of a germplasm collection of hulless barley, Journal of genetics and molecular Research: Genetics and Molecular Research 14(4):18356-18369.

Appendix 1. The name of the cultivar, place of collection and the type.

Code	Local name	County	Sub-County	Ward	Type habit
1	*Kira kieru* 1	Meru	Igembe South	Maua	Tree
2	*Kira kieru* 2	Meru	Igembe Central	Kangeta	Tree
3	*Kira kieru* 3	Meru	Igembe West	Kianjai	Tree
4	*Kira kiiru* 1	Meru	Igembe South	Maua	Tree
5	*Kira kiiru* 2	Meru	Igembe Central	Kangeta	Tree
6	*Kira gitune* 1	Meru	Igembe South	Maua	Tree
7	*Kira gitune* 2	Meru	Igembe South	Gaiti	Tree
8	*Muchuri*	Meru	Tigania West	Kianjai	Tree
9	*Kithara*	Meru	Tigania east	Muthaara	Tree
10	*Mutimutiri*	Embu	Mbeere South	Mavuria	Shrub
11	*Mugiza*1	Embu	Runynjes	Kagaari south	Shrub
12	*Mugiza* 2	Embu	Manyatta	Kithimu	Shrub
13	*Mugumo* 1	Embu	Mbeere South	Mavuria	Shrub
14	*Mugumo* 2	Embu	Mbeere North	Nthawa	Shrub
15	*Mugumo* 3	Embu	Mbeere South	Mbeti south	Shrub
16	*Mugumo* 4	Embu	Manyatta	Kithimu	Shrub
17	*Mugumo* 5	Embu	Runyenjes	Kaagari north	Shrub
18	*Muguka* 1	Embu	Mbeere South	Mavuria	Shrub
19	*Muguka* 2	Embu	Mbeere North	Muminji	Shrub
20	*Muguka* 3	Embu	Mbeere North	Nthawa	Shrub
21	*Muguka* 4	Embu	Manyatta	Mbeti north	Shrub
22	*Muguka* 5	Embu	Runyenjes	Kaagaari north	Shrub
23	*Muguka wa karimi*	Embu	Mbeere South	Mavuria	Shrub
24	*Gitu*	Embu	Mberee South	Mbeti south	Shrub
25	*Mutamucii*	Embu	Mbeere South	Mavuria	Shrub
26	*Mukurukuru*	Embu	Mbeere North	Nthawa	Shrub
27	*Muruti*	Embu	Mbeere North	Muminji	Shrub
28	*Muceke*	Embu	Mbeere South	Mavuria	Shrub
29	*Gitune*	Embu	Mbeere South	Mbeti south	Shrub
30	*Mumbu*	Embu	Mbeere North	Muminji	Shrub

Appendix 2. Phenotypic traits of 30 khat cultivars.

Genotype	Leaf length	Leaf width	Leaf margin	Petiole length	Inflorescence length	Peduncle length
Gitu	74.00±1.15^{ef}	34.00±0.58^{edfg}	92.00±1.35^{c}	2.67±0.33^{cd}	20.00±0.57^{ab}	5.33±0.33^{abcd}
Gitune	73.00±0.58^{efgh}	37.33±0.88^{cde}	71.33±1.20^{ghi}	4.33±0.33^{abc}	15.33±0.67^{ef}	4.67±0.33^{bcd}
Muguka 2	67.33±1.45^{fghi}	30.67±0.67^{gh}	63.67±0.88^{jk}	5.67±0.33^{a}	20.00±0.58^{ab}	5.33±0.33^{abcd}
Kira gitune 1	60.00±1.00^{jkl}	26.33±0.88^{ijk}	72.67±1.45^{gh}	4.33±0.33^{abc}	15.00±0.58^{f}	7.33±0.08^{a}
Kira gitune 2	75.33±1.45^{de}	21.33±0.88^{lm}	82.00±1.53^{de}	4.33±0.33^{abc}	19.67±0.33^{abc}	7.00±0.29^{ab}
Kira kieru 1	72.00±1.53^{efgh}	19.33±0.67^{m}	74.67±1.20^{fg}	4.67±0.33^{ab}	21.00±0.58^{a}	6.00±0.58^{abcd}
Kira kieru 3	94.33±1.76^{a}	30.33±0.88^{ghi}	99.00±0.58^{b}	4.67±0.33^{ab}	14.68±0.88^{f}	6.00±0.58^{abcd}
Mugumo 4	72.33±1.67^{efgh}	24.00±0.58^{kl}	80.33±0.88^{ef}	4.33±0.33^{abc}	20.33±0.33^{a}	6.00±0.58^{abcd}
Kira kieru 2	73.67±1.86^{ef}	30.00±0.57^{ghi}	87.67±1.45^{cd}	4.67±0.33^{ab}	17.00±0.58^{bcdef}	5.33±0.33^{abcd}
Kira kiiru 1	73.33±1.45^{efg}	35.33±0.67^{def}	71.67±0.88^{ghi}	4.67±0.33^{ab}	17.00±1.15^{bcdef}	5.33±0.33^{abcd}
Kira kiiru 2	71.33±0.88^{efgh}	24.00±1.00^{kl}	87.33±1.20^{cd}	4.33±0.33^{abc}	19.67±0.33^{abc}	6.83±0.44^{ab}
Kiithara	82.00±1.15^{cd}	23.00±1.15^{klm}	88.00±1.15^{cd}	4.67±0.33^{ab}	16.33±0.88^{def}	6.67±0.88^{abc}
*Muchur*i	56.00±1.15^{kl}	25.00±0.58^{jkl}	72.33±0.88^{ghi}	4.67±0.33^{ab}	16.67±0.88^{def}	6.33±0.33^{abcd}
Muguka 5	91.67±0.88^{b}	41.68±0.88^{ab}	87.67±1.20^{cd}	3.33±0.33^{bcd}	20.67±0.33^{a}	4.33±0.33^{cd}
Muguka 4	99.00±0.58^{a}	45.67±0.33^{a}	107.33±1.45^{a}	4.33±0.33^{abc}	19.33±0.33^{abc}	4.67±0.33^{bcd}

Appendix 2. Contd.

Mugumo 5	72.33±1.20^{efgh}	30.67±0.67^{gh}	71.67±0.88^{ghi}	5.00±0.57^{ab}	19.67±0.33^{abc}	4.67±0.33^{bcd}
Muguka 1	73.00±1.73^{efg}	33.67±0.88^{efg}	73.00±1.00^{gh}	5.67±0.33^{a}	16.67±0.88^{cdef}	4.33±0.33^{cd}
Muguka 3	72.00±1.53^{efgh}	32.00±1.15^{fgh}	82.33±1.45^{de}	2.33±0.33^{d}	20.67±0.67^{a}	4.33±0.33^{cd}
Muceke	70.00±0.58^{efgh}	40.00±0.58^{bc}	69.33±0.88^{ghij}	5.33±0.33^{a}	15.33±0.88^{ef}	4.67±0.3^{bcd} 3
Mugiza 2	73.33±1.76^{efg}	38.00±0.58^{bcd}	74.33±0.88^{fg}	4.33±0.33^{abc}	16.67±0.88^{cdef}	4.67±0.33^{bcd}
Muguka wa karimi	81.00±1.00^{d}	31.33±0.88^{fgh}	102.67±1.20^{ab}	4.67±0.33^{ab}	19.33±0.33^{abcd}	4.67±0.33^{bcd}
Mugiza 1	70.67±1.20^{efgh}	41.67±0.88^{ab}	73.00±0.88^{gh}	2.33±0.33^{d}	19.67±0.33^{abc}	4.00±0.58^{d}
Mugumo 1	88.67±1.20^{bc}	41.67±0.88^{bc}	102.33±1.20^{ab}	3.33±0.33^{bcd}	18.33±0.33^{abcde}	4.00±0.58^{d}
Mugumo 2	61.33±0.88^{hij}	28.67±0.88^{ijk}	63.33±0.88^{jk}	3.33±0.33^{bcd}	20.67±0.33^{a}	4.00±0.58^{d}
Mugumo 3	62.33±0.33^{ijk}	33.67±0.33^{efg}	61.67±0.33^{jk}	4.33±0.33^{abc}	15.67±0.88^{ef}	5.00±0.58^{abcd}
Mukurukuru	70.00±1.15^{efgh}	38.00±0.58^{bcd}	75.33±0.33^{fg}	4.33±0.33^{abc}	15.33±0.33^{ef}	4.67±0.33^{bcd}
Mumbu	66.33±0.33^{hij}	28.33±0.88^{hij}	66.33±0.88^{ijk}	4.33±0.33^{abc}	20.33±0.33^{a}	4.67±0.33^{bcd}
Muruti	66.67±0.88^{ghij}	33.00±0.58^{fg}	65.00±1.00^{jk}	4.33±0.33^{bcd}	19.67±0.33^{abc}	4.67±0.33^{bcd}
Mutamucii	73.33±1.76^{efg}	33.00±0.58^{fg}	68.00±0.58^{hij}	4.33±0.33^{abc}	16.33±0.33^{def}	4.67±0.33^{bcd}
Mutimutiri	53.67±1.20^{l}	21.33±0.33^{lm}	55.00±0.58^{l}	2.67±0.33^{cd}	14.33±0.33^{f}	4.33±0.33^{cd}

Values are expressed as Mean±SEM. Values with the same superscript letter are not significantly different (one way ANOVA followed by Tukey's test ($p>0.05$).

Vol. 18(29), pp. 875-881, September, 2019
DOI: 10.5897/AJB2019.16899
Article Number: A762ECB61946
ISSN: 1684-5315

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Potential of *Opuntia stricta* Haw (Mexican elephant ear) in removing cyanobacteria in surface water

Janiele F. Nery*, Gleydson K. M. Nery, Adriana G. Magalhães and Salomão S. Medeiros

Water Resources Division, Brazilian National Semiarid Institute, P. O. Box 10067, Paraíba, Brazil.

Received 21 June 2019; Accepted 26 August 2019

In this study, assays were carried out to evaluate the efficiency of cactus *Opuntia stricta* cladodes as a coagulant for removal of cyanobacteria cells. To carry out the coagulation assays, water from eutrophic reservoir Bodocongó, in Brazilian semiarid, with cyanobacteria bloom of *Microcystis aeroginosa, Plankthotrix isothrix* and *Cylindrospermopsis raciborskii* were sampled. This water was submitted to coagulation with different forms of *O. stricta* cladodes (crude, pulverized and solution), concentration (10; 50 and 100 mg/L) and time (5, 15, 30, 60 and 120 min). In order to assess cell removal, readings were made in aliquots of 5 mL of samples analyzed by triplicates counting on sedimentation chambers using an inverted optical microscope, according to the Utermöhl method. Dosage of 100 mg/L from the solution form of *O. strica* cladodes gave the best cell and turbidity removal. Colonial species were fast removed than filaments. The most removal occurred in the first 30 min of experiments. No significant differences were observed for pH changes in the experiments. *O. stricta* cladodes gave satisfactory results in reducing cyanobacteria cell in water; however, complete removal was not obtained; further studies are necessary to evaluate the best concentrations and the mechanisms of cyanobacteria removal.

Key words: Eutrophication, biobased coagulants, Cactus.

INTRODUCTION

Eutrophication is considered the Earth's most important water quality problem (Schindler, 2012). It often results in blooms of potentially toxic cyanobacteria that complicate the use of lakes and reservoirs and can cause potential public health risk (Azevedo et al., 2002; Huisman et al., 2018). Cyanobacteria have adapted to survive in a variety of aquatic and terrestrial environments and have been found globally (Rigosi et al., 2014). Harmful cyanobacteria have been increasingly gaining the attention of scientists and government agencies because they are known to produce various bioactive compounds, and some of them show beneficial therapeutic effects; thus, used as dietary supplements as well as mood enhancers (Jensen et al., 2001). Other cyanobacteria can produce cyanotoxins, which are harmful to humans, animals, and plants and fall into five different types of toxins: (i) hepatoxins (cylindrospermopsin, microcystins and nodularins); (ii) neurotoxins (anatoxin-a and saxitoxins); (*iii*) dermatotoxins; (*iv*) cytotoxins; (*v*) and irritant toxins [lipopolysaccharides (LPSs)] (Graham et al.,

*Corresponding author. E-mail: janiele.biologa@gmail.com.

2010).

Cyanotoxin can be accumulated into aquatic products via contaminated feeds, direct contact with contaminated water (living environment) and biomagnification through the food web (Ibelings and Chorus, 2007; Vasconcelos et al., 2013). Plants are not usually killed by the environmentally relevant concentration, but their growth and crop yields are affected (Babica et al., 2006; Bittencourt-Oliveira et al., 2016; Svirčev et al*.,* 2017). In addition, the soil may retain toxins when extra water flows through, and it can bioaccumulate toxins during non-bloom seasons (Pflugmacher et al., 2007; Corbel et al., 2014). Humans are exposed to cyanotoxin through drinking water, inhalation, dermal contact and foods (Carmichael et al., 2001; Cheung et al., 2013; Lee et al., 2017).

Thus, controlling eutrophication and mitigating potential toxic cyanobacteria is an essential task (Lürling et al., 2016). Conventional chemicals, like algicidies are used to remove cyanobacterial blooms but their application may come with shortcomings such as toxins and nutrient release or unwanted ecotoxicological side effects (Jančula and Maršálek, 2011; Merel et al., 2013). Removal of cyanobacteria from the water column using a combination of coagulant and ballast are a promising technique for controlling cyanobacterial bloom. The flock and lock and flock and sink techniques remove cyanobacteria out of the water column while remaining as intact cells where after the cyanobacteria and their toxins can be degraded on the sediment (Pan et al., 2011; Noyma et al., 2016; Magalhães et al., 2017). For this, the use of local soils, clays or waste products can be a faster, cheaper and easy to handle alternative.

In this perspective, natural plant-based coagulants have been tested (Miller et al., 2008; Nishi et al., 2011; Camacho et al., 2017). The main advantages of using this for water treatment are apparent; they are cost-effective, unlikely to produce treated water with extreme pH and highly biodegradable (Daza et al., 2016). These advantages are especially augmented if the plant from which the coagulant is extracted is indigenous to a rural community (Yin, 2010). In the era of climate change, depletion of the earth's natural resources and widespread environmental degradation, the application of these coagulants is a vital effort, aligned with global sustainable development initiatives.

Application of cacti species for water treatment is rather recent compared to other natural coagulants such as common bean (*Phaseolus vulgaris*) (Antov et al., 2010) nirmali seed (*Strychnos potatorum*) (Babu and Chaudhuri, 2005) or *Moringa oleífera* (Muthuraman and Sasikala, 2014; Oladoja and Pan, 2015). The most commonly studied cactus genus for water treatment is Opuntia, which represents one of the most diverse and distributed genera of plants (Zhang et al., 2006; Miler et al., 2008; Ortiz et al., 2013; Oladoja, 2015). It has since been introduced all over the world and can be found in temperate, subtropical and tropical regions (Izuegbuna et al., 2019). Besides Opuntia, other cactus species including *Cactus latifaria* have also been successfully used as natural coagulants (Diaz et al., 1999).

The high coagulation capability of Opuntia is most likely attributed to the presence of mucilage which is a viscous and complex carbohydrate stored in cactus inner and outer pads that has great water retention capacity (Saenz et al., 2004). Previous studies have established that mucilage in Opuntia cactis contains carbohydrates such as l-arabinose, d-alactose, l-rhamnose, d-xylose, and galacturonic acid (Trachtenberg and Mayer, 1981). Miller et al. (2008) reported that galacturonic acid is possibly the active ingredient that affords the coagulation capability of *Opuntia spp*. though it should be noted that it only accounts for only 50% of turbidity removal.

Among Opuntia species*,* only a few works are found in literature using *O. stricta* for water treatment (Zhang et al., 2006), however, this species is resistant to the carmine cochineal pest (*Dactylopius* spp.) being widely cultivated, especially in the Brazilian semiarid (Santos et al., 2018). It is noteworthy that no study was carried out to evaluate the coagulation potential of Opuntia species for the removal of cyanobacteria. Therefore this study analyzed the potential efficiency of *O. stricta* cladode as a coagulant for the removal of cyanobacteria cells. These coagulants are not only naturally reproducible but may also offer many other advantages like local availability, adaptability, and lesser health hazards than residual mineral coagulants or synthetic polymers.

MATERIALS AND METHODS

Study site

Bodocongó reservoir is located in Campina Grande, Brazilian semiarid (7º13'11" S, 35º52'21" W), at an altitude of 548 m above de sea level. The climate conditions are a warm semiarid (BSwh in the Köppen system). The temperature of annual mean is between 25 and 31ºC and rainfall of 700 mm/year. The reservoir is part of Paraíba river basin; it has a surface area of 371897 m^2, a mean and maximum depth of 3.5 and 7.0 m, respectively, a mean total water volume of 1019830 m^3, which may vary considerably depending on climate conditions. The urbanization processes around the reservoir promote frequently sewerage and wastewater discharge into Bodocongó's waters, and also a decline in riparian vegetation. It is a hipereutrophic reservoir with mean concentration of a total Potasium of 396.0 µg/L (Abílio et al., 2006).

For the assays, water samples were taken from different sites in the reservoir on January, 2019. At this time there was a cyanobacterial bloom, with contributions of *Microcystis aeruginosa* (Kützing) Kützing 1846, *Sphaerocavum brasilense* De Azevedo & C.L. Sant' Anna 2003*, Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju in Desikachary 1997 *and Plankthotrix isothrix (*Skuja) Komárek and Komárková 2004*.*

Preparation of coagulant

The cactus *O. stricta* were collected from experimental campus of Brazilian National Semiarid Institute (INSA, for this acronym in

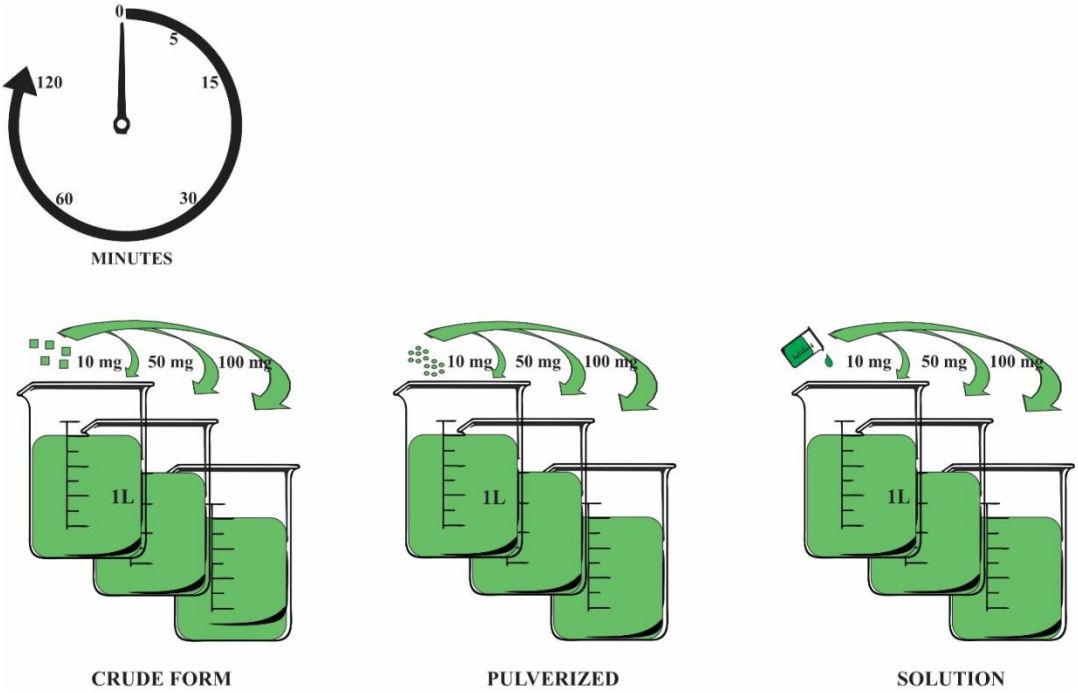

Figure 1. Schematic representation of experimental design.

Portuguese). To assays, the cladodes were used in three forms: Crude form, pulverized and pulverized solution. The collected cladodes were washed with ultrapure water, spines were removed and then stored in refrigerator at 4ºC, to assays with crude form. Other part of cactus was sliced into strips 1 cm wide, freeze and lyophilized at -80ºC. The dried cactus was milled using a Wiley mill and sieved with the aid of sieves (ABNT, 2010); a powder was obtained with particles of approximately 100 µm in diameter (Miller et al., 2008). A part of this is used in pulverized assays and others were diluted to 1% stock solution (concentration = 1000 mg/L) to pulverized solution assays. The solution of the lyophilized was used because studies of Subramonian et al., (2014) pointed out different absorption sites between the solid and diluted strata.

Experimental design

The efficiency of *O. stricta* to remove cyanobacteria was tested in a laboratory scale. The experiments were composed by three treatments (form, concentration and time) with 3 to 5 levels repeated three times (Figure 1).

Aliquots of 1000 mL water of Bodocongó reservoir were transfered to 2000 mL graduated beckers. Water was treated with designated treatment (final concentration of 10, 50 and 100 mg/L) or left untreated (controls), at 25ºC. After 5, 15, 30, 60 and 120 min, pH, turbidity and total dissolved solids (TDS) were measured with HORIBA U52 multiparameter. Samples with 5 mL were fixed with 2% lugol solution for quantitative analyzes using inverted microscope (Zeiss Axiovert), as described by Utermöhl (1958).

Data analyzes

Results were examined by analysis of variance (ANOVA), followed by Fisher's multiple comparison test for all measured parameters (pH, turbidity, TDS and cyanobacteria cells), for each treatment. Normality was assessed by Kolmogorov-Smirnov test and the homoscedasticity by Fisher's test. The effects of combined treatments were estimated by two-way ANOVA. For this statistical analyzes, values with $p<0.05$ were considered significant. The program 'Statistica' version 7.0/2004 (Statsoft) was used.

RESULTS

Significant differences were observed in the density of cyanobacteria, TDS and turbidity, when compared to the forms of *O. stricta* used after 120 min exposure. The best results occurred when the solution was used (Table 1). Among the concentrations tested, significant differences were observed in all forms used, for all variables except pH and TDS. The best coagulant effects were observed at dose C2 (50 mg/L), except when solution was used, when C3 dosage were more effective, however, not significantly different. The effects of the interaction between form and concentration were observed only for the turbidity variable (Table 1).

Significant statistical interaction was observed regarding exposure time and *O. stricta* forms to all variables, except pH ($F_{cyanobacteria\ cell}$= 8.3, $p<0.01$; F_{TDS}=40.9, $p<0.01$; $F_{Turbidity}$=5.9, $p<0.01$).

For all treatments were observed Cyanobacteria cell removal, that ranged from 30(±4.3) to 70(±2.7). Considering the time factor, significant differences were observed to coagulation process, however cell removal occurred majorly in the first 30 min of the experiments (Figure 2A). Colonial species were fast removed in all

Table 1. Mean and standard deviation of Cyanobacteria cell, pH, total dissolved solids (TDS) and turbity for different forms and concentration of *O. stricta* treatments after 120 min.

Variable	Control	Crude form			Pulverized			Solution			Form*	Interation (Form x Concentration)
		C1	C2	C3	C1	C2	C3	C1	C2	C3		
Cyanobacterial cell (Cell/mL)	106570 (±1150)^{a}	61668 (±1100)^{b}	56486 (±920)^{c}	76220 (±930)^{b}	58944 (±543)^{b}	34592 (±341)^{c}	60475 (±568)^{b}	50510 (±786)^{b}	31998 (±552)^{c}	30994 (±540)^{c}	CF=P≠S	**
pH	8.0 (±0.1)	8.04	7.92	7.7	8.1	8.1	8.0	7.8	7.8	7.7	**	**
TDS (g/L)	1.7(±0.1)^{a}	1.5(±0.1)^{a}	1.4(±0.1)^{a}	1.5(±0.1)^{a}	1.7(±0.1)^{a}	1.6(±0.1)^{a}	1.7(±0.1)^{a}	1.5(±0.1)^{a}	1.2(±0.1)^{b}	1.0(±0.1)^{a}	CF=S≠P	**
Tubidity (NTU)	58.1 (±1.5)^{a}	31.7 (±2.0)^{b}	28.7 (±2.2)^{c}	30.6 (±1.5)^{b}	36.8 (±2.7)^{b}	48.8 (±7.2)^{b}	47(±7.0)^{b}	28.7 (±2.8)^{b}	25.9 (±1.7)^{c}	23.0 (±2.6)^{c}	CF=S≠P	<0.05

Data with same letter did not differ significantly (p>0.05) among concentrations applied. *ANOVA for Form treatment – CF (crude form), P (Pulverized), S (Solution)
** Not differ significantly (p>0.05)

treatment. Filaments were optimally removal after 30 min of experiment.

pH in water increased in pulverized treatments and decreased in crude form and pulverized solution treatments, but no significant differences were observed (Table 1). These results indicated that the use of *O. stricta* does not alter pH of water and this efficiency is not strictly dependent on pH.

The optimal conditions to remove TDS of water occurred with pulverized solution in C3 concentration (Figure 2B), however the efficiency were lower than 30%. In pulverized treatments an increase in TDS were observed in the first 5 min of the experiment to C2 and C3 concentration.

Turbidity removal ranged from 19(±10.9) to 52(±5.8)%. The optimal results were obtained to solution treatment at C3 concentration (Figure 2C). Significant effect was observed in the interaction between form and concentrations, and form and time of experiment in the efficiency of turbidity removal (Table 1). In Pulverized treatments, a gradual increase in turbidity occurred after the first 15 min of experiments when C3 dosage was applied. Significant turbidity removals were observed after 30 min of the experiments using crude form and 60 min using solution treatments (Figure 2C).

DISCUSSION

O. stricta is a viable alternative for the removal of cyanobacteria in water. Considerable cyanobacteria cell (30-70%) and turbidity (19-52%) were removed after 120 min of the experiments. In this case the coagulation activity was qualified as present, especially in pulverized solution form. Miller et al. (2008) considered coagulation activity qualified as “absent” if turbidity removal is below 30%.

The predominant coagulation mechanism for *Opuntia* spp. is adsorption and bridging, whereby clay particles do not directly contact one another but are bound to a polymer-like material from *Opuntia* spp. It was also thought that adsorption may occur through hydrogen bonding or dipole interactions and this possibility was ascribed to the likelihood that natural electrolytes from within the *Opuntia* spp. pad, particularly the divalent cations, which are known to be important for coagulation with anionic polymers facilitated by the adsorption (Oladoja, 2015).

The findings from studies on the screening of green biobased materials as coagulants for water and wastewater purification showed that these evolving type of coagulants hold a lot of potential as substitute to the conventional synthetic metal or polymer based coagulants in water and wastewater treatment operations (Yin, 2010).
Regarding cyanobacteria removal by coagulation, good results have been reported, depending on the characteristics of organic matter present in water, the prevalent cyanobacteria species, and the type and concentration of coagulant (Heng et al., 2009; Henderson et al., 2010; Shen et al., 2011). The results demonstrated a good potential of O. stricta to remove colonial and filamentous organisms. According to Li et al., (2018), colonial species are first removed because they are free of protruding appendages, or have mucilage (polymeric substances), while, filamentous could not be wrapped by coagulants.

Natural coagulants exhibit highly effectual turbidity removal capabilities, with some of them removing up to 99% of initial turbidity (Nish et al., 2011; Oladoja and Pan, 2015). In the results, turbidity removal was about 52%, not exceeding

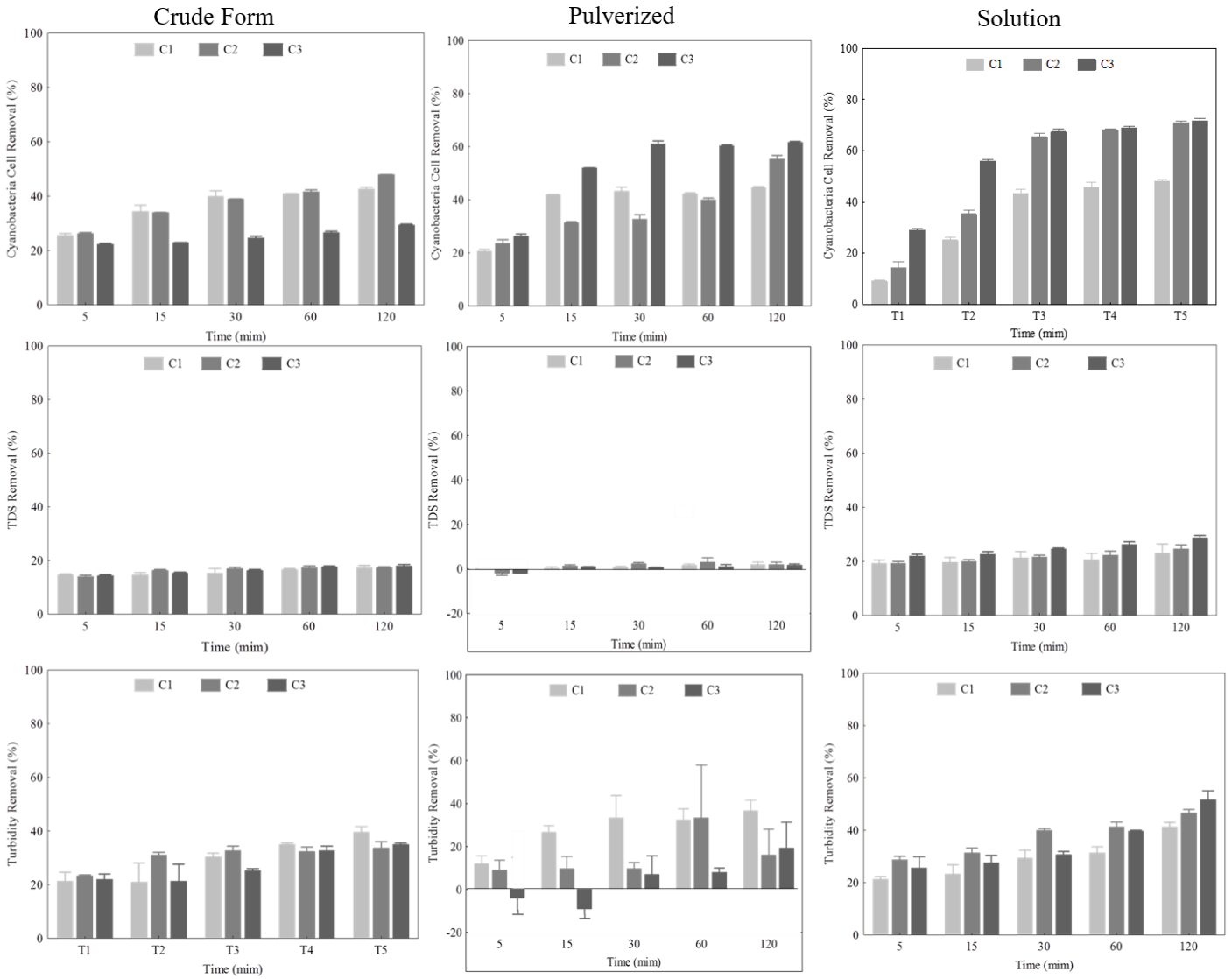

Figure 2. Removal efficiency of cyanobacteria cells, TDS and turbidity at different concentrations and forms of *O. stricta* along time.

21 NTU.

The absence of removal and increase in water turbidity observed may have occurred due to the addition of the *O. stricta* in pulverized form, increasing the organic load. Similar increases in color and turbidity in water treated with green biobased coagulants have been observed in other studies, using *M. oleifera*, particularly when the initial color and turbidity are relatively low (Ndabigengesere and Narasiah, 1998).

It was observed that coagulation activity reduced when the *O. stricta* dose is too low or too high, with best results at intermediate dosage (50 mg/L); this is consistent with a bridging removal mechanism. The bridging mechanism required a stoichiometric relationship between particle concentration and coagulant dose (Oladoja, 2015). Such efficiencies are certainly comparable to the established chemical coagulants (e.g. aluminum). Optimum dosages are generally within the range of 10 to 60 mg/L. Natural coagulants are most effective at basic waters as evident by the optimum pH values from 7 to 10 (Zhang et al., 2006; Miller at al., 2008). Furthermore, the pH of the water is not affected during coagulation and the pH adjustment may not be necessary for subsequent treatment processes.

The presence of humic substances in natural surface water may significantly alter Opuntia dose for optimal coagulation (Zhang et al., 2006). Therefore, *O stricta* may prove useful as primary coagulant for subsequent treatment through slow sand filters; however, its efficiency should also be further investigated. Analysis of size and nature of flocks achieved through coagulation using *O. strict* is coherent with the previous suppositions that potential mechanism of coagulation through Opuntia is adsorption and inter-particle bridging (Oladoja, 2015).

One of the snags of the use of green biobased coagulants is the substantial increase in the organic load of the treated water, which may result in the possibility for undesired and increased microbial activities. Organic matter is regarded as the source of odor, color, and taste, and a precursor of disinfection by-products in drinking water treatment, so, considering our results, we suggest the use of *O. stricta* to water before irrigation practice.

This is the first record of use of *O. stricta* cladodes to removal cyanobacterial cell. Other studies are necessary to evaluate the performance of *O. stricta* to remove cyanotoxins as well as to improve its efficient and compare it to inorganic coagulants.

Conclusion

Cactus *O. stricta* was an abundant natural product, cost effective, safe for human health that can be used to remove cyanobacteria in water used for irrigation. The potential to remove Cyanobacterias can be explored for water treatment for consumption associated with other coagulants-flocculants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abílio FJP, Fonseca-Gessner AA, Leite RL, Ruffo TLM (2006). Gastrópodes e outros invertebrados do sedimento e associados à macrófita *Eichhornia crassipes* de um açude hipertrófico do semiárido paraibano. BioTerra 1(supl.):165-178

Azevedo SM, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK (2002). Human intoxication by microcystins during renal dialysis treatment in Caruaru—Brazil. Toxicology 181:441-446.

Babica P, Bláha L, Maršálek B (2006). Exploring the natural role of microcystins—A review of effects on photoautotrophic organisms 1. Journal of Phycology 42(1):9-20.

Babu R, Chaudhuri M (2005). Home water treatment by direct filtration with natural coagulant. Journal of water and health 3(1):27-30.

Bittencourt-Oliveira M, Cordeiro-Araújo MK, Chia MA, Arruda-Neto JDT, Oliveira, ÊT, Santos F (2016). Lettuce irrigated with contaminated water: Photosynthetic effects, antioxidative response and bioaccumulation of microcystin congeners. Ecotoxicology and Environmental Safety 128:83-90.

Camacho FP, Sousa VS, Bergamasco R, Ribau-Teixeira M (2017). The use of Moringa oleifera as a natural coagulant in surface water treatment. Chemical Engineering Journal 313:226-237.

Carmichael WW, Azevedo, SM, An JS, Molica RJ, Jochimsen EM, Lau S, Eaglesham GK (2001). Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environmental health Perspectives 109(7):663-668.

Cheung MY, Liang S, Lee J (2013). Toxin-producing cyanobacteria in freshwater: A review of the problems, impact on drinking water safety, and efforts for protecting public health. Journal of Microbiology 51(1):1-10.

Corbel S, Mougin C, Bouaïcha N (2014). Cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96:1-15.

Daza R, Barajas-Solano AF, Epalza JM (2016). Evaluation of the eiciency of bio-polymers derived from desertic plants as flocculation agents. Chemical Engineering Transactions 49:234-239

Diaz A, Rincon N, Escorihuela A, Fernandez N, Chacin E, Forster CF (1999). Apreliminary evaluation of turbidity removal by natural coagulants indigenous to Venezuela. Process Biochemistry 35:391-395.

Graham JL, Loftin KA, Meyer MT, Ziegler AC (2010). Cyanotoxin mixtures and taste-and-odor compounds in cyanobacterial blooms from the Midwestern United States. Environmental Science and Technology 44(19):7361-7368.

Henderson RK, Parsons SA, Jefferson B (2010). The impact of differing cell and algogenic organic matter (AOM) characteristics on the coagulation and flotation of algae. Water Research 44:3617-3624.

Heng L, Jun N, Wen-jie H, Guibai L (2009). Algae removal by ultrasonic irradiation–coagulation. Desalination 239:191-197.

Huisman J, Codd GA, Paerl HW, Ibelings BW, Verspagen JMHH, Visser PM (2018). Cyanobacterial blooms. Nature Reviews Microbioogy 16:471-483.

Ibelings BW, Chorus I (2007). Accumulation of cyanobacterial toxins in freshwater “seafood” and its consequences for public health: a review. Environmental pollution 150(1):177-192.

Izuegbuna O, Otunola G, Bradley G (2019). Chemical composition, antioxidant, anti-inflammatory, and cytotoxic activities of Opuntia stricta cladodes. PloS One 14(1):e0209682.

Jančula D, Maršálek B (2011). Critical review of actually available chemical compounds for prevention and management of cyanobacterial blooms. Chemosphere 85(9):1415-1422.

Jensen GS (2001). Blue-green algae as an immuno-enhancer and biomodulator. Journal of the American Nutraceutical Association 3:24-30.

Lee J, Lee S, Jiang X (2017). Cyanobacterial toxins in freshwater and food: important sources of exposure to humans. Annual Review of Food Science and Technology 8:281-304.

Li H, Pei H, Xu H, Jin Y, Sun J (2018). Behavior of Cylindrospermopsis raciborskii during coagulation and sludge storage–higher potential risk of toxin release than Microcystis aeruginosa?. Journal of Hazardous Materials 347:307-316.

Lürling M, Mackay E, Reitzel K, Spears B M (2016). Editorial–A critical perspective on geo-engineering for eutrophication management in lakes. Water Research 97:1-10

Magalhães L, Noyma NP, Furtado LL, Mucci M, van Oosterhout F, Huszar VL, Lürling M (2017). Efficacy of coagulants and ballast compounds in removal of cyanobacteria (Microcystis) from water of the tropical lagoon Jacarepaguá (Rio de Janeiro, Brazil). Estuaries and Coasts 40(1):121-133.

Merel S, Walker D, Chicana R, Snyder S, Baurès E, Thomas O (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environment International 59:303-327.

Miller SM, Fugate EJ, Craver VO, Smith JA, Zimmerman JB (2008). Toward Understanding the Efficacy and Mechanism of Opuntia spp. as a Natural Coagulant for Potential Application in Water Treatment. Environmental Science and Technology 42(12):4274-4279.

Ndabigengesere A, Narasiah KS (1998). Quality of water treated by coagulation using *Moringa oleifera* seeds, Water Research 32(3):781-791.

Nishi L, Madrona GS, Guilherme ALF, Vieira AMS, Araújo AA, Ugri MCBA, Bergamasco R (2011). Cyanobacteria Removal by Coagulation/Floculation with Seeds of the Natural Coagulant *Moringa oleifera* Lam. Chemical Engineering Transactions 24:1129-113

Noyma N, Magalhaes L, Furtado LL, Mucci M, van Oosterhout F, Huszar VL, Lürling, M (2016). Controlling cyanobacterial blooms through effective flocculation and sedimentation with combined use of flocculants and phosphorus adsorbing natural soil and modified clay. Water Research 97:26-38.

Oladoja NA (2015). Headway on natural polymeric coagulants in water and wastewater treatment operations. Journal of Water Process Engineering 6:174-192.

Oladoja NA, Pan G (2015). Modification of local soil/sand with Moringa oleifera extracts for effective removal of cyanobacterial blooms. Sustainable Chemistry and Pharmacy 2:37-43.

Ortiz ÁV, Astudillo ICP, García JM (2013). Caracterización de la Opuntia ficus-indica para su uso como coagulante natural. Revista Colombiana de Biotecnología 15(1):137-144.

Pan G, Zou H, Chen H, Yuan X (2006). Removal of harmful cyanobacterial blooms in Taihu Lake using local soils III. Factors affecting the removal efficiency and an in situ field experiment using chitosan-modified local soils. Environmental Pollution 141(2):206-212.

Pflugmacher S, Hofmann J, Hübner B (2007). Effects on growth and physiological parameters in wheat (*Triticum aestivum* L.) grown in soil and irrigated with cyanobacterial toxin contaminated water. Environmental Toxicology and Chemistry: An International Journal 26(12):2710-2716.

Rigosi A, Carey CC, Ibelings BW, Brookes JD (2014). The interaction between climate warming and eutrophication to promote cyanobacteria is dependent on trophic state and varies among taxa. Limnology and Oceanography 59:99–114. 10.4319/lo.2014.59.1.0099

Saenz C, Sepulveda E, Matsuhiro B (2004). *Opuntia* spp mucilage's: a functional componente with industrial perspectives. Journal of Arid Environment 57:275-290.

Santos EF, Lima WB, Silva CT, Araújo JS, Pereira, DD, Lira EC (2018). Cultivo de palma forrageira (Opuntia Stricta) irrigada com água salinizada. Brazilian Applied Science Review 2(6):1869-1875.

Schindler DW (2012). The dilemma of controlling cultural eutrophication of lakes. Proceedings of the Royal Society B: Biological Sciences 279(1746):4322-4333.

Shen Q, Zhu J, Cheng L, Zhang J, Zhang Z, Xu X (2011). Enhanced algae removal by drinking water treatment of chlorination coupled with coagulation. Desalination 271(1-3):236-240.

Subramonian W, Wu TY, Chai SP (2014). A comprehensive study on coagulant performance and floc characterization of natural Cassia obtusifolia seed gum in treatment of raw pulp and paper mill effluent. Industrial Crops and Products 61:317-324.

Svirčev Z, Drobac D, Tokodi N, Mijović B, Codd GA, Meriluoto J (2017). Toxicology of microcystins with reference to cases of human intoxications and epidemiological investigations of exposures to cyanobacteria and cyanotoxins. Archives of Toxicology 91(2):621-650.

Utermöhl H (1958). Zur Vervollkomrnnung ver quantitativen Phytoplankton Methodic. Mitt. Verhandlungen des Internationalen Verein Limnologie 9:1-38

Vasconcelos JF, Barbosa JEL, Lira W, Azevedo SMFO (2013). Microcystin bioaccumulation can cause potential mutagenic effects in farm fish. The Egyptian Journal of Aquatic Research 39(3):185-192.

Yin CY (2010). Emerging usage of plant-based coagulants for water and wastewater treatment. Process Biochemistry 45(9):1437-1444.

Zhang J, Zhang F, Luo Y, Yang H (2006). A preliminary study on cactus as coagulant in water treatment. Process Biochemistry 41(3):730-733.

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