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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Addressing the issue of horizontal gene transfer from a diet containing genetically modified components into rat tissues

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Genetically modified (GM) food crops are considered to have the potential of providing food security especially in developing countries. Scientists have raised concern over the hazards associated with the consumption of genetically modified organisms (GMOs). One of these hazards, which have great controversy reports, is the possible horizontal gene transfer from GM-food or feed to human or animal tissues. Many researches were conducted to investigate the presence of some transgenic sequences in animal tissues fed on GM- crops. Many of the inserted genes in the GM-crops are under the control of the promoter of the Cauliflower mosaic virus (CaMVP35S) and produce insecticidal proteins. Health hazards are suggested to accompany the ingestion of this promoter. CaMVP35S can function in a wide range of organisms (plants and animals). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter. The present work was conducted to evaluate the possibility of horizontal gene transfer from a diet containing DNA segments from Cauliflower mosaic virus -35S promoter (CaMVP-35S) to the cells of different organs of rats fed for three months on diets containing genetically modified components. Analysis of the results revealed that: 1) ingested fragments from the CaMV-35S promoter incorporated into blood, liver, and brain tissues of experimental rats, 2) The total mean of transfer of GM target sequences increased significantly by increasing the feeding durations, and 3) The affinity of different transgenic fragments from the ingested GM-diet, to be incorporated into the different tissues of rats varied from one target sequence to the other.

Key words: Genetic modification, transgenic sequences, GM-crops, gene flow, CaMV P-35S.

INTRODUCTION

Agricultural biotechnology encompasses a variety of technologies used in food and agriculture, for a range of different purposes such as the genetic improvement of plant varieties and animal populations to increase their yields or efficiency. One of these biotechnologies is genetic modification (FAO/WHO, 2001). Among the many

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Table 1. Primers position on CaMV genome, sequences, amplicon lengths and annealing temperatures

Primer	Position on CaMV genome ‡	Sequences (5'-3')	Amplicon length (bp)	Annealing Temp (°C)
GT88	7117-7138 7183-7206	5'-TCCGGAAACCTCCTCGGATTCCAT-3' 5'-GGCATTTGTAGGAGCCACCTTCCT-3'	88	54
GF70	7222 – 7249 7273 - 7293	5'-GCCATCGTTGAAGATGCCTCTGCCG-3' 5'-CACGATGCTCCTCGTGGGGTGG-3'	70	57.5
P-35S	7190-7209 7364-7384	5´- GCTCCTACAAATGCCATCA -3´ 5´-GATAGTGGGATTGTGCGTCA-3´	195	57

‡Positions relevant to Ca.

plants that are genetically modified are soybean and maize. Apart from human consumption, soybean and maize are also used in livestock and fish feeds. Genetically modified (GM) varieties of both are extensively traded internationally for this purpose (Nowicki et al., 2010). One of the hazards accompanying consumption of GM- plants is the possible horizontal gene transfer (HGT) which is the transfer of genetic material directly to a living cell or an organism (van den Eede et al., 2004; Keese, 2008). The consequences of horizontal gene transfer may have significant effects on some human-health conditions. It has been shown that ingested DNA from food is not completely degraded by digestion, and that small fragments of DNA from GM foods can be absorbed by either the gut microflora or somatic cells lining the intestinal tract (Netherwood et al., 2004; Nielson and Townsend, 2004; van den Eede et al., 2004). Some of the animal studies support the idea that small fragments of nucleic acids may pass to the blood stream and even get to various tissues (Rizzi et al., 2012).

Recent study (Spisak et al., 2013) found that meal-derived DNA fragments which are large enough to carry complete genes can avoid the total degradation in the gastrointestinal tract, and through an unknown mechanism enter the human circulation system. Foreign DNA fragments were detected by polymerase chain reaction (PCR) based techniques in the digestive tract and leukocytes of rainbow trout fed by genetically modified soybean (Chainark et al., 2008), and other studies reported similar results in goat (Tudisco et al., 2010), pigs (Mazza et al., 2005, Sharma et al., 2006) and mice (Schubbert et al., 1998). Many GM crops produce an insecticidal protein (for example Cry1Ab). The gene coding for this protein is under the control of the 35S promoter of the Cauliflower mosaic virus (CaMV-P35S). This promoter is derived from the common plant virus Cauliflower mosaic virus, which was reported to be closely related to human hepatitis B virus (Doolittle et al., 1989, Xiong and Eikbush, 1990). It can cause cancer through over-expression of oncogenes (Ho et al., 1999).

The CaMVP-35S is a component of transgenic constructs in more than 80% of genetically modified (GM) plants (Cankar et al., 2008). It can function in a wide range of organisms (plants and animals) and can direct the expression of the bacterial neomycin phosphotransferase II (*nptII*) gene in *Escherichia coli* (Assaad and Signer, 1990). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter (Zheng et al., 2007).

Since most of the studies were investigating the transfer of GM-DNA fragments from transgenic genes for example *Cry1Ab* (Walsh et al., 2012) and *cp4epsps* (Sharma et al., 2006), the aim of the conducted study was to evaluate the transfer of dietary DNA segments from Cauliflower mosaic virus -35S promoter (CaMVP-35S) to the cells of different organs of weanling Wistar Albino rats fed for three months on diets containing genetically modified components. We used three primers to amplify different segments from Cauliflower mosaic virus-P35S (CaMV-P35S) promoter representing nearly 80% of this promoter.

MATERIALS AND METHODS

The experimental material consisted of laboratory chow containing mainly 60% of yellow maize and 34% of soybeans. The presence of genetic modification in this diet was tested in our laboratory using the PCR assay (Rose, 1991). DNA extracted from samples of the laboratory chow diet fed to rats during the three months period of the experiment were screened for the presence of genetic modification; using a pair of primers specific for the Cauliflower mosaic virus (P-35S) promoter that amplify a segment of 195 bp from the CaMV-35S promoter (Hemmer, 1997). DNA was also extracted from another well balanced diet that was fed to the animals of the control group and was tested for the presence of any GM- sequences.

Primer designing

The fact that plant DNA is fragmented to small sizes after the process of digestion lead to the assumption that designing primers to amplify smaller amplicons would increase the chance to detect transgenic fragments, which could transfer to the animal genome. Therefore, two primers GT88 and GF70 (Table 1) were designed using Primer-Blast Program (NCBI). These two primers were

designed to amplify DNA segments of the sizes 88 and 70 bp of the cauliflower mosaic virus promoter, respectively. The Primer 3 software (Rosen et al., 2000) was employed with manual editing as needed, using the Cauliflower mosaic virus (CaMVP-35S) promoter sequence available from GenBank (accession no.emb|V00141.1|). All combinations including forward-reverse primer pair, forward-forward as well as reverse-reverse pairs were avoided by the primer 3 software. Primers were then submitted to BLAST search against bacteria, mice and rat database to confirm the specificity of the designed primers to CaMVP-35S. Along with these two designed sets of primers, a third pair of primers P-35S (Table 1) which amplify a segment of 195 bp from the cauliflower mosaic virus promoter was also used. The P-35S primers were also used for evaluating the possible horizontal gene transfer from the genetically modified diets to the experimental animal's genome applying the polymerase chain reaction technique. All primers were synthesized by Metabion.

Animals, housing and diets

Twenty nine male Wistar Albino rats (*Rattus norvegicus*), obtained from the animal house of the National Research Center immediately after weaning (age of three to four weeks), were divided into two main groups. One group was fed on laboratory chow containing GM ingredient for three months (GM-diet group) and were further divided into three subgroups (6, 6 and 7 animals). Animals from GM-diet groups were euthanized after 30, 60 or 90 days, respectively. The other group (control) was fed on a balanced non-GM diet for the same period (non-GM-diet group). The animals were housed in standard cages and under standard conditions. Temperature was maintained at 22 to 25°C and relative humidity at 55 to 60% during the experiment. Exposure to light was maintained for 12 h. Diet and water were provided *ad libitum*. Animals of the GM-groups were euthanized at three intervals; namely at 30, 60 or 90 days, respectively. Control animals were euthanized at the end of the experiment. The protocol applied throughout this study comply with the NRC Ethical Committee's guidelines (reference: 12142), and all animals received humane care.

Sampling procedures

Three types of tissue samples (blood, liver and brain) were collected from each animal in both groups. Blood samples were collected on EDTA (0.5%) from all animals of both groups at the end of each interval (30, 60 or 90 days) of the experiment. Samples from liver and brain were excised and stored at -20°C.

DNA isolation

DNA extraction and purification from fresh blood samples from GM-diet and non-GM-diet groups were carried out using Wizard Genomic DNA Purification Kit (Promiga) according to the manufacturer's manual. The excised tissue samples of the liver and brain were grinded in liquid nitrogen, and genomic DNA was extracted from tissue samples from animals in both groups using the same extraction kit used above. The quality and concentration of DNA were determined using the Nano Drop 1000/Thermo Scientific spectrophotometer (USA).

PCR conditions and profile

A total of 30 DNA samples extracted from non-GM diet group (three tissue types from 10 animals), and 57 DNA samples extracted from the GM diet group (three tissue types from 19 animals) were screened for the presence of small segments from the cauliflower mosaic virus 35S promoter using the three sets of primers presented in Table 1. Double-stranded amplifications were carried out in a total volume of 25 µl. Each reaction mixture contained 100 ng template DNA, 2 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP, 2.5 pmol of each primer and 2.5 units of taq DNA polymerase in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 0.001% BSA. All polymerase chain reactions were conducted in duplicates and accomplished in TM Thermal cycler (MJ Research PTC-100 thermocycler, USA) programmed to perform a denaturing step at 98°C for 2 min, followed by 40 cycles consisting of 30 s at 95°C for denaturing, 40 s at annealing temperature specific for each primer (Table 1), and 45 s at 72°C for extension. A final extension step of 7 min at 72°C was performed.

Agarose gel electrophoresis

Following completion of the cycling reaction, each PCR product was analyzed by electrophoretic separation of a 10 µl aliquot on a 2% (W/V) agarose gel containing ethidium bromide (1 µg/ml ethidium bromide). PCR products were analyzed, using SYNGENE Bio Imaging Gel Documentation System, for the presence of a fluorescent band of the expected level for each primer (Table 1).

Sequencing analysis

For further confirmation of the presence of CaMV-P35S promoter in the diets used in this study, and to confirm the presence of transgenic DNA in genomic DNA of rats fed on the GM diet, the internal sequencing of the PCR amplicons obtained from the amplification of P-35S promoter primers in the diet DNA and liver and brain tissues of some rats has been performed by MWG-Biotech AG. PCR products of the expected size (195 bp) were purified using total fragment DNA purification kit (MEGA quick-spin, iNtRON Biotechnology, Inc). Sequencing was followed by Blast analysis with the GenBank to test for alignment using NCBI-BLASTN Program version 2.2.28+.

Statistical analysis

Percentages of transfer frequencies of the GM target sequences of the GM-DNA into rats after 30, 60 and 90 days were calculated according to the following equation:

$$\text{Transfer frequency \%} = \frac{\text{No of positives}}{\text{No of samples}} \text{X } 100$$

Multiple analysis of data was conducted applying the analysis of variance (ANOVA) test to evaluate the mean values of the number of GM-DNA segments from the diet that were transferred to different tissues in the experimental GM-diet groups.

RESULTS AND DISCUSSION

PCR amplification of the laboratory chow diet samples

The laboratory chow diet samples used all through this experiment gave positive results when it was screened with primers for the presence of Cauliflower mosaic virus promoter (CaMV-P35S). A band of the size 195 bp was detected when amplifying P-35S promoter primers

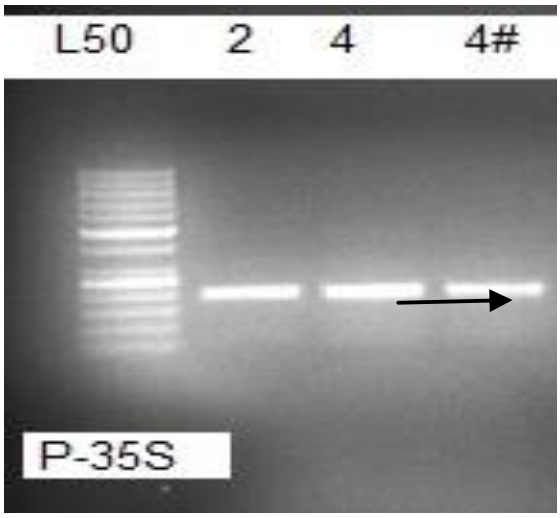

Figure 1. Confirmation of amplification of CaMV-P35S fragment (195 bp) in the diet used for this experiment. (2, 4 and 4#) are samples from the laboratory chow diets used in this study.

(Figure 1) in all the samples of the used diet. No amplification occurred in the control diet. There are several plant seeds and potato tubers that are genetically modified sold for cultivation in Egypt (Oraby et al., 2005). These products are squash, tomato, canola seeds, yellow maize and potato tubers which are used as diet for human and animals. GM varieties of yellow maize and soybeans were reported to be produced worldwide for animal feed (Nowicki et al., 2010).

Sequencing results analysis

As mentioned before and for further confirmation of the presence of Cauliflower mosaic virus-35S (CaMV-P35S) promoter in the diets used in this study, the internal sequencing of the PCR amplicons obtained from the amplification of P-35S promoter primers has been performed. Sequence alignment analysis was carried out using NCBI-BLASTN Program version 2.2.28+. BLASTN sequence alignment showed a 100% homology of the amplified segment of the expected size (195 bp) with CaMV-P35S promoter (emb|V00141.1|) at nucleotide (nt) coordinate 7190 to 7384 (Figure 2). It also shared 100% sequence homology with a number of binary vectors (for example gb|JX992842.1|, gb|JQ974028.1|, gb|JQ436739.1|, dbj|AB684633.1|, gb|JQ305140.1| and dbj|AB752377.1|). Binary vectors are usually used in the process of gene transfer (de Framond et al., 1983; Hoekema et al., 1983).

PCR amplification of different tissues

PCR amplification using the three primers (GF70, GT88 and P-35S) revealed amplicons of the expected sizes; 70, 88 and 195 bp, respectively in some of the DNA samples of blood, liver and brain in rats fed on GM diet after 30, 60 or 90 days. Representatives of some of these amplifications are presented in Figures 3, 4 and 5. Whereas, none of these primers were amplified in DNA samples from the second group of rats fed on a non GM-diet. To confirm the presence of transgenic DNA in genomic DNA of rats fed on the GM diet, further analysis of the internal sequencing of the large amplicon (195 bp) obtained from the amplification of P-35S promoter primers in some DNA samples of liver and brain in rats fed on GM diet was performed against GenBank data base. Results reveal that these sequences showed 100% similarity with Cauliflower mosaic virus whole genome (emb|V00141.1|) at the same nucleotide coordinates 7190 to 7384 which represents 195 nt of the CaMV-P35S promoter that gave 100% similarity with the PCR amplicon obtained by the amplification of the same set of primers (P-35S) from the GM-diet. It also aligned with numbers of binary vectors including those that were aligned with the sequence of the segment obtained from the diet used in this study. The binary vectors are widely known to be used for gene transfer (de Framond et al., 1983; Hoekema et al., 1983).

The total frequencies of transfer of the GM-target sequences (GF70, GT88 and P-35S) from the GM-diet into the examined DNA samples from blood, liver and brain tissues in rats fed on the GM-diet increased from 33.3 to 37% after feeding rats with GM-diet for 30 or 60 days, respectively, and reached the highest level 52.8% after 90 days of the GM-diet regime. ANOVA statistical analysis of the results of this work revealed that feeding rats with GM-diet for 30, 60 or 90 days increased the mean transfer of GM target sequences significantly (8.0 ± 0.0000, 12.3±1.2018 and 16.7±1.4529, respectively) by increasing the feeding durations (Figure 6). Mazza et al. (2005) reported a progressive decrease in DNA detected in the target tissues. Therefore, they exclude the possibility that organs like liver, kidney and spleen elicit an accumulation effects; whereas, results of the present work indicate an accumulation effects in blood, liver and brain tissues.

Many researchers reported the presence and the fate of GM-DNA in gastrointestinal tract (GIT) of experimental animals and how GM-DNA survive digestion in the oral cavity (Duggan et al., 2000), passing the stomach, intestine (Duggan et al., 2003, Sharma et al., 2006) and out with the faeces. Other researchers reported the transfer of DNA fragments from the GM and non-GM diets to different tissues of the experimental animals. Small fragment of transgenic *Cry1Ab* was detected in blood, liver, spleen and kidney of animals raised with transgenic feed (Mazza et al., 2005). Transfer of transgenic fragments of the *cp4epsps* was detected at low frequencies among GIT tissues, except the oesophagus (Sharma et al., 2006). Our findings

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CaMV-P35S  7190  GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCG  7249
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GM diet    1     GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCG  60

CaMV-P35S  7250  ACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTC  7309
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GM diet    61    ACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTC  120

CaMV-P35S  7310  CAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG  7369
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GM diet    121   CAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG  180

CaMV-P35S  7370  CACAATCCCACTATC  7384
                 |||||||||||||||
GM diet    181   CACAATCCCACTATC  195
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Figure 2. DNA sequence alignment of the PCR products obtained from GM diet DNA together with the sequence of cauliflower mosaic virus (emb|V00141.1|).

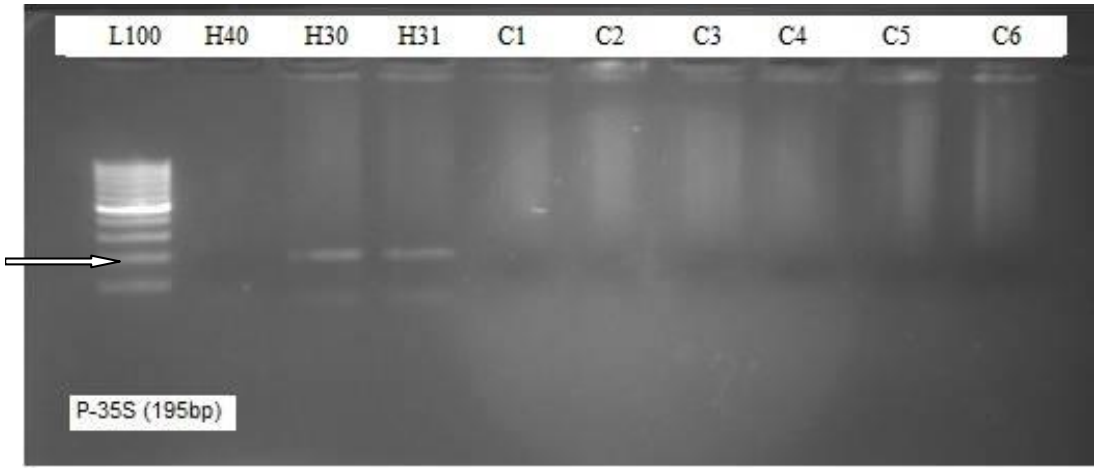

Figure 3. Representative of PCR products of P-35S (195 bp) amplification in blood (H40) and two brain samples (H30, H31) in rats fed on GM diet for 60 days. C1 to C6 are samples from rats fed on the non-GM diet.

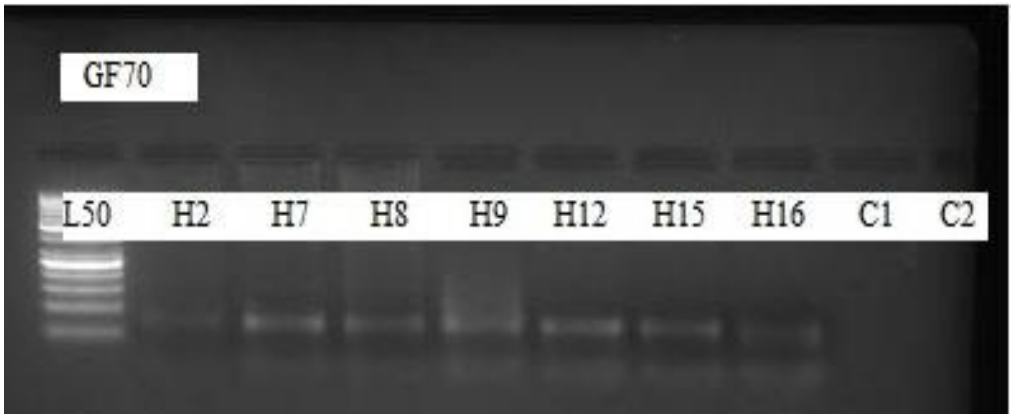

Figure 4. Representative of PCR products of GF70 (70 bp) amplification in blood samples (H2 to H9) and liver samples (H12 to H16) in rats fed on GM diet for 30 days. C1and C2 are blood samples from control rats.

Figure 5. Representative of PCR products of GT88 (88 bp) amplification in blood sample (H40, H41, H43 and H44) of rats fed on GM diet for 90 days, C is a blood sample from a control rat.

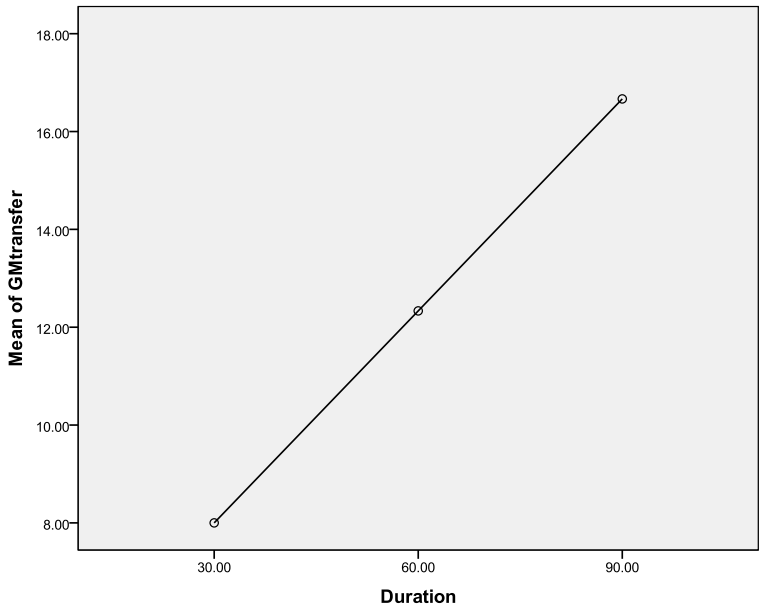

Figure 6. Mean transfer of the three GM target sequences (GF70, GT88 and P-35S) in tissues of rats fed on GM-diet for 30, 60 and 90 days.

moreover, confirm that ingested GM-DNA fragments from the Cauliflower mosaic virus P-35S promoter do incorporate into blood, liver, and brain tissues of experimental rats.

The fate of DNA from GM corn and soybean has been extensively monitored in cattle, sheep, pigs and poultry (Aeschbacher et al., 2005; Beagle et al., 2006; Chowdhury et al., 2004; Deaville and Maddison, 2005; Duggan et al., 2003; Einspanier et al., 2001; Mazza et al., 2005; Sharma et al., 2006). Most of these studies were investigating the transfer of GM-DNA fragments from transgenic genes (for example *Cry1Ab* and *C4epsps*). The authors of these studies suggested that it is unlikely that small segments from a gene will transmit genetic information (Duggan et al., 2003). In a recent study conducted by Sipsak et al. (2013), it showed that meal-derived DNA fragments are able to avoid the total degradation in the gastrointestinal tract and enter the circulation. They found that DNA fragments large enough to carry complete genes can pass from the digestive tract to the blood. Foreign DNA fragments were detected by PCR based techniques in the digestive tract and leukocytes of rainbow trout fed by genetically modified soybean (Chainark et al., 2008), and other studies reported similar results in goat (Tudisco et al., 2010), pigs (Mazza et al., 2005; Sharma et al., 2006) and mice (Schubbert et al., 1998). Many GM crops produce an insecticidal protein (for example Cry1Ab) which is under the control of the promoter of the Cauliflower mosaic virus.

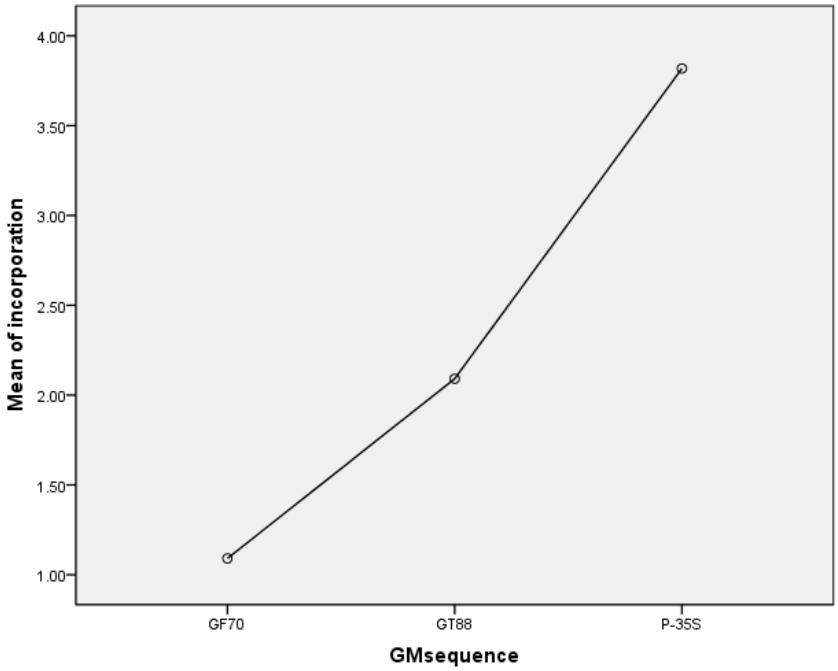

Figure 7. Affinity of incorporation of different GM-sequences (GF70, GT88 and P-35S) into the genome of rats fed on transgenic diet (GM-diet).

In our investigation, we evaluated the transfer of CaMV-P35S promoter from transgenic diet into experimental animals. For this purpose, we designed three primers that amplify three segments of CaMV-P35S promoter (accession no.emb|V00141.1|) from nucleotide coordinate 7117 to 7384 (Table 1) which represents nearly 80% of the whole promoter (343 bp). In some cases in the present work, the three segments were all amplified in the same animal. This finding may raise concern that the whole promoter might have been transferred into the genome of those animals, and this where the risk lies, since, even a promoter containing only 46 bp of 5' sequence from the CaMV-P35S promoter was previously reported to be sufficient for accurate transcription initiation (Odell et al., 1985).

Results of the present work suggest a health hazards accompanying the ingestion of diets containing Cauliflower mosaic virus 35S promoter since Cauliflower mosaic virus has been reported to be closely related to human hepatitis B virus (Doolittle et al., 1989, Xiong and Eikbush, 1990). It is also known that CaMV-P35S promoter can direct the expression of the bacterial neomycin phosphotransferase II (*NPTII*) gene in *Escherichia coli* as reported by Assaad and Signer (1990). They also reported that, it can function in a wide range of organisms (plants and animals). It has also been demonstrated that the CaMV-P35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter (Zheng et al., 2007). Previously, Ho and his colleagues (Ho et al., 1999) deduced that the CaMVP-35S promoter could combine to active dormant viruses, create new viruses, and cause cancer by over expression of normal genes. Contrary to that, others (Hull et al., 2000) claimed that there is no evidence that the CaMV 35S promoter will increase the risk over those already existing from the breeding and cultivation of conventional crops.

Comparing tissue selectivity of the three tissues (blood, liver, and brain) examined through the whole duration of this work (90 days), no significant differences were observed in the rate of transfer of GM-DNA into these tissues. GM-DNA transfer to blood tissue was barely higher (12.66 ± 2.6034) than the GM-DNA transfer to the other tissues: liver (12.00 ± 3.6055) and brain (12.00 ± 2.0000). In a study conducted by Mazza et al. (2005) blood tissues was found to be the tissue with the highest transfer frequency, followed by organs rich in blood vessels and involved infiltration, such as liver and kidney. They concluded that blood is the main tissue involved in the uptake of short DNA fragments since it collects macromolecules directly absorbed by the intestinal epithelium and the cells of the immune system and that DNA molecules may be transported in the organism via blood circulation.

Our results have demonstrated that the frequency of uptake for the larger segments was greater than that for the smaller segment. This conclusion was based on the fact that the efficiency of transfer of GM-DNA segments (70 bp) amplified by GF70 primers was the lowest (1.09 ± 0.4161) compared to that of P-35S (3.8 ± 0.8069) and GF88 (2.09 ± 0.7318) (Figure 7). The frequency of transfer of GF70 segment was only calculated when GF70

primers were amplified in the animals with no traces of amplification of P-35S primers in these animals. The affinity of incorporation of GF70 segment into the rats' genome was statistically significantly lower compared to that of the P-35S. Palmen and Hellingwef (1997) and Meier and Wachernagel (2003) postulated that the shorter the fragments are, the lower is the uptake efficiency.

Conclusion

Our results support the suggestion that monitoring for transgenic flow is a way to measure the possible environmental impacts of GMO and to serve as a warning system for deleterious effects (NRC, 2002).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Screening of *Abelmoschus esculentus* and *Abelmoschus callei* cultivars for resistance against okra leaf curl and okra mosaic viral diseases, under field conditions in South Eastern Nigeria

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Of all the several factors that limit okra production in West Africa, okra mosaic virus disease (OMVD) and okra leaf curl disease (OLCD) are rank among the most severe. Twenty three (23) *Abelmoschus esculentus* and *Abelmoschus callei* cultivars retrieved from the local farmers were screened in the open field for the resistance against OMVD and OLCD over two cropping seasons in Nsukka, South Eastern Nigeria. The degree of susceptibility was determined by the calculation of yield decline (YD) due to changes in the fruit yield with exposure to the disease causing agents as well as virtual scoring of the exposed plants. The results show that all the *A. esculentus* cultivars were susceptible to the two diseases. The findings indicate that contrary to general belief, not all *A. callei* cultivars are resistant to these viral diseases. Any cultivar with a YD score of less than 10% was identified as a potential candidate for the disease resistant genes. Only Ebi Ogwu, Ojo ogwu, Tongolo, VLO, Oru ufie and Ogolo met this criterion. The potentials of transferring these resistant genes from *A. callei* cultivars to the vulnerable *A. esculentus* cultivars are discussed.

Key words: Okra, screening, resistance, viral diseases.

INTRODUCTION

In West Africa, two distinct species of okra, *Abelmoschus esculentus* (L.) Moench, which is the conventional or early okra and *Abelmoschus caillei* (A. Chev) Stevels, late okra (Siemonsma and Hamon, 2004) are grown. Late okra is also named West African okra, since its distribution is restricted to the West African region (Martin et al., 1981; Siemonsma, 1982; Hamon and Hamon 1991). These okra types respond differently to changes in natural photoperiod and this has been one of the major means of distinction between them. Early okra has a Critical Day Length (CDL) of 12.5 h whereas late okra has a CDL of 12.25 h (Njoku, 1958; Oyolu, 1977; Nwoke, 1980; Siemonsma, 1982; Udengwu, 1998). World production of okra (both species) as fresh fruit-vegetable

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is estimated at 6 million t/year. Okra production in West and Central Africa is estimated at 500,000 to 600,000 t annually based on available consumption data. West African okra makes up half of this amount, which is about 5% of total world production of okra (Siemonsma and Hamon, 2004). Of the several factors that limit okra production in West Africa, Okra Mosaic Virus Disease (OMVD) and Okra Leaf Curl Disease (OLCD) diseases rank among the most severe. They are caused by a complex of monopartite begomoviruses and a promiscuous betasatellite with an associated parasitic DNA1 satellite in Mali (Kon et al., 2009). They can occur singularly or in combination in plants and when they occur in combination in okra plants their effects are often devastating (Kon et al., 2009). Givord and Hirth (1973) identified the okra mosaic virus (OMV) and noted that it occurs naturally in the field. Its symptoms on okra include chlorosis, mosaic and vein banding. Okra mosaic virus (OMV), transmitted by flea beetles (*Podagrica*), is widespread but damage is much less important than that caused by leaf curl, transmitted by whitefly (*Bemisia tabaci)*. These viruses must be controlled through control of the vectors (Siemonsma and Hamon, 2004).

Venkataravanappa et al. (2011) isolated from okra exhibiting typical begomovirus symptoms (leaf curl and yellow vein) four groups of beta satellite sequences- Okra Leaf Curl Beta Satellite (OLCuB), Bhendi yellow vein beta satellite (BYVB); Bhendi yellow vein India beta satellite (BYVIB) and Croton yellow vein mosaic beta satellite (CroYVMB). Their detailed analysis of the sequences showed that OLCuB, BYVB and BYVIB share highest identity with respect to βC1 gene. βC1 being the only gene encoded by beta satellites, the product of which is the major pathogenicity determinant of begomovirus-beta satellite complexes and is involved in overcoming host defenses based on RNAi.

In West Africa, climate influences (i) virus disease outbreaks, (ii) the rate of development and activity of virus vectors, and also of their migration, and (iii) the phenology of crops, weeds and wild hosts that habour plant viruses (Atiri et al., 2000). These viral diseases have been implicated in declined okra production in many okra growing regions of the world. The disease YVMV transmitted by the whitefly (*Bemisia tabaci*) is reported to be one of the most destructive plant diseases in India causing great loss by affecting quality and yield of fruits, as high as 93.80% depending on age of plant at the time of infection (Sastry and Singh, 1974). Additionally it has been stated that the unavailability of quality seed and heavy incidence of biotic stresses particularly yellow vein mosaic virus (YVMV) are the most important reasons for low yield of okra in India (Chattopadhyay et al., 2011). Prabu et al. (2007) reported that the incidence of Yellow Vein Mosiac Virus (YVMV) in wild and cultivated lines of okra was observed more in summer as compared to kharif season at hot spots in western Maharashtra. Rainfall, temperature and wind are identified as key weather components in virus pathosystems involving maize (cereal), okra (vegetable) and cassava (tuber crop), and are therefore important in determining the most suitable period in which to undertake crop protection measures (Atiri et al., 2000). According to Siemonsma and Hamon (2004), West African okra which is known to be resistant to yellow vein mosaic virus (YVMV), a major cause of crop failure of common okra in Asia, whose vector is the whitefly (*B. tabaci*), has already been introduced into several American and Asian countries for research purposes through germplasm exchange.

In their screening studies for four seasons to identify the source of resistance to okra yellow vein mosaic virus in Maharashtra, Prabu et al. (2007) reported that the wild species *Abelmoschus angulosus* was found completely free from yellow vein mosaic virus symptoms. The wild *Abelmoschus tetraphyllus* lines, *Abelmoschus moschatus* lines (1, 2, 3, 4 and 5), *A. caillei*-2 and *Abelmoschus manihot* spp. tetraphyllus were found highly resistant while the wild lines *A. tetraphyllus*-5, *A. manihot* (L.) Medikus and *A. manihot* spp. *manihot* were found to be resistant whereas *Abelmoschus tuberculatus* lines (1, 2 and 3), *Abelmoschus ficulneus* lines (1 and 2) and *A. caillei* -1 were found moderately susceptible to YVMV. Among the cultivated *A. esculentus* types, none was found resistant to YVMV. Despite the fact that several workers had conducted serious research works on how to reduce the incidence of the disease; through the use of insecticides (Sastry and Singh, 1973; 1974; Chakraborty and Mukhopadhyay, 1977; Khan and Mukhopadhyay, 1985; Ramachandran and Summanwar, 1986; Bhagat et al., 1997; Raghuraman et al., 2007; Aktar et al., 2009; Gupta et al., 2009), screening for resistant cultivars (Arora et al., 1992; Sharma et al., 1993; Singh and Gupta, 1991; Bora et al., 1992; Nath and Saikia, 1992; Rashid et al., 2002); inducing mutation for resistance to yellow vein mosaic virus disease of okra (Dalve et al., 2012); screening of plant extracts for antiviral properties against okra vein mosaic virus infection of okra (Pun et al., 1999) and management of yellow vein mosaic and leaf curl disease by manipulation of agronomic practices (Singh et al., 1989; Amoatey and Acquah, 2010; Kalita and Dhawan, 2010), the incidence of the disease is estimated to still be on the increase in important okra growing regions of the world. The threat posed by the diseases to global okra production calls for coordinated action to identify elite resistant cultivars that can facilitate production of durable resistant cultivars. There is urgent need to screen available okra land races for resistance to these two severe okra viral diseases with a view to identifying cultivars, which are highly immune to the two viral diseases. Such a move may not only minimize, but can even eliminate, the use of chemical insecticides for the control of the vectors; with the concomitant impact of boosting okra production in the region. This present study reports on field screening of 23 selected landraces of

Table 1. Details of the scale used.

Virtual infestation classification	Score	Virtual Infestation Classification	Score
Very highly immune	9	Mildly susceptible	5
Highly immune	8	Susceptible	3-4
Immune	7	Highly Susceptible	2
Slightly immune	6	Very Highly Susceptible	1
		Hyper-susceptible	0
Immune - very highly immune	6-9	Hyper-susceptible - Mildly Susceptible	0-5

$$\text{Yield decline (YD)} = \frac{\text{Mean weight of protected plants} - \text{mean weight of un-protected plants}}{\text{Mean weight of un-protected plants}} \times 100$$

both early and late okra types, for the identification of cultivars with the viral resistant genes. These will be used in the improvement of promising but susceptible cultivars, for the prevention of further erosion of okra germplasm and thus ensure maintenance of a broad genetic base for the crop.

MATERIALS AND METHODS

Fifteen (15) *A. esculentus* cultivars: Ogbu Oge, Nnobi fat, Obimo girdle, Osukwu, Mpi ele, Ogba mkpe, Iloka, Awgu early, Nwa obala, Lady finger, Kpum zua, Hawzua, Kano dwarf, Odiche Nnobi long and eight late okra cultivars: Ojo ogwu, Tongolo, AOAU, Oru ufie, Ebi ogwu, Ogolo, VLO and Ala nwagboho were used in screening studies. The preliminary field work was carried out in the Botanic garden, University of Nigeria, Nsukka in the late nineties while the substantive work was carried out in the Department of Crop Science experimental fields, University of Nigeria, Nsukka, in 2008 and 2009 following standard agricultural practices. The viral diseases were confirmed by the Plant Pathology and Virology unit of our department. The 23 cultivars were grown in four replications using Randomized Complete Block Design (RBCD). Two sets of plantings were done in different locations, 50 m apart, to ensure the absence of interactions between the two locations based on treatment. In one location, the plants were protected through weekly spraying with Vetox, foliar insecticide to control the *Bemisia* flies and other insects that might be involved in the transmission of the causative viral organisms. The second location apart from being unprotected (no spraying) had three replicated rows of Iloka, the *A. esculentus* cultivar that had chronically shown susceptibility to both okra viruses, over several generations, in each block. Stands of the cultivar were also as used as boarder crops, all aimed at ensuring adequate availability of both the vectors and the viral organisms in each of the blocks. All the plantings were done on flat beds measuring 6.5 x 6.0 m for the protected plots and 7.5 x 7.0 m for the un-protected plants. Well cured poultry manure was worked into the beds at the rate of 20.5 kg per plot, one week before planting. The 23 cultivars were planted in randomized rows for the four blocks using the table of random numbers. Plants were spaced 30 x 30 cm. Three pre-germinated seeds of each cultivar were planted inside holes 2.5 cm deep. The plants were later thinned down to one per stand, nine days after the emergence of the first two opposite juvenile leaves. There were 20 experimental plants per row giving rise to 460 plants per block. There were 1840 experimental plants per set of planting. Data were not collected from the additional replicated stands of the chronically infested cultivar as well as the border crops that served as rich sources of both the viruses and the *Bemisia* flies.

The plants were rain-fed throughout the period of the studies. Harvesting of fresh marketable fruits from each of the locations was carried out every five days. Upon termination of the experiment the data on yield per plant was subjected to ANOVA and the mean yield per cultivar for each of the two groups of planting were compiled and used to determine the yield decline (YD) as shown in the formula presented above.

Based on the calculated YD the cultivars were categorized using the following YD scale scores and symbols; i) immune scale (%): 000 = very highly immune: 0-5; 00 = highly immune: above 5-10; 0 = slightly immune above: 10-30; 2) susceptibility scale: + = Susceptible: above 30-50; ++ = highly susceptible: above 50-60; +++ = very highly susceptible: above 60-70; ++++ = hyper-susceptible: above 80.

Scoring based on virtual evaluation for resistance was carried out at the transitional stage between the vegetative and reproductive phases of growth, which was approximately 30 days after seed germination (ASG) for early okra and 90 days after seed germination (ASG) for late okra, since they have different critical day length and consequently different times for transition from vegetative to reproductive phase (Udengwu, 1998). Scores were recorded for both Okra Leaf Curl (OLC) and Okra Mosaic (OM) viruses using a scale of 0-9. The most susceptible scored 0 while the most immune scored 9. Details of the scale are shown in Table 1.

RESULTS

Table 2 shows that cultivars Nos. 1-8 (*A. callei* cultivars) had a yield decline (YD) range of 4.70% (Ebi Ogwu) to 28.57% (AOAU) for the first year of planting. The second planting YD ranged from 4.34% (Ebi Ogwu) to 29.40% (AOAU). On the basis of the YD assessment the categorization of the *A. callei* cutivars for both diseases are shown in Table 3. Ebi Ogwu whose YD is within the 0-5%, is designated as Very Highly Immune (VHI), 000. Ogolo, Oru Ufie, Ojo Ogwu, Tongolo and VLO with YD range of 5-10% are classified as Highly Immune (HI), 00; Alanwanghoho and AOAU with YD range of 10-30% are

Table 2. Mean yield (t/h) of 23 cultivars of early and late okra, exposed to *Bemisia* white flies (unprotected) and protected (*Bemisia* flies controlled), under field conditions for 2 years.

Cultivar	Yield (t h^{-1}) 1st year of planting			Yield(t h^{-1}) 2nd year of planting		
	Protected plants (PP)	Non protected plants (NPP)	Yield decline (%)	Protected plants (pp)	Non protected plants (npp)	Yield decline (%)
Ogolo (L)	3.60±0.21	3.40±0.12	5.88	0.72±0.08	0.66±0.11	6.06
Ebi Ogwu (L)	3.09±0.13	2.95±0.22	4.74	0.48±0.07	0.46±0.07	4.34
Oru Ufie (L)	2.20±.09	2.08±0.32	5.76	0.82±0.14	0.76±0.09	7.89
Ojo Ogwu (L)	2.48±0.17	2.30±0.42	7.82	0.79±0.19	0.73±0.12	8.21
Tongolo (L)	3.00±0.16	2.75±0.21	9.09	0.55±0.06	0.51±0.17	7.84
VLO(L)	3.10±0.19	2.87±0.31	8.01	0.92±0.05	0.85±0.02	8.23
Alanwa. (L)	2.00±0.11	1.60±0.13	25.00	0.52±0.01	0.42±0.14	23.81
AOAU (L)	3.60±0.13	2.80±0.35	28.57	1.10±0.24	0.85±0.14	29.40
Nnobi long (E)	5.87±0.08	3.20±0.18	83.40	2.00±0.07	1.13±0.21	76.99
Lady finger (E)	2.53±0.07	1.42±0.27	78.16	1.80±0.06	1.04±0.11	73.07
Ogba mkpe (E)	3.00±0.26	2.01±0.39	49.25	1.20±0.11	0.75±0.13	60.00
Awgu Early (E)	5.03±0.35	2.88±0.25	74.65	2.20±0.32	1.30±0.04	69.23
Iloka (E)	3.02±0.25	1.36±0.15	122.05	1.82±0.12	0.86±0.08	112.79
Ogbu oge (E)	2.26±0.08	1.50±0.07	50.66	1.10±0.08	0.72±0.21	52.77
Odiche (E)	2.20±0.08	1.30±0.21	69.23	0.90±0.14	0.56±0.09	60.71
Kano Dwarf (E)	3.00±0.18	1.92±0.18	56.25	1.30±0.14	0.85±0.07	52.94
Hawzua (E)	2.00±0.06	1.10±0.17	81.81	1.00±0.07	0.56±0.05	78.54
Obimo Girdle (E)	5.87±0.25	3.50±0.05	67.71	2.40±0.18	1.34±0.10	79.10
Nnobi fat (E)	2.53±0.09	1.50±0.11	68.66	1.92±0.21	1.20±0.17	60.00
Osukwu (E)	3.00±0.14	1.80±0.18	66.66	1.04±0.13	0.58±0.12	79.30
Mpi Ele (E)	5.03±0.28	3.10±0.24	62.25	2.10±0.24	1.25±0.08	68.00
Nwa Obala (E)	3.00±0.35	1.70±0.12	76.47	1.30±0.08	0.76±0.17	71.05
Kpum Zua (E)	2.26±0.41	1.40±0.07	61.42	1.20±0.14	0.76±0.12	57.89
$LSD_{(0.05)}$	0.74	0.28		0.20	0.01	

classified as slightly immune (SI), 0. For the *A. esculentus* cultivars, Nos. 9-23, the YD ranged from 49.25% (Ogba mkpe) to 122% (Iloka), for the first year of planting and second year of planting 52.77% (Ogbu Oge) to 112.79% (Iloka) for the second year of planting. Their categorization was as follows: Ogba mkpe and Awgu Early (first year for OLC disease) with a YD range of 30-50% was categorized as susceptible (SB), +; Lady finger, Awgu Early (first year OM disease and second year), Ogbu Oge, Odiche, Kano Dwarf, Nnobi fat, Osukwu, Mpi Ele and Kpum Zua are grouped as Highly susceptible (HS), ++. Nnobi long. Hawzua and Nwa obala with YD range of 60-70% were grouped as very highly susceptible (VHS), +++. Lastly, Iloka with YD above 80% was classified as Hyper Susceptible (HPS), +++. The responses of the cultivars for the two years were also scored based on virtual inspection of the incidence of both diseases, Table 4. This was carried out using a virtual scale range of 0-9. Scores between 0 to 4 were grouped as hyper susceptible - susceptible, those that scored from 5 - 9 were categorized as Immune - very highly immune. For cultivars nos. 1-8 representing the *A. callei* cultivars, Ebi Ogwu with a score of 9 is designated as Very Highly Immune (VHI). Alanwangboho, AOAU and Ojo Ogwu with score range of 5 - 6 are designated as Slightly Immune. The rest with score range of 7-8 are designated as Highly Immune (HI).

For the *A. esculentus* cultivars, Nos. 9-23, essentially for the two years and for the two diseases, Iloka with a virtual score of 0 is designated as Hyper-susceptible (HPS). Lady finger with a score of 1 is classified as Very Highly Susceptible (VHS). Nnobi long, Hawzua and Nwa obala, with a score of 3 are classified as Susceptible (SC). The other nine cultivars with a score of 2, are classified as highly susceptible (HSP). The yield of the 23 okra cultivars for the two years studies, for both the protected and the un-protected plants, were subjected to analysis of variance. The ANOVA table is shown in Table 5. The results indicate that the variance ratios were very highly significant for both the unprotected and the protected plants for the two years. Figure 1 shows Alanwangboho, an *A. callei* cultivar with curled leaves and mosaic pattern (CLMP) as well as aborted fruits (ABF) due to the OLC and OM diseases. The plants were still able to bear fruits (FF) despite the attack though there were evidences of abortion of fruits (ABF). An *A.*

Table 3. Summary of responses of 23 okra cultivars to exposure to okra leaf curl and okra mosaic viral diseases under field conditions, for two cropping seasons.

Cultivar		First cropping season		Second cropping season	
	Okra type	*Okra leaf curl*	*Okra mosaic*	*Okra leaf curl*	*Okra mosaic*
Ogolo	Late	00	00	00	00
Ebi ogwu	,,	000	000	000	000
Oru ufie	,,	00	00	00	00
Ojo ogwu	,,	00	00	00	00
Tongolo	,,	00	00	00	00
VLO	,,	00	00	00	00
Ala nwagboho	,,	0	0	0	0
AOAU	,,	0	0	0	0
Nnobi Long	Early	+++	+++	+++	+++
Lady finger	”	++	++	++	+++
Ogba mkpe	”	+	+	+	+
Awgu early	”	+	++	++	++
Iloka	”	++++	++++	++++	++++
Ogbu Oge	”	++	++	++	++
Odiche	”	++	++	++	++
Kano dwarf	”	++	++	++	++
Hawzua	”	+++	+++	+++	+++
Obimo girdle	”	++	++	++	++
Nnobi fat	”	++	++	++	++
Osukwu	”	++	++	++	++
Mpi ele	”	++	++	++	++
Nwa obala	”	+++	+++	+++	+++
Kpum zua	”	++	++	++	++

Score	Yield decline (%)	Score	Yield decline (%)
000 =	Very Highly Immune:	0-5	+ = Susceptible: Above 30-50
00 =	Highly Immune:	Above 5-10	++ = Highly Susceptible: Above 50-60
0 =	Slightly Immune:	Above 10-30	+++ = Very Highly Susceptible: Above 60-70
			++++ = Hyper-susceptible: Above 80

Key to Table 2 legend

Classification	Leaf curl disease	Leaf mosaic disease
Very highly immune	Very few veins affected, leaf shape still intact	Faint localized yellow and green dots on leaf surface
Highly immune	Many veins affected, leaf shape still intact	Faint yellow and green dots covering about one quarter of the leaf surface.
Slightly immune	Mid-rib affected with distortion of leaves	Yellow and green dots covering more than half of the leaf surface
Susceptible	Mid-rib and veins affected, leaves highly distorted, apical bud affected	Almost all the surface of the leaves is covered but no chlorosis.
Highly susceptible	Curling of leaves observed, apical bud severely affected. Intense curling and distortion of leaves, apical buds, severe abortion of flower buds, drastic reduction of leaf size, defoliation.	Entire leaf surface covered with yellow and green dots, slight chlorosis observed Entire leaf surface covered with yellowish green patches, leaf chlorosis, reduction of leaf size, defoliation
Very highly susceptible Hyper susceptible	Most intense curling and distortion of leaves, apical buds. Very severe abortion of flower buds. Distortion of shape of fruits	Very severe dis-colouration of leaves and fruits, fruits creamy in colour and shaped like e-plant fruits. Severe abortion of fruits

callei cultivar, AOAU, also showing CLMP with many healthy looking flower buds (HFB) but virtually no fruits (AFB) is shown in Figure 2.

A hyper susceptible *A. esculentus* cultivar, Iloka is shown in Figure 3. The curled and wrinkled leaves with mosaic pattern (LCMP); the destroyed apical bud (DAB), the discoloured and drastically reduced leaves (DDLR); the complete abortion of all flower buds (AFB) coupled with premature leaf abscission (PLA) are shown as devastating effects of the two diseases. The normal healthy *A. callei* cultivar is shown with the cultivar, VLO in Figure 4a while Kpum Zua (Figure 4b) showed a typical

Table 4. Summary of Virtual inspection of scores of 23 okra cultivars to exposure to okra leaf curl and okra mosaic viral diseases under field conditions, for 2 cropping seasons.

Cultivar	Okra type	1st Cropping season		2nd Cropping season	
		Okra leaf curl	*Okra mosaic*	*Okra leaf curl*	*Okra mosaic*
Ogolo	Late	7	7	7	7
Ebi ogwu	,,	9	9	9	9
Oru ufie	,,	8	7	7	8
Ojo ogwu	,,	6	6	7	6
Tongolo	,,	7	8	8	7
VLO	,,	7	7	6	7
Ala nwagboho	,,	5	5	5	5
AOAU	,,	5	5	5	5
Nnobi Long	Early	3	3	3	3
Lady finger	”	1	1	2	1
Ogba mkpe	”	4	4	4	4
Awgu early	”	4	2	2	2
Iloka	”	0	0	0	0
Ogbu Oge	”	2	2	2	2
Odiche	”	2	2	2	2
Kano dwarf	”	2	2	2	2
Hawzua	”	3	3	3	3
Obimo girdle	”	2	2	2	2
Nnobi fat	”	2	2	2	2
Osukwu	”	2	2	2	2
Mpi ele	”	2	2	2	2
Nwa obala	”	3	3	3	3
Kpum zua	”	2	2	2	2

Virtual inspection legend
0-5 Hyper-susceptible - mildly susceptible
6-9 Immune - very highly immune

Virtual infestation classification	Score	Virtual infestation classification	Score
Very Highly Immune	9	Mildly susceptible	5
Highly Immune	8	Susceptible	3-4
Immune	7	Highly Susceptible	2
Slightly Immune	6	Very Highly Susceptible	1
		Hyper-susceptible	0

example of a normal healthy *A. esculentus* cultivar.

DISCUSSION

In the field both viral diseases were observed to occur together on vulnerable cultivars. According to Kon et al. (2009), they can occur singly or in combination in plants and when they occur in combination in okra plants their effects are often devastating. This is the prevailing situation among vulnerable *A. esculentus* cultivars in the region.

From the results out of the eight late okra cultivars screened, only Ebi Ogwu with less than 5% YD was categorized as VHI. AOAU was more susceptible than Alanwangboho. The important point to note here is that not all the screened *A. callei* cultivars were immune to these viral diseases, contrary to the belief held by some scholars working outside the region. Consequently, screening of any *A. callei* germplasm for vulnerability to these diseases is imperative before declaring it as a resistant cultivar.

However, the degree of vulnerability of the vulnerable *A. callei* cultivars, Alanwangboho and AOAU, was nowhere near to the devastating situation found among the *A. esculentus* cultivars, especially Iloka. The acquisition of resistance to these viral diseases by *A. callei* whose distribution is restricted to the West African region (Singh and Bhatnagar, 1976) and which is believed to have evolved from the region (Harlan, 1971), might not be unconnected with their larger number of chromosomes (2n=194) as against the smaller chromosome number of (2n=130) for *A. esculentus* cultivars (Singh and Bhatnager, 1976). *A. callei* which is

Table 5. Analysis of variance of yield of 23 cultivars of early and late okra, exposed to *Bemisia* white flies (unprotected) and protected (*Bemisia* flies controlled), under field conditions for 2 years.

Item	Sum of squares	Degree of freedom	Mean square	Variance ratio
ANOVA of protected plants for first year of planting				
Total	96.95	68		
Block	0.50	2	0.25	1.25 NS
Variety	87.62	22	3.98	19.91 ***
Error	8.83	44	0.20	
ANOVA of un- protected plants for first year of planting				
Total	39.40	68		
Block	0.03	2	0.013	0.44 NS
Variety	38.08	22	1.73	59.65 ***
Error	1.29	44	0.03	
ANOVA of protected plants for second year of planting				
Total	22.53	68		
Block	0.06	2	0.03	2.0 NS
Variety	21.79	22	0.99	66.0 ***
Error	0.68	44	0.02	
ANOVA of un-protected plants for second year of planting				
Total	5.35	68		
Block	0.02	2	0.01	1.00 NS
Variety	4.88	22	0.22	22.0 ***
Error	0.44	44	0.01	

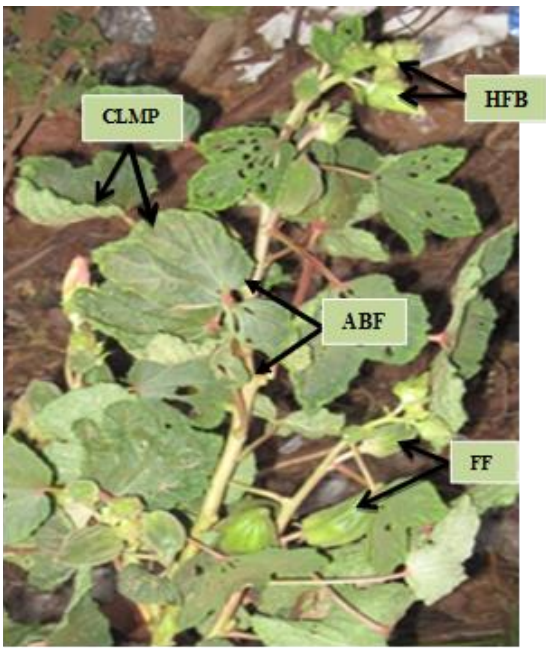

Figure 1. Late Okra Alanwagboho showing moderate attack of Leaf Curl and mosaic virus. HFB, Healthy looking flower buds; FF, Fresh fruits; CLMP, Curled leaves with mosaic pattern; ABF, Aborted fruits.

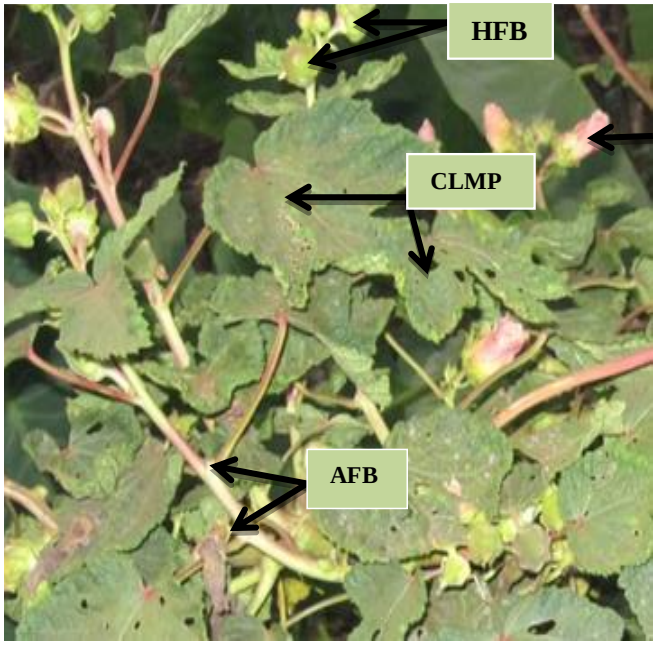

Figure 2. Late Okra AOAU showing more serious moderate attack of Leaf curl and mosaic virus with abortion of flower buds. CLMP, Curled leaves with mosaic pattern; Heal HFB, Healthy looking flower buds; ABF, Aborted fruits.

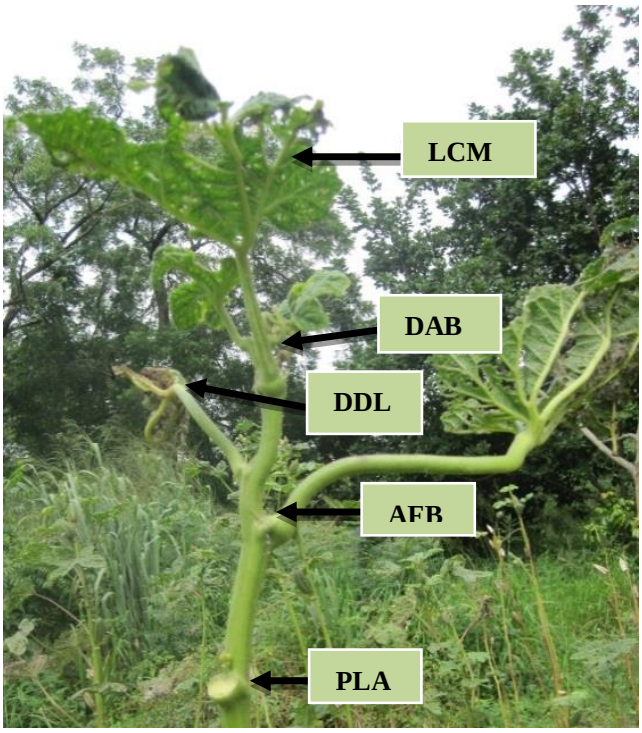

Figure 3. Chronic infestations of Iloka (Early Okra cultivar) by Okra Leaf curl and mosaic viral disease. LCMP, Leaf showing curling and mosaic pattern; DAB, destruction of apical bud; DDLR, discolouration and drastic leaf reduction; AFB, Abortion of flower buds; PLA, premature leaf abscission.

indigenous to West Africa is suspected to have conserved some gene constellations during the course of evolution which possibly conferred on them resistance to these diseases among many others. The acquisition of these disease resistant genes are thought to have occurred early in the evolution of *A. callei* in the equatorial jungles of West Africa, where this edible species can still be found growing wild (an ability which *A. esculentus* lacks) among weeds and other wild plants.

Martin and Ruberte (1978) stated that the exact origin of okra is not known but the presence of wild varieties in Ethiopia, in the region of the Upper Nile river, and the presence of primitive perennial varieties in West Africa, suest an African origin. The possession of this exceptional wild resistant ability by *A. callei* is similar to the report of Prabu et al. (2007), who noted that the five wild *Abelmoschus* species they screened showed strong resistance to Okra Yellow Vein Mosaic Virus in India. Further studies will make it possible for these resistant genes to be identified and sequenced for easy transfer to the more disease prone *A. esculentus* cultivars as well as other vulnerable plants. The existence of several wild species of *Abelmoschus* that possess genes resistant to these viruses as reported by Prabu et al. (2007) obviously broadens the source of resistant genes for the improvement of *A. esculentus*. On the other hand, the occurrence of some *A. callei* cultivars that showed varied degrees of susceptibility to these viral diseases is viewed as a recent event probably occasioned by over domestication which might have failed to provide the natural wild environment needed to stimulate the synthesis of the pertinent mRNA(s) from the genome of the plant, for the production of the necessary protein(s), needed for the conferment of resistance. This strongly argues in support of conserving the rainforests and their resources which are repositories of numerous wild genes that can have positive impact on the problem of food security in Sub-Saharan Africa. In the words of Atiri et al. (2000), the intricate interrelationships among cropping seasons, intercrop periods and virus incidence in West Africa must be thoroughly understood in order to develop ecologically based and sustainable management practices.

The vulnerability of the *A. esculentus* cultivars may not be unconnected with their lower chromosome number of 2n=130 (Singh and Bhatnager, 1976). It could be that *A. esculentus* genome lack these disease resistant genes that *A. callei* had evolved that provides protection against these viral diseases as well as other diseases. This view is supported by the observation that *A. esculentus* cultivars do not survive in the wild. They easily succumb to even weeds when not fully attended to under cultivation, talk less thriving in the wild like the robust *A. callei* cultivars. Worthy of mention is the chronic manifestation of these diseases by Iloka, an *A. esculentus* cultivar. The situation was so serious that planting of this cultivar was discontinued because of its hyper degree of manifestation of the two diseases in the field. The cultivar was seen as a repository of the viral causing organisms, since the disease always manifested on this cultivar for up to four generations. It is suspected that the viruses had become seed borne in this cultivar from where viral reserves are supplied from generation to generation. The cultivar however served the useful purpose of serving as a rich and ready source of the viruses throughout the studies. Among the other *A. esculentus* cultivars screened, the degree of susceptibility varied with Ogba mkpe showing the least vulnerability. This however does not qualify the cultivar to serve as a source of genes for conferring resistance to the diseases on other early okra cultivars. More extensive screenings involving more early okra cultivars is required for the possible identification of better resistant early okra cultivar. In their own report Prabu et al. (2007), noted that among the cultivated *A. esculentus* types they screened, none was found resistant to YVMV.

This present study was carried out during the traditional planting season of April to September which is known to be more humid than the other months of the year. The season of growth is known to be favourable to the multiplication of the *Bemisia* vector of the two diseases as well as to insects generally. By extension the season could therefore be said to favour the viruses. Reduction

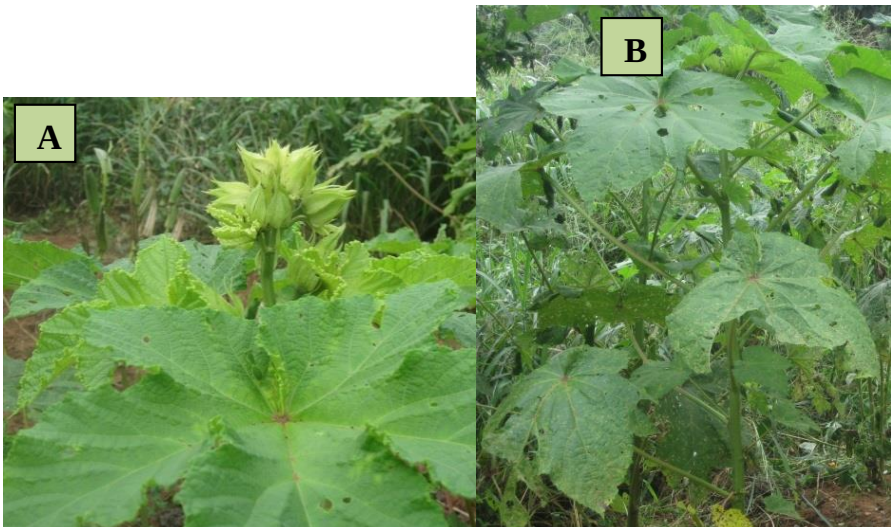

Figure 4. A. Normal healthy late okra (VLO) with a crown of inflorescence**. B.** Normal healthy early okra plants (Kpum Zua).

of the incidence of the two diseases could be based on the cultural practice of growing *A. esculentus* cultivars essentially during the hotter non-growing season, under irrigation. There is however the need to conduct a study to compare the response of the cultivars to the incidence of these diseases during the growing and non-growing seasons, to confirm this assertion. Martin and Ruberte (1978) reported that okra is a warm-season crop. It prospers in the hot, dry tropics and in the hot, humid tropics, but it is out of place and unproductive in cool highlands. High temperatures are necessary for seed germination and growth. Prabu et al. (2007) noted that the incidence of yellow vein mosiac virus (YVMV) in wild and cultivated lines of okra was observed more in summer as compared to kharif season at hot spots in western Maharashtra.

In their own report, Atiri et al. (2000) noted that rainfall, temperature and wind are key weather components in virus pathosystems involving maize (cereal), okra (vegetable) and cassava (tuber crop), and are therefore important in determining the most suitable period in which to undertake crop protection measures. Furthermore, they noted that in West Africa, climate influences (i) virus disease outbreaks, (ii) the rate of development and activity of virus vectors and also of their migration, and (iii) the phenology of crops, weeds and wild hosts that habour plant viruses. These factors need to be taken into consideration in efforts geared towards the control of the incidence of the diseases in the region.

Meanwhile, any cultivar with a YD score of less than 10% is a possible candidate for the disease resistant genes. Consequently, only the following late okra cultivars have been earmarked as late okra cultivars with promising genes that can resist these viral diseases; Ebi Ogwu, Ojo ogwu, Tongolo, VLO, Oru ufie, and Ogolo. More local land races when collected from the local farmers will still be screened to further identify more resistant cultivars. Identification of the resistant cultivars is just one part of the solution. Transferring the disease resistant genes from one *A. esculentus* resistant cultivar (if identified), to a vulnerable one may not provide a lasting solution. Nerkar (1999) reported that a Pusa Sawani cultivar tolerant to Okra Yellow Vein Mosaic disease was developed in India using a strain from I.C. 1542, which stabilized okra cultivation in the country in the 1970s.

However, later Pusa Sawani became severely affected by Okra Yellow Vein Mosaic virus. The reason for this is not quite clear. Transferring of these genes from the more rugged *A. callei* to *A. esculentus* invariably becomes a research imperative which incidentally is almost impossible, adopting the conventional breeding protocols, because of the well-known existence of barrier to gene flow between these two edible *Abelmoschus* species (Fatokun et al., 1979; Martin et al., 1981; Siemonsma, 1982; Hamon and Hamon, 1991; Siemonsma and Hamon, 2004).

This calls for utilization of modern biotechnological gene transfer techniques for rapid molecular breeding for cultivars resistant to these viral diseases. These viral diseases of okra constitute forms of abiotic stress. In the words of Nicot et al. (2005); plants exposed to virulent and avirulent pathogens respond with a range of defense and damage-limiting mechanisms. Many of the responses are also induced by other stress-causing agents, which reflect the common elements in different stress situations (Collinge and Boller, 2001). It is an established fact that numerous studies on the defense and stress mechanisms in plants have been based on gene expression (Kirch et al., 1997; Collinge and Boller, 2001; Bezier et al., 2002; Dean et al., 2002). Transcriptome studies could be helpful in providing a better under-

standing of plant stress responses due to these viral diseases and through these studies, numerous novel stress-responsive genes could be discovered (Nicot et al., 2005).

Through the use of cDNA, the genes responsible for resistance to these viral diseases can be isolated from *A. callei* and used for rapid transfer to vulnerable *A. esculentus* cultivars using *Agrobacterium* mediated, or other gene transfer protocols. This could provide fast and effective means of trouble-shooting the menace of the two diseases in the region.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Minituber production potential of selected Potato (*Solanum tuberosum* L.) genotypes in different propagation media

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Potato farmers in Malawi and other developing countries lack healthy and quality potato seed. This is mainly due to limited seed multiplication programmes to provide farmers with clean potato seed. A study to evaluate minituber production potential for selected genotypes in different media types would assist in planning for future selection of good high yielding varieties. It will also assist in planning for rapid seed multiplication programme of pathogen free planting materials to increase production of potato in the country. Three different propagation media (vermiculite, sand and sawdust) and seven different genotypes (two local genotypes- *Magalabada* and *Rosita,* five introduced genotypes-Up to date, Buffelspoort, Van der plank, Lady Rosetta and Bp 1 2007) were investigated. Plantlets grown on vermiculite performed better with higher mini tuber yield of 1740 g/m^2 for all genotypes while 850 and 292 g/m^2 in sand and sawdust, respectively. Among the genotypes 'Up to date' and Lady Rosetta produced more tubers of between 12 tubers/plant and 10 tubers/plant. Thus, vermiculite increases the number and size of Minitubers which is a very important step for rapid multiplication of potato seed. The study findings provide valuable information for potential genotypes and propagation media to assist in scaling up seed multiplication programmes for pathogen free planting materials.

Key words: Propagation media, genotype, potato, minituber.

INTRODUCTION

Potato (*Solanum tuberosum* L.) plays nutritional, economic and industrial roles in Malawi and it can supplement the food needs in the country in a substantial way (FAO, 2008). Despite potato being an important food and cash crop in Malawi, the actual yield and quality is low. The present average yield ranges from 7 to 10 tonnes/ha, but yields up to 40 tonnes/ha is achievable (MoAIFS, 2007; FAO, 2008). A major limiting factor to realizing high yields and good quality of potatoes in Malawi is inadequate availability of quality seed. This is due to limited multiplication programme to provide farmers with clean potato seed of high yielding varieties

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suitable for different agro ecological zones. Consequently, farmers use low quality and sometimes infected seed from previous potato crop and without proper selection. This practice has led to high degeneration of seed tubers due to systemic viral and bacterial diseases resulting in poor quality and quantity of potato tubers (Demo et al., 2007; Vanaei et al., 2008). As a result national potato production is below market demand of 88 kg per capita (FAO, 2008). One strategy towards improving the quality and quantity of potato tubers in Malawi is to develop a seed system that will increase multiplication of clean potato seed. Tissue culture, through micropropagation, offers a feasible propagation technique. Micropropagation is a technique of regenerating plants using small pieces of plants or tissues that are cultured on an artificial media under controlled environment and sterile conditions. Micropropagation enables the rapid multiplication of large number of potato seed tubers; in contrast with conventional means of propagation, micropropagation is faster and plants produced through micropropagation are usually free from infection by pathogens (Singh, 2002; Altindal and Karadogan, 2010).

Planting materials from tissue culture can be further multiplied in growth media for minituber seed production under greenhouse conditions. Although, potato minituber production can be useful for potato seed multiplication, response may vary among different genotypes (Sharma and Pandey, 2013). Minitubers are small seed potato tubers produced after acclimatization of plants propagated *in vitro* and planted at high density in the glasshouse in seed beds or in containers using different propagation media. Minitubers can be produced throughout the year and are principally used for the production of pre-basic or basic seed by direct field planting (Lommen and Struik, 1999; Ritter et al., 2001). The size of minitubers may range from 5 to 25 mm although in current systems larger minitubers have become common. This size range correlates to a weight range of 0.1 to 10 g or more (Struik, 2007). The quality of the transplant is very important for the production of minitubers (Jami-Moeini et al., 2001; Struik, 2007).

Media is one of the most important factors to consider for clean seed tuber (minituber) production. Due to the relatively shallow depth and limited volume of a container, potting media must be amended to provide the appropriate physical and chemical properties necessary for plant growth. Field soils are generally unsatisfactory for the production of plants in containers. Albajes et al., (2002) stated that this is primarily because soils do not provide the aeration, drainage and water holding capacity required. To improve this situation several "soilless" potting media gained more and more importance because they eliminate or reduce the need for soil disinfestations. Reaching a commercial scale in soilless culture is motivated by a potential for increasing the crop productivity and efficiency. Among soilless substrates, rock wool has been widely used in northern Europe, while in the tropics and sub-tropics cheaper substrates have been exploited (Mobini et al., 2009). Widespread adoption of soilless potting media production in global food and plant production has been reported as the technical solution for problems including root diseases, root zone oxygen deficiency, fertility control which occurs in other systems. Jami-Moeini et al. (2001) emphasized that the advantages of container production systems over ground production systems are greater water and nutrient efficiencies, with more food production, better cropping with higher salinity levels than soil grown.

Although, research has shown that different media (perlite, pumice, sand, styroform, compost, peat and sphagnum) can be used to grow *in vitro* potato plantlets in the greenhouse the most preferred media used is vermiculite (Balali et al., 2008). However, vermiculite is very expensive because of energy cost for heating to prepare suitable product. Due to high cost associated with vermiculite other alternative can be used such as sand, rice hulls and sawdust. Among these sand is a cheap media and easy to use.

Therefore, the aim of the study was to evaluate the growth and seed yield response of micropropagated potato planting materials of locally adapted and newly introduced potato genotypes in different propagation media in order to optimize clean potato seed production and increase availability to farmers in Malawi.

MATERIALS AND METHODS

The study was conducted in the greenhouse at Bunda College of Agriculture in Lilongwe, Central Malawi. The experiment was conducted from April 2011 to November 2011 (eight months).

Plant materials

Healthy (clean) tubers of locally adapted genotypes (Kwapata et al., 2007, unpublished): Rosita and Magalabada were collected from different growing areas in Malawi (Jenda in Mzimba, Dedza, Ntcheu, Bvumbwe and Mulanje). These genotypes were cleaned and certified as disease free plantlets using ELISA test. Certified seed stock of newly introduced genotypes in plantlets form were obtained from Agriculture Research Council (ARC-LNC) in South Africa they were certified as disease free plantlets using ELISA test by suppliers. These genotypes include: Van der plank, Lady Rosetta, Bp 1 2007, Buffel spoort07 and Up to date.

Bp1 2007 is a popular cultivar in South Africa and Lesotho. It produces oval tubers with white flesh and firm texture suitable for making chips. Van Der plank, a white fleshed, pear shaped and firm textured. Good for roasting, chips and stews. Lady Rosetta is used mostly by chips industries or factories because it absorbs less oil. Buffelspoort has a cream flesh with firm texture tubers and is suitable for chips and roasting. Up to date, a white fleshed with floury used mainly for mashed and baked potatoes (ARC.LNC, 2010?). Among local genotypes, Rosita and Magalabada are popular genotype in Malawi and most preferred by farmers (Demo et al., 2007). They have cream flesh with pinkish texture, oval shaped and are good for making chips.

Plant materials used in this study were raised in tissue culture at

Table 1. Characteristics of different media.

Substrate	pH	% organic carbon	% Nitrogen
Vermiculite	7.5	0.65	0.084
Sand	7.3	0.89	0.042
Saw dust	7.6	20.05	0.070

Bunda College and were sub cultured for multiplication using nodal cutting technique. The media was prepared using full strength MS mixture powder, containing all the required basal salts as outlined by Murashige and Skoog (1993), 4.4/l g MS powder. It was supplemented with 25 g/l sucrose and 7 g/l agar (a gelling agent). The medium was measured for its pH and adjusted to 5.7 (with NaOH or HCl). Four weeks old *in vitro* plantlets were subjected to hardening off for about 10 days to prepare them for *in vivo* environment. During hardening off, the following environmental factors were regulated: increase in temperature, increase in light intensity and humidity was reduced. Since plantlets were transferred to plastic magenta tubes, relative humidity (RH) was reduced by making small holes on the lid and keeping them in a sterile place for few days before transferring them to a green house. This gradually reduced the relative humidity (ARC.LNR, 2010). After hardening off, the *in vitro* produced plantlets were then used in the green house for production of minitubers.

Propagation media collection, sterilization and analysis

River sand was collected from shores of Lake Malawi to obtain pure sand. Vermiculite was sourced from South Africa and saw dust was collected from carpentry shops around Lilongwe, Malawi. Each type of media was filled in the covered aluminium jars and autoclaved at 121˚C for 1 h. After autoclaved each propagation media was then analyzed for pH, total nitrogen and Organic Matter in the laboratory (Table 1).

Potato minituber production in greenhouse

The rooted *in vitro* plantlets were transplanted to a greenhouse in trays of 28 L volume filled with different substrates (sand, vermiculite and sawdust) at a volume of 15 L. The plantlets were spaced at 10 cm between plants and 10 cm between rows, corresponding to 40 plants per tray as a gross plot and 20 plants were used as net plot. The irrigation unit was placed at the top of the tray. The inlet of this unit was connected to the water pump through a solenoid valve. The nozzles of the drip irrigation unit were fixed 10 cm equidistant to direct water and nutrient solution towards the root zone at required time intervals (Farran and Mingo-Castel, 2006; CIP, 2008; Chiipanthenga et al., 2012). NPK fertilizer was used (23:21:0 +4S), at the rate of 1.2 g per plant every 10 days throughout the plant growth. The rate was adopted from Alsadon and Knutson (1994). The day and night temperatures in the green house were regulated and the average day temperatures were 20 ± 2°C and night temperatures of 18 ± 2°C.

Statistical analysis

The study had two factors; one factor composed of three propagation media (sand, vermiculite and saw dust) also referred to as growth media or substrate. Second factor composed of seven genotypes: Bp1 2007, Van der plank, Lady Rosetta, Buffelspoort, Up to date, Rosita and Magalabada. These treatments were arranged in a randomized complete block design (RCBD) with three replicates (each block represent a replicate containing 21 treatments/trays). Data collected include; shooting percent which was determined by counting the number of plants that have survived and established against the total plantlets two weeks after transplanting. Days to mini tuber setting was determined by counting number of days plant took to start forming tuber. Tuber set was checked by lifting the media on a net plot and putting the propagation media back. Harvesting of mini-tubers started 9 weeks after transplanting when the mini tubers showed its maturity. The skin of the mature mini-tuber was thick and easy to skin off. Minituber diameter was determined by measuring the width of the tuber using a calliper and minituber yield was determined by measuring the weight of tubers in grams per square metre. Analysis of Variance (ANOVA) was performed using GenStat Statistical package version 13, using General Linear Model procedure. Mean values were compared using the least significance difference (LSD) method at the 5% level of significance.

RESULTS AND DISCUSSION

Effect of media type and genotype on the survival and the establishment of tissue culture plantlets planted in three different growing media 2 weeks after transplanting

Results have shown highly significant (P≤0.001) variation on survival and establishment percentage among the three propagation media. Higher plant establishment percentage of 95% was observed in genotypes planted in vermiculite than in sand (77%) and in sawdust (49%). There was a significant (P≤0.01) variation observed among the seven genotypes on shooting percentage. BP 1 2007, Rosita and Lady Rosetta had highest percentage of plants to shoot at two weeks after transplanting (Table 2). There was no media x genotype interaction with respect to plant survival and establishment (Table 2). The variation in plant survival and establishment percentage in different growth media agrees with the findings by Hassanpanah and Khodadadi (2009) who reported the advantage of vermiculite medium against other media which led to the maximum tuber yield under greenhouse conditions. Vermiculite has excellent ex-change and buffering capacities as well as the ability to supply potassium and magnesium (Badon and Chauhan, 2010). Among the growth media variations could be attributed to difference in the composition and characteristics of media. For instance organic carbon in sawdust was about 20% (Table 1). The results are supported by Öztürk and Yildirim (2010) who found that the C: N ratio of sawdust is such that it is not readily decomposed possibly and the conditions are not conducive for the growth of microbes.

Table 2. Effect of media type and genotype on the survival and the establishment of tissue culture plantlets planted in three different growing media, 2 weeks after transplanting.

Genotype	Propagation media			
	Sand	Vermiculite	Sawdust	Mean for genotypes
Magalabada	75.00±0.00	90.0±0.00	53.0±1.67	72.80^{bc}
Rosita	73.00±3.33	93.3±5.78	55.0±8.82	76.10^{ab}
Lady Rosetta	75.00±5.74	100.0±0.00	50.0±8.66	75.00^{ab}
Bp 1 2007	83.30±1.67	100.0±0.00	56.7±4.41	80.00^{a}
Up to date	78.00±4.41	91.7±1.67	36.7±1.67	68.90^{c}
Van der plank	80.00±5.00	88.3±4.41	45.0±2.87	71.10^{bc}
Buffelspoort	73.33±1.67	93.3±5.78	50.0±1.67	72.20^{bc}
Mean	76.90^{b}	94.52^{a}	49.52^{c}	

Values in the table are presented as mean ± standard error, Means in the main effects have been compared separately, Means with different letter superscripts within the same column and rows are significantly different at P<0.05.

Figure 1. Bp 1 2007 genotype at five weeks after transplanting in green house (a) vermiculite, (b) sand, (c) sawdust.

The high cellulose and lignin content along with insufficient Nitrogen supplies creates depletion problems which can severely restrict plant establishment and growth. However, supplemental applications of nitrogen at the required rate can reduce this problem. In this study, all the media used where supplemented with the similar rate and type of fertiliser. However, it can be observed that probably fertiliser used and the rate could not be enough for sawdust media to retain the required nutrients to the plants and causing poor growth of plants. Apart from being influenced by the genetic constitution, high plant shooting percentage is also influenced by the type of the media used among other environmental factors (Kuria et al., 2008).

Observation on shooting percentage explains the establishment of the plants in the growth media which include well developed roots and shoots (Figure 1). Other reports show that a well-established root and shoot system is important for subsequent growth which later influence tuber bulking stage in potato plant (Dwelle and Love, 2000). In this study the results implies that genotypes Bp 1 2007, Rosita and Lady Rosetta were well established in the propagation media than other genotypes.

Number of days to tuber formation as affected by propagation media and genotype

There was significance differences (P≤ 0.05) observed on interaction between propagation media and genotype (Table 3) on number of days to tuber formation. Plants in vermiculite started forming tubers earlier than plants in sand and sawdust. Among the genotypes BP 1 2007 and Buffelspoort started forming tubers at an average of 33 and 34 days after transplanting, respectively. Rosita (41 days) and Magalabada (40 days) were late in forming tubers than the introduced genotypes. The variations in tuber formation could be attributed to the media types and probably genetic constitution of the genotypes. According to a review on South Africa genotypes Black (2008) found that some genotypes were bred for early maturing others medium or late maturing depending on the agro-ecological zone which might influence time for

Table 3. Effect of Media and genotype on number of days to tuber set.

Genotype	Propagation media			Mean for genotypes
	Sand	Vermiculite	Sawdust	
Magalabada	41±0.00^{c}	40±0.33^{c}	45±0.00^{a}	42^{a}
Rosita	42±0.33^{b}	41±0.01^{c}	45±0.67^{a}	43^{a}
Lady Rosetta	37±0.00^{ef}	36±0.00^{fg}	38±0.00^{de}	37^{b}
Bp 1 2007	36±0.00^{fg}	33±0.67^{h}	38±0.00^{de}	35^{c}
Up to date	36±0.00^{fg}	35±0.33^{g}	39±0.00^{d}	36^{b}
Van der plank	37±0.00^{ef}	36±0.00^{fg}	37±0.00^{ef}	36^{b}
Buffelspoort	35±0.00^{g}	34±0.33^{h}	37±0.00^{ef}	35^{c}
Mean for media	37^{b}	35^{c}	40^{a}	

Values in the table are presented as mean ± standard error, Means in the main effects have been compared separately, Means with different letter superscripts within the same column and rows are significantly different at P<0.05.

Table 4. Minituber yield per genotype and propagation media on yield grams per m^2 at harvest.

Genotype	Propagation media			Mean
	Sand	Vermiculite	Sawdust	
Magalabada	954^{f}	1059^{d}	259^{i}	757^{d}
Rosita	962^{f}	1490^{b}	262^{i}	904^{c}
Lady Rosetta	640^{g}	2146^{a}	244^{i}	1010^{b}
Bp 1 2007.1	1136^{e}	2140^{a}	431^{h}	1238^{a}
Up to date	643^{g}	2012^{a}	291^{hi}	982^{bc}
Van der plank	708^{g}	1263^{c}	318^{hi}	779^{d}
Buffelspoort	700^{g}	1364^{bc}	240^{i}	770^{d}
Mean	820^{b}	1740^{a}	292^{c}	

Means in the main effects have been compared separately.

tuber set in a green house. Other studies have further indicated that tuberisation may be influenced by several factors such as media type, genotype, nutrition among others (Alexopoulos et al., 2007). Khodadadi et al. (2011) reported that under appropriate growth conditions, the tips of stolons will “hook” and begin to swell, resulting in initiation of new tubers. For many cultivars, including Van der plank and up to date, this occurs during early flowering. Dwelle and Love (2000) further illustrates that although there is no causal relationship between the two events. Potatoes need moderate availability of nitrogen and cool nights for good tuber growth. The results in the study indicate that media influence potato tuber formation and bulking. Therefore, good selection of media and genotype would assist in mass potato mini tuber production.

Minituber yield as affected by genotype and propagation media (yield grams per m^2) at harvest

There was a significant interaction between genotype and propagation media with respect to yield. Lady Rosetta, Bp 1 2007 and Up to date genotypes transplanted in vermiculite produced relatively higher yields of 2146, 2140 and 2012 g/m^2, respectively, among the seven genotypes tested. However, the trends were different for genotypes planted in sand Bp 1 2007, Lady Rosetta and Magalabada outperformed other genotypes having higher yields of 1136, 962 and 954 g/m^2, respectively (Table 4). The variation in yield per genotype could be attributed to the influence by media and genetic composition. Findings by Khurana et al. (2003) suggest that different potato genotype adapt differently to the planting bed. In addition, some external factor such as temperature may influence tuber initiation and bulking. The results on yields from this study are in line with findings of Akoumianakis et al., (2000); Khodadadi et al., (2011) who also reported yield range of 300 to 2000 g per square metre depending on the varieties and growth media under greenhouse conditions. Yields were lower in sawdust due to poor growth and this affects photosynthetic area which subsequently affects tuber formation of potato plant. In addition some studies have also indicated that the source of the sawdust compromise its quality and ability to return nutrients to the plants (Palacios et al., 2009). This obser-

Table 5. Number of minitubers per plant as affected by propagation media types and genotypes.

Genotype	Propagation media			Mean for genotype
	Sand	Vermiculite	Sawdust	
Magalabada	5±0.33^{fgh}	8±0.00^{cd}	2±0.00^{j}	5.02^{c}
Rosita	5±0.78^{fgh}	9±0.08^{bc}	2±0.33^{j}	5.32^{bc}
Lady Rosetta	6±0.17^{e}	10±0.79^{b}	3±0.44^{ij}	6.13^{b}
Bp 1 2007	4±0.22^{gh}	6±0.29^{ef}	3±1.33^{hi}	4.59^{c}
Up to date	7±0.29^{de}	12±0.77^{a}	3±0.68^{ij}	6.99^{a}
Van der plank	3±0.22^{ij}	4±0.11^{hi}	2±0.15^{j}	2.76^{d}
Buffelspoort	5±0.21^{efg}	7±0.17^{de}	2±0.47^{j}	4.60^{c}
Mean for Media	4.94^{b}	7.81^{a}	2.39^{c}	

Values in the table are presented as mean ± standard error, means in the main effects have been compared separately.

vation agreed with the findings by Donnelly et al. (2008) who reported that the species of tree from which sawdust is derived largely determines its quality and value for use in a growth media. In this study, saw dust that was used was a mixture from different trees which could have an effect on plant growth and tuber yield. The main role of physical characteristics of media is having suitable air-filled porosity for efficient oxygen diffusion and maintaining favourable water content for supplying water and nutrients and respiration of root (Richard et al., 2004). This implies that for high multiplication of minitubers in the greenhouse media is a very important factor to be considered as it affects both growth and minituber yield of potato.

Effect of propagation media and genotypes on number of tubers per plant

There was a significant (P≤ 0.001) interaction between genotype and propagation media with respect to number of tubers per plant. (Table 5). Up to date and Lady Rosetta genotypes planted in vermiculite registered the highest number of tubers per plant (12 and 10, respectively). Alsadon and Knutson (1994) reported that tuber number rather than weight is important for reporting yield under greenhouse conditions. Green house may permit an expression of tuber number potential but limitations in media space and possibly radiation may not permit maximum tuber size development. Up to date and Lady Rosetta recorded higher number of mini tubers per plant indicating superiority over other genotypes. This could be attributed to the interaction between genetic constitution of the genotypes and environment. High number of minitubers per plant indicates high multiplication potential of the genotypes under greenhouse conditions. The results agree with Black (2008) that Up to date and Lady Rosetta were bred for high yielding and are most popular varieties and make up 77% of the potatoes grown in South Africa. These findings by Black (2008) were also reflected in this study by having these two genotypes outperforming the other genotypes. The study findings imply that Up to date and Lady Rosetta demonstrated potential traits for high yield that would assist breeders for selection and improvement.

Asghari-Zakaria et al. (2009) reported that nutrient uptake of plant is determined by the growth media. Vermiculite despite being expensive is still the best media indicating superiority among the media tested. This could be attributed to its ability to hold and retain nutrients to the plants. Other studies suggest that generally the mixture of two or three different media is better (Khodadadi et al., 2011) This therefore indicates that with good manipulation and management of other alternative media tested such as sand could equally do well in minituber production as vermiculite and this can limit the use of this expensive media.

Potato tuber diameter as affected by propagation media and genotype

There were significant (P≤0.05) variation among the interaction between genotype and media. The introduced genotypes transplanted in vermiculite had more tubers of bigger sizes than local genotypes (Figure 2). The same trend was experienced in sand and sawdust growth media.

However, Van der plank, Lady Rosetta and Bp 1 2007 outperformed all genotypes having more tubers of diameter up to 2 cm. It was observed that Van der plank had little number of tubers per plant (Table 3) but the tubers were of bigger sizes among the genotypes possibly due to compensatory growth. According to the results sand media has also a potential to produce minitubers of the average sizes and can still work in replacing vermiculite as the best media for potato production. Sand is relatively cheaper to source than vermiculite which makes it easier to use sand than vermiculite.

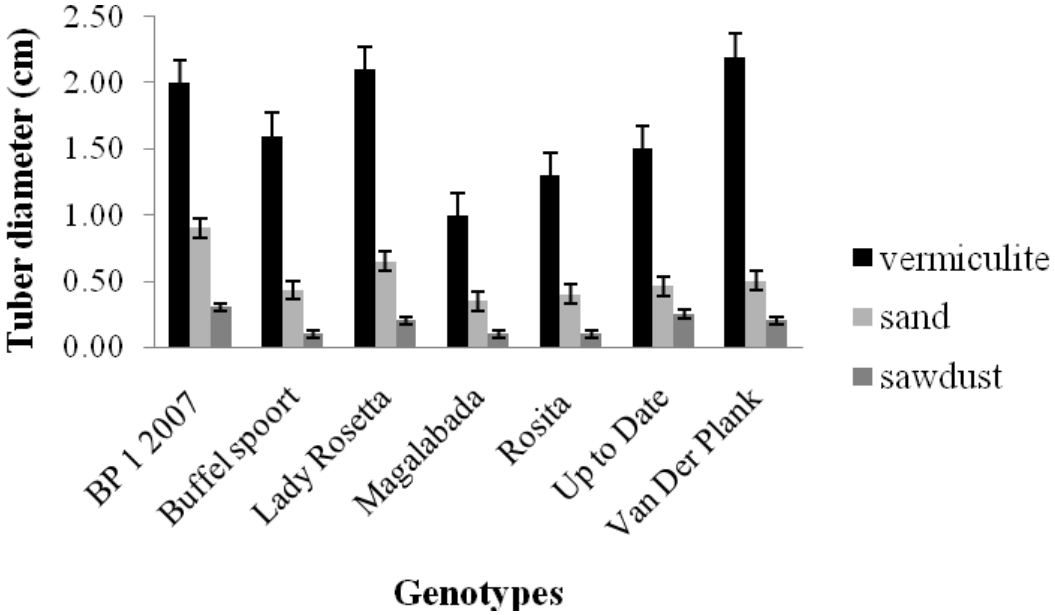

Figure 2. Tuber diameter for genotypes in different media in greenhouse at harvest.

Conclusion

The results have shown that minituber yield vary among local and introduced potato genotypes under greenhouse conditions. Genotypes with high shooting percentage and vigorous growth in a particular media represent a potential for establishment and production of minituber potatoes. Introduced genotypes grew vigorously indicating good establishment than the local genotypes. The findings of this study have revealed genotypes with a potential for minituber seed production of potato. Introduced genotype Bp 1 2007, Lady Rosetta and Up to date and Van der plank have demonstrated valuable characteristics that could assist in selection for multiplication and breeding purposes. The results have also shown that performance of potato genotypes vary with propagation media under greenhouse conditions. Among the propagation media tested vermiculite was still found to be superior in supporting the plants followed by sand. Thus, vermiculite has positive effect on potato growth and tuber formation. However, sand would also be a potential media if it can be manipulated and managed by either media combination or proper nutrient supplementation so that it replaces the use of vermiculite for optimum production of minitubers. The use of sawdust can be improved with high nutrient supplement, combined with other media and proper selection of tree species that may not have an effect to the plantlets. The study has provided valuable information on potential genotypes and propagation media which will be a bench mark on minituber production research and up scaling programmes for making available pathogen free seed tubers to increase potato production in Malawi.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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In vitro propagation of Ethiopian mustard (*Brassica carinata* A. BRAUN)

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***Brassica carinata* (A. Braun) is an amphi-diploid species that originated from interspecific hybridization between *Brassica nigra* and *Brassica oleracea* in the highlands of Ethiopia. The crop has many desirable agronomic traits but with oil quality constraints like high erucic acid and glucosinolate contents. In this study, two genotypes and two types of explants were tested for callus induction, shoot and root regeneration in Murashige and Skoog (MS) medium under different concentrations of naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), and 6-benzyl amino purine (BAP). Cotyledon proved to be most responsive for callus induction at a higher rate in a short period of time. Growth regulator type and concentration had a significant effect on the callus induction and physical appearance. The highest frequencies of callus growth (80.7 and 95%) were observed on hypocotyl and cotyledon explants, respectively, cultured on MS basal medium supplemented with 0.5 mg/L 2,4-D in Yellow Dodola. Two types of calli were obtained: white and friable callus with large cells; green and compact callus with smaller cells. For shoot induction, successful shoot regeneration from white/friable callus was achieved when MS medium was supplemented with 6-benzyl amino purine (2 mg/L). Significant genotypic difference was observed between the genotypes, Yellow Dodola giving the highest response. Maximum shoot induction was recorded in the hypocotyls of Yellow Dodola (90%) when MS medium with 2 mg/L BAP was used. Highest percentage of shoots with roots (98.7%) and highest mean number of roots per shoot (9) occurred on medium with 0.3 mg IBA, while the maximum root length (4.7 cm) was attained on MS medium without plant growth regulator (MSO) in Yellow Dodola. Plantlets were successfully acclimatized in potting medium containing a mixture of 25% sand, 50% red soil and 25% compost on acclimatization pots (1:2:1). The *in vitro* regeneration protocol developed can be used for further undertaking of other tissue culture and genetic engineering work on *B. carinata*.**

Key words: Auxin, callus, cotyledon, cultivars, cytokinin, *in vitro* regeneration, hypocotyls.

INTRODUCTION

In Ethiopia, among the oilseeds, Ethiopian mustard stands third next to niger seed and linseed in total production and area coverage (CSA, 2009). Its area and production are estimated to be 34,580 hectares and 47,721 tons, respectively, at private peasants holdings level, with an average productivity of 0.68 tones/ha (CSA, 2009). This

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low production is attributed to a number of production constraints such as lack of high yielding, early maturing varieties, high erucic acid (C22:1) content in seed oil and high glucosinolate content in the meal (EARO, 2000).

Ethiopian mustard, *Brassica carinata* A. Braun (BBCC, 2n=4x=34) is believed to have originated in the Ethiopia plateau through natural hybridization of *Brassica nigra* (BB, 2n=2x=16) with *Brassica oleracea* (CC, 2n=2x=18), followed by the chromosome doubling of the hybrids, and has several agronomical important traits such as non-dehiscent siliques, much more developed and aggressive root system than *B. napus*. It is resistance to a wide range of diseases, pests and tolerant to many abiotic stresses (Katiyar et al., 1986; Malik, 1990), which makes it a suitable candidate as a food security crop in Ethiopia.

Plant transformation systems have been developed for many economically important species of the genus *Brassica* such as *B. napus* (Moloney et al., 1989), *B. oleracea* (Deblock et al., 1989), *Brassica juncea* (Barfield and Pua, 1991), *Brassica rapa* (Radke et al., 1992), *Brassica nigra* (Gupt et al., 1993) and *B. carinata* (Babic et al., 1998) which facilitates obtaining transgenic plants with modified agronomic traits. Many genetic improvements, such as herbicide tolerance, improved oil quality and production of pharmacological and industrial products, have been achieved by genetic transformation in the *Brassica* species. For example, in *B. napus* seeds, high levels of gamma-linoleic acid were obtained by the introduction of δ12-desaturase genes from the fungus *Mortierella alpine* (Liu et al., 2001). In addition *B. carinata* used to produce biodiesel, and their erucic acid contents are used as chemical additives in plastic, tannery and cosmetic industries (Bozzini et al., 2007), and also as luminant or lubricant in soap making (Greville, 2005).

Genetic improvement of *Brassica* spp. has been mainly achieved by conventional breeding methods. Recently, genetic engineering opened a new avenue for plant improvement (Hansen and Wright, 1999). Regeneration in *Brassica* is highly genotyped, age dependent and has been reported in several species (Guo et al., 2005). *B. napus* cultivar GSL-1 showed better regeneration efficiency than Westar (a standard cultivar for transformation) in a study by Phogat et al. (2000).

In vitro regeneration is one of a key factor in developing an efficient transformation method in plants. In *Brassica spp. in vitro regeneration* is highly genotype-dependent for *B. napus* (Ono et al., 1994; Phogat et al., 2000), *B. rapa* (Zhang et al., 1998) and *B. oleracea* (Sparrow et al., 2006). In addition, Dietert et al. (1982) compared 6 species of the genus *Brassica* for callus growth and plant regeneration and reported a high influence of the genotype in the *in vitro* culture. However, the available information on the genotype and explant variability for *in vitro* culture and shoot regeneration in *B. carinata* is limited to a small number of genotypes, thus a limiting factor for the application of genetic engineering to a wide number of genotypes. For that reason, it is important to identify highly regenerant genotypes that can be used in transformation *via Agrobacterium tumefaciens.*

However, no work has been done on the effects of various growth regulators on the callus formation from the hypocotyl and cotyledon explants of Yellow Dodola and Holleta-1 *B. carinata* genotypes. But there are a number of studies carried out in the direct regeneration to increase the regeneration frequency of *Brassica* spp. and remarkable progress has been achieved. Hence, the main objective of this study was to establish an *in vitro* regene-ration and propagation protocol for the two rapseed (*B. carinata*) cultivars, Yellow Dodola and Holeta-1 using hypocotyl and cotyledon explants.

MATERIALS AND METHODS

The experiment was conducted in Holleta Agricultural Biotechnology Laboratory. Two varieties of *B. carinata* namely Holeta-1 and Yellow Dodola obtained from Highland Oil Crops Research Department of Holleta Agricultural Research Centre, were used for this study.

Surface sterilization and germination

Seeds of *B.carinata* genotypes Holeta-1 and Yellow Dodola were first washed by soaking in tap water for 5 min to remove dust and dirt. The seeds were then transferred to 70% ethanol for 1 min followed by 1% sodium hypochlorite (NaOCl) for 20 min. The surface sterilized seeds were then rinsed with sterile distilled water for 2 to 3 times. The pH of the culture medium was adjusted to 5.8 using 1.0 M HCl or 0.5 M NaOH before autoclaving and autoclaved at 15 psi at 121°C for 15 min. The seeds were plated for germination on the previously prepared hormone free full strength MS media containing 2% sucrose with 0.6% agar under sterile condition and were incubated at 25±2°C in a 16/8 h day/night photoperiodic regime under cool white fluorescent lights (2700 -Lux) µmol quanta m^{-2} s^{-1} for 5–7 days.

Cotyledon leaves and hypocotyls were excised from 5-7 days old seedlings under sterile condition and were cut into 0.5-1 cm pieces of explants excluding the meristematic axillary bud. Five explants were then placed horizontally in each magenta box with callus induction MS medium supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA) and 6-benzyl amino purine (BAP).

Callus induction medium

Explants were cultured on the basal MS-medium supplemented with three hormones 2,4-D, BAP and NAA at different concentrations (0.1, 0 .25, 0.5, 1.0 and 1.5 mg/L) to enhance callus initiation. All growth conditions were maintained with 3 replications in complete dark for one month. The explants were then sub-cultured every 4-5 weeks on the freshly prepared MS medium until callus was formed. The percentage of explants (%) that induced calli and their weights (gm) were recorded for each treatment.

Shoot induction medium

After about 30-48 days of callus culture, the calli were transferred aseptically onto a sterile Petri dish and were cut into convenient size by a sterile scalpel or blade, then transferred onto a freshly

prepared media supplemented with 0, 1, 2 and 3 mg/L BAP. Each culture box contained 5 pieces of calli and all the treatments were performed with three replications and sealed with parafilm in a growth room under 16 h light at 2700 µmol quanta m^{-2} s^{-1} lux light intensity and 8 h dark cycle at 25 ±2°C. The explants were sub-cultured every 2-3 weeks intervals on the freshly prepared medium to obtain shoot regeneration. The parameters recorded were the percentage of explants producing shoots (%), the mean number of shoots produced per explants and shoot height attained (cm).

Root induction medium

Eight weeks later, regenerated shoots were carefully removed from the culture box and cultured in freshly prepared MS medium containing different hormonal supplements like IBA and NAA at different concentrations (0.0, 0.1, 0.3 and 0.5 mg/L) for root initiation. The MS media contained 2% sucrose, 0.6% agar and the pH was 5.8. Parameters recorded were the percentage of explants producing root (%), the mean number of roots produced per explants, root length attained (cm) and the fresh and dry weight of shoots attained (cm) after four weeks of culture. Plantlets with approximate height of 4-7.7 cm, with well-developed roots were transferred to a small acclimatization pot, containing sterilized soil mix with a proportion of 1:2:1 sand, red soil and compost, respectively.

Experimental design and statistical analysis

The experiments were arranged in a complete randomized design (RCD), with three replications and each replication per treatment contained 5 explants. Data were analyzed using analysis of variance (ANOVA) using statistical analysis system (SAS versions) software and the least significant differences among mean values were compared using DMRT at $p \leq 0.05$.

RESULTS AND DISCUSSION

Callus induction on hypocotyl and cotyledon explants

The *in vitro* morphogenetic responses of cultured plants are affected by different components of the culture media and therefore, it is important to evaluate their effects on plant callus induction and regeneration (Gubis et al., 2004). The surface sterilized germinating seeds were used as sources of explants for callus induction. The explants were inoculated on MS medium with variable ranges of 2, 4-D, BAP, NAA.

It was observed that the explants showed an initial swelling followed by callus formation within 2 - 3 weeks of incubation, in treatment without plant growth regulators. Callus proliferation started from cut ends of hypocotyls and cotyledon on MS medium supplemented with different concentrations of 2, 4-D, BAP and NAA and eventually extended all over the explants. Callus initiation began from cut edges after 12 days in cotyledon explants and after 15-20 days in hypocotyl explants.

Callus produced from cotyledon explants grew faster during the first 7-30 days of culture in both varieties. Approximately, after 3 weeks of culture, the explants were almost completely converted into callus. Similar results have been described in other reports. Ullah et al. (2004) reported that on *B. napus* cv. Rainbow explants, callus proliferation started from the cut ends of the hypocotyls and cotyledon. Al–Naggar et al. (2010) reported different results in *B. napus*; 2,4-D induced callus production over the entire surface of the cotyledon and hypocotyls.

The type and quantity of callus and callogenesis efficiency depends on the duration of their exposure to hormone and on the type of explants (Bano et al., 2010; Khan and Rashid, 2002). In this experiment, the highest quantity of callus was formed in cotyledons after short induction on MS with 2,4-D. BAP and NAA required more time to produce callus and caused poor formation of compact calli, with many necrotic sites. Abellatef et al. (2008) and Dietert et al. (1982) obtained similar results in cotton and *B. napus* cv. Evitain on MS media supplemented with 2,4-D and BAP, respectively. Highly significant difference in percentage of callus formation was observed in between two types of explants, PGRs and the genotypes used (Table 1). These results are in agreement with those reported by Sayed et al. (2010) and Khan et al. (2010).

Effects of 2,4-D on callus induction

2,4-D is among the most widely used auxin for *in vitro* callus induction in a wide range of plant species (Al-Naggar et al., 2010; Khan and Rashid, 2002). The highest percentage of callus (95%) was obtained on cotyledon explants cultured on MS medium supplemented with 2,4-D at 0.5 mg/L with 1.5 g mean weight (Table 1), followed by from the same explants on 1 mg/L 2,4-D (86%). However, for the hypocotyl explant, 80.7% of callus was obtained on MS medium supplemented with 2,4-D at 0.1 mg/L (Table 1), followed by using 0.25 mg/L 2,4-D (63.9%) within the genotype of Yellow-Dodola. In Holeta–1, The highest percentage of callus (88.9%) was obtained on cotyledon explants cultured on MS medium supplemented with 0.5 mg/L, 2,4-D followed by 80% from the same explants by using 1 mg/L of 2,4-D. However, for the hypocotyls explants, 66 and 73% of callus were obtained respectively with the same concentrations of 2,4-D as mentioned in cotyledon (Table 1). In line with the present results, Magdoleen et al. (2010) also reported that the most efficient hormone concentration depends on the genotype used and the type of explants. The cotyledon in particular was found to be the best for callus proliferation in both genotypes.

The Yellow Dodola genotype in particular was found to be the best for callus initiation in both explants types with 0.5 mg/L 2,4-D. It had the highest callus initiation frequency (CIF) and produced calli with more weight than the Holleta-1 within the different concentration levels of 2,4-D. These results are in harmony with those reported by Ali et al. (2007) in *B. napus.*

Table 1. Effect of different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic acid (NAA) and benzyl adenine purine (BAP) on callus induction and mass of callus in Yellow Dodola and Holeta-1.

PGR	Concentration (mg/L)	Yellow– Dodola				Holeta–1				NC
		Callus induction (%)		Mean Weight (g)		Callus Induction (%)		Mean weight (g)		
		Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	
BAP	0	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{e}	0.0±0.0^{f}	0.0±0.0^{f}	0.0±0.0^{e}	0.0±0^{e}	NO
	0.1	44.3±1.30^{b}	53.4±0.96^{c}	0.45±0.05c	0.56±0.04^{b}	38.6±0.52^{c}	44.3±0.23^{d}	0.5±0.08^{c}	0.37±0.03^{b}	W /F
	0.25	50±1.00^{a}	66.8±1.15^{b}	0.65±0.13^{b}	1.0±0.00^{a}	41.8±0.21^{b}	58.2±0.18^{b}	0.4±0.05^{b}	0.9±0.09^{a}	W/F
	0.5	50±2.0^{a}	80.5±0.50^{a}	0.89±0.09^{a}	1.2±0.40^{a}	44.3±0.23^{a}	72.1±0.12^{a}	0.9±0.04^{a}	0.9±0.01^{a}	Y/F
	1	33.4±1.2 5^{c}	0.0±1.00^{d}	0.37±0.03^{c}	0.55±0.05^{b}	27.6±0.51^{d}	50.±0.0^{c}	0.2±0.05^{c}	0.6±0.04^{b}	W/F
	1.5	16.3±0.35^{d}	27.3±0.35^{e}	0.23±0.05^{d}	0.27±0.04^{cb}	16.8±0.17^{e}	22.1±0.12^{e}	0.12±0.07^{d}	0.21±0.10^{d}	Y/F
	Mean	32.4	46.4	0.54	0.6	28.2	41.1	0.44	0.65	
	CV (%)	3.6	1.68	2.4	3.8	1.2	0.33	1.9	6.4	
2,4–D	0	0.0±0.0 ^{f}	0.0 ± 0.0^{f}	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{f}	0.0±0.0^{f}	0.0±0.0^{e}	0.0±0.0^{d}	NO
	0.1	52.3±0.35^{c}	66.7±0.58^{d}	0.63±0.08^{b}	0.6±0.15^{c}	52.8±0.15^{e}	61.1±0.06^{e}	0.47±0.03^{d}	0.92±0.10^{cb}	C/G
	0.25	63.9± 0.10^{b}	83.5±0.68^{c}	0.89±0.10^{ba}	0.79±0.08^{c}	58.2±0.17^{c}	77.8±0.15^{c}	1.1±0.1^{b}	0.7±0.02^{c}	C/G
	0.5	80.7±0.29^{a}	95.1±0.812^{a}	1.5±0.60^{a}	2.1±0.12^{a}	66.8±0.21^{b}	88.9±0.10^{a}	1.27±0.20^{a}	1.3±0.25^{a}	C/G
	1	33.2±0.17^{d}	86.2±0.17^{b}	0.83±0.57^{b}	0.96±0.06^{b}	73.2±0.17^{a}	80.5±0.50^{b}	0.17±0.81^{c}	0.96±0.16^{b}	C/G
	1.5	16.2±0.4^{e}	27.6±0.51^{e}	0.95±0.06^{ba}	0.7±0.05^{c}	55.7±0.3^{d}	75.0±1.0^{d}	0.3±0.5^{d}	0.78±0.12^{c}	C/G
	Mean	41.1	59.9	0.97	0.85	51.1	63.8	0.82	0.9	
	CV (%)	0.64	0.9	1.2	10.7	0.35	0.72	3.8	3.1	
NAA	0	0.0±0.0^{f}	0.0±0.0 ^{f}	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{f}	0.0±0.0^{f}	0.0±0.0^{e}	0.0±0.0^{c}	W/F
	0.5	30.2±0.29^{e}	50.2±0.29^{e}	0.40±0.01^{b}	0.37±0.04^{c}	33.2±0.17^{e}	58.2±0.17^{e}	0.17±0.5^{c}	0.9±0.044^{b}	W/F
	1	41.6±0.51^{d}	52.8±0.17^{d}	0.53±0.06b^{a}	0.86±0.13^{bc}	47.1±0.12^{d}	61.1±0.06^{d}	0.17±0.5^{b}	0.9±0.15^{b}	W/F
	1.5	66.8±0.17^{b}	50.3±0.58^{b}	0.29±0.09d^{c}	1.4±0.40^{a}	58.2±0.17^{b}	73.2±0.17^{b}	0.17±0.71^{b}	0.96±0.35^{b}	W/F
	2.5	44±0.23^{c}	58.2±0.17^{c}	0.78±0.0^{a}	0.9±0.07^{b}	50±1.00^{c}	69.3±0.23^{c}	1±0.88^{a}	1.1±0.1^{b}	W/F
	Mean	37.4	50.5	0.6	0.95	42.5	57.5	0.47	1.4	
	CV (%)	0.95	0.57	1.4	6.6	1.01	0.27	7.2	0.85	

The experiment was arranged in randomized complete design, with three replications and each experiment contained five explants. Means with in a column followed by the same superscript letters are not significantly at P≤0.05 according to DMR. PGR = Plant growth regulators; NC = nature of callus; W = white; F = Friable; C = compact; G = green and Y = yellow.

Effects of BAP on callus induction

In study on the effects of different concentrations of BAP on callus initiation from hypocotyl and cotyledon, the results show that MS medium supplemented with 0.5 mg/L of BAP was the most effective in callus induction for both types of explants and genotype. 0.5 mg/L BAP was more effective in inducing callus from cotyledon explants than hypocotyls. A higher concentration of BAP (1.5 mg/L) inhibited callus proliferation. BAP at low concentrations were more effective for

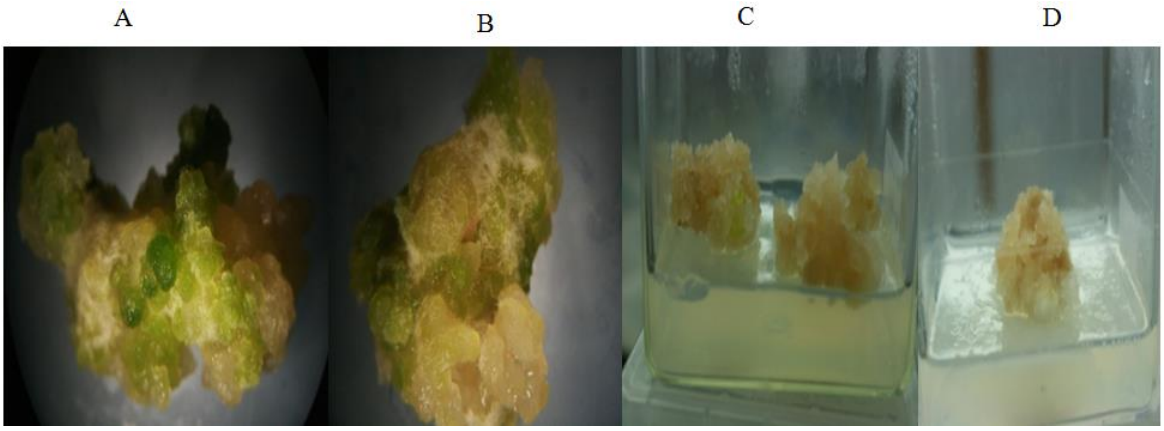

Figure 1. Proliferation of callus from hypocotyl and cotyledon explants of *Brassica carinata* cultivars after 5 weeks of culture on full MS medium supplemented with 0.5 mg/L 2,4-D. **(A).** Compact/green callus formation from cotyledon of Yellow Dodola. **(B).** Compact/green callus formation from cotyledon of Holeta–1. **(C).** White/friable callus formation from the hypocotyl of Yellow Dodola. **(D).** White/friable callus formation from the hypocotyl of Holeta–1.

induction of callus. The present study showed that callus initiation frequency (CIF) was 80 and 50% for Yellow Dodola cotyledon and hypocotyls explant, supplemented with concentration of 0.5 mg/L of BAP respectively (Table 1). Whereas, Holetta–1 had CIF of 72 and 44% on cotyledon and hypocotyl explants supplemented with the same concentration of BAP, respectively (Table 1). Among the explants types, the cotyledon explants was more responsive than the hypocotyls.

Comparison of different explants types and lines in terms of callus production revealed a genotypic variation between the types of explants and lines with Holleta-1 being the least responsive while Yellow Dodola was the most productive. This finding correlated with the result of various research reports in *B. napus* (Datta and Conger, 1999; Fang et al., 2005; Khan et al., 2010; Pathirana and Eason, 2006, 2004; Rao et al., 2006.

Effects of NAA on callus induction

In study on the effects of different concentrations of NAA on callus initiation from hypocotyl and cotyledon, the results show that MS medium supplemented with 1.5 mg/L of NAA was the most effective in callus induction for both types of explants and genotype. 1.5 mg/L NAA was more effective in inducing callus in explants hypocotyls than cotyledon. The present study showed that callus initiation percentage was 66.8 and 52.8% for Yellow Dodola hypocotyls and cotyledon explants, supplemented with concentration of 1.5 mg/L NAA respectively (Table 1) whereas, in Holetta–1, the best callus induction was observed in cotyledonary explants and it was 73 and 69% at the concentration of 1.5 and 2.5 mg/L of NAA in MS medium, respectively. The second best callus was 58% at the concentration of 1.5 mg/L of NAA from the hypocotyls explants (Table 1). For the comparison of explants types, the cotyledon explants was more responsive than the hypocotyls (Table 1). These results are in agreement with those reported by Magdoleen et al. (2010) and Chamandosti et al. (2006). Comparison of different explants types and lines in terms of callus production revealed a genotypic variation between the types of explants and lines with Holleta-1 being the best responsive while Yellow Dodola was the least productive.

Two morphological types of calli were obtained after 30 days of culture; white friable and compact green. Calli derived from hypocotyls became soft or friable, sticky, yellow-white to cream colored and nodular type, and mostly consisted of large and translucent cells (Figure 1C and D). Whereas calli from cotyledons were nodular, green, organogenetic, compact green calli and non-friable, with small and green cells often developed on media containing 0.5 mg/L 2,4-D and formed round masses (Figure 1A and B). This result is in consistence with the result obtained for sugar beet by Kamal et al. (2007) and Chamandosti et al. (2006) which reported similar findings in *B. napus;* three morphologically distinct types of calli were induced by using different concentrations of 2,4-D and BAP.

Shoot initiation and regeneration via indirect organogenesis

Shoot proliferation occurred through a callus phase. The development of shoots (Figure 2) from white friable and compact callus (regardless of the explants type) was observed after 3-4 weeks of culture. Shoot development was achieved at all concentrations of BAP except for the treatment without plant growth regulator (Table 2). The percentage of explants forming shoots varied between type of explants (hypocotyl and cotyledon) as well as between the genotypes, Holleta–1 and Yellow Dodola

Figure 2. Shoot initiation and regeneration from the callus of hypocotyl and cotyledon explants of *Brassica carinata* cultivars after eight weeks of culture on full MS medium supplemented with 2 mg/L BAP and root induction in MS medium supplemented with 0.3 mg/L IBA. **(A and B).** New shoot initiation from hypocotyl and cotyledon explant of Yellow Dodola respectively after 8 week of culture. **(C and D).** New shoot initiation of hypocotyl and cotyledon explant of Holleta–1 respectively after 8 weeks of culture. **(E and F).** Shoot regeneration of hypocotyl derived calli on MS medium after 10 weeks of culture from Holeta–1 and Yellow Dodola, respectively, **(G and H).** Root induction after four weeks culture from Yellow Dodola and Holeta–1 respectively, **(I and J)**. Acclimatization of the *in vitro* plantlets of Yellow Dodola and Holetta–1 respectively in the greenhouse after two weeks of removing the plastic bag, **(K and L).** Well-developed plantlets one month after acclimatization and plantlets flowering after a month in the green house, respectively.

Table 2. Shoot regeneration frequencies from hypocotyls and cotyledon explants of *Brassica carinata* cultivars obtained from callus using different concentrations of BAP after eight weeks of culture.

PGR	Concentration (mg/L)	Hypocotyl			Cotyledon		
		Calli regenerated shoots (%)	No of shoots per callus	Shoot length	Calli regenerated shoots (%)	No of shoots per callus	Shoot length (cm)
	Yellow Dodola						
BAP	0	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}
	1	39.6±1.2^{c}	3±0.87^{c}	3.9±0.53^{b}	22±1.0^{c}	3.1±0.63^{b}	3±1.0^{b}
	2	90±2.0^{a}	7.7±1.52^{a}	7.7±1.52^{a}	45±0.81^{a}	5.4±1.53^{a}	5.4±0.38^{a}
	3	46.4±1.8^{b}	5±1.53^{b}	5.7±1.44^{ba}	29±1.53^{b}	3.8±1.04^{b}	3.8±1.0^{b}
	Mean	44	5	5.8	24.2	3.32	4
	CV (%)	3.4	16	3.8	3.6	17	4.7
	Holeta–1						
BAP	0	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{c}
	1	31±0.76^{c}	2.3±0.57^{b}	3.7±0.58^{b}	19.8±1.06^{c}	1.3±0.58^{bc}	2.9±0.1^{b}
	2	56.9±1.21^{a}	6±1.53a	6.7±0.85^{a}	34±2.08^{a}	3.7±1.0^{a}	6±1.0^{a}
	3	37±0.68^{b}	3.7±1.15^{b}	4.3±1.53^{b}	22.4±0.53^{b}	2.3±0.58^{ba}	4±1.0^{b}
	Mean	31.3	4	33.7	19.1	2.4	4.3
	CV (%)	2.6	11	12	3.8	13	1.9

The experiment was arranged in randomized complete design, with three replications and each experiment contained five explants Per Magenta Box. Means with in a column followed by the same superscript letters are not significantly at P≤0.05 according to DMRT.

on MS media supplemented with different concentrations of the BAP. These findings exhibited a varied response (22-90%) of shoot regeneration from both explants of both cultivars. Similar results were obtained by Moghaieb et al. (2006) and Kamal et al. (2007) in *B. napus.*

The explants developed as tiny patches of pale green callus bearing several hump-like structures within a period of 20-30 days. At the concentration of 0.5 mg/L 2, 4-D subsequently, they differentiated into shoot buds. Best shoot elongation was achieved when regenerating segment of the callus was cut into smaller pieces containing three or four shoots and sub cultured in the basal medium supplemented with 2 mg/L BAP. These results are correlated with those reported by Moghaieb et al. (2006), Zeynali et al. (2010) and Kamal et al. (2007) in *B. napus.* Results of the effect of BAP on shoot initiation in the two varieties of *B. carinata* are presented in Table 2 and Figure 2. The result reveals that all varieties respond to all treatments. Morphologically, best shoots were induced on the medium that contained 2 mg/L BAP (Figure 2). Highest percentage of survival rate of cultured explants was obtained at 2 mg/L of BAP. This holds true for both the varieties. Shoot regeneration frequency (SRF) was 90% for hypocotyl explants and 45% for cotyledonary explants of Yellow Dodola at 2 mg/L of BAP. In Holleta–1 there is a significance difference among the concentrations. The highest recorded SRF is 56% from hypocotyls explants and 34% from cotyledonary explants (Table 2) at 2 mg/L of BAP. Based on this result, 2 mg/L BAP concentration was used as an optimum PGRs for shoot initiation. Similar results have been reported by Ravanfar et al. (2009) in *B. oleracea*.

The same results have been reported by Khan and Rashid (2002) where they have shown that the important factors for shoot regeneration were explant type and genotype. This is also in agreement with Moghaieb et al. (2006), Yang et al. (1991) and Yu et al. (2005). Thus, it is clearly shown that the regeneration depends on explants type and genotype.

Among the four different BAP, 2 mg/L of BAP produced significantly maximum number of shoots for both varieties (Table 2). Yellow Dodola gave maximum mean number of shoots (7.7) followed by Holleta–1 (6) from the hypocotyl explants. The maximum shoot height attained at 2 mg/L of BAP for Yellow Dodola and Holleta–1 were 7.7 and 6.7 cm, respectively from the hypocotyl explants (Table 2). Medium supplemented with 2 mg/L of BAP was the best in many of the parameters recorded. However, there was a significant difference between the cultivars in number of shoots developed and shoots length. Yellow Dodolla was best in both cases (Table 2).

The responses of explants for shoots regeneration were different. Various authors (Munshi et al., 2007; Gubis et al., 2004) reported that hypocotyl explants were superior to cotyledon in shoot regeneration efficiency and shoot length. MS medium supplemented with 2 mg/L BAP was the most effective in adventitious shoot formation and shoot length in both explants and cultivars. This result agreed with those of George et al. (2008) who showed that one of the *B. napus* cultivars named RK–7 had low shoot regeneration (18%) from cotyledonary explants while the same cultivar had a higher (27%) shoot regeneration from the hypocotyls explants.

Genotypic effects

Genotypic effect is well established in tissue culture response. Different genotypes had different physiological requirement of plant growth regulators for *in vitro* shoot and root regeneration (Malik et al., 2005).

The response of various genotypes to callus formation varied considerably. Similarly, the effect of hormones on callus formation of the two genotypes was different (Table 1). A differential response in callus induction and survival was noticed when calli were cultured in media supplemented with auxins (2,4-D and NAA) and cytokinin (BAP) at different concentrations. The genotype, Yellow Dodola was found more responsive to callus production than Holleta–1 (Table 1). Such results can be attributed to the genetic differences in the two varieties. Genotypic influence on *in vitro* morphogenesis in *Brassica* has been documented previously (Khan and Rashid, 2002; Dietert et al., 1982; Fazekas et al., 1986; Khehra and Mathias, 1992). Such reports indicated that the developmental processes reflected by *in vitro* response are genetically controlled. Total number of shoots regenerated varied significantly among the two genotypes. During this study, a better consistent response and maximum number of shoots (90%) was produced from Yellow Dodola.

Root organogenesis

Effect of NAA and IBA on root induction

The rooting results reveal that plantlets cultured on rooting media induced roots in all media whether or not supplemented with NAA and IBA. The primary roots were observed after two weeks of culturing on MS medium supplemented by different concentrations of IBA and NAA. However, well developed and mature roots were obtained after a month. Rooting of plantlets was usually attained at half MS medium with low concentrations of auxins and sucrose. Similar result was reported by Khan and Rashid (2002). Among the different auxins, IBA was the best for root induction. The treatment containing 0.3 mg/L IBA and the control (MSO) gave the highest percentage of plantlets producing roots and mean number of roots per plantlet in both genotypes (Table 3). The highest percentage of explant producing roots (98%) and mean number of roots produced per explants (9) was observed on Yellow Dodela supplemented with IBA (0.3. mg/L) and the lowest frequency of response (41%) on Yellow Dodola fortified with NAA (0.5 mg/L). Whereas, Holleta–1 had the highest and lowest root formation at 92 and 38% on media fortified with 0.3 and 0.5 mg/L of IBA and NAA, respectively. NAA supplemented medium resulted in low root induction and shoots in the medium had a tender callus at the base of shoots and produced stumpy and thick roots. These results are in agreement with the findings of Ravanfar et al. (2009). Caboni and Tonalli (2002) reported that IBA is the most effective auxin for root induction in a wide range of plant species. IBA is superior in stability than NAA. The present study also proved that IBA was better than NAA for root induction. Similarly, Salman (2002) as well as Ali et al. (2007) reported a similar result by using 0.3 mg/L of IBA in *B. oleracea* and *B. napus* (Westar) respectively where 90% root formation was recorded in the latter.

The second maximum frequency of root formation (91%) of Yellow Dodola was achieved on half strength MS medium without growth regulators along with maximum root length (4.7 cm). It is assumed that root formation on auxin free medium may be due to the availability of higher quantity of endogenous auxin in shootlets raised *in vitro*. Similar results were reported by Ali et al. (2007) in *B. napus*. Both treatments showed significant difference with respect to the percentage of shoots forming roots, mean number of roots produced per shoot, root length, fresh and dry weight. In this study, the highest increase in fresh and dry weight was observed in medium supplemented with 0.3 mg/L IBA. There was a significant difference between the root length attained with IBA and NAA treatment. NAA produced fewer roots than IBA. Similar results were obtained by various authors (Moghaieb et al., 2006; Chamandosti et al., 2006; Kamal et al., 2007).

Genotypic effect

The response of different genotypes to root induction varied considerably. Similarly, the effect of hormones on root formation of the two genotypes was different (Table 3)

Table 3. Effect of auxin types on percentage of shoots with root, mean number of roots produced per shoot, root length (cm) and fresh and dry weight (gm) of *B. carinata* after four weeks of culture.

PGR	Concentration (mg/L)	Shoots with root (%)	Number of roots per shoot	Root length (cm)	Fresh weight (g)	Dry weight (g)
Yellow Dodola						
MSO	0	91.6±0.513^{b}	7.3±1.16^{b}	4.7±0.57^{a}	1.3±0.15b	0.17±0.058^{a}
	0.1	80.50±0.50^{c}	3.7 ±1.54^{c}	2.3±0.57^{b}	1.3±0.21^{b}	0.13±0.0^{a}
	0.3	98.7±0.65^{a}	9.0±1.0^{a}	4±1.7^{ba}	2.0±0.0^{a}	0.22±0.07^{a}
IBA	0.5	66.0±1.0^{d}	3.0±1.0^{c}	1.9±1^{b}	0.93± 0.6^{b}	0.11 ±0.09^{a}
	Mean	84.2	5.75	3.2	1.4	0.43
	CV (%)	0.67	9.6	12	10	8.6
	0.1	66.7±0.51^{c}	1.6±0.63^{b}	1.3±0.57^{b}	1.1± 0.17b^{a}	0.13 ±0.061^{ba}
	0.3	77.9±0.15^{b}	2.3±0.58^{b}	1.7±0.57^{b}	1.4±0.32^{a}	0.17 ±0.058^{a}
NAA	0.5	41.5±0.50^{d}	1.50±0.50^{b}	1.3±0.57^{b}	0.57±0.45^{b}	0.08 ±0.029^{b}
	Mean	69.4	3.2	2.2	1.1	0.12
	CV (%)	0.35	13	19	3.8	2.8
Holeta–1						
MSO	0.0	88.6±0.49^{a}	7.0±1.0^{a}	3.9±0.13^{a}	1.17±0.29^{ba}	0.16± 0.21^{a}
	0.1	77.9±0.15^{b}	3.3 ±1^{b}	3.3±0.57^{a}	1.0±0.10^{b}	0.10± 0.0^{a}
	0.3	92.0±4.13^{a}	8.0±1.0^{a}	1.7±0.57^{b}	1.37±0.12^{a}	0.13 ±0.06^{a}
IBA	0.5	43.5±3.01^{c}	2.3 ±0.58^{b}	1.6±1.23^{b}	0.8±0.39^{b}	0.09±0.01^{a}
	Mean	75.5	5.2	2.6	1.1	0.12
	CV (%)	3.74	1.97	5.4	9.09	0.4
	0.1	62.0±0.95^{c}	2.0±1.00^{b}	0.83±0.14^{c}	0.77±0.21^{ba}	0.086±0.03^{a}
	0.3	66.8±0.21^{b}	2.7±0.58^{b}	0.98±0.028^{c}	1.1±0.15^{a}	0.10± 0.00^{a}
NAA	0.5	38.6±0.53^{d}	1.33±0.58^{b}	0.77±1.15^{c}	0.5±0.36^{b}	0.08±0.035^{a}
	Mean	63.7	3.08	7.4	0.87	0.1
	CV (%)	1.26	9.9	8.8	3.8	4.7

The experiment was arranged in randomized complete design, with three replications and each experiment contained five explants. Means with in a column followed by the same superscript letters are not significantly at (P≤0.05) according to DMRT.

Variations in percentage and root length among the tested cultivars indicated that the differences in responses were due to the differences between the genotypes. Genotypic variations in *in vitro* culture system are frequently observed in *B. carinata* cultivars. During the present study, it was revealed that the varieties varied significantly for their regeneration capacity on the similar medium. This might be due to their genetic differences. Similarly, genotypic influence on *in vitro* morphogenesis in *Brassica* spp. has been documented previously (Dietert et al., 1982; Khan et al., 2010). After thorough washing of the well-developed roots with tap water, *in vitro* raised plantlets were then transferred to the pot containing sterilized soil mixed with a proportion of 1:2:1 sand, red soil and compost, respectively, at green house with 80% humidity, 25°C

temperature and 16 h light of about 2700 lux of light intensity and 8 h dark. About 100% plantlets were successfully established.

Conclusion

The present study describes an effective indirect regeneration protocol for *in vitro* propagation of two genotypes of *B. carinata.* Cotyledon explants gave higher callus initiation percentage than hypocotyls explants in both genotypes at the concentration of 0.5 mg/L 2, 4-D. The BAP and NAA took longer time to produce callus, and caused the formation of poor and compact calli with many necrotic sites. Successful shoot regeneration was achieved from white compact callus. 2 mg/L BAP is better for shoots induction in both genotypes. The highest percentage of shoots producing roots and mean number of roots per explants was achieved at the concentration of 0.3 mg/L of IBA. The survival rate of regenerated plantlet was 100%.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology

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The present study was conducted to optimize the media composition through response surface methodology (RSM) for extracellular polysaccharide (EPS) production in *Halobacillus trueperi* AJSK strain isolated from the salt pan. *Halobacillus trueperi* was identified with morphological, biochemical characteristics as well as 16S rRNA gene sequencing method. Production medium was optimized through central composite design. In the present study, the maximum EPS production was achieved under the optimal culture conditions of peptone, glucose, NaCl and $MgSO_4$ and they were 15.50, 22.24, 61.56 and 2.33 g/L, respectively at pH 9.0, 35°C in 72 h. An EPS production of 12.93 (g/L) which was well in agreement with the predicted value was achieved by this optimized procedure. Results of the present study proved that statistical media composition analysis with RSM enhanced the EPS production in *Halobacillus trueperi.*

Key words: Extracellular polysaccharide, *Halobacillus trueperi,* response surface methodology, salt pan bacteria.

INTRODUCTION

Marine bacteria are known to produce extracellular polysaccharides for their thriving fitness such as adhering purpose and surviving in adverse conditions. Microbial exopolysaccharides (EPS) are a heterogenous matrix of polymers comprised of different biological molecules such as polysaccharides, proteins, nucleic acids, phospholipids and other polymeric compounds thereby carrying different organic functional groups such as acetyl, succinyl or pyruvyl and some inorganic constituent like sulfate (Mishra and Jha, 2013; Nielsen et al., 1999).

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Microbial EPS are commonly present in two forms either in capsular or slime. In capsular form the polysaccharides are closely bound to the cell surface and in slime the polysaccharides are freely associated with the cell surface (Shauna and Reckseidler-Zenteno, 2012; Costerton, 1999). These EPS either remain attached to the cell surfaces or get released into the extracellular medium. Microbial EPS owing to their interesting physico-chemical and rheological properties has a wide range of industrial applications such as the production of textiles, detergents, adhesives, cosmetics, pharmaceuticals, food additives as well as applications in brewing, microbial enhanced oil recovery, wastewater treatment, dredging and various downstream processing processes, cosmetology, pharmacology and as food additives. The EPS also contribute to various physiological activities in human beings as anti-tumor, anti-viral and anti-inflammatory agents and can act as inducers for interferon, platelet aggregation inhibition and colony stimulating factor synthesis (Manivasagam et al., 2013; Lin and Zhang, 2004; Sutherland, 1999).

Optimization of bioprocess plays crucial role in reducing the production cost of all biotechnological commercial products. The optimization process using the 'one variable at a time' approach gives non reliable results and inter-active effects of different variables for the production also cannot be resolved by this approach. Statistical experimental strategies including factorial design and response surface methodology (RSM) are more reliable than classical experiments. Central composite design (CCD) is one of the most conventional experimental designs among different classes of RSM and this strategy particularly helps us to predict the better concentrations of substrates with less accidental errors (Sathiyanarayanan et al., 2013). Statistical optimization methods have been successfully employed for the optimization of EPS production through fermentation process (Fang et al., 2013).

The EPS production depends on several factors such as species employed, cultivation conditions and age of the cultures. The design of fermentation conditions is very vital (Allard and Tazi, 1993). Statistical design of experiments provides an economic and efficient method of optimizing several conditions at a time. Furthermore, the production of EPS is not species specific and each strain of same species may produce different kinds of EPS with different biotechnology properties. In the past decade more prominent research has been done in search for novel microbial EPS and EPS producing strains (Manivasagam et al., 2013). Still the search for EPS among halophilic bacteria and bacteria from saline soils holds to be pristine. In view of that, in this study, we report a strain of *Halobacillus trueperi* isolated from the Tamilnadu Salt and Marine Chemicals (TSMC) salt pan, Tuticorin, India. The production of EPS by *Halobacillus trueperi* AJSK was optimized by classical method followed by statistical experimental design.

MATERIALS AND METHODS

Isolation and identification of EPS producing halophilic bacteria

The soil samples collected from the TSMC salt pan, Tuticorin were brought to the laboratory within 6 h. Serially diluted samples were plated on Zobell marine agar plates (10% NaCl). Potential EPS producing strain was selected by observing for better mucoid colony morphology (Fusconi and Godinho, 2002) and the selected potential EPS producing strain was identified based on morphological and biochemical characteristics according to the Bergey's manual of determinative bacteriology (Garrity et al., 2001) and also confirmed through molecular characterization. Briefly, the bacterial genomic DNA was extracted by phenol chloroform method (Marmur, 1961) and the 16S rRNA gene was amplified by using forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'- GGGCGGTGTGTACAAGGC -3'). PCR was performed under the following conditions; initial denaturation at 95°C for 5 min followed by 35 cycles consisting of, denaturation at 95°C for 30 S, annealing at 55°C for 30 S and followed by final extension of 5 min at 72°C. The 16S rRNA forward and reverse sequences was obtained by an automated DNA sequencer (Megabace, GE) and homology was analyzed with sequences in the Gene Bank by using CLUSTAL X software. The phylogenetic tree was constructed by the neighbor-joining method (Saitou, 1987).

Microbial exopolysaccharides (EPS) analysis

EPS production was carried out with the production media consisting of peptone, 10 g/L; glucose, 15, g/L and NaCl, 75 g/L and the culture was incubated at 28°C, pH 10.0 for 72 h. The EPS were precipitated from the cell free liquid culture by adding two volumes of cold ethanol. Then the precipitates were collected by centrifugation, dissolved in distilled water and the EPS concentrations were determined by phenol-sulfuric acid method using glucose as standard (Dubois et al., 1956).

One-factor-at-a-time experiments

Classical method was used to investigate the EPS production by the strain *H. trueperi* AJSK. EPS production was carried out with the production media consisting of peptone, 10 g/L; glucose, 15, g/L, NaCl 75 g/L and $MgSO_4$ 1.5 (g/L). The time course of experiment was carried out for 72 h in 1 L flask containing 250 ml of

Table 1. Experimental design and EPS results of central composite design optimization experiment.

Run order	Peptone (g/L)	Glucose (g/L)	$MgSO_4$ (g/L)	NaCl (g/L)	EPS producion (g/L)	
					Observed	Predicted
1	10	10	1.0	40	5.1920	5.1859
2	20	10	1.0	40	6.7521	6.8232
3	10	30	1.0	40	6.5422	6.6587
4	20	30	1.0	40	7.8761	7.9104
5	10	10	4.0	40	5.4691	5.7321
6	20	10	4.0	40	6.5732	6.7648
7	10	30	4.0	40	7.1462	6.9673
8	20	30	4.0	40	7.7872	7.6145
9	10	10	1.0	90	5.3210	5.7463
10	20	10	1.0	90	6.3822	6.5946
11	10	30	1.0	90	8.0312	7.8731
12	20	30	1.0	90	8.3462	8.3358
13	10	10	4.0	90	4.0621	4.0614
14	20	10	4.0	90	4.1691	4.3052
15	10	30	4.0	90	5.7691	5.9506
16	20	30	4.0	90	5.7691	5.8088
17	5	20	2.5	65	4.3018	4.1236
18	25	20	2.5	65	5.7271	5.6191
19	15	0	2.5	65	4.3271	3.8239
20	15	40	2.5	65	6.5832	6.8003
21	15	20	0.5	65	6.1654	5.9660
22	15	20	5.5	65	4.0719	3.9852
23	15	20	2.5	15	11.0630	11.0467
24	15	20	2.5	115	10.0713	9.8015
25	15	20	2.5	65	12.1732	12.2035
26	15	20	2.5	65	12.1790	12.2035
27	15	20	2.5	65	12.1807	12.2035
28	15	20	2.5	65	11.9507	12.2035
29	15	20	2.5	65	12.5794	12.2035
30	15	20	2.5	65	12.3811	12.2035
31	15	20	2.5	65	11.9807	12.2035

culture medium. The optimum initial pH for EPS production was determined by adjusting various pH (4 to 11) with 1 M HCl and 1 M NaOH before sterilization and the optimum temperature for EPS production was evaluated by incubating the liquid cultures at various temperatures (20 to 45°C).

Central composite design (CCD)

Physical factor such as pH and temperature was selected by one-factor-at-a-time experiments and chemical factors were selected based upon the available literature, the media ingredients namely peptone, glucose, NaCl and $MgSO_4$ are the significant variables for statistical optimization (Mata et al., 2007; Liu et al., 2011; Nahas et al., 2011; Lu et al., 2011; Cerning et al., 1994). All the above said independent variables were evaluated at five different levels (-2, -1, 0, +1, +2) conducting 31 experiments. The central values of all variable were coded as zero. The minimum and maximum ranges of the variables and the full experimental plan with regard to their values in actual and coded form are presented in Table 1.

The data derived via RSM on production of EPS were analyzed with the analysis of variance (ANOVA). The results of the experiments were subjected to the response surface regression procedure with the given second order polynomial equation:

$$Y = \beta 0 + \sum \beta_i X_i + \sum \beta_{ii} X^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response**,** xi and xj are inde-

Table 2. Biochemical characteristics of the strain *H. trueperi* AJSK.

Characteristic	Activity
Indole	-
Methyl red	-
Voges Proskaevuer	-
Citrate	-
Catalase	+
Oxidase	+
Gelatin	+
Casein	-
H_2S	-
Fructose	+
Glucose	+
Maltose	+
Sucrose	+

pendent factors, *β*0 is the intercept, *βi* is the linear coefficient, βii is the quadratic coefficient and βij is the interaction coefficient. The response values (Y) in each trial were presented as average of the triplicates. The statistical software package 'Minitab' (Version 16.0) was used to analyze the experimental design.

RESULTS AND DISCUSSION

Isolation and identification of *H. trueperi* AJSK

In the present study, a total of eight morphologically different isolates were investigated for potential EPS production. Among these, strain T7 produced significant mucoid colony in the preliminary screening. Furthermore, the strain was identified as *H. trueperi* Gram positive, rod shaped through biochemical characteristics (Table 2) and 16S rRNA analysis. Phylogenetic analysis revealed that the strain T7 belongs to the Firmicutes, Bacillaceae, *Halobacillus*. Evolutionary relation with other *Halobacillus* sp. is explained with the phylogenetic tree created by neighbor joining method (Figure 1). BLAST analysis with NCBI database retrieved a 96% similarity for strain T7 with *H. trueperi*. The 16S rRNA gene sequence of strain T7 was submitted to GenBank as *H. trueperi* AJSK with the accession number KC699491.

Effect of pH and temperature on EPS production

A series of experiments were carried out to study the effects of physical factors such as pH and temperature on EPS production in *H. trueperi* AJSK. Experiments were conducted using basal medium containing peptone 10 (g/L), glucose 15 (g/L) and NaCl 75 (g/L) and $MgSO_4$ 1.5 (g/L) for 72 h. The optimization of pH and temperature for EPS production revealed that pH 9.0 (3.73 g/L) and temperature 35°C (2.98 g/L) were found as optimum culture condition for maximum EPS production (Figures 2 and 3), respectively. The pH is an essential physical factor in EPS biosynthesis that may affect the uptake of various nutrients and EPS biosynthesis (Kim, 2005). Kanekar et al. (2008) has reported that 1.2 g/L of EPS production at an alkaline pH of 10 in *Vagococcus carniphilus* an alkalophilic bacterium isolated from alkaline Lonar Lake, India. *Pseudomonas polymyxa* EJS-3 is also reported to produce EPS at a slightly alkaline pH 8 (Liu, 2009). The present study reveals a good EPS production in an alkaline pH which is an industrially desired property. These results did not comply with the report of maximum EPS production at pH 7 by *P. fluorescens* (Raza et al., 2012). *Bacillus megaterium* RB-05 from the river sediment is reported to produce 0.895 g/L EPS at a neutral pH of 7.0 (Chowdhury et al., 2011). Mata et al. (2011) have reported a maximum EPS production at 32°C in *Alteromonadaceae* sp. a halophilic bacterium which is likely in agreement with the present study. In contrary, Liu et al. (2011) reported maximum production of EPS at 9.8°C from *Zunonwangia profunda*, a deep sea bacterium. Also reports of maximum EPS production at temperatures ranging from 28 to 37°C were reported (Raza et al., 2012; Chowdhury et al., 2011; Kaur et al., 2013).

Optimization of variables using central composite design (CCD)

Four variables such as peptone, glucose, NaCl and $MgSO_4$ were selected based on the results of previous literature reports for the CCD experiments. The values of the response (EPS) obtained under different experimental conditions are given in Table 1. Experiments were done as per the CCD experimental plan. The F value is a measure of variation of the data about the mean. High F value and a very low probability (p>F=0.00) indicates that the present model is in a good prediction of the experimental results.

The corresponding analysis of variance (ANOVA) is presented in Table 3. The regression equation is represented in the three-dimensional graphical response surface plots (Figure 4). The interest of using response surface methodology is to efficiently find out the accurate optimum values of the variables, with the maximized response.

The surface plots confirmed that the objective function is unimodal in nature, which shows an optimum in the centre. Also significant P-values (0.000) suggested that the obtained experimental data was a good fit with the model and it is also checked by the determination of coefficient (R^2) with R^2 (multiple correlation coefficient) of 99.52%. The predicted R^2 and the adjusted R^2 was

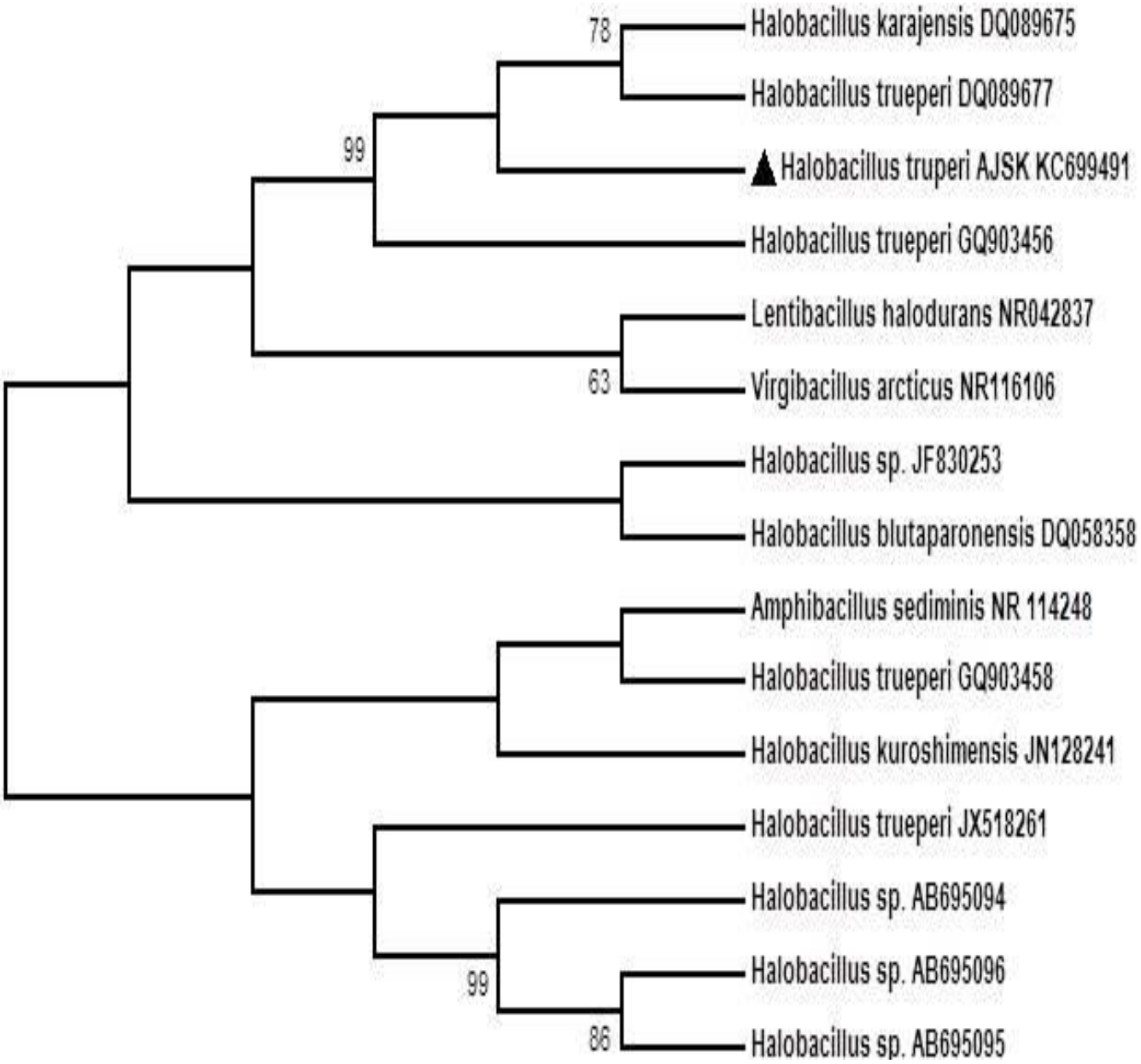

Figure 1. Phylogenetic tree of *Halobacillus trueperi* AJSK 16S rRNA gene sequence with other *Halobacillus* species/strains

Table 3. Analysis of variance for the fitted quadratic polynomial model for optimization of EPS production.

Source	DF	Seq SS	Adj SS	Adj MS	*F*- value	*P*- Value
Linear	4	24.855	101.778	25.4444	327.19	0.000
Square	4	226.280	226.280	56.5699	727.44	0.000
Interaction	6	6.599	6.599	1.0998	14.14	0.000
Residual error	16	1.244	1.244	0.0778		
Lack-of-Fit	10	0.956	0.956	0.0956	1.99	0.207
Pure error	6	0.288	0.288	0.0481		
Total	30	258.978				

DF, Degree of freedom; Seq SS, sequential sums of squares; Adj SS, adjusted sums of squares; Adj MS, adjusted mean square.

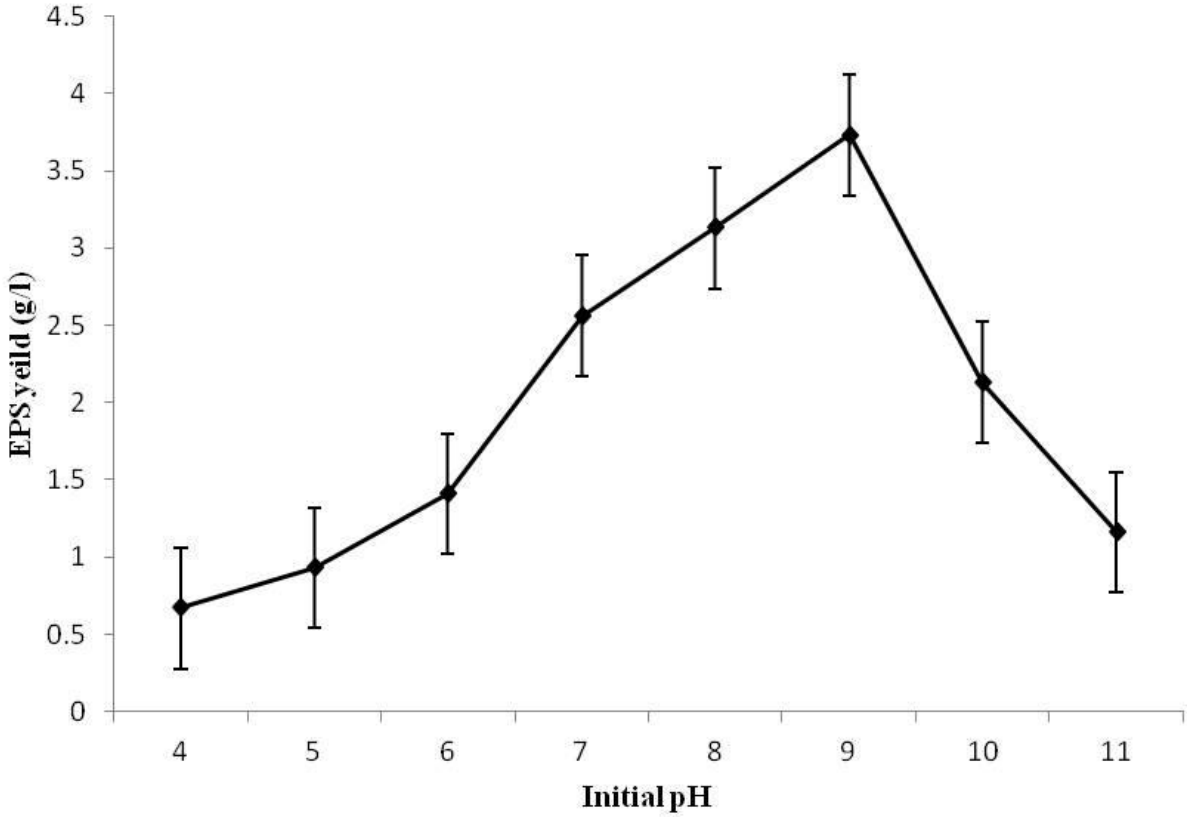

Figure 2. Effect of various pH on extracellular polysaccharide production.

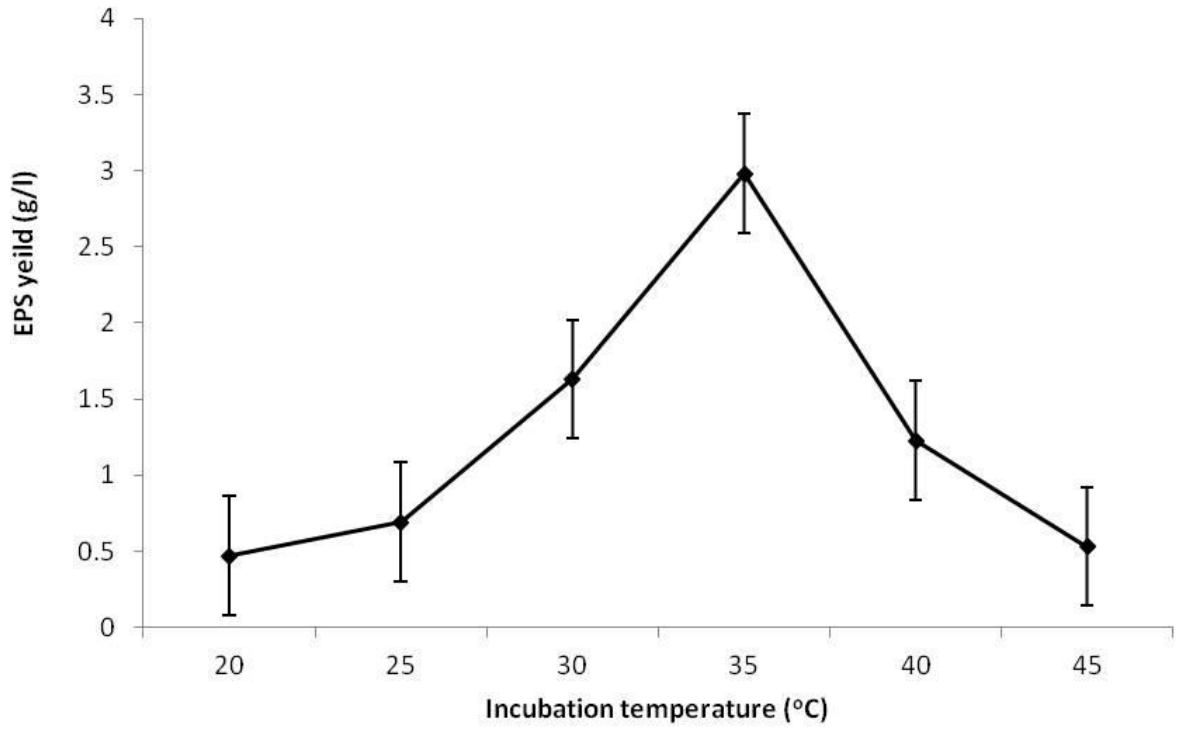

Figure 3. Effect of various temperatures on extracellular polysaccharide production.

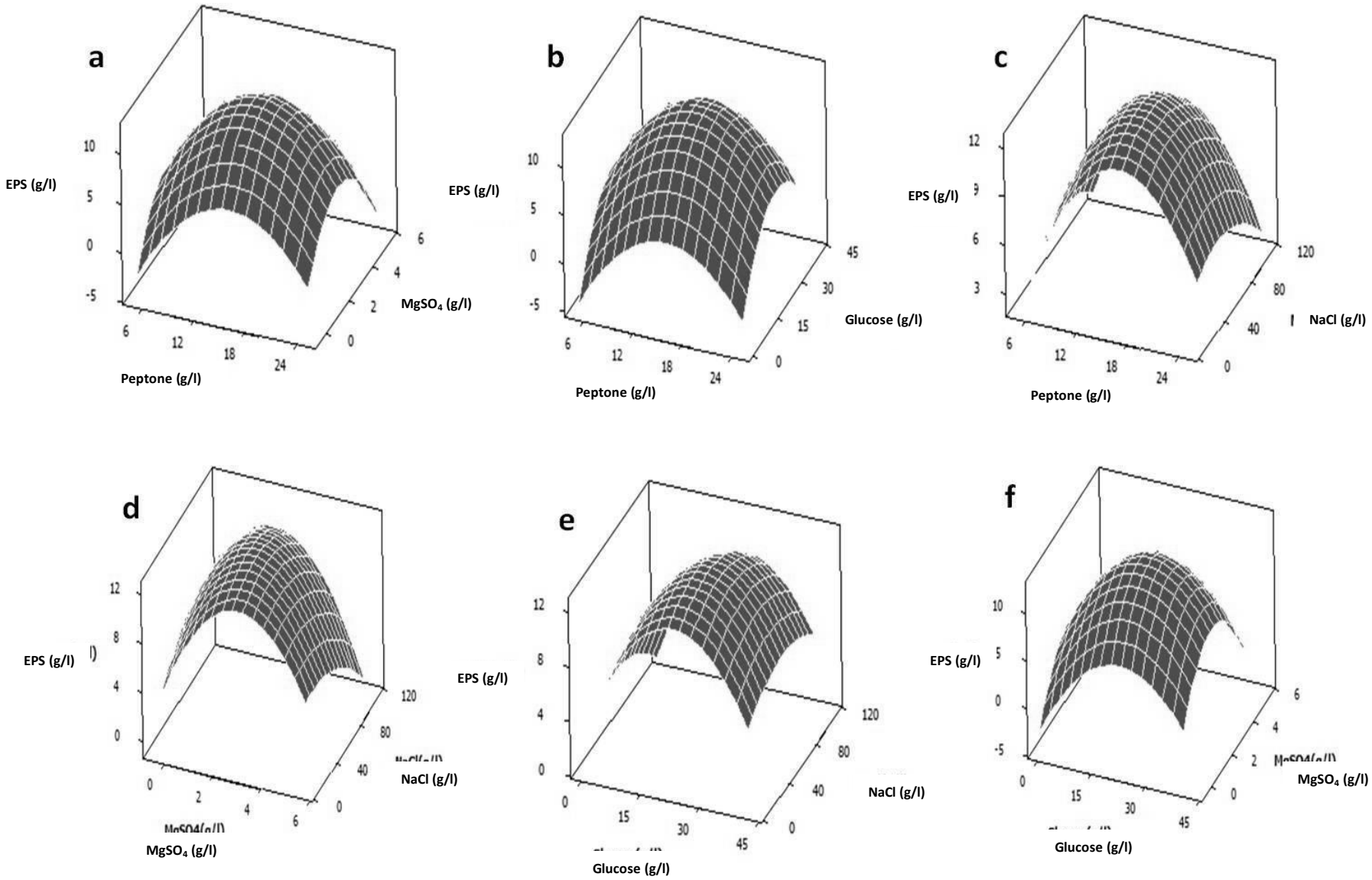

Figure 4. Three dimensional response surface plot for extracellular polysaccharide production showing the interactive effects of (a), peptone and $MgSO_4$ (b), Peptone and glucose (c), peptone and NaCl (d), $MgSO_4$ and NaCl (e), glucose and NaCl (f), glucose and $MgSO_4$.

Table 4. Results of regression analysis of the second-order polynomial model for optimization of EPS production.

Term	Coefficient	Estimate coefficient	*t*-Value	*p*-Value
Constant	-24.8245	1.15772	-21.443	0.000
X_1	2.4659	0.08172	30.177	0.000
X_2	0.7599	0.03699	20.541	0.000
X_3	5.0337	0.23990	20.982	0.000
X_4	0.1279	0.01567	8.159	0.000
$X1^2$	-0.0733	0.00209	-35.150	0.000
X_2^2	-0.0172	0.00052	-33.038	0.000
X_3^2	-0.8031	0.02318	34.651	0.000
$X4^2$	-0.0007	0.00008	-8.531	0.000
X_1x_2	-0.0019	0.00139	-1.383	0.186
X_1x_3	-0.0202	0.00930	-2.168	0.046
X_1X_4	-0.0016	0.00056	-2.829	0.012
X_2X_3	-0.0040	0.00465	-0.852	0.407
X_2X_4	0.0007	0.00028	2.345	0.032

about 97.72 and 99.10%, respectively (Table 4). The central optimum poin was evaluated by using gradient method in the direction of steepest ascend of media for the EPS production evaluated from the surface plots.

The response surface plots provide a visual interpretation of the interaction between variables and assist in determining optimal conditions by revealing the significance of the interaction among the variables. In this study the interaction between the variables glucose and NaCl is significant. Similarly Manivasagam et al. (2013) demonstrated a significant interaction between the variables glucose and NaCl using RSM in EPS production by *Streptomyces violaceus*. The optimal values of peptone, glucose, NaCl and $MgSO_4$ were estimated in actual units and they were 15.50, 22.24, 61.56 and 2.33 (g/L), respectively, with a predicted exopolysaccharide production of 12.35 (g/L). Conformation experiment was conducted for these predicted optimum conditions and extracellular polysaccharide production from the experiment was 12.93 g/L. This was little higher than the predicted value which reveals the higher accuracy of the model. A maximum EPS production of 9.01 g/L by a marine bacterium with glucose as best carbon source at pH 7 in seven days was reported by Nahas et al. (2011). In the present study we used glucose as the sole carbon source. Chowdhury et al. (2011) in *B. megaterium* RB-05 reported that glucose is the better substrate over fructose, sucrose, maltose and lactose for high EPS yields. Liu et al. (2011) reported a maximum of 8.90 g/L EPS production in *Zunonwangia profunda*, a deep sea bacterium with peptone as more influential than yeast extract. As reported by Srinivas and Padma (2014) the organic nitrogen sources were much more suitable than inorganic nitrogen sources for the microbial EPS production. Peptone with its peptide and amino acid composition serves an excellent nitrogen source for EPS production. Similarly, Wang et al. (2011) reported that beef extract, maltose, peptone and NaCl gave a maximum of 20.19 g/L EPS production in *B. thuringiensis* isolated from desert sand biological soil crusts using optimization by orthogonal matrix method. A maximum of 3.34 g/L EPS production in *Paenibacillus polymyxa* with galactose was reported by (Raza et al., 2011).

Conclusion

The present study lead to the optimization of key culture conditions with CCD designs for increased EPS production in halophilic bacterium, *H. trueperi* AJSK with promising properties for industrial exploitation. The RSM yielded a maximum of 12.93 (g/L) EPS production. Further investigation will identify the most befitting field of application. Numerous halophilic bacteria should be explored to reveal their potential for novel exopolysaccharides with biotechnolgically important properties to efficiently replace the synthetic polymers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article titled “Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology”.

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Characterisation and evaluation of the efficiency of petroleum degrading bacteria isolated from soils around the oil exploration areas in western Uganda

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Contamination with petroleum and its products is an environmental threat in oil producing countries. Microbes have been used to clean up petroleum contaminated environments, which has been demonstrated to be an appropriate and more practical alternative compared to the mechanical and chemical techniques. In this study, crude oil degrading indigenous bacteria were isolated from soils around the oil exploration sites in Western Uganda and their efficiency of oil biodegradation and presence of the catabolic gene *xylE* in the isolates to relate with their biodegradation efficiency were determined. The organisms with oil degrading activity were screened by isolating them from crude oil supplemented mineral salts medium (MSM). The isolates were tentatively identified phenotypically and confirmed genotypically by 16S ribosomal ribonucleic acid (rRNA) gene sequencing. Hydrocarbon degradation in the culture fluids was analyzed by a gas chromatography mass spectrometer (GC/MS); and amplification of catabolic gene *xylE* by polymerase chain reaction (PCR) was done in order to relate with the degradation activity. Forty four indigenous oil degrading bacteria were isolated and categorized into eight groups based on their morphological and biochemical properties; and were identified as belonging to the genera; *Corynebacterium, Pseudomonas, Moraxella, Bacillus, Enterobacteriaceae, Nocardia, Serratia* and *Rhodococcus*. Eight isolates that exhibited a relatively higher biodegradation activity in the first five days of incubation were selected for a detailed analysis. Based on 16S rRNA gene sequence analyses, the eight selected isolates were identical to *Stenotrophomonas maltophilia, Burkholderia* sp., *Delftia tsuruhatensis, Pseudomonas aeruginosa, Acinetobacter* sp., *Curtobacterium* sp. and *Paenibacillus agaridevorans.* The selected isolates; Ngara-T1, Kig5-1, Kig5-T2, Kig3-T3, Kig3-T4, Kas2-T7, Kas2-T5 and Gat-3 degraded the total petroleum hydrocarbons (TPHs) up to: 91.9, 79.9, 89.2, 73.1, 94.2, 83.2, 77.0 and 67.2%, respectively, by the end of 21 days of incubation as compared to 29.7% degradation in the control experiment (without bacteria). The catabolic gene *xylE* was detected in two of the selected isolates, Ngara T-1 and Kig5-T2. It could be concluded that the oil degrading bacteria identified in this study showed diverse and varying capacities to degrade the crude oil; with some degrading up to 90% and can be exploited for cleaning up hydrocarbon contaminated sites in western Uganda.

Key words: Bacteria, bioremediation, degradation, oil, hydrocarbons, Uganda.

INTRODUCTION

The term petroleum is used as a common denotation for crude oil and natural gas, which are the hydrocarbons from which various oil and gas products are made (JICA and EEAA, 2008). Crude oil is a complex mixture of thousands of different chemical components, mainly organic compounds which usually make up about 95%. At the refinery, crude oil is separated into light and heavy fractions, which are then converted into various products, such as petrol, diesel oil, jet fuel and lubricating oils through processes such as catalytic cracking and fractional distillation (JICA and EEAA, 2008; Zhu et al., 2001).

During routine operations of extraction, transportation storage, refining and distribution, accidental spills of crude oil and its refined products occur on a frequent basis despite the counter measures put in place (Zhu et al., 2001). It is approximated that five million tons of crude oil and refined oil enter the environment each year and most of it is directly related to human activities including deliberate waste disposal (Malatova, 2005). The oil spill incident from British Petroleum in the Gulf Coast of Mexico from April to July, 2010, caused almost 600,000 tons of crude oil spilled along the Gulf Coast (Wang et al., 2011). South Africa which is situated on one of the world's major shipping routes, is constantly sub-jected to contamination of its coastal waters by oil spills that leak from passing traffic (Moldan and Dehrman, 1989). Also, the National Petroleum Corporation in Nigeria placed the quantity of petroleum jettisoned into the environment at 2,300 cubic meters of oil spilled in 300 separate incidents annually (Manby, 1999).

Petroleum contains polycyclic aromatic hydrocarbon (PAHs) compounds such as benzene, toluene, ethyl benzene, *xyle*nes and naphthalene, which are among the most toxic components to plants and animals (Atlas and Hazen, 1999; Liebeg and Cutright, 1999). The aromatic hydrocarbons are more resistant against biodegradation than aliphatic compounds and they often cause serious problems during bioremediation; hence, it is very important to minimize contamination of the environment with the petroleum constituents (Rajaei et al., 2013).

The mechanical strategies for containment of oil spills typically recover no more than 10-15% of the oil after a major spill (Zhu et al., 2001). Bioremediation has emerged as one of the most promising secondary treatment options for oil removal which is often used as a polishing step after conventional cleanup options have been applied (Bragg et al., 1994; Zhu et al., 2001).

Bioremediation is done by adding materials such as microbes and nutrients (phosphorus and nitrogen) to contaminated environments, such as oil spill sites, to cause an acceleration of the natural biodegradation processes (Zhu et al., 2001). Hydrocarbons are biodegraded using microbial organisms such as bacteria, protozoa and fungi, which degrade contaminants completely to biomass, carbon-dioxide and water and utilize the resulting compounds as nutrients and energy sources for growth and reproduction (Frick et al., 1999). Several genes associated with bacteria have been evaluated for bioremediation processes. The catabolic gene *xylE* which codes for catechol 2,3-dioxygenase that cleaves the aromatic rings in PAHs is one of the genes identified in hydrocarbon degrading bacteria that are evaluated for use in hydrocarbon contaminated sites for bioremediation processes (Malkawi et al., 2009; Rajaei et al., 2013).

Bioremediation has a proven track record and it has been used successfully to clean up spills of oils and other hydrocarbons for more than 20 years (Frick et al., 1999). Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution because the majority of molecules in the crude oil and refined products are biodegradable and oil-degrading microorganisms are ubiquitous (Aislabie et al., 1998). According to Mittal and Singh (2009), the most direct measure of bioremediation efficacy is by monitoring of hydrocarbon disappearance rates. In addition to measuring Total petroleum hydrocarbons (TPH) in samples, the gas chromatography/flame ionization detector (GC-FID) chromatograms provide a distribution pattern of petroleum hydrocarbons fingerprints of the major oil components and information on the biodegradation extent of the spilled oil.

Since oil exploration and production is a new activity in Uganda, little or no research has been done in the country to investigate the presence of oil degrading bacteria in soils. Therefore, there is need to explore and prepare the techniques for thorough cleanup of oil spills as the latter cannot be predicted when and where they would occur despite any measures put in place. Hence, the aim of this study was to explore the possibility of bioremediation by isolating bacteria that occur in the soils in oil exploration and production sites in Western Uganda, and to determine their oil biodegradation capabilities.

METHODOLOGY

Study sites

Samples of soil for this study were collected from four petroleum exploration fields; Ngara, Kigogole 5, Kigogole 3 and Kasamene 2 all found in Buliisa district in western Uganda. Soil samples contaminated with oil were also collected from Gatsby motor garage located in Makerere University, Kampala, Uganda. Samples of

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petroleum, earlier collected from Ngara-1 exploration site, were obtained from the Petroleum Exploration and Production Department (PEPD) located at Entebbe, Uganda.

Sample collection

Subsurface soils (500 g) contaminated with petroleum were picked from the study sites, labeled and transported in pre-sterilized polythene bags in a cooler box. The samples (in 20% glycerol as a cryopreservative) were stored at -20°C in the microbiology laboratory at the College of Veterinary Medicine, Makerere University.

Preparation of media

The enrichment medium (MSM) consisted of the following salts: $NaNO_3$ (7 g/l); K_2HPO_4 (1 g/l); KH_2PO_4 (0.5 g/l); KCl (0.1 g/l); $MgSO_4$-$7H_2O$ (0.5 g/l); $CaCl_2$ (0.01 g/l) and $FeSO_4$-$7H_2O$ (0.01 g/l). The medium was supplemented with trace elements solution (0.05 ml) composed of the following salts: H_3BO_3 (0.25 g/l); $CuSO_4$-$5H_2O$ (0.5 g/l); $MnSO_4$-H_2O (0.5 g/l); $MoNa_2O4$-H_2O (0.06 g/l) and $ZnSO_4$-$7H_2O$ (0.7 g/l) (Kebria et al., 2009). The petroleum agar was prepared by adding 10 ml of crude oil and 20 g of bacteriological agar in a liter of prepared mineral salts medium.

Isolation of oil degrading bacteria

The isolation of petroleum degrading bacteria was done according to the method described by Mittal and Singh (2009). Briefly, 10 g of soil sample were suspended in 30 ml of distilled water in a falcon tube and mixed thoroughly for two minutes by vortexing. The suspension was allowed to settle down for five minutes and 5 ml of supernatant were inoculated in 100 ml of mineral salt medium (MSM) supplemented with crude oil (1%) as the sole source of carbon and energy. The flasks were incubated at room temperature (25 ± 2.0°C) on a rotary shaker at 150 rpm for 48 h. Successive sub-culturing was done by transferring 5 ml of MSM broth culture onto a fresh mineral salt medium supplemented with crude oil (1%). The sub-culturing was done to ensure that only oil-tolerant and -degrading bacteria would be isolated. After three sub-culturing steps, the oil-degrading bacteria were isolated by spreading out 1 ml of the broth culture onto petroleum agar plates. The inoculated plates were incubated aerobically at room temperature in an incubator for four days.

Phenotypic identification of the bacterial isolates

The bacterial colonies that grew on petroleum agar plates were selected based on the colony shape, colour, size and elevation. The selected colonies were sub-cultured on nutrient agar for pure culture preparation and identification. The bacterial isolates were identified on the basis of their colonial and cellular morphology; and biochemical characteristics. The colony colour, margin, form and elevation of the isolates were noted. Gram staining and biochemical tests including; catalase, oxidase, glucose and maltose fermentation, indole, urease, citrate and haemolysis tests were also performed to identify the pure colonies. The pure colonies were preserved in glycerol broth (25% v/v). For the day to day experiments, the bacteria were maintained on nutrient agar plates at 4°C in a refrigerator and sub-cultured at an interval of two weeks.

Screening for the efficient oil degrading bacteria

A crude oil biodegradation experiment was carried out to select the efficient oil degraders for a detailed examination. The 44 isolated bacteria were each grown in a culture flask of 250 ml capacity containing 99 ml of mineral salts medium supplemented with sterile crude oil (1 ml) as the sole source of carbon and energy. For each of the selected pure isolates, a bacterial colony was pre-grown in 5 ml of peptone water for 24 h before seeding into the culture flasks. Control flasks were added and subjected to the same experimental conditions as the samples, except for the absence of bacterial culture. The experiments were carried out in two replicates for each isolate and the inoculated flasks were incubated in an orbital shaker at room temperature (25±2.0°C) at 150 rpm for five days. The concentration of crude oil in the culture fluids was analyzed using a GC/MS machine before inoculating the pure isolates and after five days of incubation. The isolates which showed better crude oil degradation during the five days of incubation were selected for a detailed analysis.

The selected bacterial isolates (Ngara-T1, Kig3-T4, Kig5-T2, Kas2-T7, Kig5-1, Kig3-T3, Kas2-T5 and Gat-3) were incubated for 21 days in culture bottles of 500 ml capacity containing 198 ml of enrichment medium supplemented with 2 ml of sterile crude oil. The residual oil in the culture fluids was extracted with chloroform every week and analyzed using a GC/MS. The residual oil was extracted by pipetting 10 ml aliquots of thoroughly shaken culture fluids into a separating funnel of 100 ml capacity followed by10 ml of chloroform. The funnel was vigorously shaken for one to two minutes and then allowed to settle for two minutes for the aqueous and organic phases to separate. The oil-solvent layer was removed using a separator funnel and the solvent was evaporated using a water bath at 40°C. The oil was reconstituted to 0.5 ml with chloroform and 1 µL of the extracted crude oil was analyzed by GC/MS.

Analysis of un-degraded crude oil by GC/MS

The total petroleum hydrocarbons were analyzed according to the method by Wongsa et al. (2004) with a GC/MS (GC model 6890N and MS model 5975 - Agilent Technologies, USA). The GC/MS was equipped with a column from Agilent Technologies (19091 S-433) comprising of a length of 30 M, internal diameter of 0.25 mM and film thickness of 0.25 µM. The injector temperature was set at 250°C, while the column temperature was first set at 40°C and held for three minutes before raising it to 220°C at a rate of 5°C per minute. The total area under the resulting chromatograms were noted and the chromatograms were analyzed by an automatic mass spectral deconvolution and identification system (AMDIS) to identify the petroleum components and compared against the National Institute of Standards and Technology (NIST) library in the GC/MS.

Analysis of Biodegradation data

The percentage of total hydrocarbons un-degraded after a given time was calculated by comparing the area of the peaks with that of the corresponding peaks shown by a control that was subjected to the same experimental conditions as the samples, except for the absence of a bacterial culture (Wongsa et al., 2004). Statistical analysis of variance was done at a 95% confidence limit to compare degradation efficiency among the isolates during the 21 days of incubation.

Bacterial genomic DNA extraction

The genomic DNA of the efficient oil degrading bacteria was

extracted by the CTAB method as described by William and Copeland (2004). The isolates were each grown in 10 ml of peptone water for overnight and 3 ml of the bacterial culture were transferred to a 15 ml tube and centrifuged at 10,000 rpm for five minutes. The supernatant was discarded and the cells were resuspended in 1 ml of Tris-EDTA (TE) buffer and transferred to a clean centrifuge tube of 1.5 ml. 40 µL of lysozyme were added, mixed and solution incubated for 30 min at 37°C. 40 µl of 10% sodium dodecyl sulphate (SDS) were added and mixed; and then 8 µL of 10mg/ml Proteinase K were added, mixed and mixture incubated for 1hr at 56°C. 100 µL of 5M sodium chloride were added and mixed. 100µL CTAB/NaCl were added, mixed and incubated at 65°C for 10 min. 0.5 ml of Phenol: chloroform: isoamyl (25:24:1) were added, mixed and spun at maximum speed for ten minutes at room temperature. 0.5ml of chloroform: isoamylalcohol (24:1) were added, mixed well and spun at 13,200 rpm at room temperature. 500 µL of the aqueous phase were transferred to a clean centrifuge tube and 3 volumes ofcold absolute ethanol at -20°C were added followed by 10% of 3 M sodium acetate and precipitated overnight at -20°C. The tubes were spun at 13,200 rpm for 30 min, at 4°C and the supernatant was discarded. The pellet was washed with 500 µL of cold ethanol (70%), spun at 13,200 rpm for 15 min; and the supernatant was discarded. The pellet was dried at room temperature for 20 min and then eluted with 50 µL of the TE buffer. 0.2 µL of RNAse were added, mixed and incubated at room temperature for one hour, and the extracted DNA was stored at -20°C till used.

Genomic amplification of 16S rRNA

The 16S ribosomal RNA gene for the selected oil degrading bacteria was amplified by PCR using universal primer pair; 16S rRNA forward 5'-GAGTTTGATCCTGGCTCAG-3' and 16S rRNA reverse 5'-AAGGAGGTGATCCAGCC-3' (Wongsa et al., 2004), which correspond to positions 9 to 27 and 1525 to 1541, respectively, in the 16S rRNA gene sequence of *Escherichia coli* (Wongsa et al., 2004). The DNA was amplified using a Taq PCR kit (Thermo Scientific,® Finland) containing the following stock solutions; 10× PCR buffer, 25 mM of $MgCl_2$, 10 mM each of dNTPs, 5 U/µL of *Taq* polymerase and 10 µM of each primer. The amplification was done in a final volume of 50 µL containing 5 µL of genomic DNA. The conditions for amplification consisted of 30 cycles of 94°C (30 s), 52°C (30 s) and 72°C (30 s), plus one additional cycle with a final five minute chain elongation. All amplifications were performed in a GeneAmp PCR System (model 9700, Applied Biosystems,® USA). The PCR products were separated on agarose gel electrophoresis (1%) and the sizes of the fragments were estimated based on a 50 base pair DNA ladder (Sigma,® USA).

DNA Sequencing

The PCR products were purified and submitted to Inquaba Biotechnical Industries in Pretoria, South Africa, for sequencing.

Analysis of sequence data

The isolates were identified by comparing the 16S rRNA consensus sequence data with those in the National Center for Biotechnology Information (NCBI) GenBank data bases using the n-BLAST program Sequences with more than 98% identity with a GenBank sequence were considered to be of the same species (Kumar et al., 2006).

Amplification of *xylE* gene for biodegradation efficiency

The *xylE*gene was amplified by PCR in the selected oil degrading bacteria using the primer pair; DEG-Forward 5'-CGACCTGATCATCGCATGACCGA-3' and DEG-Reverse 5'-TCTAGGTCAGTACACGGT CA-3' which plays a key role in the metabolism of aromatic rings of aromatic compounds (Malkawi et al., 2009). Genomic DNA was amplified using a Taq PCR kit (Thermo Scientific®, Finland) containing the following stock solutions; 10× PCR buffer, 25 mM of $MgCl_2$, 10 mM of each dNTPs, 5 U/µL of *Taq* polymerase and 10 µM of each primer. The amplification was done in a final volume of 25 µL containing 2.5 µL of genomic DNA. The PCR conditions were; 30 cycles of 94°C (30 s), 50°C (30 s) and 72°C (30 s), plus one additional cycle with a final 5 min chain elongation. All amplifications were performed in a GeneAmp PCR System (model 9700, Applied Biosystems®). The PCR products were separated on agarose gel electrophoresis (1%) and the sizes of the fragments were estimated based on a Sigma 50 base pair DNA ladder (Sigma, USA) and the presence of *xylE* gene in the selected oil degrading bacteria was related to their biodegradation efficiency.

RESULTS

Phenotypic identity of oil degrading bacterial isolates

Mixed colonies of bacteria grew on the surface of petroleum agar plates and some colonies formed depressions on the petroleum agar plates (Figure 1). A total of 44 crude oil degrading bacteria were isolated from the soil samples. Based on their colonial and cellular morphology and biochemical characteristics, the bacterial isolates were categorized into eight (8) groups. The colony characteristics of the isolates are summarized in Table 1. The cell morphology and biochemical characteristics; and the number of isolates in each of the eight groups are shown in Table 2. The genera *Corynebacterium* and *Pseudomonas* were the most common in the soils contaminated with crude oil, followed by *Moraxella*, *Bacillus* and *Enterobacteriaceae*. The genera *Nocardia, Serratia* and *Rhodococcus* were the least abundant isolates. Some isolates (23) could not be placed in a particular genus due to the phenotypic characteristics they exhibited and therefore they remained un-identified.

Oil biodegradation activity of the bacterial isolates

From the crude oil biodegradation experiment that was initially carried out (for five days), the bacterial isolates that degraded over 20% of the crude oil in the liquid medium (Figure 2) were selected for a detailed analysis. A relatively higher degradation activity was exhibited in the test bottles than in the negative control set-up.

The selected strains degraded the total petroleum hydrocarbons to over 60% by the end of the third week of incubation; and in all experiments inoculated with bacteria, the percentage of oil degradation was observed to increase with increase in the incubation period (Figure 3).

Among the eight isolates analyzed, Kig3-T4 and Ngara-

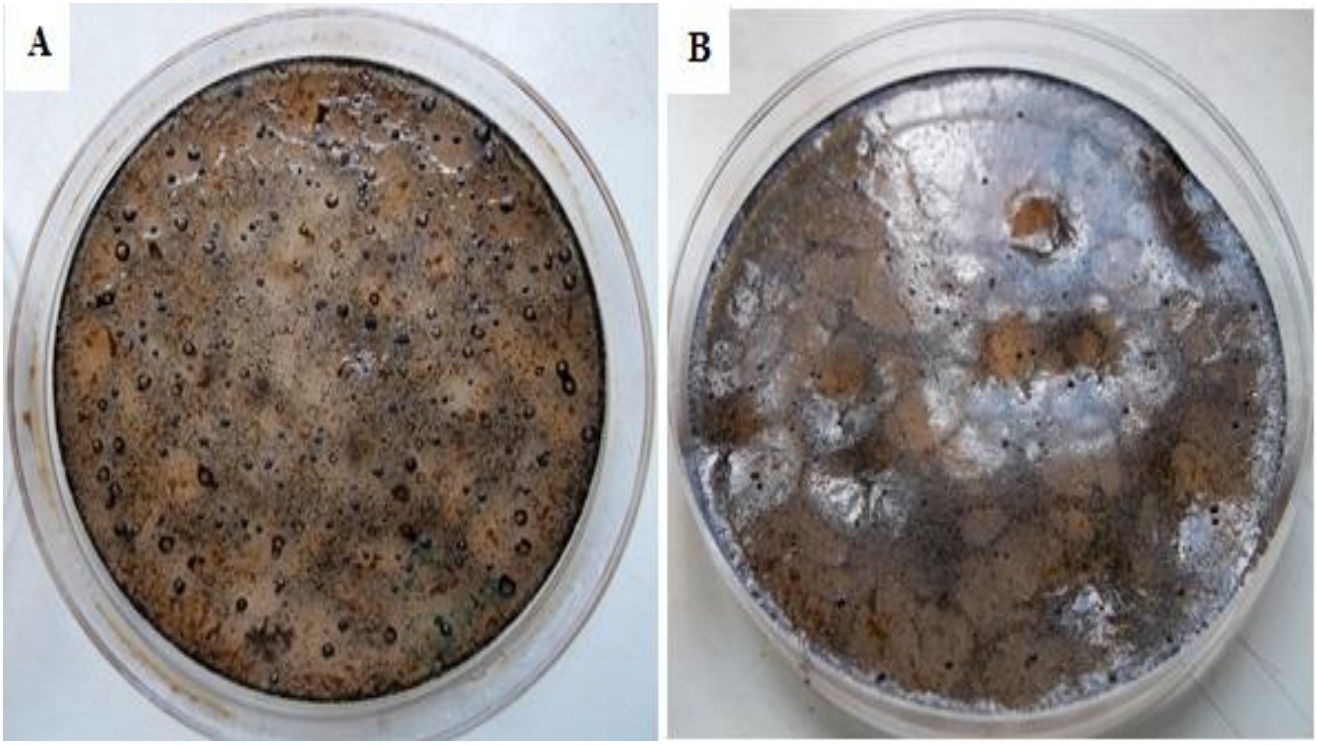

Figure 1. Appearance of the oil degrading bacterial colonies on petroleum agar plates. Arrows show the regions of bacterial colonies. The dark appearance in the plates was due to the crude oil mixed with agar medium. Plate A – The arrows indicate bacterial colonies surrounded by a dark film of crude oil on surface of petroleum agar plate; Plate B – The arrows indicate bacterial colonies formed in depressions on the surface of petroleum agar plate.

T1 exhibited the best degradation activity (91.9 and 94.2%, respectively) followed by isolate Kig5-T2 and Kas2-T7 (89.2 and 83.2% respectively). These were followed by Kig5-1, Kig3-T3 and Kas2-T5 with a biodegradation of 79.9%, 73.1 and 77.0% respectively. Gat-3 was the least crude oil degrader with 67.2% as shown in Figure 3. The rate of hydrocarbon degradation in the control experiment was relatively low (29.7%) as compared to the rest of the experiments inoculated with bacterial isolates.

The statistical analysis of variance revealed that the percentage of oil degradation at the seventh day of incubation, was not statistically significant ($P>0.05$) for Gat3. It was, however, significant ($P<0.05$) for Kas2-T7 and Kig3-T3; very significant ($P<0.01$) for Kas2-T5, Kig5-T2 and Kig5-1; and extremely significant ($P<0.001$) for Kig3-T4 and Ngara-T1. During the 14th day of incubation, the degradation was very significant ($P<0.01$) for Gat3; and extremely significant ($P<0.001$) for Kas2-T7, Kas2-T5, Kig5-T2, Kig5-1, Kig3-T3, Kig3-T4 and Ngara-T1. The degradation at the 21st day of incubation was extremely significant ($P<0.001$) for Gat-3, Kas2-T7, Kas2-T5, Kig5-T2, Kig5-1, Kig3-T3, Kig3-T4 and Ngara-T1.

The GC/MS analysis of the crude oil sample revealed both aliphatic chain hydrocarbons (C9 to C24) and polycyclic aromatic hydrocarbons such as; o-*xyl*ene, cyclohexane, benzene, 1-ethyl-3-methyl, benzene 1, 2, 4-trimethyl and benzene 1,3,5-trimethyl in the crude oil sample (Figure 4). As indicated in Figure 5A2 and E2, the hydrocarbon components in the crude oil were reduced in abundance after the three weeks of incubation in experiments that were inoculated with bacteria; compared to a minimal reduction in the control experiment (Figure 5G1).

Amplification of *xylE* by PCR

The catabolic gene *xylE* was detected in two of the eight selected bacterial isolates; that is in lane A and C which corresponded to the isolates Ngara-T1 and Kig5-T2 respectively (Figure 6). The degradation of crude oil by these two isolates (Ngara-T1 and Kig5-T2) after three weeks of incubation was very close, that is 91.4 and 89.2% for Ngara-T1 and Kig5-T2 respectively. The isolate Kig3-T4 which recovered most of the crude oil (94.2%) and other isolates with moderate and least crude oil degradation percentages did not possess the gene *xylE*.

Genotypic Identification

The 16S rRNA gene sequences were accessed from the public GenBank data bases using the n-BLAST program. Ngara-T1 which was Gram positive cocci and indole

Table 1. The colonial characters used to identify some of the bacterial isolates.

Isolate identity	Colony colour	Colony size	Colony form	Colony elevation	Colon margin	Genus identity
Ngara-T4, Kig3-4, Kas2-T2, Gat-T5, Kig3-T3	Blue-green, beige brown	Medium	Irregular, circular	Slightly raised	Undulated	*Pseudomonas*
Gat-3, Kig5-1, Gat-1, Kig5-T3, Kig5-T1, Kas2-T5 Kas2-T4	Cream, grey, white	medium	Circular	Raised, convex	Entire	*Corynebacterium*
Ngara-T2, Kig3-T2	Beige	Small	Punctiform	Convex	Entire	*Moraxella*
Ngara-3 Kig5-T2	Beige, cream	Medium	Irregular	Flat	Entire, undulated	*Bacillus*
Gat-T4	White, chalky	Small	Irregular	Raised	Entire	*Nocardia*
Ngara-T3, Kig5-T4	Cream	Medium	Circular	Convex	Entire	*Enterobacteriaceae*
Kig3-T1	Orange	Medium	Circular	Slightly elevated	Entire	*Serratia*
Kas2-1	White/buff	Medium	Circular	Flat	Entire	*Rhodococcus*

Table 2. The gram's staining and biochemical parameters used to identify the bacterial isolates.

Isolate Identity	Number of Isolates	Gram's reaction and cell shape Test	Catalase Test	Oxidase Test	Indole Test	Glucose Ferm	Maltose Ferm	Lactose Ferm	Citrate Test	Urease Test	Haemolysis Test	Genus Identity
Ngara-T4, Kig3-4, Kas2-T2, Gat-T5, Kig3-T3	5	– rods	+	+	–	–	NA	NA	+	NA	NA	*Pseudomonas*
Gat-3, Kig5-1, Gat-1, Kig5-T3, Kig5-T1, Kas2-T4, Kas2-T5	7	+ rods	+	+ / –	NA	NA	–	NA	NA	NA	NA	*Corynebacterium*
Ngara-T2, Kig3-T2	2	– cocci	+	–	NA	NA	NA	NA	NA	–	+	*Moraxella*
Ngara-3 Kig5-T2	2	+ rods	+	NA	NA	+	NA	NA	NA	NA	NA	*Bacillus*
Gat-T4	1	+ rods	–	–	NA	NA	NA	NA	NA	NA	NA	*Nocardia*
Ngara-T3, Kig5-T4	2	– rods	+/ –	+ / –	NA	NA	NA	+	–	–	NA	*Enterobacteriaceae*
Kig3-T1	1	– rods	–	–	–	+	NA	–	NA	NA	NA	*Serratia*
Kas2-1	1	+ cocci	–	+	NA	+	NA	NA	NA	NA	NA	*Rhodococcus*
Ngara-1, Ngara-2, Gat-T3, Ngara-4, Ngara-T1, Gat-2 Gat-T1, Gat-T2, Kig5-2, Kig5-3, Kig5-4, Kas2-2, Gat-4, Kig3-3, Kas2-3, Kas2-4, Kas2-T1, Kas2-T3, Kas2-T6, Kas2-T7, Kig3-1, Kig3-2, Kig3-T4,	23	+ / –	+ / –	+ / –	NA	NA	NA	NA	NA	NA	NA	Un-identified

NA, Not applicable; Ferm, fermentation

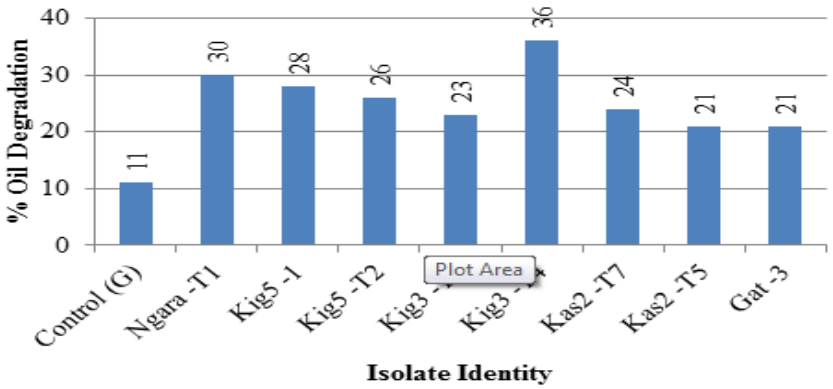

Figure 2. Crude oil degradation by the bacterial isolates during the five days of incubation in MSM supplemented with crude oil (1%) as the sole source of carbon. The experiments were incubated aerobically in duplicates on a rotary shaker (150 rpm) at room temperature (25 ± 2.0°C). Isolates that exhibited a relatively higher degradation (over 20%) were selected for a detailed analysis.

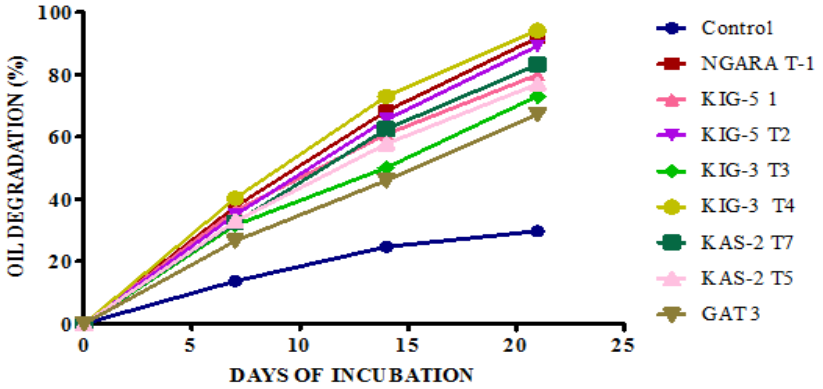

Figure 3. The percentage of degraded crude oil by selected bacterial isolates during three weeks of incubation. The experiments were incubated aerobically in duplicates on a rotary shaker (150 rpm) at room temperature (25 ± 2.0°C).

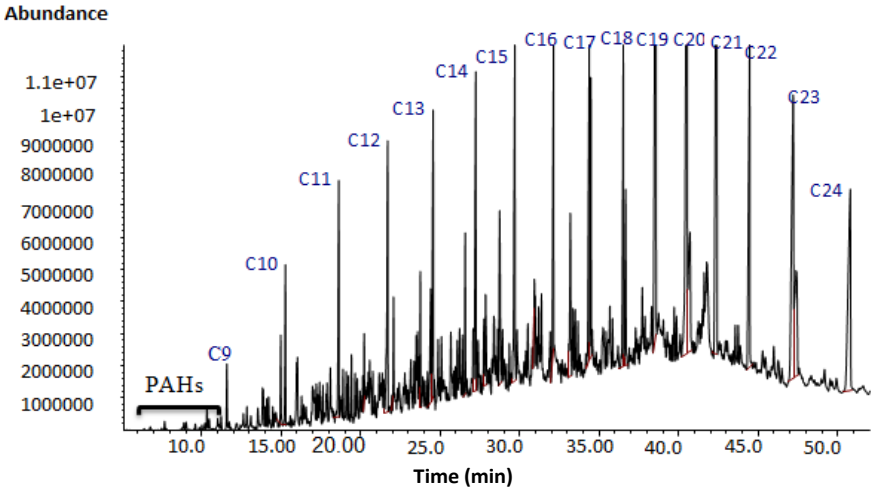

Figure 4. The crude oil degradation profiles as revealed by GC-MS analysis. The abundance of the polycyclic aromatic hydrocarbons (PAHs) was relatively low as compared to the aliphatic hydrocarbons (C9-C24).

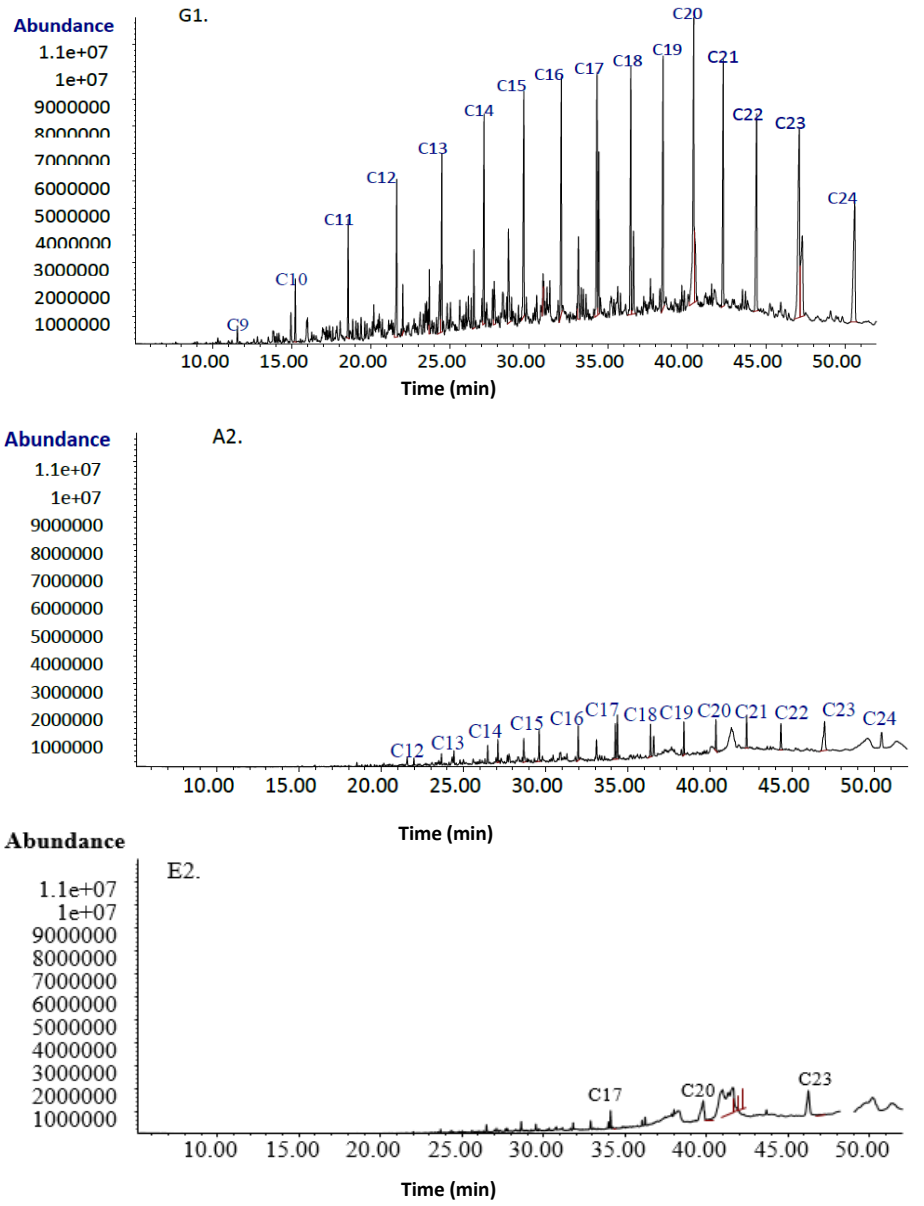

Figure 5. The GC/MS profile of crude oil components in culture medium after three weeks of incubation. G1, Control experiment (without inoculum). A2, hydrocarbon degradation by Ngara-T1. E2, hydrocarbon degradation by Kig3-T4. All PAHs and most aliphatic hydrocarbons were degraded in experiment A2 and E2 by the inoculated bacteria whereas they were still detectable in G1.

negative was 99% identical to *Stenotrophomonas maltophilia,* suggesting that Ngara-T1 was a strain of this species. Kig5-1 was Gram negative rod and positive for oxidase and catalase as it is for *Burkholderia* sp.; and it was 94% identical to *Burkholderia* sp., suggesting that Kig5-1 was a strain of *Burkholderia* sp. The strain Kig5-T2 was 82% identical to *Delftia tsuruhatensis* and the phenotypic characters were Gram negative rods, oxidase positive and catalase negative, similar to *Delftia tsuruhatensis*; suggesting that Kig5-T2 was *Delftia tsuruhatensis*. The isolate Kas2-T5 was 95% identical to *Curtobacterium* sp. and its taxonomic characteristics were similar to *Curtobacterium* sp.; that is, Gram positive rods, positive for oxidase and catalase. These characters suggest that Kas2-T5 was *Curtobacterium* sp.

The alignment for Kas2-T7 was 98% identical to that of *Acinetobacter junii*. Kas2-T7 was Gram positive cocci, oxidase negative and catalase positive as it is for *Acinetobacter* sp., implying that Kas2-T7 was *Acinetobacter junii*. Similarly, the sequence alignment for

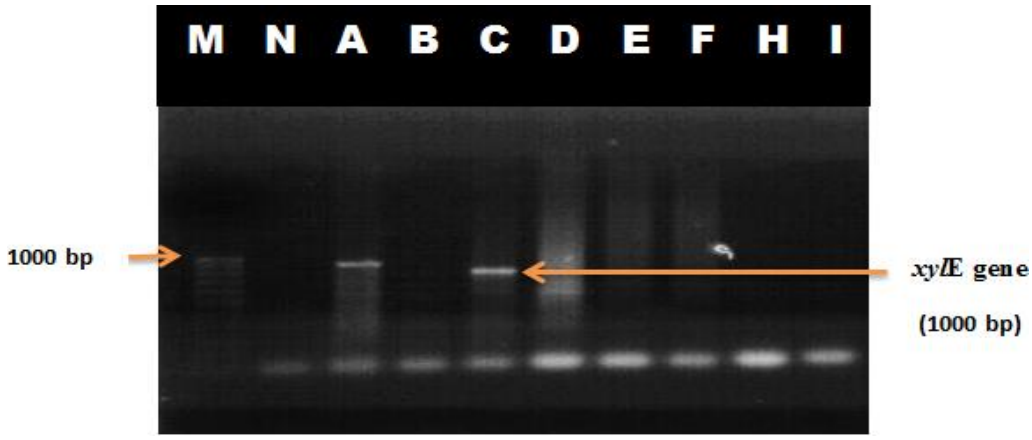

Figure 6. PCR amplification of the catabolic gene *xylE* in the selected isolates. M is the DNA marker (bands not clearly visible), N is the negative control (without DNA) and A, B, C, D, E, F, G and H lanes loaded with DNA from the selected isolates; Ngara-T1, Kig5-1, Kig5-T2, Kig3-T3, Kig3-T4, Kas2-T7, Kas2-T5 and Gat-3 respectively. The catabolic gene *xylE* was detected in lane A and C.

Table 3. The 16S rRNA gene sequence of the selected isolates in compulsion to the 16S rRNA gene sequences in the public gene Bank data base.

Isolate identity	Genus identity	Genotypic identity	Querry cover (%)	Max Identity (%)	Accession number
Ngara-T1	Un-identified	*Stenotrophomonas maltophilia*	100	99	EU741084.1
Kig5-1	*Corynebacterium*	*Burkholderia* sp.	98	94	JX845723.1
Kig5-T2	*Bacillus*	*Delftia tsuruhatensis*	95	82	AY684785.1
Kig3-T3	*Pseudomonas*	*Pseudomonas aeruginosa*	100	99	NR_074828.1
Kig3-T4	Un-identified	*Uncultured Acinetobacter* sp.	100	87	FJ191646.1
Kas2-T7	Un-identified	*Acinetobacter junii*	100	98	HE651918.1
Kas2-T5	*Corynebacterium*	*Curtobacterium* sp.	100	95	KC466122.1
Gat-3	*Corynebacterium*	*Paenibacillus agaridevorans*	99	95	NR_025490.1

Kig3-T4 was 87% identical to *Acinetobacter* sp. and the strains were Gram positive cocci and oxidase negative as it is for *Acinetobacter* sp. Kig3-T4 had some resemblance to *Acinetobacter* sp.

Gat-3 was a strain of *Paenibacillus agaridevorans*. The sequence alignment for Kig3-T3 was 99% identical to that of *Pseudomonas aeruginosa* and its taxonomic characteristics were typically the same. The isolates of Kig3-T3 were Gram negative rods, oxidase positive/negative, catalase positive and with production of a greenish pigmentation, which suggests that Kig3-T3 was a strain of *Pseudomonas aeruginosa.* The BLAST search for the 16S rRNA consensus sequence of the selected isolates are summarised in Table 3 (Figure 7).

In this study, strains of *Pseudomonas* sp. were among the most numerous crude oil degrading bacterial isolates, and were selected among the isolates that demonstrated relatively higher crude oil degradation.

DISCUSSION

In the present research, crude oil degrading bacteria were isolated from the oil exploration areas in Western Uganda. Such organisms are naturally present in these environments and play a very important role in the removal of the pollutants (Obayori et al., 2012). The crude oil degrading bacteria were phenotypically identified as belonging to the genera; *Corynebacterium, Pseudomonas, Moraxella, Bacillus, Enterobacteriaceae, Nocardia, Serratia* and *Rhodococcus*. These genera have previously been reported as containing oil degrading species (Bicca et al., 1999; Iwabuchi et al., 2002; Malkawi et al., 2009; Obayori et al., 2012; Rajaei et al., 2013; Wongsa et al., 2004).

In this study, GC/MS analysis revealed hydrocarbon components with carbon numbers as high as C24. All components of the crude oil were biodegraded and

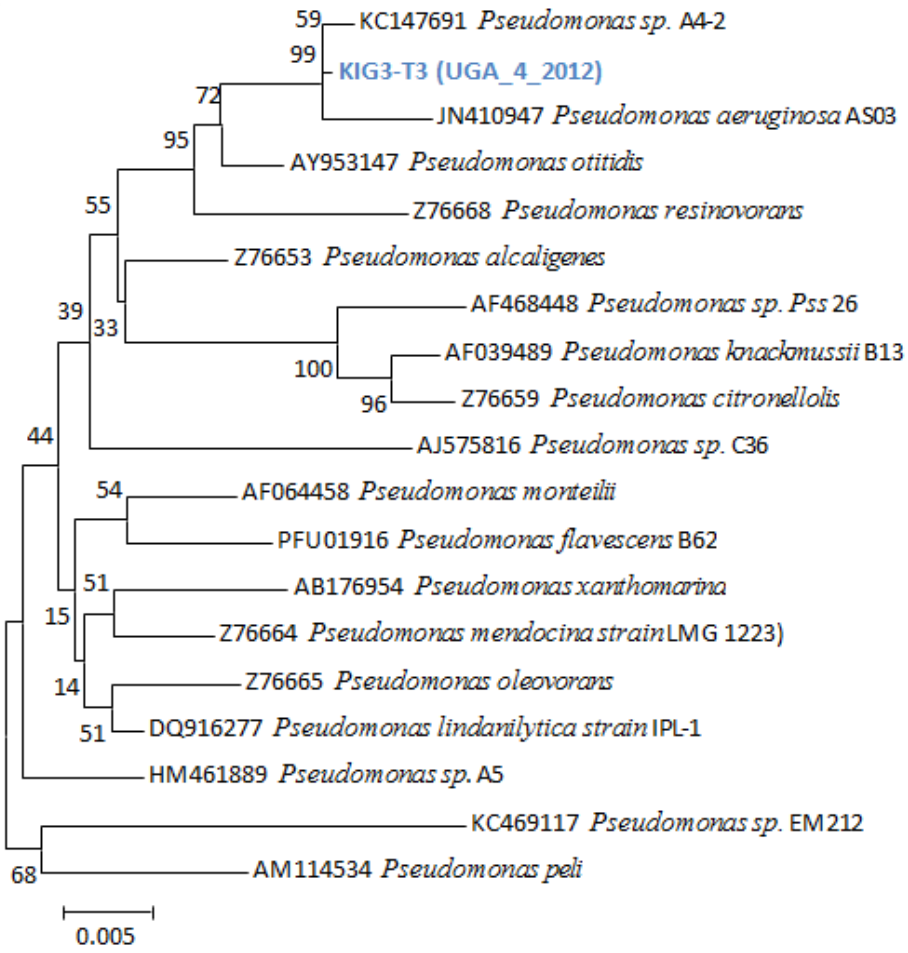

Figure 7. The phylogenetic tree showing isolate Kig3-T3 and its related Pseudomonas sp. The tree was constructed on the basis of 16S ribosomal RNA gene sequence using the neighbor-joining method. The evolutionary analyses were conducted using MEGA5 software. The analysis indicated that the closest relatives of strain Kig3-T3 were Pseudomonas sp. Strain A4-2 and Pseudomonas aeruginosa strain AS03.

reduced by the bacterial isolates as compared to the control. The crude oil was the sole source of carbon and energy in the liquid growth medium (MSM), indicating that these strains were capable of degrading and utilizing crude oil hydrocarbons. This is in agreement with a previous study by Wongsa et al. (2004) that observed a reduction in the hydrocarbon quantities after incubation of the oil bio-degrading bacteria in a diesel enriched mineral salts medium. The crude oil was dissolved into the culture broth in the experiments inoculated with bacteria, while it remained suspended above the Un-inoculated broth in the control experiment, suggesting that the bacteria may have produced biosurfactants and enzymes which emulsified the crude oil. Production of bio-surfactants is one of the ways the microbes can take up hydrophobic substrates and it has been reported in organisms such as *Pseudomonas aeruginosa* Acinetobacter sp. and Bacillus polymyxa (Rajaei et al., 2013).

The degradation of hydrocarbons is aided by catabolic genes carried by the oil degrading organisms, and identification of such genes is commonly performed to evaluate the microbes for bioremediation processes (Rajaei et al., 2013). In this study, the catabolic gene *xylE* was detected in two of the eight selected bacterial isolates; that is, Ngara-T1 and Kig5-T2. The total petroleum hydrocarbons degraded by these two isolates (Ngara-T1 and Kig5-T2) was very close; that is, 91.4 and 89.2%, respectively, suggesting that presence of *xylE* gene may have played a similar role in the degradation of crude oil hydrocarbons in the two bacterial isolates.

However, isolate Kig3-T4, which degraded most of the crude oil in the liquid medium (94.2%); and the other isolates with moderate and least crude oil degradation did not possess the catabolic gene *xylE*. This suggests that the presence of *xylE* gene is not the sole factor that contributes to crude oil degradation by bacteria. There

are seven catabolic genes that encode enzymes involved in a variety of known bacterial hydrocarbon degradative pathways that have been reported (Malkawi et al., 2009).

According to Harayama and Rekik (1989), hydrocarbon degrading bacteria possess oxygenases which catalyze reactions involving both biosynthesis and biodegradation. Such enzymes are classified into two groups: dioxygenases that catalyze the incorporation of both atoms of oxygen into substrates; and monooxygenases that catalyze the insertion of one atom of oxygen. The enzyme catechol 1,2-dioxygenase, cleaves the aromatic ring of catechol and the substrates of the ring-cleavage dioxygenases usually contain two hydroxyl groups on two adjacent aromatic carbons. The degradation of alkanes involves formation of an alcohol, an aldehyde and a fatty acid, which is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation (Malatova, 2005). *Acinetobacter* sp. possesses an alkane monooxygenase which oxidizes the second carbon atom leading to the production of a secondary alcohol; and the subsequent ketone is further metabolized to a primary alcohol for further breakdown (Malatova, 2005).

The alignment of the 16S rRNA gene sequence of the selected isolates revealed that the strains were identical to *Stenotrophomonas maltophilia*, *Burkholderia* sp., *Delftia tsuruhatensis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter junii*, *Curtobacterium* sp. and *Paenibacillus agaridevorans*. Except for *Delftia tsuruhatensis,* the rest of these strains have commonly been isolated and reported as oil degrading species (Laurie and Lloyd-Jones, 1999; Rajaei et al., 2013; Urszula et al., 2009). *Delftia tsuruhatensis* first described in 2003, was isolated from activated sludge collected from a domestic wastewater treatment plant in Japan, and it was able to degrade various (hazardous) aromatic hydrogen carbon compounds (Preiswerk et al., 2011). The Gram negative, oxidase positive and catalase negative strains of *Delftia* sp. isolated in this study, were able to degrade crude oil in the liquid medium as the sole source of carbon and energy.

Pseudomonas sp. have frequently been reported as crude oil degrading bacteria; and so far, they are the most studied of all the hydrocarbon degrading bacteria (Mittal and Singh, 2009). It is not surprising that strains of *Pseudomonas* sp. were among the most numerous crude oil degrading bacteria identified in this research. The isolates were Gram negative rods, oxidase positive-/negative, catalase positive and with production of a greenish pigmentation.

A study in 2009 by Obuekwe and others identified *Paenibacillus* sp. as a prominent crude oil degrader in the Kuwait desert environment; and it was able to survive the prevalent high soil temperatures (40-50°C) (Ganesh and Lin, 2009). The same study also showed that both *Paenibacillus* sp. and *Stenotrophomonas* sp. were effective in degrading diesel up to 83%, making them prominent hydrocarbon degraders. In this study, a Gram positive cocci and indole negative *Stenotrophomonas maltophilia* was the most efficient crude oil degrader; while the Gram positive and catalase positive strains of *Paenibacillus* sp. identified in this study were not prominent crude oil degraders. The difference in crude oil degradation could be attributed to the differences in environments where the two strains were obtained.

Curtobacterium sp. were listed among the genera of hydrocarbon degrading bacteria which grow in the presence of PAHs or alkanes (Verania, 2008). Previous researchers reported isolating *Curtobacterium* sp., with crude oil degrading activity from rhizosphere-bound soil samples (Rajaei et al., 2013). Strains of *Acinetobacter* sp. have been shown to degrade the hydrocarbons in crude oil in many studies. These same studies demonstrated the ability of *Acinetobacter* sp. to utilize crude oil hydrocarbons in liquid media and to compete with indigenous soil microorganism (Hanson et al., 1997; Rajaei et al., 2013). Similarly, strains of *Acinetobacter* sp. which were able to degrade the crude oil in liquid media were isolated during this study.

Conclusion

The oil degrading bacteria that were encountered in the soils around the oil exploration fields in Western Uganda were identified as; *Stenotrophomonas maltophilia*, *Burkholderia* sp., *Delftia tsuruhatensis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter junii*, *Curtobacterium* sp. and *Paenibacillus agaridevorans*. The oil degrading bacteria showed diverse and varying capacities to degrade the crude oil; with some degrading up to 90% and can be exploited for cleaning up hydrocarbon contaminated sites. There is need for more research on these isolates to establish whether it is possible to carry out crude oil bioremediation either through inoculation of specifically isolated microbial communities with higher degradation potential or targeted stimulation of the indigenous soil microbes. The catabolic gene *xylE* was not detected in all of the isolates that exhibited oil degrading because it is not the only gene that plays a role in hydrocarbon degradation by microbes. Hence, presence of other genes associated with this activity need to be investigated. To the best of our knowledge, this study on isolation, identification and determination of crude oil degradation efficiency of the bacteria was the first of its kind in Uganda. Therefore, the study is an important step towards the development of bioremediation strategies for cleaning of sites contaminated with oil pollutants and wastes. There is need to do further research in order to test the degradation activity of the isolates in combination and establish the safety of the isolates and of the by-products of oil metabolism in the environment.

Furthermore, there is need to screen for biosurfactant production, investigate mechanism of oil reduction and screen for other reported genes to establish the mechanism of biodegradation. There is also need to determine the optimum growth requirements of the isolates, test oil biodegradation in the field by the microbes and compare the isolates with well-known characterised organisms.

Conflict of Interests

The author(s) have not declared any conflict of interest.

Authors' contributions

Andrew Wedulo contributed to the conception of the idea, design, data collection, laboratory analysis and writing of the manuscript. David Kalenzi Atuhaire contributed to laboratory analysis, data analysis and writing of the manuscript. Sylvester Ochwo contributed to data collection, laboratory analysis and writing of the manuscript. Vincent Muwanika contributed to the conception of the idea, design and writing of the manuscript. Abel John Julian Rwendeire contributed to the conception of the idea and writing of the manuscript. Jesca Lukanga Nakavuma contributed to the conception of the idea, design, data analysis and writing of the manuscript. All read and approved the manuscript.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Isolation and partial characterization of the active metabolite of ascidian, *Polyclinum madrasensis* from the Palk Bay Region, Southeast coast of India

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Ascidians are rich source of bioactive agents which could be used for novel antimicrobial drugs. The present investigation inspects the antibacterial potential of ascidian, *Polyclinum madrasensis* collected from Mandapam, the Palk Bay region, Southeast coast of India. The crude extracts were tested for inhibition of bacterial growth against human pathogens. Antibacterial assay was carried out by agar well diffusion method. The maximum inhibition zone (12.0±0.5 mm) was observed against the *Staphylococcus aureus* in crude methanol extract. The consequent zone of 6.5±0.1 mm was observed against *S. aureus in* ethanol extract and minimum inhibition zone (3.2±0.5 mm) was noticed with *Pseudomonas aeruginosa*. Molecular weight of tissue protein was determined through sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE) and active metabolites were characterized by Fourier Transform Infrared spectroscopy (FTIR) analysis. The protein bands were at 36.5, 20.5, and 10.5 kDa, in SDS-PAGE and O-H stretch carboxylic acid compounds identified at the peak 3533.59 cm^{-1}. It could be concluded from the present study that crude extract of the ascidian, *P. madrasensis* has potential antimicrobial effect against human pathogens.

Key wards: *Polyclinum madrasensis,* bioactive compounds, Fourier Transform Infrared spectroscopy (FT-IR), sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

It is a real fact that the importance of marine organisms as a source of new substances is growing. With marine species comprising approximately a half of the total global biodiversity, the sea offers an enormous resource for novel compounds (Blunt et al., 2011). A very different kind of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive and aggressive surrounding and are very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules (De Vries, 1995). Peptidic compounds analyzed are obtained from very different marine organisms exhibiting different chemical structures and displaying a large variety of

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pharmacological effects on specific targets (Aneiros and Garateix, 2004). In marine invertebrates so far, approximately 7,000 marine natural products have been reported (Venkataraman and Wafer, 2005). Ascidians have attracted attention as a source of antimicrobial proteins because they are marine sessile, filter feeding invertebrate which hold a phylogenetically strategic position close to the origin of the vertebrate line. The group of ascidians is one of the most compelling sources of metabolites both of chemical and biomedical interest in the marine environment (Blunt et al., 2011). Antitumor, antiviral and immunosuppressive active compounds are primarily isolated from tunicates (Schmitz et al., 1993).

Bacterial infection cause high rate of mortality in human population (Kandhasamy and Arunachalam, 2008). During the last few decades, the number of upcoming infectious disease is increasing in the developing countries (Murugan and Mohan, 2011). *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, are well known to be causative agents for boils skin infections, abscesses, dysentery and diarrhoea (Levin, 1987; Ananthanarayanan and Paniker, 2008). Presently, various ascidians such as *Botryllus* sp., and *Didemnum* sp. have been reported for producing anti-cancer drugs (Azumi et al., 1990). Halocyamine A, an antimicrobial substance was isolated from haemocytes of the solitary ascidians *Halocynthia roretzi* (Azumi et al., 1990). Ascidian-based products may be a new boon to the development of antibiotics effective against antibiotic resistant strain. Then, attention has focused on ascidians because of their biologically active metabolites and the chemistry of ascidians has become one of the most active fields of marine natural products; it has been amply demonstrated that these sea creatures are prolific producers of unusual structures with significant bioactivities (Faulkner, 2002; Blunt, 2010).

In the arena of marine habitat the colonization process is affected by organic metabolites produced by the host organism. Such metabolites may affect bacteria in a number of ways, ranging from the induction of chemo tactic responses to the inhibition of bacterial growth or cell death (Bell and Mitchell, 1972). In this study, we evaluated the antimicrobial potential of the ascidian, *Polyclinum madrasensis* and partially characterized its biologically active metabolites through sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE) and Fourier Transform Infrared spectroscopy (FT-IR).

MATERIALS AND METHODS

Collection

The ascidian, *P. madrasensis* was collected from Mandapam (Lat. 0957' N: Long79 11' E) Palk Bay region, Southeast coast of India. The sample was collected by hand-picking during low tide season and transported to the laboratory with safe condition. Molluscan shell, calcrete rock fragments attached to the foot of the animal was carefully removed. They were identified using key to identification of Indian ascidians (Meenakshi, 1997).

Preparation of ascidian extract

The fresh tissues of *P.madrasensis* were freezed at -20°C and used for the extraction of antimicrobial metabolites. Their soft bodies were removed by breaking the outer layer. The whole body tissues of the sample (50 g) were cut into small pieces and the tissue sample was used for extraction using methanol and ethanol. The extracts were cold steeped over night at -18°C and filtered with What man No. 1 filter paper. The filtrate was evaporated to dryness in rotary evaporator (Becerro, 1994; Wright, 1998). The dried crude extracts were used for antibacterial assay against human pathogens (*P. aeruginosa*, *E. coli*, *S. aureus*, *Salmonella typhi*, *Vibrio cholera*, *Vibrio parahaemolyticus*, *Klebsiella pneumoniae* and *Proteus mirabilis)*. All the pathogenic bacterial strains were obtained from the Department of Clinical Pathology, Raja Muthiah Medical College, Annamalai University, Tamil Nadu, India.

Antibacterial assay

The ascidian, *P. madrasensis* crude extracts were tested for inhibition of bacterial growth against human human pathogens. Antibacterial assaywas carried out by agar well diffusion technique (El-Masry et al., 2000). Human pathogens were inoculated in sterile nutrient broth and incubated at 37°C for 24 h. Pathogens were swabbed on the surface of the Muller Hinton agar plates and wells were punched out using a sterile cork borer (6 mm diameter). About 50 µl of the different solvent of ascidian, *P. madrasensis* extracts, were transferred in to each well. For each pathogen, controls were maintained where pure solvents were used instead of the ascidian extract.

The plates were incubated at 37°C for 24 h (Mtolera and Semesi, 1996). The results were obtained by measuring the inhibition zone diameter for each well and expressed in millimetre (Mohamed et al., 2010).

Muscle protein molecular weight determination by SDS-PAGE

Molecular weight of the muscle protein of *P. madrasensis* was determined using SDS-PAGE following the procedure by Sambrro et al. (2006). Glass plates were assembled and 20 ml of 15% resolving gel was prepared and poured immediately to the notch plate. It was over laid with butanol. After polymerization was completed, it was poured off and the top layer was washed with deionized water. Then it was over laid with 8 ml of stock gel. Approximately 1 ml of 1% SDS gel loading buffer and sample were taken and it was heated at 100°C for 3 min. Then, it was carefully fixed in electrophoresis apparatus. 15 µL of samples with different molecular weight markers (14.0 to 97.4 kDa) were loaded, respectively in the well, run in the gel and stained with coomassive brilliant blue.

Fourier transform infrared (FT-IR) spectral analysis

The lyophilized methanol extract of *P. madrasensis* (10 mg) were mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc. The disc was then read spectrophotometrically (Bio-Rad FTIR-40-model, USA). The frequencies of different components present in sample were analyzed.

Table 1. Antibacterial assay of ascidian, *Polyclinum madrasensis* against human pathogens.

Human pathogens	Zone of Inhibition(mm)	
	Methanol extract	Ethanol extract
S. aureus	12.0±0.5	5.0±0.8
E. coli	8.2 ±0.5	5.0±0.5
P. aeruginosa	6.5±0.2	3.2±0.5
V. parahaemolyticus	6.5±0.8	5.0±0.1
V. cholera	5.5±0.6	3.4±0.8
K. pneumonia	5.5 ±0.5	3.3±0.5
P. mirabilis	5.5 ±0.8	3.4±0.2
S. typhi	4.3±0.7	3.3±0.5

Values are the mean of triplicates ± SD $p < 0.05$

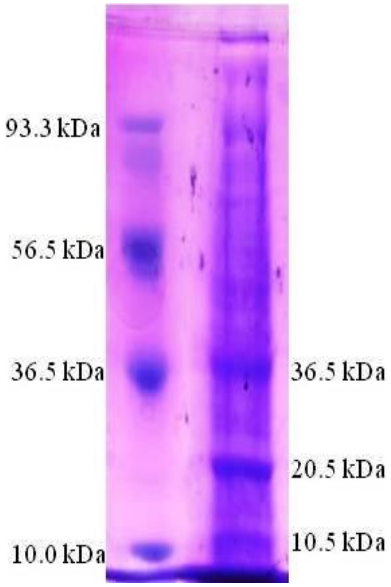

Figure 1. Molecular size of ascidian, *Polyclinum madrasensis* tissue protein determined by SDS. Lane 1: Standard protein molecular weight marker. Lane 2: Crude protein sample of ascidian, *Polyclinum madrasensis*.

RESULTS

Antibacterial activity

Two crude extracts of ascidian, P. madrasensis were screened against eight human bacterial pathogens for testing their antibacterial activities. The inhibition zones of methanol and ethanol extracts against the specific test organisms were shown in Table 1. The methanol extracts showed high antibacterial activity against *S. aureus* (12.0±0.5 mm), followed by *E. coli* (8.2 ±0.5 mm), *P. aeruginosa* (6.5±0.2 mm), *V. parahaemolyticus* (6.5±0.8 mm), *V. cholera*, (5.5±0.6 mm) *K. pneumonia* (5.5±0.8 mm) P. mirabilis (5.5 ±0.8 mm) and S. typhi (4.3±0.7 mm). The minimum inhibition zone (3.2±0.5 mm) was noticed with *P. aeruginosa* in ethanol extract. However, maximum zone of inhibition range between 5.0±0.1 - 5.0±0.8 mm against *E. coli*, *S. aureus* and *V. parahaemolyticus* in ethanol crude extract. Other indicated organisms were recorded as having meagre activity in ethanol extract.

Muscle protein molecular weight determination by SDS-PAGE

Crude protein sample of ascidian, *P. madrasensis* yielded three bands ranging from 25.0 to 110.0 kDa with well defined. The bands were at 36.5, 20.5 and 10.5 kDa, respectively. Ascidian, *P. madrasensis* sample was compared with the standard protein molecular weight marker (14.4 to 97.4 kDa) (Banglore Genei, India) (Figure 2).

FT- IR spectral analysis

The FTIR spectrum of the ascidian methanol extracts revealed characteristics of functional groups (Figure 1) such as O-H stretch carboxylic acid compounds, identified at the peak 3533.59 cm^{-1} and C-H stretching peak of alkanes group at 2954.95, 2922.16 and 2850.79 cm^{-1}, respectively. The –C (triple band) C–stretch peaks of alkenes were at 1641.42 and 1631.78 cm^{-1}. The peaks of 1458.18 and 1402.25 cm^{-1} indicate C-C stretch aromatic. Peak values were recorded at 1014.56 and 1083.99 cm^{-1}. Absorption indicated C-N stretch aliphatic amines.

DISCUSSION

Marine organisms have been recognized as a rich source of novel compounds that are of potential interest to mankind (Faulkner, 2000). They produce secondary metabolites and other compounds to repel and deter predators

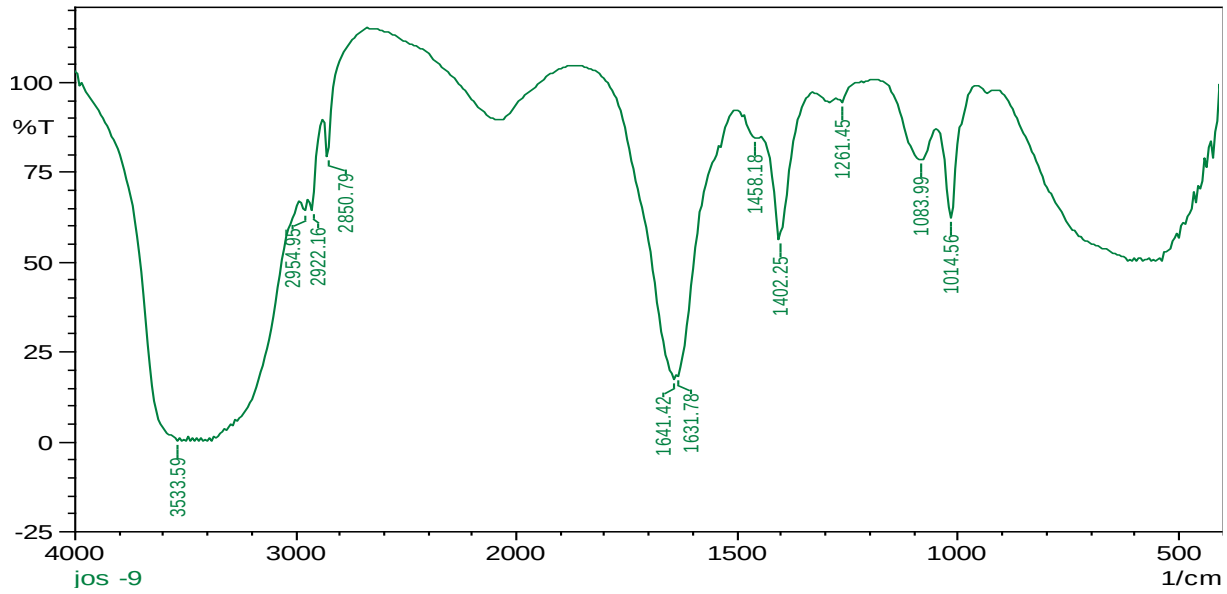

Figure 2. The FT-IR Spectra of lyophilized sample in *Polyclinum madrasensis*.

(Pawlik et al., 2002). Most of these pristine resources have not been explored for bio prospecting and microbial ecological studies. The ascidians are a potential chemical, ecological phenomenon, which provides sustainable source of supply for developing novel pharmaceutical leads. Antibacterial activity has been previously reported from extracts of some ascidians. Overall, ascidian extracts caused growth inhibition in gram positive and gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganism (Thompson et al., 1985).

In the present study, a pronounced antimicrobial effective has been observed against some human pathogens. Both methanol and ethanol crude extract of *P. madrasensis* inhibit growth of human clinical pathogens. The maximum activity was observed against *S. aureus* (12.0±0.5 mm) and minimum activity was observed against *P. aeruginosa* (3.2±0.5 mm). Similarly, maximum activity was exposed by methanol extract in *S. aureus* and minimum activity was showed by ethanol extract in *P. aeruginosa* which was collected at Tuticorin coast (Bragadeeswaran et al., 2010). Selva Prabhu et al. (2012) have reported that the crude methanol extract of *P. madrasensis* of the maximum inhibition zone of against *E. coli* and the minimum of *P. aeroginosa* and ethanol extract produced *E. coli* and *P. aeruginosa.* It supports this work. In this present study, we used -18°C cold steep extract.

In the present investigation, the tissue extraction from *P. madrasensis* showed antimicrobial activity and it was subjected to SDS-PAGE in order to estimate the molecular weight. After electrophoresis, clear bands were detected in the gel which represented molecular weight of proteins ranging 11 to 97 kDa. Green et al. (2003) reported that the molecular weight of protein from hemolymph of the solitary ascidian, *Styela plicata* from Australian waters is 43 kDa. The endoderm specific alkaline phosphate proteins with molecular masses of 86 and 103 kDa were likewise reported by Kumano et al. (1996). The amount of carbohydrate, protein, lipid and minerals such as phosphorous and calcium contents in these ascidians were previously reported by Rajesh and Ali (2008) and Natarajan et al. (2011). FT-IR analysis reveals the presence of bioactive compound signals at different ranges. Previously, aliphatic chain was identified as the peak of 3394.43 and 2920.40 cm^{-1} in ethanol extract of *P. madrasensis* by Meenakshi et al. (2013). FT-IR spectroscopy is proved to be a reliable and sensitive method for detection of biomolecular composition (Komal Kumar and Devi Prasad, 2011). This study shows that the medicinal value of the ascidian *P. madrasensis* tissue may be due to high quality of bioactive metabolite.

The present study revealed that the species of *P. madrasensis* showed antibacterial activities against the human pathogens. So, they possess potential pharmacological action.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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