African Journal of Biotechnology

Volume 15 Number 39, 28 September 2016

ISSN 1684-5315

Academic Journals

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African Journal of Biotechnology

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Vol. 15(39), pp. 2124-2131, 28 September, 2016
DOI: 10.5897/AJB2016.15444
Article Number: 3158EF060671
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular identification of *Anopheles gambiae sensu stricto* Giles (formerly *Anopheles gambiae* Savannah Form) in Kamuli District, Uganda

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Received 1 May, 2016; Accepted 25 July, 2016

Anopheles gambiae sensu stricto **Giles (formerly *A. gambiae* S molecular form), the largely anthropophilic species, is reportedly the most important malaria vector in Uganda among the *A. gambiae* complex species. Indoor and outdoor human-biting mosquitoes were caught for four consecutive nights in each of 48 households in Kamuli district using human-baited bed net traps for subsequent identification of the principal *Anopheles* sibling species responsible for transmitting malaria. Sibling species under the *A. gambiae* complex were characterized by polymerase chain reaction using species specific single nucleotide polymorphism (SNPs) in the intergenic spacer region (IGS) with primers specific for *A. gambiae s.s., Anopheles arabiensis*, *Anopheles melas, Anopheles merus* and *Anopheles quadriannulatus*. Molecular forms of the *A. gambiae s.s.* were further discriminated using primers specific for Mopti and Savannah forms. Out of 300 *A. gambiae s.l.* amplified, 98% (n= 294) were *A. gambiae s.s.* Out of 142 *A. gambiae s.s.* samples analyzed for molecular forms, 78.9% (n=112) were identified as *A. gambiae s.s.* Giles *(A. gambiae* Savannah (S) form, while the other 21.1% were not identifiable. the presence of *A. gambiae s.s. Giles* in Kamuli was also reported. Considering the anthropophilic, endophagic and endophilic behavior of *A. gambiae s.s.* (and of the molecularly similar *A. gambiae s.s.* Giles), the combined use of insecticide-treated nets (ITNs), indoor residual spraying, larval source management and improved house design in the context of integrated vector management, may be the appropriate vector control strategies in the area. There is also need for regular monitoring of the vector species composition, distribution and behavior for proper planning of appropriate vector control interventions in the future.**

Key words: Sibling species, molecular forms, *Anopheles gambiae* complex, anthropophily, IPM.

INTRODUCTION

Malaria is endemic in Uganda and is the leading cause of illness especially among young children (Echodu et al., 2010; Lanier, 2012). The 2011 World Health Statistics showed that Uganda's malaria mortality rate of 103 per

100,000 was more than seven fold that of Kenya (12/100,000), 18% more than that of Tanzania and 9% more than that of sub-Saharan Africa (Ministry of Health, Uganda, 2011). In Eastern Uganda, malaria is endemic, with perennial and high levels of transmission, despite the widespread distribution of insecticide-treated nets (ITNs), in addition to other anti-malarial interventions in the region (Helinski et al., 2015; Ojuka et al., 2015). In Kamuli district too, malaria has remained the leading cause of morbidity and mortality in all age groups with 38.4% of all diagnosed patients being malaria cases. The district is one of the few districts in Uganda with a good number of Non-Government Organizations (E.g. Plan International, Christian Children's Fund, etc) that have intervened with large supplies of free ITNs since the late 1990s to curb the high malaria challenge. Nevertheless, malaria has remained a very big challenge (Kamuli District Health Sector Strategic Plan, 2005/06-2010; Kamuli District Health Status Reports, 2005/06-2009/10, Unpublished). Members of *Anopheles gambiae* complex, known for being one of the most efficient vectors of human malaria in the world (Cohuet et al., 2003; Mayagaya et al., 2009), are the most common vectors in most parts of Uganda (Echodu et al., 2010). *A. gambiae sensu stricto,* one of the recognized sibling species of the complex and known for being the most anthropophilic malaria vector in Africa (Toure' et al., 1994) is the most important vector of human malaria parasites in Uganda (MoH, Uganda, 2005; Echodu et al., 2010).

The major vector control interventions like the rapid scale-up of ITNs and IRS have been deployed without a detailed understanding of the species composition, distribution and behaviour dynamics of the local vectors. This may complicate impact monitoring (Coetzee et al., 2000).

The principal mosquito species under the *A. gambiae* complex responsible for transmission of malaria parasites in Kamuli district were not yet known, while the molecular forms of *A. gambiae s.s.* that occurred in this area and Uganda as a whole, that is, *Anopheles coluzzii* and *A. gambiae s.s.* Giles (formerly "*A. gambiae* molecular "M" and S" forms, respectively) (Coetzee et al., 2013; Sawadogo et al., 2013), were also not yet known. Perhaps there was a particular species/molecular form responsible for the transmission.

The knowledge of which form, *Anopheles coluzzii*, *A. gambiae s.s.* Giles, or both of them occur in Uganda will have epidemiological implications in terms of vectorial capacity, distribution range and susceptibility to the currently available vector control interventions. The two molecular forms, *A. coluzzii* and *A. gambiae s.s.* Giles have high vectorial capacity but differ in their susceptibility to pyrethroids (Lehmann and Diabate, 2008), the preferred class of insecticide for treatment of long lasting bed nets (Harris et al., 2013). Thus, identification of the sibling species and molecular forms using molecular methods can have important implications in subsequent planning and implementation of the most appropriate vector control measures (Coetzee et al., 2000; Fanello et al., 2002; Koekemoer et al., 2002).

This study was therefore aimed at establishing the principal *Anopheles* mosquito sibling species and molecular forms within the *A. gambiae* complex responsible for transmission of malaria parasites in Kamuli District, Uganda, as part of a major study to investigate the effect of long-term use of insecticide-treated bed nets on the biting behaviour and vectorial capacity of *A. gambiae s.l.* and *A. funestus* group in this part of the country.

MATERIALS AND METHODS

Study area and mosquito sampling

The study was conducted in Kamuli district in Eastern Uganda, located at (01° 05'N 33° 15' E), 68 km North of the source of River Nile. Kamuli district was chosen because several villages were well supplied with Insecticide Treated Bed Nets (ITNs) that were given to the population by NGOs in an effort to supplement government efforts to control malaria among particularly vulnerable groups–pregnant mothers, children under five years and the People Living with HIV/AIDS.

Mosquitoes were collected from forty-eight households randomly selected from ten villages with five villages in Kamuli Town Council and Nabwigulu Sub County where ITNs had been in use for over five years, with 69% of the households having at least one ITN. These were the intervention villages. The other five villages were located in Bugaya and Buyende sub counties where ITNs had not been in use before the entomological survey and comprised the non-intervention zone. These were in the North East of Kamuli Town Council, and well over twenty kilometers away from the intervention zone.

Both intervention and non-intervention zones were in the same climatic and ecological zone (NEMA, 2007) and were surrounded by a variety of vegetation types including swamps, crop fields and grazing lands. Therefore, at the time of entomological sampling, ITN use was taken to be the only unique factor between the two study zones.

From December 2009 to November 2010, hourly indoor biting mosquitoes were collected from 19:00 to 07:00 h for four consecutive nights per month by a two-person team of trained catchers using bed net traps (Okello et al., 2006). The bed net trap was made by making a 3 x 3 inch hole on each of the sides of an untreated bed net, making a total of 4 to 6 holes on the net. The catcher sat under the bed net trap which gave him some protection which is denied when the human-landing catch method is used. This method was preferred to the CDC light trap (used initially) which was more costly to run overnight, requiring replacement of batteries after a few days. Outdoor human biting catches were carried out concurrently using the same method at the same household ten meters away (Okello et al., 2006).

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People living in a room were protected with an untreated net each, and as hungry mosquitoes persisted in their attempts to look for a blood meal, they got near the human-baited trap and were caught (Lines et al., 1991) by the human bait (collector) using an aspirator and a torch (Okello et al., 2006). It was assumed that the mosquitoes that entered a trap during any hour were those actively seeking hosts, and, in most cases, would bite human hosts in the same hour and room/house if the bed net trap was absent (Maxwell et al., 1998).

The indoor and outdoor human-biting fractions of the *Anopheles* mosquitoes were determined and recorded throughout the whole sampling period for both intervention and non-intervention zones. Each hourly catch were separately placed in a disposable polystyrene container pre-labeled with date, time and location of capture and taken to the laboratory for identification of mosquitoes collected (Curtis et al., 1998). Mosquitoes were kept alive by providing them with a 10% sugar solution to feed on through a cotton wick (Styer et al., 2007).

Morphological identification

Each hourly catch of the human-biting fractions of the mosquito population were identified morphologically using a simplified key adopted from Gillies and Coetzee (1987), while the morphological identifications were confirmed by an Entomologist at the Vector Control Division, Ministry of Health, Uganda.

Extraction of DNA, PCR amplification and species identification

To confirm and improve on the accuracy of the morphological identification, PCR techniques were used to separate the *A. gambiae complex* samples. A total of 300 mosquito samples (150 samples from each of the two zones) were characterized into sibling species under the *gambiae* complex using species specific single nucleotide polymorphism (SNPs) in the intergenic spacer region (IGS) using primers specific for *A. gambiae sensu stricto, Anopheles arabiensis*, *Anopheles melas Anopheles merus* and *Anopheles quadriannulatus* (Scott et al., 1993; Mbogo et al., 1996; Curtis et al., 1998). Same proportions of the samples caught indoors and outdoors were analyzed (n = 75) by PCR. At least fifty samples were randomly selected from each of the 1st, 2nd and 3rd (last) of the night collections in both intervention and non-intervention zones. In cases where samples caught in any third of the night were less than 50, all the samples were taken for characterization.

All the members of the *A. gambiae* complex were discriminated by the SNP based PCR with primers that bind to the SNP sites utilizing Intentional Mismatch Primers (IMPs) within the intergenic spacer region, producing unique bands for each of the species and sub-forms (Wilkins et al., 2006).

One to two legs or wings of a single adult mosquito (as DNA source) were placed in 22.0 µl of PCR reaction mixture. This mixture contained the following: 14.5 µl of double distilled water, 5.0 µl of 5X High Fusion reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM $MgCl_2$), 0.5 µl of 10 mM dNTPs,1.5 µl of UN, ME, AR, GA and QD primers (synthesized by Inqaba Biotechnical Industries, Pretoria, South Africa) and 0.5 µl of Phusion ^{TM} DNA polymerase. Finally, 3.0 µl of double distilled water was added to make the total volume 25.0 µl and the reaction mixture was agitated a few times [($MgCl_2$, Buffer, Phusion ^{TM} DNA polymerase and dNTPs (supplied by Celtic Molecular Diagnostics (Pty) Ltd, South Africa)].

Negative controls containing PCR reaction mixtures without DNA were added to each PCR experiment. The PCR reaction conditions were as follows: Initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final auto extension at 72°C for 5 min. A volume of 10.0 µl of the PCR product was mixed with 3.0 µl of ficoll dye (50% sucrose, 0.05 M EDTA pH 7, 0.1% bromophenol blue, 10% ficoll powder) and loaded on 2% agarose gel stained with 12.0 µl ethidium bromide (10 mg/100 ml) (Cat. No. 15585-011, Gibco BRL, UK), submerged in 1XTAE buffer and electrophoresed at 100 V for one hour. 5.0 µl of molecular marker (Gene Ruler TM DNA ladder Mix, Cat. No. SM0331) was loaded on the first well of the agarose gel, followed by the wild samples in the next wells, positive control in the second last well and the negative control in the last well.

The DNA fragments were visualized under ultra violet (UV) light and the size of the products was confirmed using the molecular ladder. Molecular forms of the *A. gambiae sensu stricto* were further discriminated by PCR using primers specific for *A. coluzzii* (formerly *A. gambiae* Mopti or 'M' form) and *A. gambiae s.s.* Giles (formerly *A. gambiae* Savannah or 'S' form), that is, M_3, M_5, S_3 and S_5, using the same PCR protocol as described above (Scott et al., 1993) using the following cycling conditions: Initial denaturation at 98°C for 3 min, 39 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final auto extension at 72°C for 7 min. Samples were run on a 2% agarose gel for visualization using standard markers.

Specificity of the *A. gambiae sensu lato* primers was checked by using three samples of *A. gambiae s.s.* positive controls. A sample known to contain trypanosomes was also included in checking the specificity of the primers. The *A. gambiae s.s.* DNA positive controls were obtained from the Uganda Virus Research Institute's Entomology Laboratory in Entebbe, while characterization of molecular forms was confirmed using positive controls in Benin's Ministry of Health Entomology laboratory.

Ethical issues

Prior to start of the study, approval was obtained from the Uganda National Council for Science and Technology and Health Research Ethics Committee (Reference Number: HS 263).

Household owners, village and district authorities were sensitized prior to the study and their permission was obtained, while the privacy and psycho-social needs of the individual participants and household members were highly protected. Catchers were selected from the local community to facilitate acceptance from residents. Informed consent was obtained from each catcher.

The catchers were trained to collect landing mosquitoes prior to blood feeding to minimize the risk of malaria transmission. They were given anti-malarial drugs as this geographical area has high transmission of *Plasmodium falciparum* with resistance to anti-malarial drugs (Dr. Lopita Micah, Pers. Communication). At least two bed nets (LLNS) were donated to each participating household following the study.

Statistical analysis

Comparison of the indoor and outdoor human biting catches of the *A. gambiae* complex and *A. funestus* group of mosquitoes for the whole sampling period between the intervention and non-intervention zones was done using the Kruskal-Wallis rank sum test of the R-Statistics software, version 2.15.0 (2012.03.30) (R Development Core Team, 2002), given their non-normal distribution.

RESULTS

Morphological identifications

Over 70% of the *Anopheles* species caught were *A.*

Table 1. Numbers of female *Anopheles* mosquitoes caught indoors and outdoors in both non-intervention and intervention zones over a 12-month sampling period.

Mosquito group	Non-intervention zone		Intervention zone		Total	%
	Indoor	Outdoor	Indoor	Outdoor		
Anopheles gambiae s.l	853	1079	299	346	2,577	73.2
Anopheles funestus s.l.	453	411	39	39	942	*26.8*
Total	1,306	1,490	338	385	3,519	

Other mosquito species caught but not included in this table were: *A. mouche*ti (n > 500), *Culex* species (n> 1,840) *and Aedes aegypti* (n > 150).

Table 2. Polymerase chain reaction (PCR) primers and sizes of the amplified products for species within *A. gambiae* complex (Scott et al., 1993).

Primers	Primer sequence (5' to 3')	Temperature (°C)	Identified species	Size of the PCR product (bp)
UN(F)	[GTG TGC CCC TTC CTC GAT GT]	58.3		
GA(R)	[CTG GTT TGG TCG GCA CGT TT]	59.3	*A. gambiae s.s.*	390
ME (R)	[TGA CCA ACC CAC TCC CTT GA]	57.2		
AR (R)	[AAG TGT CCT TCT CCA TCC TA]	47.4		
QD (R)	[CAG ACC AAG ATG GTT AGT AT]	42.7		

TM = Melting temperature; bp = base pairs. UN primer anneals to the same position of the rDNA of all the five species, GA anneals specifically to *An gambiae*, ME anneals to both *A*. *merus* and *A*. *melas*, AR anneals to *A*. *arabiensis* and QD anneals to *A*. *quadriannulatus.* F= Forward orientation; R= reverse orientation.

gambiae s.l, and 26.8% were *A. funestus* group. Other mosquitoes caught included *A. moucheti*, *Culex* and *Aedes* species. Based on morphological identification, there was approximately four times more *Anopheles* spp. caught in the non-intervention as compared to the intervention zone (2,796 and 723 anophelines in the respective zones (Table 1); Chi-squared = 159.894, df = 1, $P < 0.001$). For both zones, *A. gambiae s.l.* catches exceeded those of *A. funestus* (Chi-squared = 86.662, df = 1, $p < 0.001$), this trend being greater in the intervention zone. Outdoor biting apparently exceeded the indoor biting catches although with no statistically significant difference (Chi-squared = 0.227, df = 1, $p > 0.05$). Detailed biting behavior and seasonal abundances of the *Anopheles* mosquitoes were discussed in another earlier study (Kabbale et al., 2013).

PCR amplification and identification of species and molecular forms

Fifty percent (50%) of the indoor and outdoor catches from both intervention and non-intervention were amplified by PCR. A total of 300 samples of *A. gambiae s.l.* was amplified of which 98% (294 out of 300) were all identified as *A. gambiae sensu stricto* (Table 2 and Figure 1). The identity of the remaining 2% of the *A. gambiae s.l.* could not be established. Out of the 294 identified samples, 145 (49.3%) and 149 (50.7%) were from intervention and non-intervention zones, respectively. Approximately 50% of the identified *A. gambiae s.s.* samples were caught from indoors and outdoors in both intervention and non-intervention zones.

Out of 142 *A. gambiae s.s.* samples analyzed for molecular forms (50%, n= 71, from each zone), 78.9% (112 out of 142) were identified as *A. gambiae* s.s. Giles (Formerly Savannah (S) form) (Table 3 and Figure 2). The remaining 21.1% (30 out 142 *A. gambiae s.s.*) could not be identified. The results therefore showed that *A. gambiae s.s.* Giles exists in Kamuli district, and probably other parts of the country.

DISCUSSION

Morphological identifications and *Anopheles* species abundance

The difference in relative proportions of *A. gambiae s.l.* and *A. funestus* (89.2 and 10.8% respectively) in the intervention zone was observed to be much higher than the difference in the relative proportions in the non-intervention zone (69.1 and 30.9% for *A. gambiae s.l.* and *A. funestus* group, respectively. The lower mosquito abundance in the intervention zone is probably suggestive of effectiveness of the vector control intervention (ITNs/ LLINs) under use in this zone as compared to the non-intervention zone without treated bed nets. However, this

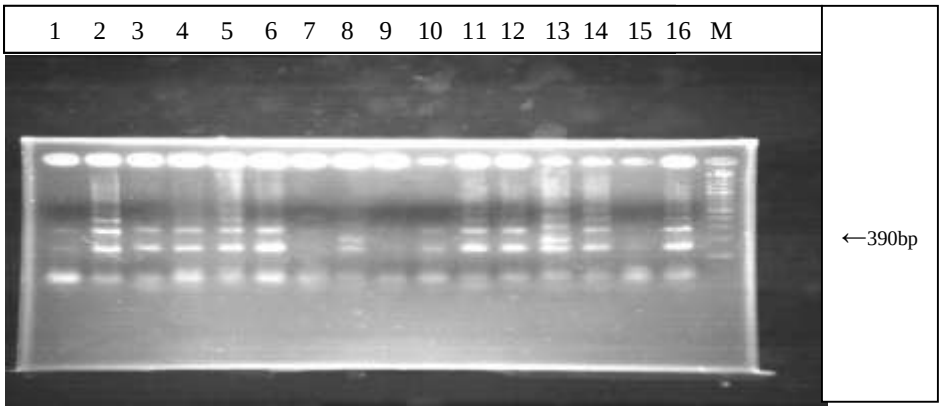

Figure 1. PCR-amplified fragments for identification of *Anopheles gambiae* complex species: Lane 1: Negative control, Lane 2: *A. gambiae s.s.* positive control, Lanes 3-16: *A. gambiae s.s.*, Lane M: 100-basepair, DNA size marker ladder.

Table 3. Polymerase chain reaction (PCR) primers and sizes of the amplified products for molecular forms within the *Anopheles gambiae sensu stricto* (Scott et al., 1993; Wilkins et al., 2006).

Primers primer sequence (5' to 3')	Temperature (°C)	Identified forms	Size of the PCR product (bp)
M5 (F) [CTT GGT CTG GAG ACC GTT CCa TA]	59.5		
M3 (R) [GAC ACG TCA ACT AAG TCA ACA CAT tAC]	58.2		
S5 (F) [GCC CCT TCC TCG ATG Ga GC]	61.5		
S3 (R) [CAA CCG GCC CAA ACG GcT T]	59.4	S (*A. gambiae s.s*. Giles)	335

TM = Melting temperature; bp = base pairs; M3, M5, S3 and S5: Specific primers for the identification of molecular forms under *A. gambiae sensu stricto.*

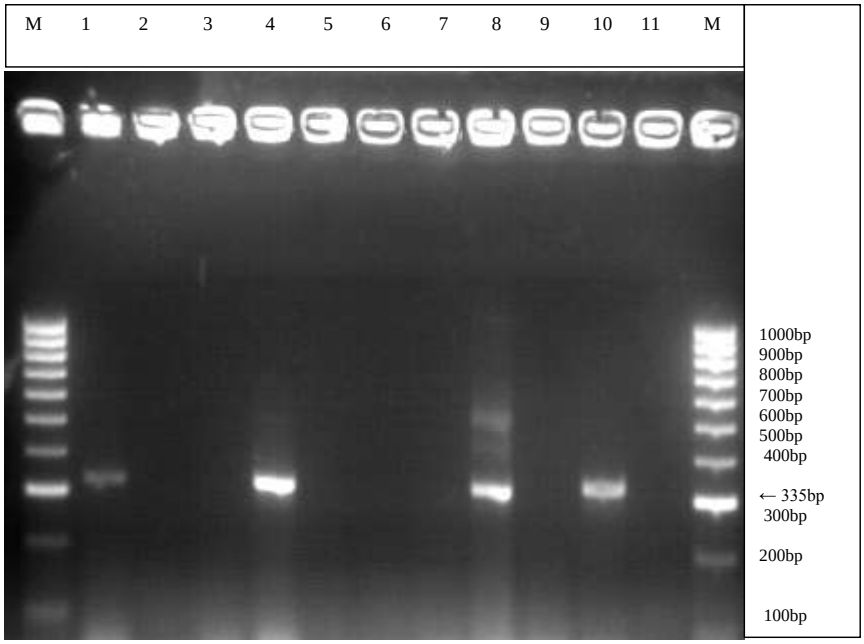

Figure 2. PCR amplified fragments using M3, M5, S3, and S5 Specific primers for the identification of molecular forms under *Anopheles gambiae sensu stricto.* Lanes M and M, 100-base pair DNA size marker ladder, lanes 1, 4, 8 and 10: *A. gambiae s.s.* Giles (formerly molecular form S).

could not rule out the fact that there could be other prevailing ecological or human behavioural factors in the intervention zone.

The relatively higher proportion of the *A. funestus* group in the non-intervention zone could be attributed to the presence of more permanent water for breeding provided by a larger swamp (Nabigaga) in this locality. *A. funestus* group is known to breed all year round and prefer permanent, stagnant water bodies such as shores of rivers and creeks, swamps or fish ponds for breeding, while *A. gambiae* complex breed in temporary/man-made water bodies e.g. pools, puddles or brick pits, fields, construction sites, hoof prints or even tyre tracks (Kabbale et al., 2013).

The presence of other mosquito species particularly *culex* species and *Aedes aegypti* poses a threat of other emerging and re-emerging parasitic and viral infections (Rozendaal, 1997).

Polymerase chain reactions

The *A. gambiae s.s.* positive control showed 2 to 3 bands. This could not be regarded as a hybrid of *A. gambiae s.s.* and *A. arabiensis* or *A. gambiae s.s.*/*A. arabiensis*/*An. melas,* nor could it have been a result of contaminations, but possibly due to unsuitability of one of the cycling conditions, or probably due to incomplete digestion of DNA by Taq polymerase. Therefore, all field samples that showed the same pattern of bands on the electrophoresis gel were regarded as *A. gambiae s.s.* positive samples. The challenge is the identity of the band below the 390 base pair band. This may call for later sequencing of the unidentified band and further characterization.

Molecular identifications and relevance to malaria epidemiology and control

In the present paper, based on molecular data, the presence of *A. gambiae s.s.* as the sole sibling species under the *A. gambiae* complex in Kamuli district, Uganda was shown. This is a species known for its highly anthropophilic, endophagic and endophilic behavior (Rozendaal, 1997; MoH, Uganda, 1999b).

These results are consistent with previous reports in Uganda that revealed the presence of *A. gambaie s.l*., (and *A*. *funestus)* as the main vectors responsible for transmission of human malaria parasites in the different regions of the country (MoH, Uganda, 2005) and the entire sub-Saharan Africa region (Cohuet et al., 2003; Mayagaya et al., 2009).

According to Ministry of Health, Uganda studies (Okello et al., 2006,), *A. gambiae s.s.* (33%) and *A. arabiensis* (39.5%) under *A. gambiae* complex and *A. funestus* (49%) under the *A. funestus* group were identified in Jinja area, which is located only 62 km away from Kamuli district, having the same ecological characteristics suitable for breeding of these species of *Anopheles* mosquitoes. However, some efforts to identify chromosomal or molecular forms of *A. gambiae s.s.* prevalent in Uganda did not yield results (Vector Control Division, Ministry of Health, Uganda, 2006-Un published report).

In the present paper, further discrimination of molecular forms under the *A. gambiae sensu stricto* revealed the presence of *A. gambiae s.s.* Giles in the study area. Therefore, it is reported here, the presence of *A. gambiae s.s.*Giles in Kamuli district.

These findings are consistent with an earlier report that *A. gambiae s.s.* Giles (formerly Savannah (S) form) is the most common and widespread in sub-Saharan Africa. This finding is also consistent with the ecological requirements for the *A. gambiae s.s*. Giles. This form is highly diverse and breeds in a wide variety of small, rain dependent habitats (Cuamba et al., 2006; Coetzee et al., 2013) including hoof prints and rice paddies. Such habitats are very predominant in the study area and most parts of Uganda, resulting from the increased economic activities for example cultivation for agricultural activities and constructions (MoH, Uganda, 2006). The *A. coluzzii* (formerly the mopti (M) form) is believed to breed in dry season and arid areas typical of the drier Northern savannah and Sahel zone of Senegal, many parts of West Africa and the Sudan (Toure et al., 1994).

The finding, however, may not completely rule out the possibility of co-existence of the two molecular forms (*A. gambiae s.s*. Giles and *A. coluzzii*), as was exceptionally reported in Kanyemba, the Zambezi valley, Zimbabwe (Masendu et al., 2004). Further analysis of more samples of *A. gambiae s.s.* mosquitoes sampled from different parts of Uganda need to be carried out to establish whether or not the *A. coluzzii* which is believed to be restricted to West Africa (Cuamba et al., 2006), contrary to other reports (Masendu et al., 2004), does exist. A fine population genetics analysis of *A. gambiae s.s.* samples using molecular markers may reveal presence of *A. coluzzii* in East Africa and suggest migration patterns (active or passive) of the mosquito populations (Samb et al., 2012; Coletta-Filho et al., 2011). This will enable mapping the distribution of the chromosomal form(s) of *A. gambiae s.s.* in the different ecological zones in the country.

The two molecular forms, *A. gambiae s.s.* Giles and *A. coluzzii* differ in ecological preference and their susceptibility to pyrethroids (Lehmann and Diabate, 2008), the preferred class of insecticide for treatment of long lasting bed nets (Harris et al., 2013). Therefore, findings of the study may provide evidence-based guidance in the planning and implementation of the most appropriate vector control interventions (Coetzee et al*.,* 2000), and monitoring insecticide resistance (Masendu et al., 2004), all geared towards malaria control and possibly

elimination (Moiroux et al., 2013).

Conclusion and recommendations

This study identified the principal sibling species under *A. gambiae* complex and the molecular form under *A. gambiae sensu stricto* that occur in Kamuli district, Uganda. Since *A. gambiae s.s.* Giles (formerly, the savannah form) identified in the study area molecularly belongs to *A. gambiae* sensu *stricto,* known for its highly anthropophilic, endophagic and endophilic behaviour, the use of long lasting insecticide treated nets, indoor residual spraying may be the appropriate vector control strategies in this part of the country. Additionally, considering the abundance of *Anopheles* breeding sites, larval source management, where appropriate and environmental hygiene strategies, and improved house design to reduce the indoor and outdoor human biting densities may be promoted in this area in the context of integrated vector management strategy. There is also need for regular monitoring of the vector species composition, distribution and behavior for proper planning of appropriate vector control interventions in the future.

Conflict of interest

The authors declare that they have no conflict of interest.

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Vol. 15(39), pp. 2132-2145, 28 September, 2016
DOI: 10.5897/AJB2016.15571
Article Number: B0B9BDF60673
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro methods for mutation induction in potato (*Solanum tuberosum* L.)

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Received 18 July, 2016; Accepted 7 September, 2016

Potato (*Solanum tuberosum* L.) is an important vegetable and staple crop worldwide and mainly propagated vegetatively. Breeding of potato is problematic and therefore induced mutation is an attractive means of improving the crop. *In vitro* culture systems, and especially the production of micro-tubers, are ideal for such purposes in potato improvement. Radio-sensitivity testing (growth reduction, GR and lethal dose, LD) allows the determination of irradiation treatments (Gy) for mutation induction. Three schemes incorporating *in vitro* techniques were tested for mutation induction in potato namely: 1) irradiation of cuttings without leaves and subsequent dissociation of chimeras to produce plantlets or micro-tubers on M_1V_2 (or further generation) plantlets, 2) irradiation of cuttings with leaves and direct induction of mutant micro-tubers, and 3) induction and irradiation of micro-tubers. Variability among the potato genotypes to gamma irradiation was recorded. Optimized irradiation treatments for mutation induction were established for the various tissues/propagules: cutting growth (GR_{50}, 9-6 to 20.6 Gy), cutting tuberization ability (LD_{50}, 7.3 to 13 Gy) and micro-tuber sprouting ability (LD_{50}, 20.6 to 54.8 Gy). Micro-tubers were found to be more resistant for *in vitro* mutation induction than *in vitro* cuttings. This study shows the susceptibility of different plant tissue/propagule and potato genotypes to gamma irradiation. Radio-sensitivity analyses showed that lower gamma doses are required when mutation induction is applied in combination with micro-tuberization.

Key words: Potato, gamma irradiation, stem cuttings, micro-tubers, *in vitro* tuberization.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important vegetable and staple food crop worldwide consumed by over one

billion people. Its annual production of over 368 million tonnes ranks fourth after maize, rice and wheat (Albiski et al., 2012; Food and Agricultural Organization (FAO), 2014). In addition to high starch levels, potato tubers contain significant amounts of antioxidants, protein, vitamins (C and E), macro- and micro- nutrients (calcium, magnesium, iron and zinc), polyphenols, carotenoids and tocopherols (Brown, 2005), which are important for the human diet.

Potato belongs to the family Solanaceae and depending on the purpose, can be propagated through seed, axillary buds, apical meristems, synthetic seeds, tubers, mini-tubers and micro-tubers (Sharma et al., 2007; Badoni et al., 2010). With over 160 potato species (wild and cultivated), the Solanaceae family has a large gene pool (Grüneberg et al., 2009). However, success in breeding new cultivars, utilising these resources, has been slow, mainly because the crop is clonally propagated and highly heterozygous. For example, potato breeding in China, which has the biggest potato production in the world, is based on a narrow genetic base due to common pedigrees of breeding materials (Cheng et al., 2010). This low genetic diversity among cultivars represents a serious limitation to crop improvement, especially in the emergence of new diseases, pests and climatic changes.

Potato is considered to be among the most important clonally propagated crops, including cassava, sweet potato, yam, taro, sugar cane, banana and plantain (Grüneberg et al., 2009).

Major problems affecting potato production are: low multiplication rates in the field under conventional (biological) seed production, and yield loss due to susceptibility to diseases and pests such as late blight disease, potato cyst nematode and Colorado beetle (Evans et al., 1992; Mahfouze et al., 2012). In developing countries, many traditional cultivars suffer from poor yield with reduced tuber size and have undesirable traits such as sunken eyes, which reduce their market value. Potato, *S. tuberosum* is a tetraploid, outbreeding species that maintains a high degree of heterozygosity; therefore, it is mainly propagated vegetatively. Consequently, biological seed, which is heterogeneous, does not present a suitable material for mutation induction in potato (Sharma et al., 2007). *In vitro* culture of vegetatively propagated crops in combination with radiation induced mutation has proven to be a valuable method to broaden genetic variability (van Harten and Broertjies, 1989; Elias et al., 2009; Cheng et al., 2010; Mahfouze et al., 2012; Yaycili and Alikamanoglu, 2012; Jankowicz-Cieslak et al., 2012). Ionizing radiation was indicated as a potent method to generate new genetic variability for crop improvement (Stadler, 1928; Ahloowalia and Maluszynki, 2001). Furthermore, the main aim and advantage of mutation induction in vegetatively propagated crops is the ability to change one or a few characteristics without changing the elite cultivar genetic background (Ahloowalia, 1995; Broertjes and van Harten, 1988), that is having a low mutational load.

Since the pioneering work of Asseyeva (1931) in potato, mutation induction in potato has produced mutants for diverse traits such as modified starch biosynthesis (Cieśla et al., 2002; Muth et al., 2008), increased yield (Al-Safadi et al., 2000; Li et al., 2005) modified histological and texture properties (Nayak et al., 2007), long shelf-life (Baskaran et al., 2007) and increased tolerance to abiotic and biotic stresses (Al-Safadi and Arabi, 2003,2007; Albiski et al., 2012). From 1931 to 2015 only 6 potato improved cultivars have been registered in the Food and Agricultural Organization/ International Atomic Energy Agency (FAO/IAEA) mutant's database (http://mvd.iaea.org). Most mutation induction, using physical and chemical mutagens, for potato improvement reported by previous studies was conducted using *in vitro* cuttings, *in vivo* tubers and mini-tubers. Today, the *in vitro* micro-tuber represents another major target for mutagenesis. This study therefore, aimed to developed mutation induction methods that target *in vitro* micro-tubers.

Prior to mutation induction, radio-sensitivity tests need to be performed to determine the optimal dose treatment for mutation induction. This consideration is even more important for vegetatively propagated crops, because of the impossibility to restore the genetic background by backcrossing. It is important to note that the mutagenesis reported by different studies on micro-tuber induction and gamma irradiation were for the purpose of enhancing the micro-tuber production with minimal genetic change (Al-Safadi et al., 2000; Li et al., 2005; Mahfouze et al., 2012). In this study, the susceptibility of 8 different potato genotypes (landraces and commercial cultivars) to gamma irradiation was determined. The data provide useful information in optimizing irradiation treatments for mutation induction, which may be applied to other genotypes.

MATERIALS AND METHODS

Plant

Eight potato (*S. tuberosum*) cultivars known to be grown in Kenya (Mpya, Sherekea and Asante), Lesotho (Basotho Pink, BP1, Up-To-

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Date and Mondial) and Morocco (Kondor) were used in this study. Important characteristics of these cultivars for improvement are given in Table 1.

Conventional tubers were used as starting material and these were supplied from FAO/IAEA Member States, National Institute of Nuclear Energy, and grown up in the greenhouse at the FAO/IAEA Plant Breeding and Genetics Laboratory (PBGL), Seibersdorf, Austria to provide shoots as donor material to initiate *in vitro* shoot cultures as described as follows.

Tissue culture conditions

Young shoots from greenhouse grown plants were harvested and used to initiate *in vitro* cultures after sterilization of axillary meristems with 70% ethanol for 10 to 20 s, 20% commercial bleach for 15mins, and three rinses with sterile distilled water, operations carried out in a laminar flow bench. The propagation medium was based on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 0.18% gelrite as gelling agent. The pH of the medium was adjusted to 5.8. One node cuttings were placed in test tubes for initiation (1 to 3 per tube) and subsequently also for micro-propagation. Developing shoots were sub-cultured every 2 to 3 weeks and maintained in controlled environment rooms with 16 h fluorescent light (65 µmol/m2/s; using cool white fluorescent tubes, Philips TLP 36/86, Philips, Amsterdam, the Netherlands) at 22° ± 2°C. Rounds of sub-culturing continued until sufficient plantlets were obtained for mutation induction.

Tuberization conditions

In order to compare genotypic differences in micro-tuber production and the susceptibility of micro-tubers to gamma irradiation, one node cuttings with one leaf were transplanted to a modified medium from Hoque (2010) consisting of MS basal medium supplemented with 4 mg/L Kinetin, 8% sucrose and 0.18% gelrite. Differential responses to culture conditions have been reported for different genotypes (Piao et al., 2003; Hoque, 2010; Nistor et al., 2010; Kodym et al., 2012). In order to minimize these differences an attempt was made to identify a common medium that would work sufficiently well for all the eight genotypes. This was selected from media with various cytokinin (kinetin, benzyl adenine purine, and chlorocholine chloride) combinations with one sucrose concentration (8%). The pH of the micro-tuber induction medium was adjusted to 5.7. The cultures were incubated in the dark at 22° ± 2°C and developing micro-tubers were harvested. Radio-sensitivity tests were carried out 5 to 6 weeks after initial culture.

Irradiation methods

Standard gamma cells (220, Atomic Energy of Canada Limited, Ottawa, Canada) with ^{60}Co source with a low emission dose rate of 2 or 7.07 Gy/min were used for irradiation. The optimal dosage for mutation induction, GR_{30} and GR_{50} (30 and 50% growth reduction, respectively) as well as LD_{30} and LD_{50} (30 and 50% lethality dose) were determined for each potato genotype, using methods described by Kodym et al. (2012), to define the susceptibility. Three *in vitro* radio-sensitivity tests applicable to potato mutation induction were developed (Figure 1) involving different target tissues for irradiation, but also different patterns of regeneration:

Scheme 1A: *In vitro* single node stem cuttings (without leaves) were irradiated with 6 different doses and subjected to several rounds of *in vitro* shoot propagation to dissolve chimeras. Plantlets at the stage of M_1V_{3-4} were used for phenotypic and genotypic screening of mutants. Alternatively, in **Scheme 1B** after the dissolution of chimeras, micro-tubers were induced on the M_1V_{2-3} cuttings. Micro-tubers were used for field evaluation. Three replications with at least 20 uniform cuttings with one axial meristem per dose was selected and used to determine the optimal dose for mutation induction, the radio-sensitivity test with dose treatments ranging from 0, 5, 10, 15, 20 and 30Gy using 2 Gy/min gamma dose rate. After subsequent growth for a cycle of 2 to 3 weeks, plant height, fresh weight and number of nodes were recorded to assess the effects and the optimal dose of gamma irradiation. The plantlet height was used to determine optimal dosage for mutation induction as growth reduction GR_{30} and GR_{50}.

Scheme 2: *In vitro* single node stem cuttings (with leaves) were irradiated and induced to produce micro-tubers *in vitro* directly. The micro-tuber induction rate was used to determine the optimal dose for mutation induction using two replications of at least 36 cuttings with one axial meristem per dose ranging from 0, 3, 6, 9, 12 and 15 Gy using 2 Gy/min gamma dose rate. Tuberization was recorded as the number, weight and size of micro-tubers developed. The tuberization rate (%) was calculated as the number of nodal induced micro-tuber/number of planted × 100. Micro-tubers induced on *in vitro* plantlets at the stage M_1V_2 were used for mutation screening. The lethality dose, given as the reduction of the tuberization response at 30 and 50% (LD_{30} and LD_{50}) of the cuttings per genotype, were determined.

Scheme 3: *In vitro* micro-tubers were irradiated with 7 different doses. As a first step micro-tubers were produced in sufficient amounts and sorted for uniformity of size (medium and large) and weight. Radio-sensitivity tests were performed with 30 micro-tubers per dose using a wide dose range of: 0, 10, 20, 30, 40, 60 and 80 Gy using 7.07 Gy/min gamma dose rate. To facilitate sprouting, micro-tubers were placed on filter paper in Petri dishes moistened with 5 mg/L GA_3 and incubated in the dark for 24 h at 22° ± 2°C. Sprouting ability was assessed as a parameter to determine the vitality of the treated tissues. Micro-tubers were considered sprouted, when after 4 weeks the sprouting shoot length was equal or longer than the size of micro-tuber. The sprouting ability rate (%) was calculated as the number of micro-tuber sprouted/number of control micro-tubers sprouted × 100. Mutant plantlets at the stage of M_1V_1 cannot be used for phenotypic and genotypic screening of mutants as they are likely to be chimeric. Typical radio-sensitivity curves show genotype difference, but an enhancement of plant growth and tuberization at low doses, lethal effects at high doses were observed.

Statistical analyses

Analysis of variance (ANOVA) and least significant differences (LSD) of means (5% level) were performed using GenStat Release 9.2 for *in vitro* cuttings and tuberization and JMP statistics packages 12 for the micro-tubers sprouting ability.

RESULTS

Mutation induction schemes

Three mutation induction schemes were developed for *in vitro* tissues and organs of potato (Figure 1). In a first

Table 1. Characteristics of potato cultivars and target traits for their improvement by mutation induction.

Country	Cultivars	Characteristics	Traits to be improved
Kenya	Mpya	- Tuber yield 35-45 t/ ha - Oval/round, large size tubers - Early tuberization - Cream white skin colour with pink eyes - Shallow eye depth - Highly tolerance to late blight - Short dormancy - Requires 90 days to maturity	Increase yield
	Sherekea	- Tuber yield 40-50 t/ha - Oblong/round tubers - High number of tubers per plant - Red skin colour - Medium eyes depth - Highly tolerant to late blight and viruses - Good storability - Intermediate dormancy - Requires 105 -120 days to maturity	- Shorten dormancy period - Shorten the maturity period
	Asante	- Tuber yield 35-45 t /ha - Fairly tolerant to late blight - Intermediate dormancy Requires 90 -120 days to maturity	- Increase late blight tolerance - Shorten the maturity period - Shorten dormancy period
Lesotho	Basotho Pink	- High yielding - Sunken eye - Resistance to diseases and pests - Palatable - Drought tolerant - Intermediate dormancy - Requires 140 days to maturity	- Decrease eyes depth - Shorten maturity period - Increase frost tolerance
	BP1	- Acceptable yields - Moderately resistant to diseases and pests - Drought susceptible - Intermediate dormancy - Requires 90-120 days to maturity	- Reduce metallic taste - Increase drought and frost tolerance - Increase yield
	Up-to-date	- Acceptable yields - Moderately resistant to diseases and pests - Drought susceptible - Requires 110-120 days to maturity	- Reduce metallic taste - Drought and frost tolerance - Increase yield
	Mondial	- High yields - Susceptible to bacterial wilt - Taste palatable - Drought susceptible - Intermediate dormancy - Requires 90-120 days to maturity	- Increase wilt resistance - Increase drought tolerance

Table 1. Cont'd.

Morocco	Kondor	- High yields - Early bulking - High resistance to drought - Good resistance to *Potato Virus Y* - Shorten dormancy - Requires 110-120 days to maturity	- Increase resistance to common scab: *Streptomyces scabies*

step radio-sensitivity tests were conducted on different tissues (one node cutting with and without leaf and micro-tuber). In a second step different patterns of regeneration were compared. *In vitro* mutant plantlets or micro-tubers may be used for phenotypic or genotypic mutant screening.

Effects of gamma irradiation on in vitro cuttings

Analysis of variance of plant height on seedlings grown from *in vitro* cuttings of six potato genotypes exposed to different gamma irradiation dose (Scheme 1 and Figure 1) was significant ($P<0.05$) among irradiation treatments, genotypes and the interactions of dose*genotypes (Table 2). The results indicated that increasing doses of gamma irradiation progressively inhibited the growth of stem cuttings. The potato genotypes showed different responses (Table 3 and Figure 2). The effects of gamma rays were more pronounced on rooting, plant height and fresh weight than number of nodes and leaves for each genotype. Since further sub-culturing and chimera dissolution could be performed only with differentiated plantlets, plant height was considered for optimum dosage determination. A significant effect of irradiation on the plantlet height was recorded in the six potato genotypes (Table 3). Plant height showed growth retardation at relatively high doses such as 15 and 20Gy whereas a relatively low dose of 5Gy enhanced the growth of cultivars Mpya, Kondor and Mondial, which exhibited better growth than their respective untreated controls (Table 3 and Figure 2). Cuttings irradiated at doses equal to or above 15 Gy exhibited a very low root induction rate, and an undifferentiated shoot growth was recorded which had further negative impact on micro-tuber production. The plantlet height was used in determination of optimal dosage for mutation induction according to Kodym et al. (2012) as growth reduction GR_{30} and GR_{50} (Table 3). The results showed an expected variation in response among the cultivars: Mpya (GR_{50} = 20.6 Gy) and Sherekea (GR_{50} = 18.0 Gy) were relatively more radio-resistant than the susceptible genotypes BP1 (GR_{50} = 9.7 Gy) and Up-To-Date (GR_{50} = 9.9 Gy); whereas the cultivars Mondial (GR_{50} = 14.5 Gy) and Kondor (GR_{50} = 13.9 Gy) exhibited moderate resistance to gamma irradiation (Figure 2 and Table 3).

Effects of gamma irradiation on the tuberization

The effects of irradiation on micro-tuberization (Scheme 2 and Figure 1) showed that the untreated stem cuttings from cultivars Kondor and Basotho Pink had about 100% tuberization whereas other cultivars reached about 80% (Figure 3). A significant effect of gamma irradiation was recorded on tuberization of five potato genotypes and gamma irradiation doses ($P<0.05$) (Table 4). Increasing the applied dose of gamma irradiation diminished the tuberization response of all genotypes (Table 5 and Figure 3). However, a relatively low dose of 3 and/or 6 Gy increased tuberization rate of all potato genotypes except of Basotho Pink. The estimated LD_{50} showed that genotype BP1 was relatively more resistant to gamma irradiation than other genotypes. While genotypes Basotho Pink, Kondor and Mondial were moderately resistant, the genotype Mpya was susceptible (Table 5 and Figure 3).

Effects of gamma irradiation on sprouting ability of in vitro micro-tuber

Micro-tuber production showed variations in shape (oval and spherical), size (small, medium and big) and skin color (cream, purple, white with red spots, yellow) (Figure 4). Variability was also observed in initiation time and micro-tuber position on the stem (basal, axial or apical). Uniform micro-tubers were selected for radio-sensitivity tests (Scheme 3, Figure 1). The sprouting ability of micro-tubers was affected significantly by gamma irradiation dose and genotype (Table 6). The size of the micro-tuber had no effect on sprouting ability and the number of eyes sprouted was not significant, while emerged eyes ranged from 1 to 4 independently of the irradiation dose applied. Analysis of variance of sprouting ability was significant with the gamma irradiation dose and genotype by least significant differences of means testing ($P<0.05$) (Table 6). Doses of 10 and 20 Gy stimulated the sprouting ability of micro-tubers of genotypes Kondor and Basotho Pink, respectively. All doses above 40 Gy were completely

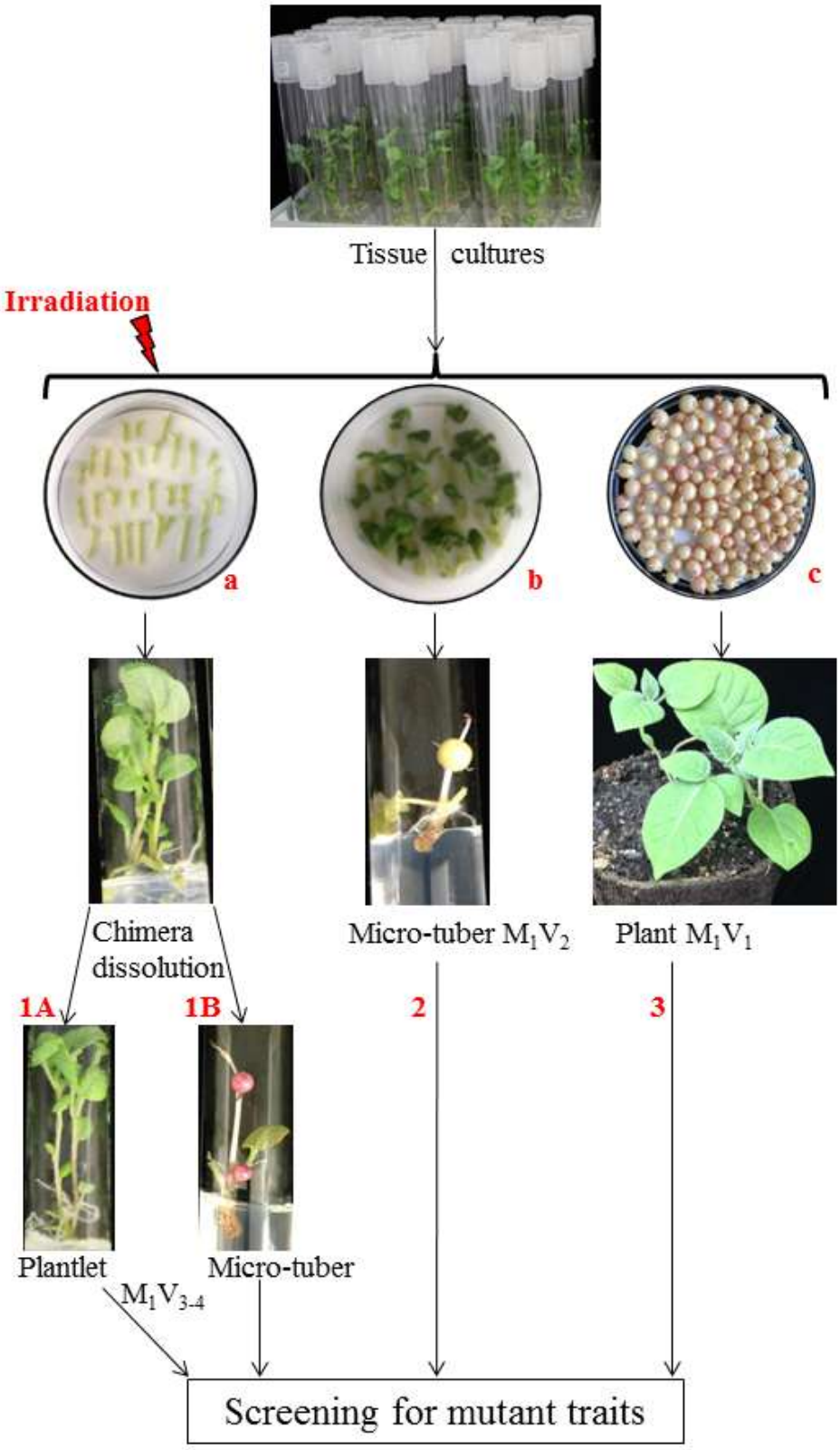

Figure 1. Mutation induction schemes for *in vitro* tissue cultures of potato: irradiation of a) cuttings with leaves and b) cuttings without leaves, c) micro-tubers; and screening of either *in vitro* plantlets or micro-tubers using strategies 1A or 1B, 2 and 3.

Table 2. Analysis of variance for effects of gamma irradiation doses on *in vitro* plantlet height of six potato genotypes.

Source of variation	DF	Mean Square	F-Value	P-value
Dose (Gy)	5	64.8253	216.78	<0.001
Genotype	5	2.3271	7.78	<0.001
Dose (Gy)*Genotype	25	1.8048	6.04	<0.001
Residual	70	0.2990		
Total	107			

Table 3. Mean and standard deviation of *in vitro* plantlet height (cm), and the respective mutation induction dose (GR_{30} – GR_{50}) for six potato genotypes.

Dose (Gy)	Genotype					
	BP1	Kondor	Mondial	Mpya	Sherekea	Up-to-Date
0	6.07±0.81^{a}	3.33±0.15^{b}	4.40±1.47^{b}	3.70±0.46^{b}	3.73±0.12^{a}	6.60±1.13^{a}
5	5.47±0.32^{b}	4.30±0.26^{a}	5.07±1.63^{a}	5.23±1.12^{a}	3.53±0.06^{a}	5.60±0.75^{b}
10	2.37±1.20^{c}	2.43±0.32^{c}	2.80±0.50^{c}	3.03±0.85^{c}	2.57±0.06^{b}	2.93±1.44^{c}
15	1.30±0.30^{d}	1.17±0.06^{d}	2.10±0.56^{d}	3.17±0.60^{c}	2.47±0.50^{b}	2.00±0.72^{d}
20	0.50±0.10^{e}	0.73±0.06^{e}	0.60±0.20^{e}	2.37±0.32^{d}	1.70±0.26^{c}	0.43±0.49^{e}
30	0.00±0.00^{f}	0.10±0.00^{f}	0.00±0.00^{f}	0.23±0.06^{e}	0.40±0.10^{d}	0.17±0.06^{e}
CV%	21.3					
LSD	0.3635					
GR_{30} (Gy)	6.8	10.5	11.5	16.5	13.4	6.7
GR_{50} (Gy)*	9.6^{c}	13.8^{b}	14.5^{b}	20.6^{a}	18.0^{b}	9.9^{c}

*Values in the same column followed by the same letter are not significantly different.

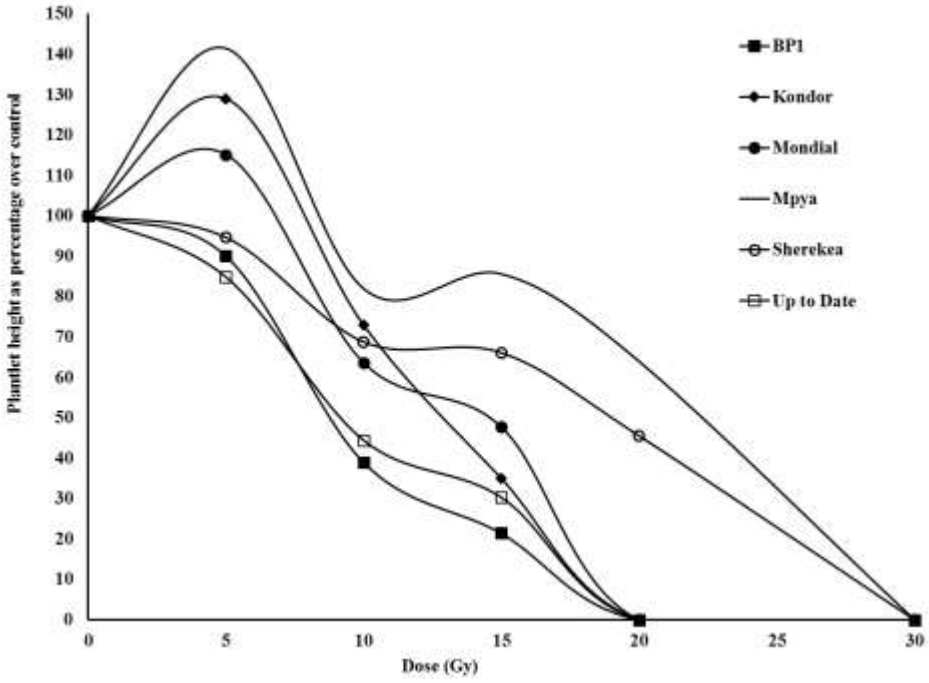

Figure 2. Effects of gamma irradiation on plant height of six potato genotypes after three weeks growth.

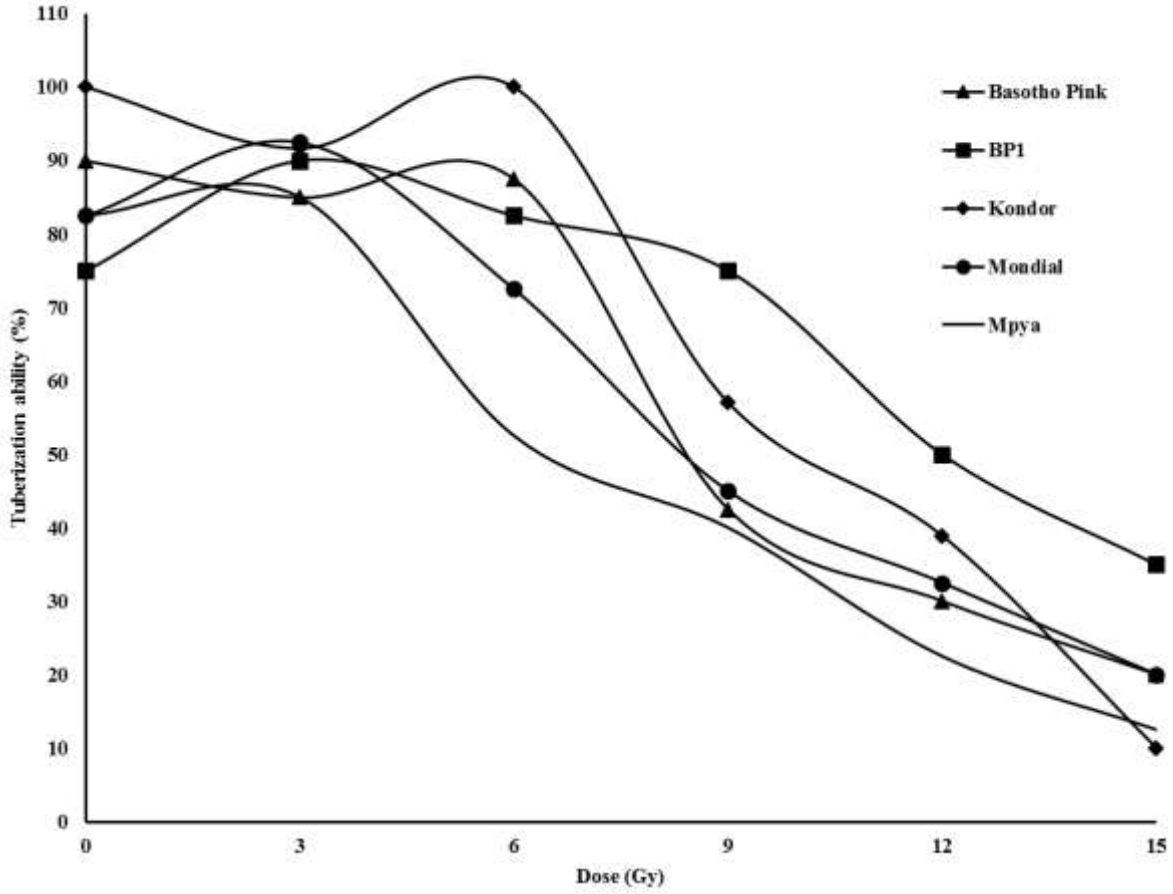

Figure 3. Effects of gamma irradiation on micro-tuber production of five potato genotypes after six weeks.

Table 4. Analysis of variance for effects of gamma irradiation doses on *in vitro* tuberization ability of five potato genotypes.

Source of variation	DF	Mean Square	F-Value	P-value
Dose (Gy)	5	0.842059	163.84	<0.001
Genotype	4	0.067864	13.20	<0.001
Dose (Gy)*Genotype	20	0.019673	3.83	<0.001
Residual	29	0.005140		
Total	59			

lethal for genotypes Mpya and Sherekea, whereas in other genotypes growth was severely retarded. Genotype differences for sprouting ability were observed among the genotypes investigated. Thus the genotypes Mpya and Up-To-Date were more radio-susceptible with observed LD_{50} of 20.6 and 26 Gy. Genotypes Asante, BP1 and Sherekea were moderately radio-resistant with LD_{50} between 32.4 and 35.5 Gy in comparison to relatively radio-resistant genotypes Basotho Pink and Kondor with 41.1 and 54.8 Gy, respectively to gamma irradiation (Table 7 and Figure 5).

DISCUSSION

Effects of increasing doses of gamma irradiation on *in vitro* cuttings of potato genotypes showed a significant growth decrease. High dose treatments of cuttings may also affect subsequent micro-tuber production when adopting Scheme 1B (Figure 1) due to difficulties in sub-culturing undifferentiated nodes. Plantlet height was negatively correlated with increasing applied dosage of gamma irradiation. These results agree with reports of Kodym et al. (2012) that plant height determined by cell

Table 5. Mean and standard deviation of *in vitro* tuberization ability rate (%), and the respective mutation induction dose (LD_{30} – LD_{50}) for five potato genotypes.

Dose (Gy)	Genotype				
	Basotho Pink	**BP1**	**Kondor**	**Mondial**	**Mpya**
0	90.00±0.00^{a}	75.00±7.00^{c}	100.00±0.00^{a}	82.50±4.00^{b}	82.50±4.00^{a}
3	85.00±7.00^{a}	90.00±0.00^{a}	91.67±4.00^{b}	92.50±4.00^{a}	85.00±7.00^{a}
6	87.50±4.00^{a}	82.50±11.00^{b}	100.00±0.00^{a}	72.50±4.00^{c}	52.50±11.00^{b}
9	42.50±11.00^{b}	75.00±7.00^{c}	57.03±0.06^{c}	45.00±7.00^{d}	40.00±14.0^{c}
12	30.00±14.00^{c}	50.00±0.00^{d}	38.89±0.07^{d}	32.50±1.00^{e}	22.50±4.00^{d}
15	20.00±7.00^{d}	35.00±0.00^{e}	10.00±0.00^{e}	20.00±7.00^{f}	12.50±10.00^{e}
CV%	11.9				
LSD	05.986				
LD_{30}(Gy)	6.4	7.6	8.7	5.7	4.0
LD_{50}(Gy)*	9.4^{ab}	13.0^{a}	10.5^{ab}	9.2^{bc}	7.3^{c}

*Values in the same column followed by the same letter are not significantly different.

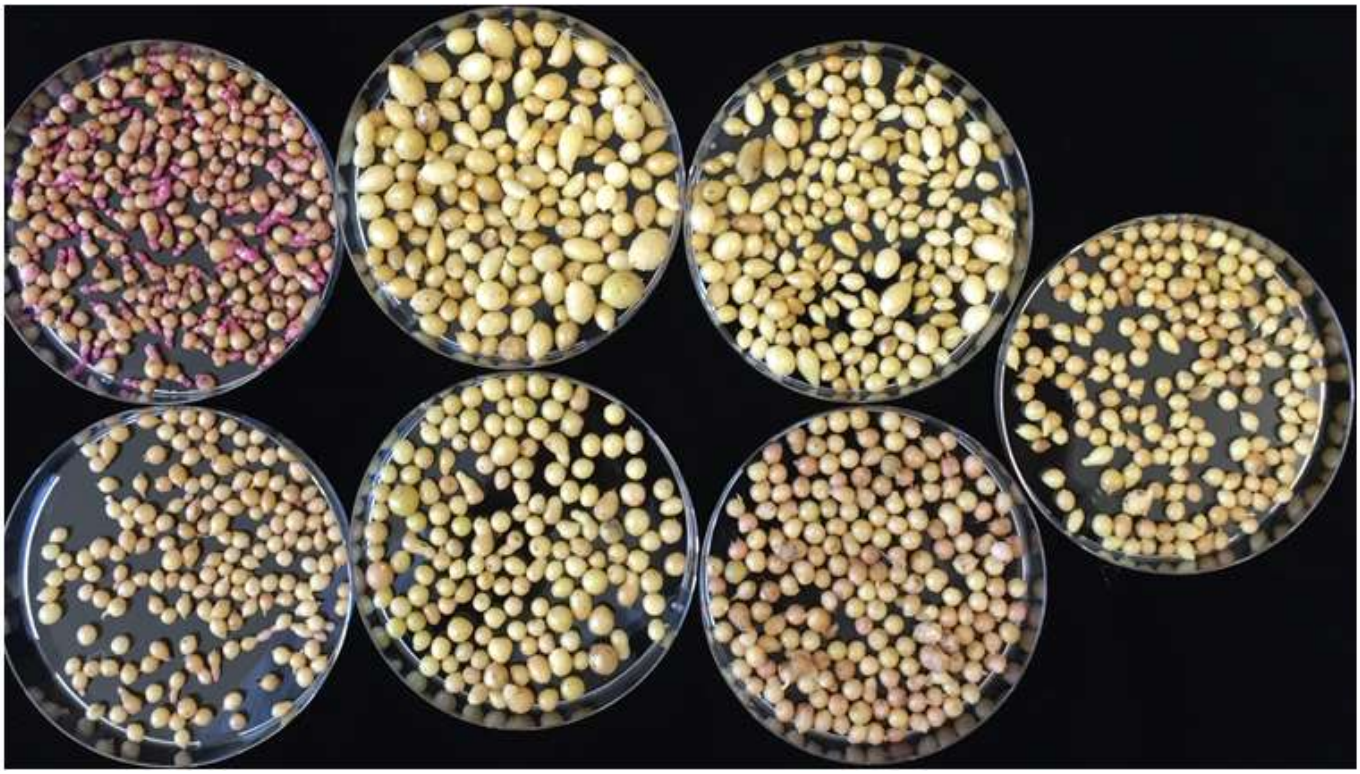

Figure 4. Micro-tubers of potato cultivars showing variation in shape, size and color): upper row, left to right: Basotho Pink, BP1, Up-To-Date, bottom row: Mpya, Asante, Sherekea and last to the right side: Kondor.

division as well as cell extension and provides a simple early measure of mutagenic treatment effects. Although reduced plant growth was recorded with increasing gamma irradiation dose for most genotypes, however the dose 5 Gy exerted a growth stimulation effect on the genotypes Kondor, Mondial and Mpya. The phenomenon of growth stimulation due to low irradiation treatments was recorded in the present study (Figure 2) and has been reported in M_1/M_1V_1 generation in many radio-sensitivity tests for seed and vegetatively propagated

Table 6. Analysis of variance for effects of gamma irradiation doses on sprouting ability of seven potato genotypes.

Source of variation	DF	ChiSquare	Prob>ChiSq	P-value
Dose (Gy)	1	83.158799	<0.001*	<0.00000
Genotype	6	18.460181	0.0052*	0.00518
Dose (Gy) *Genotype	6	1.6026054	0.9524	0.95239
Difference	13	127.0782	<0.0001	

Table 7. Sprouting ability rate (%) and the respective mutation induction dose (LD_{30} – LD_{50}) for seven potato genotypes.

Dose (Gy)	Genotype						
	Asante	Basotho Pink	BP1	Kondor	Mpya	Sherekea	Up-To-Date
0	76.92	88.00	75.00	96.97	86.96	90.48	92.31
10	75.00	88.00	65.00	100.00	78.26	80.95	83.33
20	53.85	88.46	50.00	93.55	65.22	68.42	65.39
30	57.69	65.39	40.00	75.76	21.74	42.86	46.15
40	57.69	50.00	20.00	68.75	4.17	27.27	11.54
60	28.00	21.43	10.00	34.38	0.00	23.81	7.69
80	11.54	8.33	0.00	25.00	0.00	20.00	4.00
LD_{30} (Gy)	12.9	27.9	15.4	41.3	13.9	15.7	17.05
LD_{50} (Gy)*	35.5^{bc}	41.1^{ab}	32.4^{bc}	54.8^{a}	20.6^{d}	33.8^{bc}	26.8^{cd}

*Values in the same row followed by the same letter are not significantly different.

crops (Al-Safadi and Simon, 1990; Wiendl et al., 1995; Paull, 1996; Jain et al., 2011; Cheng et al., 2010) confirming the present results. Generally, irradiation induced growth stimulation is observed with low dosage treatments, and is genotype dependant. Performing radio-sensitivity test on *in vitro* cuttings of three potato genotypes Al-Safadi and Arabi (2003) reported that 1, 5 and 10 Gy treatments of gamma rays stimulated post-irradiation plant growth. At higher dosages, DNA damage occurs more frequently and provides more mutation events but mostly lethal to plant survival (Preuss and Britt, 2003). The *in vitro* cutting radio-sensitivity test revealed resistant, moderately resistant and susceptible genotypes among the different potato genotypes. Genetic variation may explain the differential responses in different genotypes within a species, which are due to physical and biochemical characteristics of the tissue, such as propagule size, water content, DNA content, nuclear volume, etc.

In mutation induction, radio-sensitivity is performed with the purpose of selecting the optimal treatment for a specific genotype, that is, the dose that will provide the desired genotype (mutant trait in low mutational load genetic background) at a frequency that can be detected in a mutant population. This is especially important in vegetatively propagated crops - such as potato - as it is difficult to restore an elite genetic background by backcrossing. The optimal dose for mutation induction was found to be around 30 Gy for potato *in vitro* stem cuttings, which gave 50% reduction of the shoot length (Safadi and Arabi, 2003, 2007). The contrast of this high dose to observations in this study could be explained by the different genotype and also the radiation method and the application of the dose rate (chronic dose rate of 0.71 Gy/min) used. In order to produce the same relative biology effects, higher doses under chronic irradiation are needed than under acute irradiation because of plant tissue adaptation to irradiation (Esnault et al., 2010). The present data are in agreement with the results of Yaycili and Alikamanoglu (2012) on potato.

Two different schemes have been developed to induce and isolate potato mutants after mutagenesis on *in vitro* stem cuttings (Scheme 1A and B, Figure 1). A plantlet is the final product of chimera dissolution in Scheme 1A and may be subject to screening for desirable mutant traits (Safadi and Arabi, 2003, 2007; Esnault et al., 2010). Scheme 1B involves micro-tubers as the final products. The advantages of micro-tubers in comparison to plantlets are manifold (Nistor et al., 2010). In fact, the advantage of using micro-tubers in strategy 1B, 2 and 3

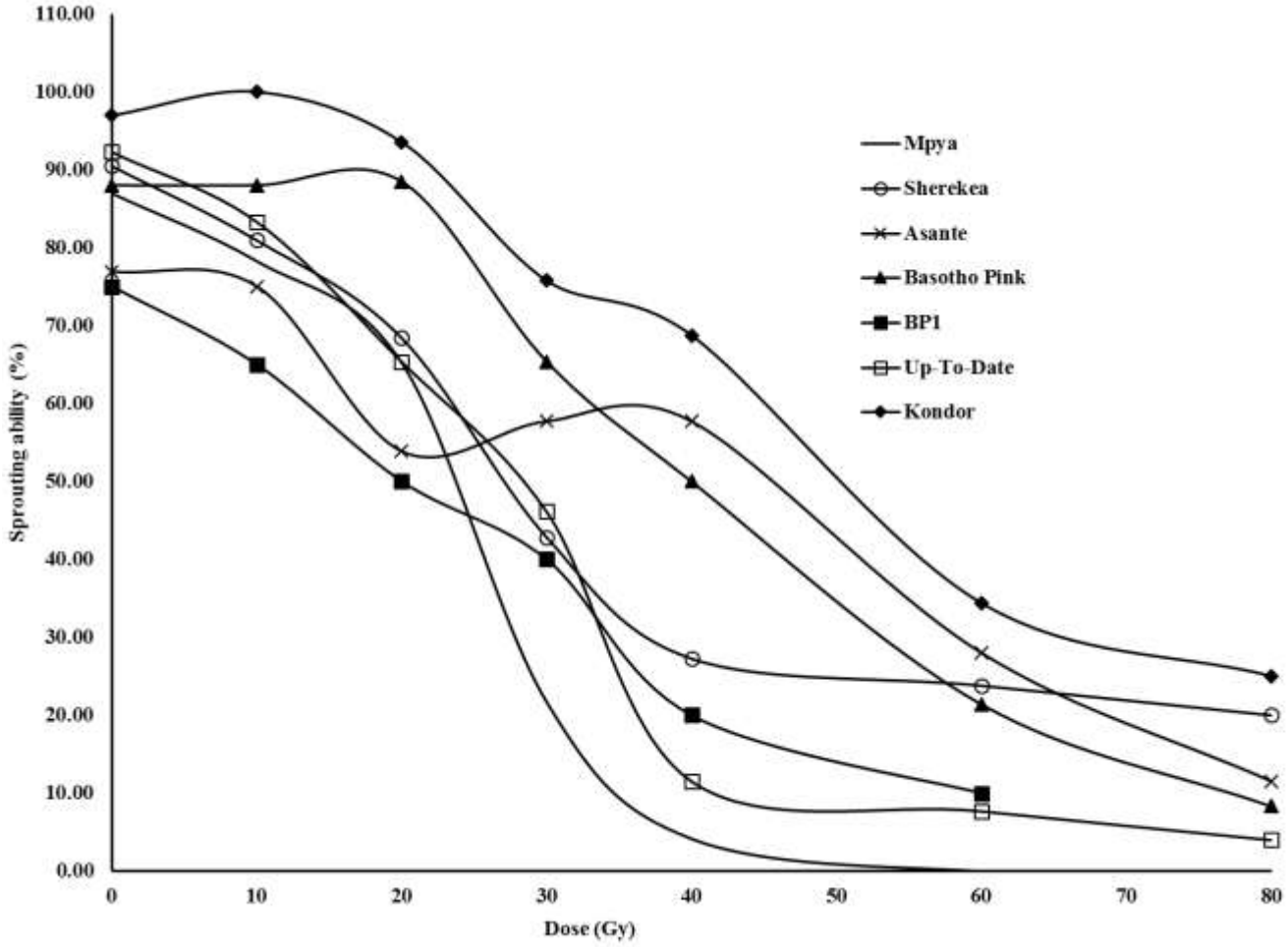

Figure 5. Effects of gamma irradiation on micro-tubers sprouting of seven potato cultivars after treatment with GA_3 and 4 weeks incubation.

over *in vitro* plantlets in strategy 1A is the higher vigour and vitality of micro-tubers. The production can be carried out all year-round; there is no need for immediate plant production as micro-tubers can be stored, and micro-tubers are easy to transport (Nistor et al., 2010) (Table 8).

The loss of *in vitro* plantlets before reaching field trials recorded during the acclimatization step is much higher, whereas micro-tubers sprouting ability can be enhanced to 100% using GA_3 and was even shown to enhance yield (Pruiski et al., 2003). Although *in vitro* plantlets can be maintained without sub-culturing for up to 2 months, they are sensitive to stress such as drought during acclimatization. However, micro-tubers withstand handling better and do not dry out as rapidly as plantlets. Micro-tubers are generally dormant and can be transported or shipped over long distances and stored for over 6 months. Additionally, micro-tubers may be used in early screening, which reduces field labour to evaluate the mutant lines. Over all, large scale handling like mutant population of plantlets requires more laboratory space and manpower for maintenance in comparison to micro-tubers (Table 8).

Advantages of micro-tubers were taken into consideration at the early stage during assessment effects of gamma irradiation on the tuberization. This study revealed genotype variation in tuberization capacity (Figure 3), as previously reported by Ahloowalia (1994, 1999) who investigated the ability of 15 potato cultivars to form mini-tubers. However, micro-tuber weight and size were also significantly determined by the genotypes (data not shown). Various weight, size and eyes of micro-tubers were recorded in each treatment and compared to untreated samples. These parameters are not recommended for Scheme 3 because the scattering of data for weight and size found for each dose makes the assessment difficult. In fact, similar findings on micro-

Table 8. Comparison of advantages and disadvantages of the three *in vitro* mutation induction schemes for potato.

Scheme	Advantages	Disadvantages
1A	1. *In vitro* screening for abiotic and abiotic stresses 2. Plant phenotyping 3. Advanced generation development	1. Chimera dissolution 2. Laborious maintenance 3. Incubation room space 4. Long period of mutant population development 5. Population size limited to original mutation induction size 6. Acclimatization losses 7. Specialized shipment
1B	1. – 3. as in 1A 4. Easy handling and transport micro-tubers	1. 5. as in 1A 6. Requires optimal tuberization conditions
2	1. Short period of population development 2. Easy handling and transport micro-tubers 3. Generation ready to be screened (nutritional, tuber shape, color) 4. Limited laboratory work	1. May require a pre-treatment for sprouting ability 2. Requires optimal tuberization conditions 3. Population size limited to original mutation induction size
3	1. 2. as in 2 3. May require less micro-tubers for large population size, since the population size is factor of number emerged eyes per tubers 4. Very limited laboratory work	1. 2. as in 2 3. Developed population is at first generation M_1V_1. 4. May be seasonal advanced in field

tuber weight and size after gamma irradiation of stem cuttings before tuberization were already reported (Al-Safadi et al., 2000; Li et al., 2005; Mahfouze et al., 2012). Rarely more than one micro-tuber was produced *per* cutting; which is convenient when adopting a 'single seed descendant' type approach in advancing potato lines from micro-tubers. Therefore, the tuberization capacity of cuttings was used to distinguish the radio-susceptibility of potato genotypes. Optimal dose established (LD_{50}) for mutation induction exhibited the relative resistance of genotype BP1, Basotho Pink, Kondor and Mondial to be moderate resistant, whereas Mpya was found to be radio-susceptible to gamma irradiation. A similar stimulation of micro-tuber induction by gamma irradiation was previously reported in different potato genotypes (Al-Safadi et al., 2000; Li et al., 2005; Al-Safadi and Arabi, 2003; Mahfouze et al., 2012). In this study the stimulation dose varied between potato genotypes, but remained below or equal to 10Gy under *in vitro* culture conditions. In addition to enhancing the tuberization rate, low gamma dose affects positively affected the content of ascorbic acid, reducing sugars and proteins of micro-tuber (Li et al., 2005). Low irradiation doses were reported to stimulate plant growth through enhanced physiological activity (Roy et al., 2009).

It is important to note that the mutagenesis reported by different studies on micro-tuber induction and gamma irradiation were for the purpose of enhancing the micro-tuber production with minimal genetic change (Al-Safadi et al., 2000; Li et al., 2005; Mahfouze et al., 2012). On the contrary, the attempt in this study was to determine the optimal dose treatments for mutation induction with the objective of generating mutant populations for screening in potato improvement, a unique approach to our knowledge. The most effective doses could be compared to stimulation doses. The optimal dose for mutation induction using scheme 2 (Figure 1) allowed the production of M_1V_2 generation micro-tubers. The advantage of this scheme is the direct production of micro-tubers at a stage, which can be screened for micro-tuber size, color, sprouting ability. In addition, micro-tubers may be evaluated for their biochemical content, but it is important to note that screening technique may be destructive (Table 8). An advantage of micro-tubers is they are easily multiplied and therefore reserve clones may be developed.

Effects of gamma irradiation on the sprouting ability of *in vitro* micro-tubers are comparable to seed mutagenesis with regard to dosage applied and shipment constraints. This represents an advantage for potato mutation

breeding, when the mutagenesis facility is not available in a given laboratory. Thus the study aimed to establish optimum dose for this type of potato propagule (Scheme 3 and Figure 1). The results of optimal dose (LD_{50}) established for the seven potato genotypes are comparable to gamma irradiation reported for mini-tubers of potato cultivar Shepody (Cheng et al., 2010). Doses of 10, 20 and 30 Gy promoted sprouting in mini-tubers whereas 60 Gy caused no sprouting. This study presents the first data on potato micro-tuber irradiation. Micro-tubers also have similar susceptibilities to gamma irradiation as mini-tubers. The radio-sensitivity test on white yam mini-tuber showed a 50% lethality dose around 40 Gy for gamma irradiation (Nwachukwu et al., 2009), which matches our findings on micro-tubers irradiation with Kondor and Basotho Pink genotypes. However, in micro-tuber gamma irradiation Asante, BP1 and Sherekea were moderately resistant and Mpya and Up-To-Date susceptible genotypes. That variable response recorded with micro-tubers exhibits the same genetic variation observed among potato genotypes with *in vitro* cuttings in the two other schemes. The M_1V_1 plants produced by scheme 3 have the advantage of increasing the population size because of the number of sprouted eyes per micro-tuber. However, the following generation is recommended for screening and selection. Thus Table 8 summarizes the advantages and disadvantages of the three strategies adopted in this study as guidelines regards to irradiation facility availability, population development, screening and selection of mutants.

The mechanisms of mutation induction caused by irradiation are complex and discussed in Lagoda (2012) and Shu et al. (2012). For mutation breeding optimal irradiation doses should be applied to induce adequate genetic changes to allow for efficient selection of desirable mutants (Nwachukwu et al., 2009; Sparrow, 1961). However, different genotypes and different tissues/propagules may show a different susceptibility to irradiation (Ahloowalia and Maluszynski, 2001). The present study corroborates previous studies by showing that cultivars respond differently depending on the tissues/organs that are subjected to irradiation. In fact, micro-tubers like mini-tubers show more resistance to gamma irradiation than most other propagules used in potato breeding.

Mutation induction optimum dose as 50% of growth reduction or lethality dose of gamma irradiation for potato mutation breeding after evaluation of the susceptibility of various genotypes are 10 to 21 Gy, 7 to 13 Gy and 20 to 55 Gy respectively for gamma irradiation of stem cuttings, tuberization and micro-tuber sprouting ability. Results in this study showed a higher susceptibility of the tuberization process to gamma irradiation than shoot growth. On the other side, micro-tuber sprouting ability was most resistant to gamma irradiation. Three schemes were evaluated here, the advantages and disadvantages for potato mutation breeding are given in Table 8. The importance of micro-tuber versus plantlets has been discussed at length with regard to development, handling of putative mutant populations and their yield enhancement reported by different researchers (Nistor et al., 2003; Pruski et al., 2003).

Conclusions

In vitro tissue culture combined with mutation induction proved to be effective in inducing useful mutants in vegetatively crops. Mutagen dose/concentration and the plant tissue/propagule used in the mutagenesis treatment are key factors for the successful improvement of potato through induced mutation. Potato currently lags behind other crops in improvement via plant mutation breeding. Three schemes have been developed to exploit *in vitro* cultures in potato mutation induction. Genotypic variation was recorded with respect to radio-sensitivity. The choice of scheme will depend on available facilities, the ability to develop, handle large mutant populations and screening for desired mutant types.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was funded by Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency through their Joint FAO/IAEA Program of Nuclear Techniques in Food and Agriculture. We would like to thank A. Draganitsch and B. Guenter for valuable support.

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Vol. 15(39), pp. 2146-2155, 28 September, 2016
DOI: 10.5897/AJB2016.15502
Article Number: B6B571E60675
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Natural occurrence and pathogenicity of *Xanthomonas* bacteria on selected plants

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Received 1 June, 2016; Accepted 8 August, 2016

The bacterial genus *Xanthomonas* consists of several species of economic importance, among which *Xanthomonas campestris pv.musacearum* (*Xcm*), the cause of enset and banana wilt is the most important in tropical Africa. However, the natural occurrence and host range of this species is yet to be clarified. The objectives of this study were to verify the presence of *Xanthomonas* bacteria on plants growing in and around enset gardens in South and Southwest Ethiopia, and to elucidate the pathogenicity of *Xcm* strains to cultivated and wild plants. Several economical and ornamental plants were assessed for wilting in South and Southwest Ethiopia. Wilting was visible on *Canna* spp. with 9.8% incidence and 30% prevalence, while reddish streak symptoms (typical of *Xanthomonas* bacteria) were observed on the leaves of sugarcane, sorghum and wild sorghum with disease incidence ranging from 20 to 80%, and prevalence varying from 30 to 100%. The pathogenicity of three *Xcm* isolates to five plant species was tested in a factorial experiment arranged in CRD with five replications. All the tested *Xcm* isolates were found to be pathogenic to banana, cultivated and wild enset, *Canna indica*, *Canna orchoides*, maize, sorghum and finger millet. The analysis of variance for incubation period and disease incidence revealed significant differences ($p<0.05$) among test plants and isolates. Results suggest marked variations among test plants' ability to resist the bacterium. Variations were also evident in the aggressiveness of the bacterial isolates. On the other hand, enset and banana did not show any symptom after being inoculated with four *Xanthomonas* isolates from other crops.

Key words: Enset, incubation period, wilt incidence, *Xanthomonas campestris pv. musacearum.*

INTRODUCTION

The genus *Xanthomonas* is composed of several species of economic importance as they affect the production of different crops all over the world. A member of this genus, *Xanthomonas campestris* pv. *musacearum* (*Xcm*), has been implicated in threatening the crop enset (*Ensete ventricosum* (Welw.) Cheesman) in Ethiopia since the 1960s (Yirgou and Bradbury, 1968; Dereje, 1985; Weldemichael, 2000).

The disease has also been implicated as causing a serious threat on banana production thereby the

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livelihood of thousands of people throughout the Great Lake Region of Africa (Wasukira et al., 2012). Previous work based primarily on DNA sequences and fatty acid data has shown that strains of *X. campestris* pv. *musacearum* have very close homology to strains of *Xanthomonas vasicola* and most likely belong to this species. Accordingly, the name *X. vasicola* has been proposed for *X. campestris* pv. *musacearum* (Aritua et al., 2007). However, we will use the previous name *Xcm* (currently still the official name) throughout this paper.

The initial symptoms by *Xcm* on enset and banana occur on the central leaf and spread to all parts. The earliest symptoms are usually loss of turgor and wilting in the spear (youngest emerging leaf) or one or more of the young leaves, sometimes preceded by yellowing and distortion, especially in young plants. Bacterial ooze exudes when a non-dry part of the plant is cut. A cut made through the petioles of newly infected enset plants reveals browning of the vascular bundles and yellowish or grayish masses of bacterial ooze come out from the vascular bundles (Tripathi et al., 2009). Cross sections at the base of the pseudostem and corm show discoloration of the vascular bundles with large bacterial pockets and grayish or yellowish exudates with brownish to black spots, respectively (Wondimagagne, 1981; Ashagari, 1985).

The main known natural host plants to *X. campestris* pv. *musacearum* are Banana (*Musa* spp.) and cultivated enset (*Ensete ventricosum*) both of which belong to the *Musaceae* family and order zingiberales (Yirgou and Bradbury, 1968, 1974). However, the host range of this pathogen appears rather controversial. While Ssekiwoko et al. (2006) reported *Xcm* as being able to only infect monocots that belong to the families Musaceae and Cannaceae, Mwangi et al. (2006) ruled out grasses like maize, sorghum and napier grass along with such crops as common beans, cassava, taro, sweet potato and tobacco as hosts to the pathogen. On the other hand, *Xanthomonas* species have been reported in sweet potato, sugar cane, maize, common beans and sorghum (Hernandez and Trujillo, 1990; Destefano et al., 2003; Mkandawire et al., 2004; De Cleene, 2008; Todorović et al., 2008). *Xcm* was also found to be pathogenic to maize and sugarcane (Aritua et al., 2008; Karamura, 2012). Wild *Musa zebrina*, *Musa ornata* and *Canna indica* were also reported as potential alternative hosts for this pathogen (Ssekiwoko et al., 2006).

Enset bacterial wilt caused by *X. campestris* pv. *musacearum* was first reported in Ethiopia by Yirgou and Bradbury (1968) and has since spread to all the enset growing regions in Ethiopia (Brandt et al., 1997). However, most of the studies conducted in Ethiopia thus far focus on surveying the disease in some areas and characterizing the pathogen based on biochemical tests (Wondimagagne, 1981; Ashagari, 1985; Spring et al., 1996; Bobosha, 2003; Addis et al., 2004).

Screening some cultivated enset clones for wilt resistance and studying the survival and dispersal of the pathogen have also been investigated although not thoroughly (Weldemichael, 2000; Addis et al., 2006; Weldemichael et al., 2008a and b). Studies on the occurrence of the disease on plants other than enset and banana are lacking under Ethiopian conditions, and are very limited even throughout Africa. Besides, the pathogenicity of *Xcm* isolates to plants growing in and around enset gardens has not been well established.

Therefore, this study was designed to: 1) verify the presence of *Xanthomonas* bacteria on plants growing in and around enset gardens in South and Southwest Ethiopia, and 2) elucidate the pathogenicity of *Xcm* strains to various plants.

MATERIALS AND METHODS

Assessing plants around enset gardens for *Xanthomonas* spp. infection

A field survey was carried out to assess some crops that is, *Canna* spp., sugar cane (*Sacharum officinarum*), cultivated sorghum (*Sorghum bicolor*), and wild sorghum (*Sorghum halepense*). The survey was carried out by visiting enset and banana producing areas in South and Southwest Ethiopia. During the survey, data were collected on the type of plants growing in and around each field; incidence of disease on each of the above plants as proportion of plants with visible symptoms. Besides, specimens were collected from each plant and brought to the laboratory for verification. Identity of the isolated bacteria was confirmed following colony growth on semi selective medium (sucrose peptone agar medium: 20 g sucrose, 5 g peptone, 0.5 g $K_2H_3PO_4$, 0.25 g $MgSO_4$ and 15 g agar in 1l sterilized distilled water) (Mwebaze, 2007; Mwangi et al., 2007), catalase (Dickey and Kelman, 1988) and Gram staining reaction tests (Schaad, 1988). In addition, physiological tests that is, gelatin liquefaction and starch hydrolysis testes as well as catalase reaction were carried out.

Pathogenicity tests

Pathogenicity tests were carried out to determine the possible host range of the pathogenic *Xcm* and the reaction of various plant species. The experiment had a factorial design with isolates as sub-factors and test plants as main factors. It was arranged in a completely randomized design with five replications.

Three *Xcm* isolates (I_1, I_2 and I_3) were isolated from naturally infected cultivated enset, wild enset and banana, respectively (Table 1), and used for the pathogenicity test on cultivated enset, wild enset, banana, *Canna* species and cereal crops (maize, sorghum and finger millet) collected from different areas (Table 2). Each isolate was collected by taking bacterial ooze in the field using a toothpick and then suspending the ooze in a test tube half filled with sterilized distilled water according to Weldemichael (2000). Before inoculation of test plants, the concentration of each bacterial suspension was adjusted using a spectrophotometer to 0.3OD at 460 nm, which is equivalent to 10^8cfu/ml bacteria cells.

Seedlings of banana, enset and *Canna* spp. were transplanted into pots (22×22 cm), filled with a sun-dried mixture of soil, sand and manure at a ratio of 3:1:1 (Quimio, 1992), then allowed to establish for three months (four to seven leaf stage). Inoculation of test plants with each bacterial isolate was done by injecting an aliquot of 3 ml of the bacterial suspension into the petiole base of the newly expanding central leaf using a 10 ml sterile hypodermic

Table 1. Description of the *Xanthomonas campestris* pv. *musacearum* isolates used for the pathogenicity tests.

Isolate code	Location	Altitude (masl)	Plant species sampled
I_1	Keffa/Gimbo/Ufundo	1.692	Wild enset (Epoo)
I_2	Keffa/Gimbo/Ufundo	1.702	Banana (Pisang awak)
I_3	Sidama/Hagerselam/Calbessa	2.752	Cultivated enset (Gullumo)

Table 2. Plant materials used for the pathogenicity tests, their designation and source.

Tested plant species	Cultivar/clone	Designation	Source
Banana	Pisang awak	Pa	Yirgalem
Cultivated enset	Meziya	Mz	Areka
Cultivated enset	Mandaluka	Ml	Areka
Cultivated enset	Hiniwa	Hn	Areka
Cultivated enset	Ashure	As	Areka
Cultivated enset	Digmaz	Dg	Areka
Cultivated enset	Zerate	Zr	Areka
Cultivated enset	Sorpie	Sp	Areka
Cultivated enset	Hiniba	Hb	Areka
Cultivated enset	Buffare	Bf	Areka
Cultivated enset	Geziwet	Gz	Areka
Wild enset	Erpa	Erp13	Areka
Wild enset	Epoo	Epo1	Kaffa collection
Wild enset	Epoo	Epo2	Kaffa collection
Wild enset	Epoo	Epo3	Kaffa collection
Wild enset	Epoo	Epo4	Kaffa collection
Wild enset	Epoo	Epo5	Kaffa collection
Wild enset	Epoo	Epo6	Areka
Wild enset	Erpa	Erp18	Areka
Wild enset	Erpa	Erp19	Areka
Canna spp.	*Canna indica*	CI	Kaffa collection
Canna spp.	*Canna orchoides*	Co	HCA
Maize	Melkessa-1	Mk	HCA
Maize	ACV6	AC	HCA
Sorghum	Gambella 1107	Gb	HCA
Sorghum	IS-18758	IS	HCA
Sorghum	RTxTAM	RT	HCA
Sorghum	BTx623	BT	HCA
Wild sorghum	*Sorghum halepense*	Sh	MARC
Finger millet	Tadess	Td	ANARC
Finger millet	Pandet	Pd	ANARC

HCA-Hawassa College of Agriculture; MARC-Melkassa Agriculture Research Center; ANARC-Arsi Negella Agriculture Research Center, Erpa and Epoo are vernacular names of wild ensets.

syringe (Ashagari, 1985). Inoculated plants were then covered with a wet plastic bag for 48 h.

For the cereals, seeds of each species were planted in plastic pots (18x18 cm) filled with a sun-dried sterile mixture of soil, sand and manure (3:1:1) and 324 mg of urea per pot; this amount of urea was re-applied six weeks after planting. About 10 seeds were planted in drills and thinned to five plants/pot two weeks after planting. The cereals were inoculated at one month old (three to four leaf stage) by wounding and spraying techniques, that is, their leaves were physically struck with very fine sterile sand paper, sprayed with 3 ml of each bacterial isolate suspension and covered with a transparent plastic bag for 48 h (Hussien, 2001). Negative

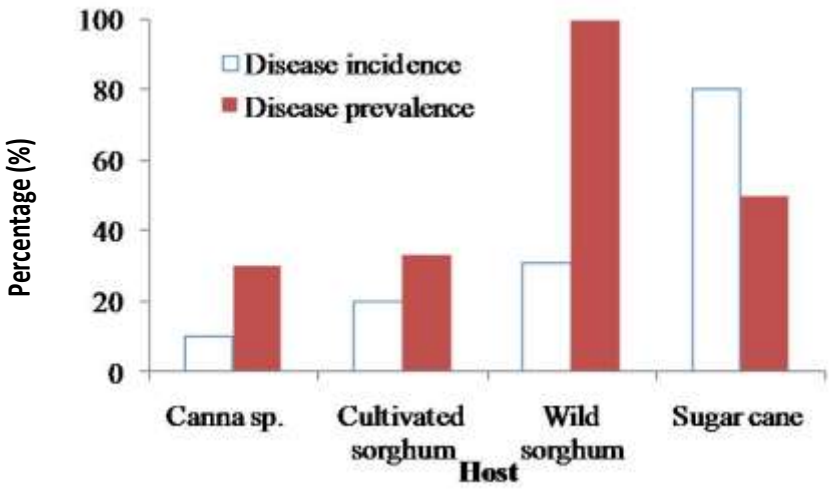

Figure 1. Mean disease incidence and prevalence of *Xanthomonas* spp. on *Canna*, sorghum, sugar cane and wild sorghum.

controls of each plant species were inoculated with the same quantity of sterile distilled water.

Disease assessment

Data were collected on incubation period (period between inoculation and first wilting symptom) and the number of plants showing disease symptoms was recorded weekly starting from one week after inoculation for four consecutive months. Disease incidence was calculated according to the following formula:

$$DI = \frac{NPCW \times 100}{NPPT}$$

Where, DI: disease incidence, NPCW: number of plants completely wilted, NPPT: number of plants assessed.

In addition, disease severity was assessed using a standard disease scale of 0 to 5 (Winstead and Kelman, 1952) where 0: no symptom; 1: only the inoculated leaf wilted; 2: 2 to 3 leaves wilted; 3: four leaves wilted; 4: all leaves wilted and 5: plant dead). The severity grades were converted into percentage severity index for analysis (Cooke, 2006).

$$\mathbf{PSI} = \frac{SNR \times 100}{NPR \times MSS}$$

Where, PSI is percent severity index; SNR is the sum of the numerical rating; NPR is number of plant rated; MSS is the maximum score of the scale. Severity from each scoring date was converted to area under percent severity index progress curve (AUPSiPC) using the formula by Jerger and Vijanen-Rollinson (2001) as follows:

$$AUPSiPC = \sum_{i-1}^{n=i} 1/2\,(x_i + x_{i+1})\,(t_{i+1} - t_i)$$

Where, n is total number of assessments, t_i is the time of the i^{th} assessment in weeks from the first assessment date, x_i is the percentage of the disease severity or disease incidence at i^{th} assessment. AUPSIC is the area under percent severity index progress curve was expressed in percent-weeks.

Data analysis

Analysis of variance was performed for data on disease parameters (wilt incidence and incubation period) using the General Linear Model of SAS computer package (SAS, Institute Inc., 2003). Means were separated by least significant difference (LSD) at 5% probability level.

RESULTS

Disease incidence on plants around enset garden and bacterial isolates characterization

Visible disease symptoms (yellowing of the leaf at margin side and tip, wilted leaf and blade folded upward and inward and also dry leaf) were evident on diseased plants. Reddish-brown streaks were also recorded on the grasses that is, cultivated and wild sorghum, and sugar cane. Disease incidence (proportion of infected plants in a field) varied from 10% on *Canna* sp. to 80% on sugar cane (Figure 1). Disease prevalence (proportion of fields with at least one diseased plant) ranges between 30% on *Canna* sp. and 100% on wild sorghum.

When diseased (wilted) plants were plated on sucrose peptone agar medium (a semi selective medium for *Xanthomonas*), deep yellow colonies grew out from sugarcane and sorghum, and yellow colored colonies were observed growing from *Canna*. All of these isolates were found to possess negative reaction to Gram staining, and positive reaction to catalase reaction. Inoculation of the isolates to enset and banana did not

Figure 2. (A left) Death of enset plant ('Astara' clone) after inoculation with an *Xcm* isolate collected from cultivated enset and (A right) healthy looking control plant of same clone; (B) wilting of the enset clone 'Sorpie' after inoculation with an *Xcm* isolate obtained from wild enset.

induce any symptom and hence the isolates were considered non-pathogenic to enset and banana.

Pathogenicity of *Xcm* isolates to various plants

The pathogenicity of *Xcm* isolates to various plants was tested in two experiments. Although, the results of both experiments were consistent; the average of the two experiments was presented in the current report.

Banana and cultivated enset

Disease assessment started a week after inoculation and the earliest typical external disease symptoms were observed two to four weeks post inoculation on 'Pisang awak' and enset clones. These included folding down of the leaf blade along the midrib, followed by scalding and dull green appearance of the central inoculated leaf. This was followed by yellowing, starting at the apex, sequential wilting of leaves, drying and wilting of the whole plant and finally plant rotting and death (Figure 2). Yellowish bacterial ooze was observed when pseudostem and leaf petiole were cut. In the current experiment, there were significant variations among clones and isolates in terms of incubation period, disease incidence and area under disease severity index progress curve (Tables 3 to 5). However, the interaction was not significant (data not shown).

Among the tested plants, the banana cultivar 'Pisang awak' was found to have the shortest incubation period followed by the enset clone 'Mandaluka'. Enset clone 'Mezya' had the longest incubation period. This clone also had the lowest average wilt incidence (27%) across

Table 3. Incubation period (IP) (weeks), percent disease incidence (%DI) and area under percent severity index progress curve (AUDSPC) for 10 cultivated enset clones and a banana cultivar, 'Pisang awak', inoculated with three *Xcm* isolates under greenhouse conditions.

Clone	IP	%DI	AUDSPC
Pisang awak	2.8^{c}	73.3^{a}	1039^{a}
Mezya	4.5^{a}	26.7^{g}	534^{f}
Mandaluka	2.9^{c}	60.0^{b}	891^{abc}
Hiniwa	3.3^{bc}	33.3^{f}	649^{ef}
Ashure	3.3^{bc}	53.3^{c}	751^{bcde}
Digmaz	3.7^{b}	33.3^{f}	611^{ef}
Zerate	3.7^{b}	33.3^{f}	693^{def}
Sorpie	3.2^{bc}	46.7^{d}	833^{bcd}
Hiniba	3.1^{bc}	53.3^{c}	728^{cde}
Geziwet	3.1^{bc}	73.3^{a}	919^{ab}
Buffare	3.1^{bc}	40.0^{e}	649^{e}
LSD	0.7011	5.288	184
Isolates			
I_1	3.95^{a}	5.5^{c}	438^{c}
I_2	3.4^{b}	56.4^{b}	857^{b}
I_3	2.7^{c}	81.8^{a}	981^{a}
LSD	0.366	2.76	95.88
% CV	29.06	15.29	23

Means in a column followed by the same letter are not significantly different at 5% level of significance. I_1: Isolate from wild enset, I_2: Isolate from banana ('Pisang awak'), I_3: Isolate from cultivated enset clone ('Gullumo'). Data represent mean of five replications.

Table 4. Incubation period (IP) (weeks), percent disease incidence (%DI) and area under percent severity index progress curve (AUDSPC) for nine wild enset types inoculated with three *Xcm* isolate under greenhouse condition.

Wild enset	IP	%DI	AUDSPC
Epoo1	3.3^{bc}	26.7^{de}	621
Epoo2	3.0^{c}	55.6^{a}	805
Epoo3	3.6^{bc}	40.0^{bc}	803
Epoo4	4.6^{a}	15.3^{f}	460
Epoo5	3.0^{c}	33.3^{dc}	537
Epoo6	3.5^{bc}	33.3^{dc}	786
Erpa13	3.4^{bc}	46.7^{b}	775
Erpa18	3.1^{bc}	60.00^{a}	747
Erpa 19	4.1^{ab}	25.00^{e}	630
LSD	0.95	7.45	357.5
Isolates			
I_1	3.90^{a}	3.45^{c}	537^{b}
I_2	3.59^{a}	48.28^{b}	815^{a}
I_3	2.83^{b}	70.00^{a}	831^{a}
LSD	0.445	3.48	165.16
CV	24.83	16.28	24

Means followed by the same letter in the column are not significantly different at 5% level of significance. I_1: Isolate from wild enset, I_2: Isolate from banana ('Pisang awak'), I_3: Isolate from cultivated enset clone ('Gullumo'). Data represent mean of five replications.

Table 5. Incubation period (IP) (weeks), percent disease incidence (%DI) and area under percent severity index progress curve (AUDSPC) for *Canna* spp. and various cereals inoculated with three *Xcm* isolates under greenhouse conditions.

Plant	Species/cultivar	IP	%DI	AUDSPC
Canna spp.	*Canna indica*	3.8^{abc}	77.8^{a}	845^{a}
	Canna orchoides	3.8^{a}	73.3^{a}	701^{ab}
Maize	Melkassa1	2.8^{efg}	40^{d}	436de
	ACV6	3.0^{defg}	66.7^{b}	700^{b}
Sorghum	Gambella 1107	2.5^{fg}	33.3^{d}	519^{c}
	BTx623	3.1^{def}	33.3^{d}	519^{c}
	IS-18758	3.2^{cde}	50^{c}	589^{bc}
	RTxTAM	2.4^{g}	25^{ef}	525^{c}
Wild sorghum	*Sorghum halepense*	2.8^{efg}	12.8^{f}	513^{c}
Millet	Tadess	3.2^{bcde}	16. 7^{f}	327de
	Pandet	3.8^{a}	8.4^{f}	292^{e}
	LSD	0.6253	12.2981	144.35
Isolates	I_1	3.6^{a}	19.6^{c}	495^{b}
	I_2	3.1^{b}	38.9^{b}	572^{a}
	I_3	2.7^{c}	55.3^{a}	593^{a}
	LSD	0.28	2.3174	74.639
	%CV	23.49	14.15	33

Means in a column followed by the same letter are not significantly different at 5% level of significance. I_1: Isolate from wild enset, I_2: Isolate from banana ('Pisang awak'), I_3: Isolate from cultivated enset clone ('Gullumo'). Data represent mean of five replications.

the three isolates, while 'Pisang awak' and 'Geziwot' had the highest wilt incidence (73%), followed by 'Mandaluka' (60%). Furthermore, the highest AUPSPC (1039) was recorded on 'Pisang awak' followed by 'Geziwet' and 'Mandaluka' in that order, while the lowest AUPSPC (534) was recorded on 'Mezya'. Thus, the banana cultivar 'Pisang awak' and enset clone 'Geziwot' were suggested to be highly susceptible to *Xcm* as compared to the other clones tested in the current experiment.

When comparisons were made across isolates, isolate I_3 caused wilting the earliest (2.7 weeks after inoculation) while the other two isolates, I_1 and I_2, took about four weeks and three weeks, respectively, to induce symptoms (Table 3). Most plantlets inoculated with isolates I_2 and I_3 completely wilted but most of the enset clones and some 'Pisang awak' plantlets inoculated with isolate I_1 did not wilt completely. Moreover, wilt incidence and area under the disease severity index progress curve were significantly the lowest for isolate I_1. On the other hand, isolate I_3 caused the earliest wilting and disease parameters after inoculation with this isolate were significantly greater than for the others. As a result, among the three isolates of *Xcm* used in this study, the wild *Xcm* isolate I_1 was found to be a weaker pathogen as compared to isolates I_3 and I_2. In contrast, isolate I_3, which was obtained from cultivated enset in Sidama zone of southern Ethiopia, was the most virulent and aggressive.

Wild enset

The first disease symptoms on wild enset plants were recorded a week after inoculation as yellowing from the apex to the edge of the inoculated leaf and water-soaked lesions along the inoculated leaf's midrib. Two to five weeks after inoculation leaf wilting and yellowing symptoms were observed on most plantlets (Figure 2). Yellowish bacterial ooze was observed when pseudostem and leaf petiole were cut. Such symptoms are similar to typical *Xanthomonads* bacterial wilt symptoms described on the banana cultivars and cultivated enset under field and experimental conditions.

Like the cultivated enset clones, the wild enset types also reacted differentially to the isolates of *Xcm*. Significant variations were observed among the wild enset types and *Xcm* isolates in terms of incubation period, disease incidence and area under disease severity index progress curve (Table 4).

The mean number of weeks required for the appearance of initial symptoms on wild enset clones varied between three and five. The incubation period was shorter on wild ensets clones such as 'Epoo5', 'Epoo2', 'Erpha18' and 'Erpha13', while 'Epoo4' had the longest incubation period among the tested wild enset clones. None of the nine wild enset types tested in the current experiment showed complete resistance to *Xcm* isolates used in this study. Among wild enset type tested in the current experiment, wilt incidence was the highest (60%) on 'Erpa18' followed by 'Epoo2', which had the highest AUPSPC (805). Thus these two wild enset types were found to be highly susceptible to *Xcm*. On the other hand, the wild enset 'Epoo4' had significantly the lowest wilt incidence and AUDSPC, making it relatively more tolerant to the pathogen.

In this experiment too, incubation period was the longest for isolate I_1, while isolate I_3 had the shortest incubation period. Symptom appearance after inoculation with I_1 was delayed by one to two weeks compared to the other two isolates. Most of the plantlets inoculated with isolates I_2 and I_3 were completely wilted 10 weeks after inoculation. On the other hand, only one plantlet of 'Epoo3' inoculated with isolate I_1 completely wilted at the same time of assessment. This difference between isolates in inducing symptoms on tested plants indicates variations in aggressiveness among the isolates. Disease incidence and severity were also high for most wild enset after inoculation with isolates I_2 and I_3. One hundred percent disease severity indexes were recorded at 5 to 9 weeks after inoculation on wild enset with isolate I_3 (data not shown). Isolate I_2 caused 60 to 100% severity at 7 to 11 weeks after inoculation, while isolate I_1 resulted in 40 to 60% severity at 8 to 14 weeks after inoculation. On average, 70% disease incidence and AUDSPC value of 831 were caused by isolate I_3. In contrast, isolate I_1 had significantly lower disease incidence and AUDSPC. This further confirmed the most aggressive nature of isolate I_3 as compared to the remaining two isolates.

Canna spp*. and cereals*

Among the suspected alternative host plants, *Canna* spp., maize, sorghum and finger millet varieties were tested for the reaction to three *Xcm* isolates. Two to three weeks after inoculation, typical external disease symptoms were observed on some plantlets of these suspected plants. On *Canna* plantlets, water soaked lesions developed along the inoculated leaf's midrib within two weeks after inoculation and after three to four weeks some inoculated leaves wilted and leaf blade folded upward and inward, turned yellow, dried and died. However, new suckers that emerged from the corm after the inoculated plantlet kept growing. This may be related to inability of the bacteria to colonize the corm of the *Canna* plants.

In maize, the first symptom observed on the inoculated leaf was necrosis and discoloration or yellowing of the leaf, starting from the tip to the bottom of the leaf, three to four weeks after inoculation. Gradual wilting along the midrib to the edge of the inoculated leaf was also observed. In sorghum varieties, lesions or discoloration initially developed at the tip of the inoculated leaf two weeks after inoculation. Thereafter, the lesions at the tip

of the leaf gradually elongated to the midrib and then to the leaf blade. Eventually, a yellowing symptom appeared on the leaf blade and, in severe cases, a burned appearance at the margin of the leaf. In addition, leaves withered and turned brown, wilted, dried and dropped off. The observed symptoms on finger millet varieties were discoloration starting from the tip to bottom of the leaf and finally turning yellow and dried.

The analysis of variance for incubation period, disease incidence and AUDSPC revealed significant differences among varieties and isolates (Table 5). The number of weeks to the appearance of first disease symptoms varied between two and half, and four among cereal cultivars. Among the tested plants, initial symptoms appeared the earliest on the sorghum cultivar 'RTxTAM' and the latest on the finger millet cultivar 'Pandet'. Each of the inoculated plant species reacted differently to the three isolates of *Xcm*. Disease incidence was in excess of 70% on *C. indica* and *C. orchoides* and reached 67% on the maize variety 'ACV6' (Table 5). Disease incidence was negligible on the finger miller variety 'Pandet'. This variety showed initial symptoms but then the disease progressed quite slowly. The second longest incubation period and lowest AUDSPC were recorded from the other finger millet variety, 'Tadess'. The current results may suggest the more resistant nature of finger millets as compared to the other cereals.

Isolates of *Xcm* differed in their ability to cause the disease on *Canna* spp. and the various cereals. Disease symptoms were induced the earliest by isolate I_3 followed by isolate I_2. The highest disease incidence was induced by isolate I_3 on *Canna indica* and *Canna orchoides*. The same isolate caused up to 60% disease incidence on maize, cultivated and wild sorghum and finger millets. On average, the highest AUDSPC value of 593 was recorded when plants were inoculated by isolates I_3 (Table 5). This was significantly higher than the AUDPSC from isolate I_1. In a trend similar to that from cultivated and wild enset, and banana, isolate I_3 was found to be the most aggressive on *Canna* spp. and cereals.

DISCUSSION

Bacterial wilt caused by *X. campestris pv. musacearum* is considered as one of the major biotic stresses threatening enset and banana (Thwaites et al., 2000). The enset-*Xcm* pathosystem remains one of the least studied pathosystems to date. The current study objectives were to determine enset bacterial wilt occurrence on various plants commonly grown in and around enset farms in South and Southwest Ethiopia and elucidate the pathogenicity of *Xcm* isolated from different group of plants.

During the field survey, different plants that is, *Canna* sp., cultivated and wild sorghum, and sugarcane were assessed for symptoms associated with the *Xanthomonas* bacteria. Results reveal the prevalence of symptoms associated with the *Xanthomonas* bacteria ranging from 30 to 100% with disease incidence varying from 10 to 80%. Thus, these plants were considered as possible alternate hosts to the *Xcm* bacteria. Ssekiwoko et al. (2006) also reported 80 to 100% disease incidence on *C. indica* in a pot experiment, while Ashagari (1985) identified *C. orchoides* as a host for the *Xcm* pathogen. In the present study, *Xanthomonas* from any of these plants did not induce observable symptoms on enset and banana, and hence the isolates were considered as non-pathogenic to enset and banana. On the other hand, the *Canna* spp. and all the aforementioned cereals crops were found to be susceptible to *Xcm* from enset and banana.

The current study reveals the pathogenicity of *Xcm* to cultivated and wild enset, banana, *Canna* spp., and several grasses. In contrast, Ssekiwoko et al. (2006) reported that *Xcm* infects only monocots belonging to the two families Musaceae and Cannaceae. Mwangi et al. (2006) has also excluded maize and sorghum from possible hosts of *Xcm*. On the other hand, Aritua et al. (2008) and Karamura (2012) have reported maize and sugarcane developing disease after being artificially inoculated with *Xcm*. Aritua et al. (2008) even reported genetic similarities between *Xcm* isolates on one hand, and isolates of *Xanthomonas vasicola* pv *holcicola* from sorghum and *Xanthomonas vasicola* pv *vasculorum* from sugarcane on the other. Inoculation of maize with *Xcm* resulted in the development of full blown yellow-brown streaks (Karamura, 2012), a result that coincides with this study findings.

Significant variations ($p<0.05$) existed among the isolates in terms of incubation period, wilting incidence and severity. In general, isolate I_3 from cultivated enset in South Ethiopia was found to be the most aggressive, while I_1 from wild enset plant was the least aggressive. Variability in terms of pathogenicity among *Xcm* isolates was also reported by Weldemichael (2000). This was contrary to a report by Aritua et al. (2007) that revealed low level of genetic variation among the pathogen isolates collected from different African countries.

However, the current pathogenicity test findings contradict those of Tripathi et al. (2009), who reported no significant differences in pathogenicity among *Xcm* isolates. Our findings thus call for more research in diversity of the pathogen populations. Besides, the results confirm the need to consider isolate variation in breeding for bacterial wilt resistance. We recommend this isolate be used in future resistance screening trials. We also suggest that molecular studies including sequencing be carried out to understand the genetic basis of variation in pathogenicity of the isolates.

The test plants also differed significantly in their degree of susceptibility to *Xcm*. The banana cultivar Pisang awak, *C. indica*, and enset clones Geziwot, Mandaluka, wild enset Epoo2 and Erpa18 showed high disease

incidence and severity, and short incubation period, and hence were considered as most susceptible. Enset clones Mezya, wild enset Epoo4, and finger millet cultivar Pandet had lower disease severity and longer incubation period, and hence were considered relatively tolerant to the pathogen. While not much work has been done to assess the susceptibility of wild enset to *Xcm*, the enset clone 'Mezya' was also found to be more tolerant to *Xcm* infection by Ashagari (1985) and Weldemichael (2000).

The current work reveals the potential various plants including wild enset may play in harboring *Xcm* pathogenic to both cultivated enset and banana. Hence, care must be taken to minimize the risk of the pathogen being spread from the wild to agricultural fields. Further characterization of the *X. campestris* pv.*musacearum* strains from wild enset, cultivated enset and banana should be carried out by using the existing available detection methods. In addition, the genetic diversity among both the host and the pathogen should be investigated further. Additional tests on the *Xcm* isolates to different plant species should be carried out to elucidate the potential of wild and cultivated plants in harboring and disseminating the pathogen.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors acknowledge the Directorate General for Development (DGD-Belgium) through the Bioversity International component of the CIALCA project for sponsoring the research.

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academicJournals

Vol. 15(39), pp. 2156-2165, 28 September, 2016
DOI: 10.5897/AJB2015.15121
Article Number: 0BA21E860677
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Development of simple kinetic models and parameter estimation for simulation of recombinant human serum albumin production by *Pichia pastoris*

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Received 23 November, 2015; Accepted 7 September, 2016

In order to describe and predict the growth and expression of recombinant proteins by using a genetically modified *Pichia pastoris*, we developed a number of unstructured models based on growth kinetic equation, fed-batch mass balance and the assumptions of constant cell and protein yields. The growth of *P. pastoris* on both glycerol and methanol could be represented by Monod kinetic equation. A simple simulation methodology and developed models were shown to satisfactorily describe both growth and production of recombinant human serum albumin (rHSA) using a genetically modified *P. pastoris* Mut^S strain. The obtained parameters from curve fitting were reasonable and could be acceptable. Moreover, the same parameter sets obtained by the experiments indicated the rigidity and consistency of the developed models and fermentation approach of this study. With correlation coefficients (r^2) exceeding 0.99, the models were able to simulate and predict the cell growth behavior and recombinant protein production by *P. pastoris* without requiring complex models.

Key words: Exponential feed, growth modeling, Monod kinetic equation, *Pichia pastoris*, recombinant human serum albumin.

INTRODUCTION

Pichia pastoris is a methylotrophic yeast which has been successfully used for the heterologous expression of a great number of recombinant proteins. The popularity of *P. pastoris* as a host for the production of recombinant

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proteins is due to its several inherent advantages as described in many reviews (Çelik and Çalik, 2012; Gao and Shi, 2013; Gonçalves et al., 2013; Vogl et al., 2013; Fickers, 2014; Byrne, 2015; Çalik et al., 2015; Looser et al., 2015). By combination of powerful genetic techniques, the ability of protein expression and recombinant protein purification at a comparatively low cost, therefore *P. pastoris* is made as a promising system for recombinant protein production. In recombinant protein production, increasing expression and productivity are desired. Therefore, a number of strategies have been employed to increase expression of the recombinant proteins in question, for example, optimizing the fermentation medium (Jungo et al., 2007b; Batista et al., 2013), improving the fermentation method (Bushell et al., 2003; Ohya et al., 2005), different feeding strategies (Sinha et al., 2003; Hu et al., 2007), mixed-substrate feeding (d'Anjou and Daugulis, 2000; Jungo et al., 2007a; Arnau et al., 2011; Zalai et al., 2012) and different oxygen supplementation strategies (Charoenrat et al., 2005; Zhang et al., 2005; Charoenrat et al., 2006). Modeling is another approach without more empirical experiments enhancing and optimizing the production of recombinant proteins. This approach can be successfully applied to describe growth behavior and is used to enhance the productivity of recombinant proteins from both *Escherichia coli* (Baheri et al., 1997) and *Saccharomyces cerevisiae* (Hardjito et al., 1992; Patkar et al., 1993). The modeling is also used for developing an improved fermentation protocol for recombinant *P. pastoris* systems (d'Anjou and Daugulis, 1997). Many model approaches are proposed such as model-based feeding strategy based on mass balance (d'Anjou and Daugulis, 1997; d'Anjou and Daugulis, 2001), macrokinetic modeling based on stoichiometric balance (Ren et al., 2003), model-based specific growth rate (Ren and Yuan, 2005), mix-feed modeling based on growth kinetic (Zhang et al., 2003), growth and protein production modeling based on metabolic flux and metabolic model (Jahic et al., 2002; Sohn et al., 2010; Nocon et al., 2014), growth model optimization using response surface methodology (RSM) (Zhang et al., 2004; Yu et al., 2014) and artificial neural networks (ANNs), fuzzy rule-based systems, or a combination of both (Jenzsch et al., 2006). On these studies, the majorities are complex and require a complicated technical knowledge. However, simple simulation of recombinant microorganisms can be taken by kinetic modeling with the aid of computer software (Vinayagam et al., 2015).

The production of recombinant proteins is normally performed in three-stage fermentation: Batch, fed-batch and induction stage (Çelik and Çalik, 2012; Potvin et al., 2012; Looser et al., 2015). The first stage is batch fermentation where *P. pastoris* is cultured with glycerol. After the initial glycerol is depleted, the glycerol is added to the culture in order to reach high cell density in the second stage. The third stage is the induction stage where expression of the recombinant proteins is induced by methanol.

In this study, the unstructured models based on growth kinetic equation, fed-batch mass balance and constancy of cell and protein yields were developed and constructed following the substrates, glycerol and methanol. The growth model on glycerol is mostly published while the growth model on methanol is rarely due to cell growth of Mut^S strain on this medium, which is very low so is neglected in the model (d'Anjou and Daugulis, 1997). Moreover, in mixed-substrate feeding, the growth on methanol is unnecessary to differentiate from growth on glycerol (d'Anjou and Daugulis, 2001). Therefore, this study proposed the model of growth not only on glycerol but also on methanol. Furthermore, a simple simulation methodology to investigate the behavior of growth and protein production of recombinant microorganisms was also introduced. A Mut^S *P. pastoris* KM71 strain genetically modified to produce and secrete human serum albumin (HSA), a major protein component of human blood plasma, was used as a model for mathematical model development. These models and parameters obtained by simulation methodology could be used as a tool to inspect the recombinant *P. pastoris* fermentation.

MATERIALS AND METHODS

Microorganism

Genetically modified *P. pastoris* KM71 capable of expressing and secreting HSA was used in all experiments. The *P. pastoris* clone created by inserting the coding DNA sequence for mature full length HSA into the expression vector pPICZαA and then integrating into the genome of *P. pastoris* was provided by Dr. Witoon Tirasophon, Mahidol University, Thailand.

Medium

Yeast extract peptone dextrose (YPD) medium contained 10 g yeast extract, 20 g peptone and 20 g dextrose per liter of deionized water. Basal salt medium (BSM) contained 26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4.7H_20$, 4.13 g KOH, 50.0 g glycerol and 6.7 ml PTM1 trace salt in deionized water made up to a total volume of 1 L. The PTM1 trace salt contained 0.5 g $CoCl_2.6H_2O$, 20.0 g $ZnCl_2$, 65 g $FeSO_4.7H_20$, 6.0 g $CuSO_4.5H_2O$, 3.0 g $MnSO_4.H_2O$, 0.1 g KI, 0.2 g $Na_2MoO_4.2H_2O$, 0.02 g H_3BO_3, 5.0 ml H_2SO_4 and 0.2 g biotin in deionized water made up to a total volume of 1 L. The PTM1 trace salt was sterilized by filtration.

Inoculum preparation

P. pastoris stored at -80°C was used to inoculate a starter culture in YPD medium which was subsequently incubated at 30°C and 250 rpm. The starter culture was then used to inoculate 100 ml BSM which was continuously incubated at the aforementioned condition until reaching an OD_{600} of 20 (total OD_{600}). The BSM inocula were then transferred aseptically to 7 L of BSM (working volume) in a 15 L bioreactor (BIOSTAT C, B. Braun Biotech International, Melsungen, Germany). The volume of inocula used in all

experiments was 10% of the working volume of the bioreactor.

Batch fermentation

During the batch fermentation stage, the temperature was set at 30°C and pH maintained at 5.00 by the addition of 25% NH_4OH and 85% H_3PO_4. Dissolved oxygen (DO) was kept above 20% saturation by using cascaded control of agitation. Aeration was supplied at 2 vvm and the air was supplemented and mixed with pure oxygen if the stirrer could not maintain the oxygen levels at the set value. Foaming was monitored by an antifoam probe and antifoam (Antifoam 204, Sigma, Deisenhofen, Germany) was added to the culture in order to prevent excessive foaming during fermentation.

Fed-batch fermentation

After depletion of initial glycerol in the BSM, the glycerol feed medium (50% w/v glycerol in water with 15 ml/l PTM1) was fed according to a predetermined exponential feeding rate described by d'Anjou and Daugulis (1997) and Jahic et al. (2002) until the cell concentration reached 100 g-DCW/l. The conditions were set to the same values as those during the batch fermentation stage.

Protein induction

After reaching the predetermined cell density (100 g-DCW/l), glycerol feed was discontinued and the culture was left for a 4-hour starvation period. Methanol with the addition of 15 ml/l PTM1 was then fed into the bioreactor in order to induce rHSA expression. The initial pulse of methanol was first fed into the bioreactor to a concentration of 4 g-methanol/l (Trinh et al., 2003; Looser et al., 2015) and left for 4 h before the continuous feeding strategies commenced. The temperature was set to 22°C (Jahic et al., 2003; Wu et al., 2012; Gao and Shi, 2013; Gonçalves et al., 2013; Anasontzis and Penã, 2014; Yu et al., 2014) and pH 6.00 (Kobayashi et al., 2000) during the induction phase.

Analysis methods

Samples were taken 5 ml at 6-hourly intervals during both the batch and fed-batch phases and every 24 h during the induction phase. The samples were centrifuged at 9000 rpm (RCF = 9055∗g), 4°C for 5 min and the supernatant was collected for further analysis. Cell concentrations were determined by measuring OD_{600} and then converted to dry cell weight by a correlation of $0.323 \times OD_{600}$ (r^2 = 0.998). Glycerol and methanol concentrations in the medium were analyzed by HPLC (Shimadzu Ltd., Tokyo, Japan) using an Aminex HPX-87H Ion Exclusion Column (Bio Rad) with 0.5 mM sulfuric acid as mobile phase and a flow rate of 0.6 ml/min at 45°C. A refractive index detector was utilized for detection. Total protein concentration in the medium was analyzed by Bradford assay (Suwannarat et al., 2013). The amount of rHSA protein was calculated from the band density, which obtained from SDS-PAGE analysis using 12% gels (according to standard protocols) stained with Imperial™ Protein Stain (Thermo Fisher Scientific), and compared to standard HSA of known concentration using Gene Tools program version 3.06.02. Western blot analysis was performed in order to verify the expression of rHSA protein and the identity of the rHSA bands.

Model development

The models describing the fermentation process were constructed by mass balance on biomass, substrate concentration, recombinant protein production and system volume. The *P. pastoris* KM71 strain used in this study was designated Mut^S which indicated the ability to grow on methanol as well as glycerol. Methanol could be also utilized as an inducer for the expression of recombinant proteins by the *AOX1* promoter (Trinh et al., 2003). The mass balance equations used in this study were described as follows:

$$\frac{dX}{dt} = \mu X \quad (1)$$

$$\frac{dS}{dt} = \frac{F_S}{V} S_0 - \frac{\mu}{Y_{X/S}} X \quad (2)$$

$$\frac{dM}{dt} = \frac{F_M}{V} M_0 - \frac{\mu_M}{Y_{X/M}} X \quad (3)$$

$$\frac{dP}{dt} = \mu_M Y_{P/X} X \quad (4)$$

Where X is biomass concentration in dry weight (g-DCW/l), V is the medium volume (l), F_S is glycerol feed rate (l/h), F_M is methanol feed rate (l/h), S is the substrate (glycerol) concentration (g-glycerol/l), S_0 is the substrate (glycerol) concentration in inlet feed (g-glycerol/l), M is the methanol concentration (g-methanol/l), M_0 is the methanol concentration in inlet feed (g-methanol/l), P is the concentration of recombinant protein secreted into the medium (g-protein/l), $Y_{X/S}$ is the yield coefficient biomass per substrate, glycerol (g-DCW/g-glycerol), $Y_{X/M}$ is the yield coefficient biomass per substrate, methanol (g-DCW/g-methanol), $Y_{P/X}$ is the yield coefficient protein per biomass (g-protein/g-DCW), μ is the specific growth rate on glycerol (h^{-1}), μ_M is the specific growth rate on methanol (h^{-1}) and t is the run time (h).

Monod kinetic equation is widely used for describing the growth of microorganisms and was therefore used to describe the specific growth rate of *P. pastoris* on glycerol (μ) in this study as shown by Equation 5. Methanol was the only energy and carbon source during the induction stage, consequently the specific growth rate on methanol (μ_M) based on the Monod equation could be described as shown in Equation 6 (Curvers et al., 2001):

$$\mu = \frac{\mu_{\max} S}{S + K_S} \quad (5)$$

$$\mu_M = \frac{\mu_{M\max} M}{M + K_M} \quad (6)$$

Where K_S is the Monod saturation constant on glycerol (g-glycerol/l), K_M is the Monod saturation constant on methanol (g-methanol/l), $\mu_{\max}$ is the maximum specific growth rate on glycerol (h^{-1}) and $\mu_{M\max}$ is the maximum specific growth rate on methanol (h^{-1}).

In order to prolong cell growth and attain higher cell density during the fed-batch stage, glycerol was fed exponentially according to Equation 7 to achieve exponential cell growth as

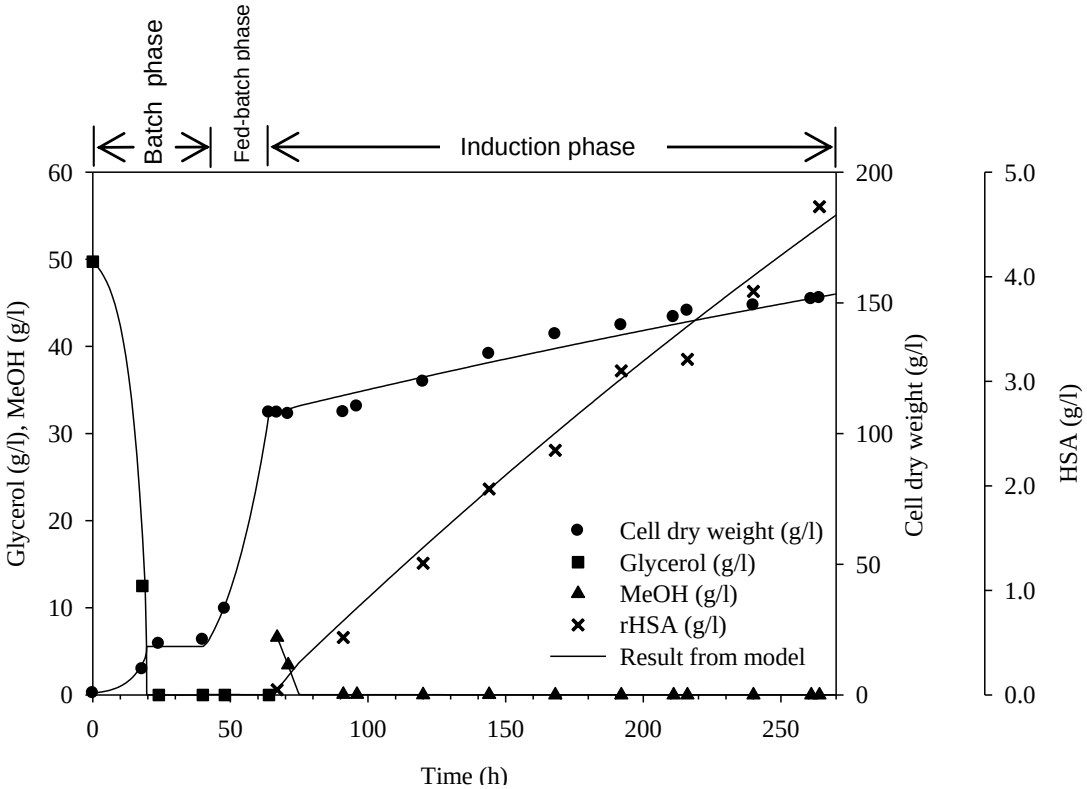

Figure 1. The growth behavior of Mut^S *P. pastoris* KM71 when producing rHSA in RUN 1 experiment. Symbols represent measured data and lines represent simulation. ■ , Glycerol; ● , yeast biomass; ▲, methanol; ×, rHSA

described by d'Anjou and Daugulis (1997) and Jahic, et al. (2002). Equation 8 was used to calculate the yeast cell concentration during the feed batch fermentation and the time required to reach the predetermined final cell concentration during the fed batch fermentation.

$$F_S = \frac{\mu_{set}}{S_0 Y_{X/S}} X_0 V_0 e^{\mu_{set}(t-t_0)} \quad (7)$$

$$X = \frac{X_0 V_0}{V} e^{\mu_{set}(t-t_0)} \quad (8)$$

Where X_0 is biomass concentration in dry weight when the exponential feeding is initiated (g-DCW/l), V_0 is the medium volume when the exponential feeding is initiated (l), μ_{set} is the specific growth rate set point (h^{-1}) and t_0 is the time when the exponential feeding is initiated (h).

A continuous feed pattern was chosen for the methanol feed during the induction stage. The methanol feed rate was calculated based on biomass and the methanol consumption rate according to Equation 9.

$$F_M = \frac{q_M X V}{M_0} \quad (9)$$

Where q_M is specific methanol consumption rate (g-methanol/g-DCW·h).

To solve these differential equations when growing on glycerol and methanol, cell yield, protein yield and the specific methanol consumption rate were assumed to be constant. The models represented as these equations were coded and computed using instructions in Berkeley Madonna program version 9.0.118.

RESULTS AND DISCUSSION

The experiments were performed in a 15 L bioreactor and were initiated as batch fermentation with a working volume of 7 L. In Run 1 experiment, as shown in Figure 1, the yeast biomass increased from 0.67 g-DCW/l at the time of inoculation to 21.04 g-DCW/l at the end of the batch fermentation. During this time period, glycerol was metabolized and was completely consumed within 40 h, decreasing from 49.72 g-glycerol/l. After the initial glycerol in the BSM had been depleted during the batch fermentation stage (at the 40th hour), the subsequent fed-batch stage started by feeding additional glycerol into the bioreactor, thereby prolonging the growth phase of *P. pastoris* and increasing cell density. In order to achieve an exponential growth rate, the feed pattern for the addition of glycerol was calculated using Equation 7. The

Table 1. The predetermined parameters used in simulation of recombinant Mut^S *P. pastoris* KM71 fermentation.

Parameter	Run 1	Run 2
Cell concentration at starting batch culture (g-DCW/l)	0.67	0.59
Glycerol concentration at starting batch culture (g-glycerol/l)	49.72	56.47
Medium volume at initial feeding, V_0 (l)	7.0	7.0
Glycerol concentration in inlet feed, S_0 (g-glycerol/l)	501.67	505.08
Methanol concentration in inlet feed, M_0 (g-methanol/l)	778.15	777.90
Initial feeding time, t_0 (h)	40	40
Specific growth rate set point, μ_{set} (h^{-1})	0.08	0.08
Cell concentration at initial feeding, X_0 (g-DCW/l)	21.04	20.64

parameters used in Equation 7 were obtained from an experiment as shown in Table 1. The yield coefficient on glycerol ($Y_{X/S}$) in Equation 7 was obtained from the change of glycerol and cell concentration over time in batch stage by estimation with the curve fitting function in Berkeley Madonna program version 9.0.118. The $Y_{X/S}$ of *P. pastoris* used in this study was 0.36 g-DCW/g-glycerol. During the exponential feed in fed-batch stage, the μ_{set} (Equation 7) was set at 0.08 h^{-1} (Jenzsch et al., 2006; Suwannarat et al., 2013) to ensure that metabolic overflow would be avoided (Looser et al., 2015). The duration of the exponential feed and the time needed to achieve a certain cell concentration could be calculated using Equation 8, the composed term in Equation 7 representing cell concentration. The cell density targeted in the fed-batch stage was set at 100 g-DCW/l, which achieved in 24 h. In order to activate the *AOX1* promoter and induce expression of rHSA, the induction phase was initiated by the addition of methanol at 4 g-methanol/l (Trinh et al., 2003). An initial pulse of methanol was first given to acclimatize the cells to metabolize methanol. Not only the inducing chemical, methanol is also poisonous to the *P. pastoris* cells because of the accumulation of formaldehyde and hydrogen peroxide, the products of methanol metabolism, inside the cells if it exists at a high concentration (Khatri and Hoffmann, 2006). However, a low methanol concentration is inadequate for protein expression (Gonçalves et al., 2013). Thus, the optimum amount of methanol should be regulated strictly (Potvin et al., 2012). For model development in this study, the continuous methanol feed based on the specific substrate uptake rate was selected (Dietzsch et al., 2011a). Four hours after the initial pulse feed, the continuous methanol feed was initiated and methanol was added to the culture at a rate equal to the specific methanol consumption rate (q_M) of the *P. pastoris* strain used in this study. The q_M had previously been determined by monitoring both methanol and cell concentrations over time in fermentations with constant methanol feed. The data obtained in those experiments gave a q_M of 0.026 g-methanol/g-DCW·h by calculation based on fed-batch mass balance. This value was similar to the study by Dietzsch et al. (2011a, b) in *P. pastoris* Mut^S KM71H strain. The medium volume was drained daily to maintain a constant at 7 L in order to avoid exceeding the capacity of the bioreactor. After the initial methanol pulse, the methanol concentration decreased during the induction phase until the residual methanol was undetectable by HPLC even though methanol was continuously added into the bioreactor according to the calculated feed rate. This was due to limitations of the pump belonging to the bioreactor and in order to avoid accumulation of excess methanol in the bioreactor, the setting had to be slightly lower than the calculation. As shown in Figure 1, the amount of rHSA (× symbol) increased during the induction phase with this methanol feed strategy. Methanol was not present while the methanol feed was operating which was an indication that yeast cells consumed methanol residuals at the feed rate added in (constant q_M, 0.026 g-methanol/g-DCW·h).

In the simulation of *P. pastoris* KM71, Equation 1 to 4 describing the yeast behavior in growth, substrate utilization and recombinant protein production were derived by fed-batch mass balance. The growth kinetic when growing on glycerol (μ) was explained by Monod kinetic equation, shown in Equation 5. In the fed-batch stage, the exponential glycerol feed pattern could be calculated by Equation 7, the cell density and length of the fed-batch stage could be calculated by Equation 8, as described previously. For the induction stage, methanol was the only energy source added into the bioreactor for *P. pastoris*, therefore, the growth of yeast also depended on methanol only. As with the growth on glycerol, Monod kinetic equation was also used for the growth on methanol due to its simplicity and the fact that it did not require complicated parameters. Hence, the kinetic growth on methanol (μ_M) was introduced by Equation 6. During the induction period, the methanol was continuously fed after the initial methanol pulse at 4 g-methanol/l for 4 h. The methanol feed (F_M) presented by Equation 9 was continuously added with constant q_M as described previously. The models represented as these

Table 2. The obtained parameters from simulation of recombinant Mut^S *P. pastoris* KM71 fermentation.

Parameter	Values
Maximum specific growth rate on glycerol, μ_{max} (h^{-1})	0.16
Maximum specific growth rate on methanol, $\mu_{M\,max}$ (h^{-1})	0.003
Yield coefficient biomass per substrate (glycerol), $Y_{X/S}$ (g-DCW/g-glycerol)	0.36
Yield coefficient biomass per substrate (methanol), $Y_{X/M}$ (g-DCW/g-methanol)	0.09
Yield coefficient protein per biomass, $Y_{P/X}$ (g-protein/g-DCW)	0.10
Monod saturation constant on glycerol, K_S (g-glycerol/l)	0.04
Monod saturation constant on methanol K_M (g-methanol/l)	0.01

equations were coded and computed using instructions in Berkeley Madonna program version 9.0.118. The parameters used in simulation are shown in Tables 1 and 2. The parameters in Table 1 were obtained from experimental measurements. As for Table 2, the parameters were obtained using the curve fitter feature in Berkeley Madonna program. The simulation and fermentation results of RUN 1 experiment are shown in Figure 1 where the symbols represent data from the experiment and the lines are derived from the simulation results. The results showed accordance of developed models and that the experiment fit very well. In the induction stage, when methanol was added, it seemed that the yeast had a lag growth due to the effect of diauxic growth where the yeast cell accommodated to methanol. This effect was ignored by the models because of the simplicity of the objective in model development; however, the r^2 between the models and the experimental results exceeded 0.99 in all data sets. In the RUN 1 experiment (Figure 1) the highest concentration of secreted rHSA present in the medium was 4.67 g/l, which occurred after 264 h (197 h of induction) with a simultaneous cell concentration of 151.81 g-DCW/l. The secreted rHSA produced by *P. pastoris* was analyzed by SDS-PAGE and showed the same molecular size as standard HSA (67 kDa), as shown in Figure 2a. By comparing band densities, the rHSA quantity was analyzed with known concentration of standard HSA using Gene Tools program version 3.06.02. Furthermore, the rHSA was also verified with the Western blot analysis and it showed specific binding with antibody as same as standard HSA (Figure 2b).

After 264 h (197 h of induction), the cells entered a stationary phase (data not shown), most likely due to the exhaustion of some essential medium components (d'Anjou and Daugulis, 2000) and/or the accumulation of some metabolites in the medium. The observed decrease in cell growth lowered methanol metabolism, which consequently resulted in the accumulation of excess methanol. It was likely, therefore, that methanol concentration in the bioreactor increased while, simultaneously, the production of rHSA decreased. The experiment was continuously operated until the cell concentration started to decrease. A potential explanation for the decline in cell concentration at these extended durations could be a slower growth rate and methanol consumption combined with a maintained methanol feed resulting in a simultaneous decrease of both cells and protein concentration due to dilution.

When the parameters obtained from this study were compared with the parameters previously reported by other researchers then a number of observations could be made. In d'Anjou and Daugulis (1997, 2000, 2001) studies, they calculated μ_{max} and $\mu_{M\,max}$ for a Mut^S GS115 strain of *P. pastoris* producing and secreting raven anti-freeze protein (SR-AFP) between 0.25 and 0.27 h^{-1} and 0.01 and 0.04 h^{-1}, respectively, where both exceed the values obtained in our experiments (0.16 and 0.003 h^{-1}, respectively). In the review by Looser et al. (2015), μ_{max} of *P. pastoris* GS115 strain were normally in range of 0.20 to 0.29 h^{-1} in all recombinant protein expressions but μ_{max} of *P. pastoris* KM71 strain was not reported. However, μ_{max} of *P. pastoris* Mut$^+$ strain expressing the heavy-chain fragment C of botulinum neurotoxin serotype C (BoNT/C(Hc)) intracellularly as determined by Zhang et al. (2000, 2003) was 0.177 h^{-1}. In addition, μ_{max} determined by Hang et al. (2009) in *P. pastoris* GS115-YY113 with phenotype of Mut^S strain expressing phytase and Cos et al. (2005) in *P. pastoris* X-33 both Mut$^+$ and Mut^S strains expressing lipase were 0.18 h^{-1} which corresponded more closely with the value reported here. For this parameter, the μ_{max} could be compared across the methanol utilization phenotype strains of *P. pastoris* (Looser et al., 2015) because this parameter did not depend on methanol utilization phenotype strains, it commonly presented in any *P. pastoris* strains grew on glycerol. In the review by Looser et al. (2015), $\mu_{M\,max}$ of *P. pastoris* in Mut^S strains in any protein expressions collected were in the range of 0.011 to 0.03 h^{-1} which were similar to d'Anjou and Daugulis (1997, 2000, 2001) studies, however, these values were obtained at 30°C induction temperature which is higher

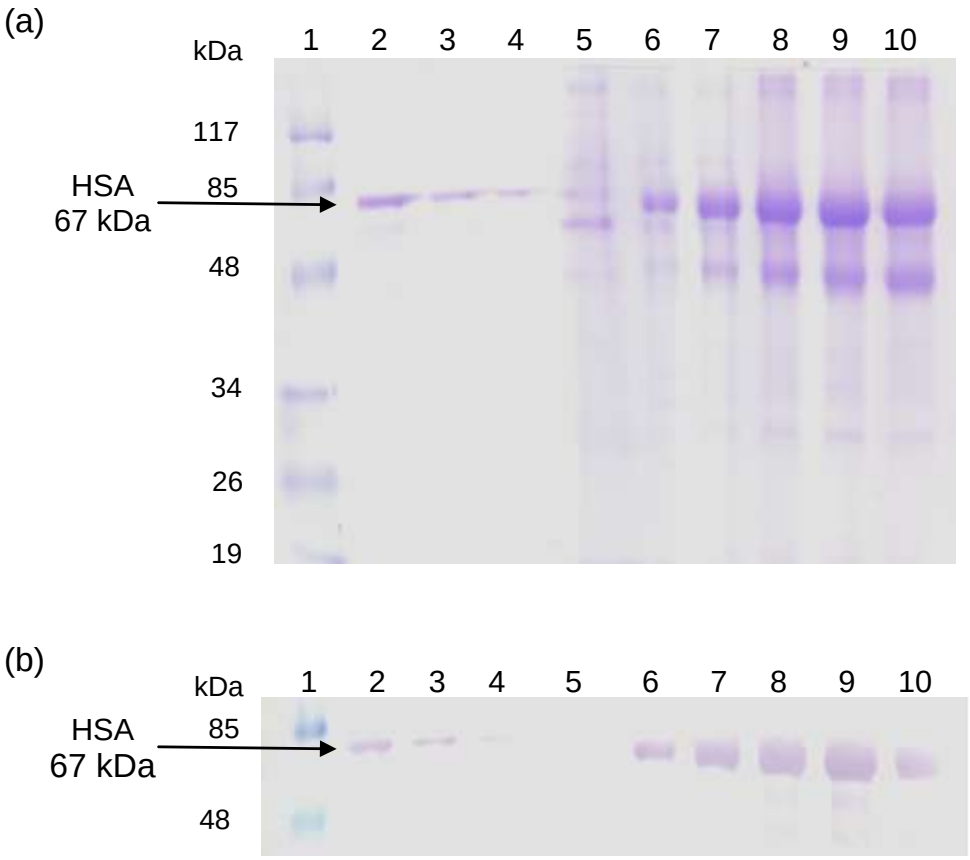

Figure 2. The analysis of rHSA produced by Mut^S *P. pastoris* KM71. (a) The SDS-PAGE analysis at different time of induction. Lane 1, molecular marker; Lane 2, standard HSA (67 kDa) at 0.0504 g/l; Lane 3, standard HSA at 0.0252 g/l; Lane 4, standard HSA at 0.0126 g/l; Lane 5, 0th h; Lane 6, 50th h; Lane 7, 122nd h; Lane 8, 194th h; Lane 9, 271st h; Lane 10, 311st h. (b) The Western blot analysis of SDS-PAGE.

than that of this study (22°C). Therefore, this parameter ($\mu_{M\max}$) could be expected to be larger than that of this study (Jahic et al., 2003). In Zhang et al. (2003) study, $\mu_{\max}$ (0.177 h^{-1}) was similar to this study; they also measured $\mu_{M\max}$ in Mut^S strain of *P. pastoris* GS115 expressing BoNT/C(Hc) at 0.008 h^{-1} which was also closer to the results here (0.003 h^{-1}) than other researchers, but the difference was still obvious. An explanation for the discrepancy could be that the induction stage in this study was performed at 22°C which is lower than the 30°C used in their publication and consequently causes slower growth rates (Curvers et al., 2001; Jahic et al., 2003) corresponding to Arrhenius plot of the growth rate (Jahic et al., 2003). Moreover, the difference in $\mu_{M\max}$ might be due to the use of different strains and the expression of different recombinant proteins. In addition, the cell yield on glycerol ($Y_{X/S}$) and methanol ($Y_{X/M}$) of *P. pastoris* Mut^S strain as described by d'Anjou and Daugulis (1997, 2000, 2001) were between 0.40 and 0.45 g-DCW/g-glycerol and 0.61 and 1.73 g-DCW/g-methanol, respectively. In the review by Looser et al. (2015), $Y_{X/S}$ and $Y_{X/M}$ of *P. pastoris* Mut^S strain were 0.5 to 0.619 g-DCW/g-glycerol and 0.3 g-DCW/g-methanol, respectively. The $Y_{X/S}$, obtained in the current study (0.36 g-DCW/g-glycerol) was fairly close to d'Anjou and Daugulis (1997, 2000, 2001) but $Y_{X/M}$ (0.09 g-DCW/g-methanol) was lower than theirs. It was not strange that the $\mu_{M\max}$ and $Y_{X/M}$ obtained (0.003 and 0.09 g-DCW/g-methanol, respectively) were low as well, because the recombinant protein induction was performed at a lower temperature. Monod saturation constant on glycerol (K_S) calculated in this study was 0.04 g-glycerol/l which differed from the obtained value (0.005 g-glycerol/l) by d'Anjou and Daugulis (1997, 2000) Finally, Monod saturation constant obtained on methanol (K_M) was 0.01 g-methanol/l. Unfortunately, K_M for a Mut^S *P. pastoris* strain was rarely reported in the publications due to overlooking in the slow growth rate on methanol of the strain (d'Anjou and Daugulis, 1997; d'Anjou and Daugulis, 2001). The published K_M parameter was

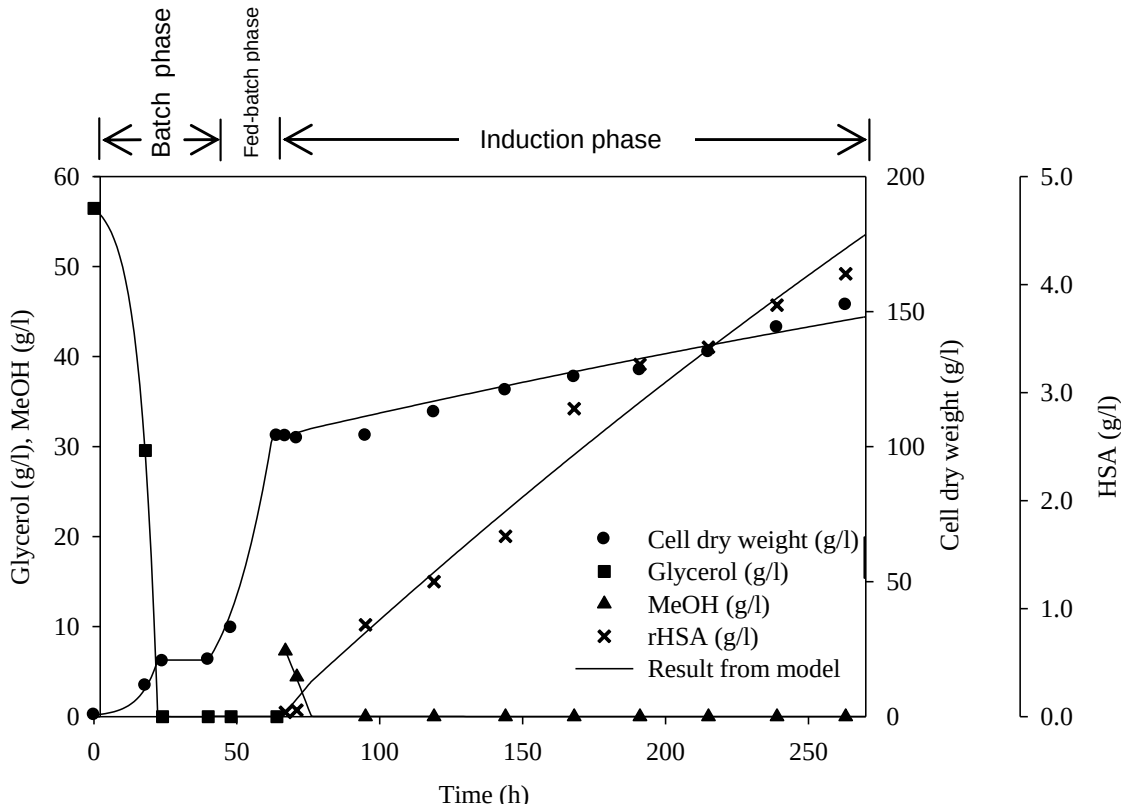

Figure 3. The growth behavior of Mut^S *P. pastoris* KM71 when producing rHSA in RUN 2 experiment. Symbols represent measured data and lines represent simulation. ■ , Glycerol; ● , yeast biomass; ▲, methanol; X, rHSA

reported for a Mut$^+$ strain and was equal to 0.22 g-methanol/l (Curvers et al., 2001).

The Run 2 experiment was operated the same as the Run 1 experiment and the results were similar. As shown in Figure 3, the biomass in batch fermentation increased from 0.59 to 21.04 g-DCW/l within 40 h with glycerol simultaneously decreasing from 56.47 g-glycerol/l until completely consumed. After the batch stage, the fed-batch of exponential glycerol feed started at 40th hour following Equation 7 with μ_{set} at 0.08 h^{-1} for 24 h (calculated by Equation 8) in order to prolong the yeast growth and achieve 100 g-DCW/l high cell density. Henceforth, the rHSA expression was induced by methanol. Four hours after the initial methanol pulse at 4 g-methanol/l, the methanol was continually added following Equation 9 with constant q_M (0.026 g-methanol/g-DCW·h). The methanol residue in the culture broth remained only at the beginning of the feed and then decreased over time until it could not be detected by HPLC; even the methanol was still fed. At the end of the experiment, the concentration of secreted rHSA (× symbol) in the medium at 263 h (196 h of induction) was 4.10 g-methanol/l with a cell concentration of 152.46 g-DCW/l. Furthermore, the results from RUN 2 experiment were used to validate the developed models and parameter set. The consequence from curve fitting showed that the obtained parameters from Run 2 experiment were same as Run 1 (Table 2). By using the parameters in Tables 1 and 2, the Run 2 experiment was simulated according to the proposed models and the results were represented by the lines in Figure 3. As shown in Figure 3, the r^2 in all data sets exceeded 0.99. The simulation results showed that the developed models and the rHSA fermentation approach for this study were rigid and consistent.

Conclusion

In this study, simple models were developed based on growth kinetic equations, fed-batch mass balance and the assumptions of constant cell and protein yields. Monod kinetic equation was used to describe both growths on glycerol and methanol. The developed models fit very well with the experiments with r^2 values exceeding 0.99 in all data sets. The obtained parameters could be reasonably acceptable. Moreover, the models and parameters

were rigid and consistent and could describe and predict cell growth, substrate (glycerol and methanol) utilization and recombinant protein production by *P. pastoris* KM71. Furthermore, the demonstrated simulation methodology in this study could also be used as a tool to study heterologous protein production by recombinant microorganisms where fermentation could be simulated using simple equations and simple methods without the requirement of complex models.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors thank Dr. Witoon Tirasophon of Shrimp Molecular Biology Research Group, Institute of Molecular Bioscience, Mahidol University, Thailand (25/25 Phuttamonthon 4 Road, Salaya, Nakhon Pathom 73170, Thailand) for supplying the recombinant cell line from Intracellular Signaling Lab, Institute of Molecular Biology and Genetics, Mahidol University. They also thank the Research and Development Institute of Government Pharmaceutical Organization (GPO), Thailand for providing the 15 L bioreactor. This work was supported by a grant from Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (NSTDA), Thailand.

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Vol. 15(39), pp. 2166-2171, 28 September, 2016
DOI: 10.5897/AJB2016.15542
Article Number: B4C8C6060679
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Improved embryogenic callus induction and plant regeneration in big bluestem (*Andropogon gerardii* Vitman), a potential bioenergy feedstock

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Received 25 June, 2016; Accepted 7 September, 2016

The objective of this study was to develop an efficient regeneration protocol in big bluestem, a potential feedstock that produces huge biomass. Embryogenic calli were induced from the seeds of cultivars, Kaw and Earl, on Murashige and Skoog (MS) medium with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 mg l^{-1}) alone or in combination with 6-benzylaminopurine (BA) (0.5 mg l^{-1}) or L-proline (2.0 g l^{-1}). In Kaw, the highest number of embryogenic calli (39.1%) was induced on MS + 0.5 mg l^{-1} 2,4-D and L-proline, whereas in Earl, the highest number of embryogenic calli (39.8%) was obtained on MS medium containing 1.0 mg l^{-1} 2,4-D and L-proline. The embryogenic calli were then transferred to regeneration media (MS medium supplemented with kinetin, 0.2, 0.5, 1.0, 3.0 and 5.0 mg l^{-1} or BA, 0.2, 0.5, 1.0, 3.0 and 5.0 mg l^{-1}). Shoots were regenerated on all of the concentrations tested and the regeneration percentage and number of shoots per calli increased with the increase in BA or kinetin concentration. Regenerated shoots were transferred to half strength MS medium for rooting. The fully developed plantlets were established in the greenhouse. The regeneration protocol established in this study may be used for the application of genetic engineering technologies in big bluestem.

Key words: Big bluestem, biomass, embryogenic calli, L-proline, regeneration.

INTRODUCTION

Big bluestem (*Andropogon gerardii* Vitman) is a warm-season (C_4) perennial grass native to North America (Boe et al., 2004). It is adapted to most native prairie ecosystems and comprises as much as 80% of plant biomass in prairies in the North American Mid-western grassland (Gould and Shaw, 1983; Knapp et al., 1998). Compared to switchgrass (*Panicum virgatum* L.) and Indiangrass (*Sorghastrum nutans* (L.) Nash), big bluestem produces twice the biomass due to its efficient nutrient utilization (Johnson and Matchett, 2001). Currently, big bluestem is considered a potential biomass feedstock for lignocellulosic ethanol production (Zhang et al., 2012). However, the biggest limiting factor in the efficient utilization of plant biomass for cellulosic bio-fuel is the

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presence of the ligno-hemicellulose complex that prevent the enzyme cellulase from effective conversion of biomass to biofuel (Sticklen, 2008). While approaches such as acid or heat pre-treatment are being used to break the ligno-hemicellulosic complex, genetic engineering technology holds great promise for reducing lignin content in biomass through down-regulation of lignin pathway genes (Hisano et al., 2009). Such an approach requires an efficient plant regeneration system, which is not available for a number of important cultivars in big bluestem.

Despite its importance as a potential bioenergy grass, very few studies have been carried out on *in vitro* regeneration of big bluestem (Chen et al., 1977; Chen and Boe, 1988; Li et al., 2009a). Chen et al. (1977) and Chen and Boe (1988) regenerated plants from calli that were induced from young inflorescence on Linsmaier and Skoog (LS) medium supplemented with 5.0 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg l^{-1} kinetin. Li et al. (2009a) obtained embryogenic calli from mature seeds on LS medium containing 2.0 to 4.0 mg l^{-1} 2,4-D for big bluestem cultivars Bison and Bonilla. Cultivars such as Bison and Bonilla are well adapted to US Midwest and Western states; however, a regeneration system is not available for cultivars that are adapted to US Southern states such as Arkansas and Texas.

The objective of this study was, therefore, to establish an efficient regeneration system from the calli of mature seeds in big bluestem cultivars, Kaw and Earl, that are well adapted to the southern US. Mature seeds are considered an important source of explants, as they are readily available and used frequently for *in vitro* regeneration (Li et al., 2009a; Lee et al., 2006, 2008).

MATERIALS AND METHODS

Plant materials

Mature seeds of big bluestem grass cultivars, Kaw and Earl, were obtained from Turner Seeds, Breckenridge, TX, USA. The seeds were sterilized with 70% ethanol for 30 s, followed by 20% commercial bleach (sodium hypochlorite, 5.25%) containing two drops of Tween 20 (MP Biomedicals, LLC, Aurora, Ohio, USA) for 45 min, and then washed three times with sterile distilled water for 5 min each.

Callus induction

Sterilized seeds were cultured for callus induction on petri plates containing MS (Murashige and Skoog, 1962) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2, 0.5, 1.0, 2.0, 3.0 and 5.0 mg l^{-1}) alone or in combination with 6-benzylaminopurine (BA) (0.5 mg l^{-1}) or L-proline (2.0 g l^{-1}). The components of MS medium as well as growth regulators, 2,4-D and BA were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Proline was obtained from Caisson Labs (Logan, UT, USA). Media were supplemented with 30 g l^{-1} of sucrose (PhytoTechnology Laboratory, Shawnee Mission, KS, USA); pH was adjusted to 5.7 with 1 N NaOH before sterilizing at 121°C (120 kPa for 20 min) and the media were solidified with 4.0 g l^{-1} Gelzan (Phyto Technology Laboratory). Callus induction was performed under dark at 28°C (Isotemp Incubator, Fisher Scientific, Hanover Park, IL, USA). Primary shoots from seed were removed 10 days after inoculation and subculture was done 3 weeks after inoculation on the same medium. Overall 18 treatments were tested for embryogenic callus induction in each cultivar. The frequency of embryogenic calli was calculated by: [the number of seeds producing embryogenic calli/the number of seeds germinated on callus induction medium] × 100.

Shoot regeneration, rooting, and plant establishment

Embryogenic calli were transferred to regeneration medium for shoot regeneration. Regeneration media contained MS medium supplemented with kinetin (0.2, 0.5, 1.0, 3.0 and 5.0 mg l^{-1}; Sigma-Aldrich) or BA (0.2, 0.5, 1.0, 3.0 and 5.0 mg l^{-1}) and 30 g l^{-1} of sucrose); pH was adjusted to 5.7 with 1 N NaOH before sterilization and the media were solidified with 4.0 g l^{-1} Gelzan. The cultures were incubated in a plant tissue culture chamber at 25°C under 16 h photoperiod (Percival Scientific, Perry, IA, USA).

After 4 weeks, the regenerated plants were transferred to rooting medium. Rooting medium contained half-strength MS medium with 30 g l^{-1} of sucrose; pH was adjusted to 5.7 with 1 N NaOH before sterilizing and the medium was solidified with 4.0 g l^{-1} Gelzan. The rooting was carried out in a plant tissue culture chamber under a 16 h photoperiod at 25°C (Percival Scientific, Perry, IA, USA). Rooted plants, after 2 to 3 weeks, were transferred to peat pellets for 1 week for hardening and then to the greenhouse for further growth.

Experimental design and statistical analysis

The experiment was conducted in six replicates with 25 seeds per treatment and data presented here represent an average of six experiments. The experiment was performed as a completely randomized design. To assess the treatment effects, the percentages of primary calli, embryogenic calli and shoot regeneration were subjected to Analysis of Variance (ANOVA) and mean separation was performed using least significant difference (LSD) with SAS software, version 9.2 (SAS Institute Inc., 2008).

RESULTS AND DISCUSSION

Seed germination

Germination of mature seeds started 3 days after inoculation in both Kaw and Earl. Germination percentage ranged from 80.0 to 82.7 for Kaw and 60.3 to 75.3 for Earl (Table 1). Germination data were recorded 10 days after seed inoculation.

Effect of 2,4-D on embryogenic calli induction

Callus was initiated 7 days after inoculation of Kaw and 10 days after inoculation of Earl on MS medium containing different concentrations of 2,4-D. The callus thus formed was white and non-nodular (Figure 1a). After 3 weeks, callus was sub-cultured onto the same medium. After 1 week of subculture, nodular structures, which were smaller in size, light yellow, friable and fast-growing, started appearing on the outer surface of the calli. The

Table 1. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D), L-proline and 6-benzylaminopurine (BA) on embryogenic callus induction in big bluestem cv. Kaw and Earl.

2,4-D (mg l^{-1})	Proline (g l^{-1})	BA (mg l^{-1})	% of seeds germinated[1]		% of embryogenic calli[2]	
			Kaw	Earl	Kaw	Earl
0.2	-	-	82.0 ± 6.1	60.8 ± 16.3	9.8 ± 3.4^{h*}	10.1 ± 3.9^{i*}
0.5	-	-	81.3 ± 9.7	61.2 ± 15.4	13.2 ± 4.3^{g,h}	14.6 ± 2.7^{g,h,i}
1.0	-	-	80.0 ± 14.1	68.8 ± 14.5	14.2 ± 2.8^{g,h}	17.8 ± 5.5^{e,f,g,h,i}
2.0	-	-	80.1 ± 12.8	60.3 ± 11.3	18.6 ± 5.6^{d,e,f,g}	24.5 ± 8.9^{c,d,e,f}
3.0	-	-	81.3 ± 12.7	64.8 ± 10.9	23.8 ± 1.9^{c,d,e}	18.3 ± 3.8^{e,f,g,h,i}
5.0	-	-	80.7 ± 11.1	66.8 ± 12.5	18.8 ± 2.7^{d,e,f,g}	14.1 ± 6.8^{h,i}
0.2	2.0	-	80.7 ± 9.9	67.3 ± 8.5	21.4 ± 7.3^{c,d,e,f,}	15.1 ± 6.6^{g,h,i}
0.5	2.0	-	82.7 ± 9.7	68.0 ± 13.6	39.1 ± 7.4^{a}	30.7 ± 9.9^{b,c}
1.0	2.0	-	82.0 ± 9.7	66.0 ± 7.5	31.0 ± 8.6^{b}	39.8 ± 7.6^{a}
2.0	2.0	-	81.3 ± 7.0	72.0 ± 9.1	24.7 ± 8.4^{b,c,d}	37.8 ± 5.6^{a,b}
3.0	2.0	-	80.7 ± 10.6	73.3 ± 6.5	21.8 ± 5.2^{c,d,e,f}	26.0 ± 7.4^{c,d,e}
5.0	2.0	-	80.0 ± 10.4	71.3 ± 15.1	15.3 ± 8.6^{f,g,h}	16.9 ± 7.6^{f,g,h,i}
0.2	-	0.5	81.3 ± 7.4	68.7 ± 6.9	12.3 ± 2.8^{g,h}	13.8 ± 7.1^{h,i}
0.5	-	0.5	80.0 ± 6.7	71.3 ± 5.9	15.9 ± 6.6^{f,g,h}	20.7 ± 4.6^{d,e,f,g,h}
1.0	-	0.5	80.0 ± 8.4	75.3 ± 7.3	17.4 ± 1.4^{e,f,g}	27.7 ± 13.8^{c,d}
2.0	-	0.5	80.7 ± 5.9	72.0 ± 5.1	21.6 ± 5.2^{c,d,e,f}	23.0 ± 6.4^{c,d,e,f,g}
3.0	-	0.5	80.0 ± 4.4	70.6 ± 5.5	26.6 ± 3.4^{b,c}	20.9 ± 8.6^{d,e,f,g,h}
5.0	-	0.5	81.3 ± 4.1	70.0 ± 6.1	17.4 ± 7.9^{e,f,g}	16.5 ± 7.8^{f,g,h,i}

[1]Seed germination percentage was calculated based on the number of seeds germinated and the number of seeds inoculated. [2] Percentage of embryogenic calli was determined based on the number of embryogenic calli formed and the number of seeds germinated. *Percentages followed by different superscript letters within a column are significantly different at P≤0.05. Each value represents the mean ±SD from six replicates.

fast growing nodular calli were recognized as embryogenic (Figure 1b), while the larger, white, slow growing non-nodular calli were identified as non-embryogenic (Figure 1a). The formation of two distinct calli, embryogenic and non-embryogenic, with different regeneration potential is an important characteristic of cereals and grasses (Pola et al., 2008). The auxin 2,4-D has been successfully used to induce embryogenic calli in grasses such as big bluestem (Chen et al., 1977; Chen and Boe, 1988; Li et al., 2009a), Indiangrass (Chen et al., 1979; Li et al., 2009b), *Brachypodium* (Zombori et al., 2011) and little bluestem (*Schizachyrium scoparium* (Michs.) Nash) (Songstad et al., 1986; Li et al., 2009a). In our study, 23.8% of germinated seed produced embryogenic calli on MS medium containing 3.0 mg l^{-1} 2,4-D in Kaw and 24.5% of germinated seed produced embryogenic calli on MS medium with 2.0 mg l^{-1} 2,4-D in Earl (Table 1). The percentage of embryogenic calli induced in our study is comparable to the percentage of embryogenic calli (23.1%) obtained with the supplementation of 2,4-D in other big bluestem cultivars, Bison and Bonilla (Li et al., 2009a).

Effect of L-proline on embryogenic calli induction

The use of L-proline in the medium has a positive effect on the frequency of callus induction in rice (*Oryza sativa* L.), a member of grass family (Chowdhry et al., 1993; Ge et al., 2006). In this study, we have tested the effectiveness of L-proline (2.0 g l^{-1}) on embryogenic calli induction in Kaw and Earl (Table 1). With the addition of L-proline, the frequency of embryogenic calli was significantly increased to 39.1% on MS medium supplemented with 0.5 mg l^{-1} 2,4-D in Kaw and 39.8% on MS medium containing 1.0 mg l^{-1} 2,4-D in Earl. The percentage of germinated seed that produced embryogenic calli in this study (39.1% in Kaw and 39.8% in Earl) was significantly higher (69 to 72%) than the percentage of embryogenic calli obtained in big bluestem cultivars, Bison and Bonila (23.1%) (Li et al., 2009a). Clearly, the increase in embryogenic calli frequency obtained in this study can be attributed to the supplementation of L-proline in callus induction medium. In switchgrass cv. Alamo, addition of L-proline increased the embryogenic calli formation efficiency by 30% (Li and Qu, 2010). The effectiveness of L-proline for the initiation and maintenance of embryogenic calli has also been reported in rice (Datta et al., 1992; Kishor et al., 1999) and alfalfa (*Medicago sativa* L.) (Shetty and McKersie, 1993). L-Proline also promoted embryogenesis in somatic tissue cultures of *Zea mays* (Armstrong and Green, 1985; Vasil and Vasil, 1986) and wheat (*Triticum aestivum* L.) (Gill and Gosal, 2015).

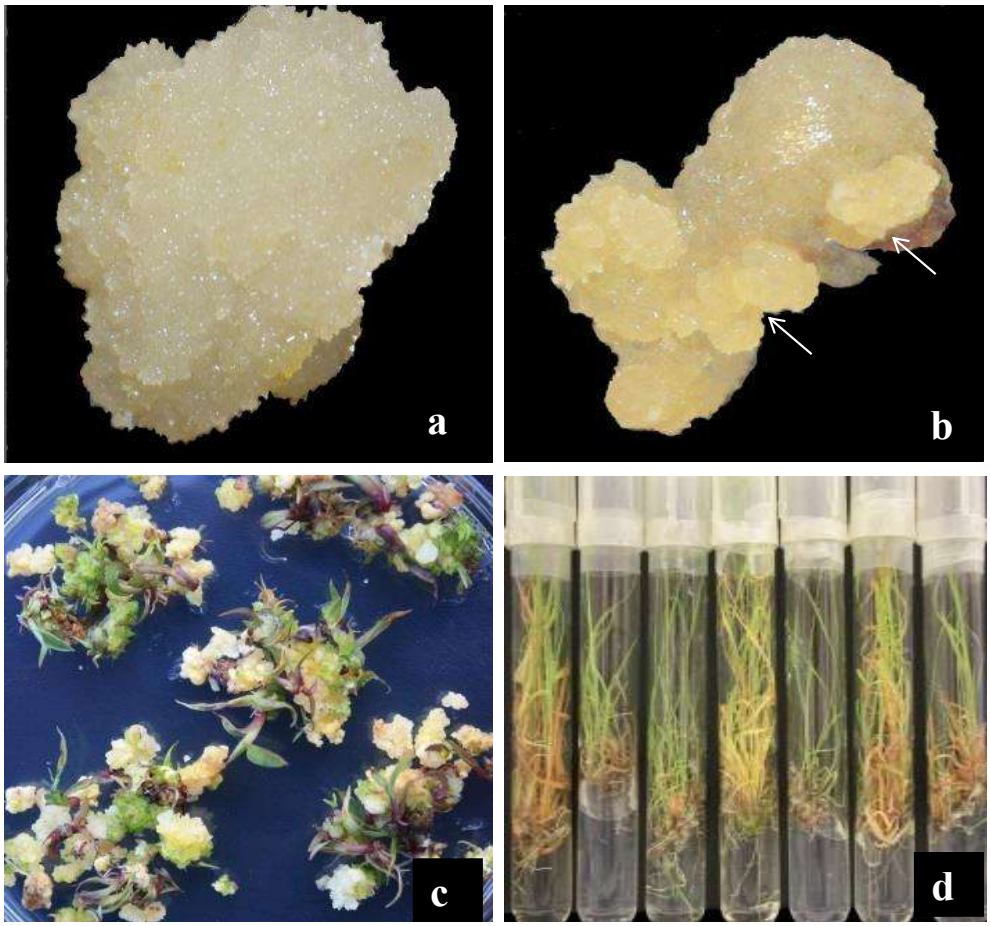

Figure 1. *In vitro* plant regeneration in big bluestem cv. Kaw: a. Non-embryogenic callus induced on MS + 3.0 mg l^{-1} 2,4-D under dark at 28°C; b. Embryogenic callus obtained on MS + 0.5 mg l^{-1} 2,4-D + 2.0 g l^{-1} L-proline under dark. Arrow indicates nodal embryogenic structures; c. Shoots regenerated from embryogenic calli 25 days after culture on regeneration medium (MS + 5.0 mg l^{-1} kinetin) under light (16/8 h photoperiod); d. Rooting of regenerated shoots on half strength MS medium under light (16/8 h photoperiod).

Effect of BA on embryogenic calli induction

In general, the addition of 0.5 mg l^{-1} BA on MS medium supplemented with 2,4-D did not significantly increase embryogenic calli formation in Kaw and Earl (Table 1). In Kaw, the maximum of 26.6% embryogenic calli was obtained on MS medium containing 3.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA, while in Earl, the maximum of 27.7% embryogenic calli was formed on MS medium containing 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. It has been reported that BA in combination with auxins such as 2,4-D has a positive effect on callus induction in grasses (Altpeter and Posselt, 2000; Chaudhury and Qu, 2000; Bai and Qu, 2001). However, BA in combination with 2,4-D did not have a positive effect on callus induction in seashore paspalum turfgrass (*Paspalum vaginatum* Swartz) (Neibaur et al., 2008). The BA requirement may be species-specific (Chaudhury and Qu, 2000) and depends on the specific endogenous hormone levels (Bhaskaran and Smith, 1990).

Shoot regeneration

The actively growing embryogenic calli were transferred to MS medium with different concentrations of kinetin or BA for shoot induction. Addition of cytokinin in the regeneration medium can significantly increase plant regeneration in grasses (Songstad et al., 1986; Fei et al., 1997). In the present study, the percentage of the shoot regeneration and number of shoots per calli increased with the increasing concentrations of kinetin or BA in both Kaw and Earl (Table 2). However, compared to BA, kinetin was better with 100% regeneration achieved in both Kaw and Earl on MS medium containing 5.0 mg l^{-1} kinetin (Table 2) Similar high percentage of regeneration was observed with the addition of 5.0 mg l^{-1} kinetin in the regeneration medium for big bluestem cultivars, Bison

Table 2. Effect of different concentrations of Kinetin or 6-benzylaminopurine (BA) on shoot regeneration from the embryogenic callus induced on MS medium in Kaw and Earl cultivars of big bluestem.

Kinetin (mg l^{-1})	BA (mg l^{-1})	% of callus forming shoots		Number of shoots per calli	
		Kaw	Earl	Kaw	Earl
0.2	-	41.7 ± 12.6^{e,f*}	45.8 ± 11.7^{e,f}	2.9 ± 0.5^{e,f}	3.1 ± 0.4^{e,f}
0.5	-	50.8 ± 12.6^{d,e}	60.0 ± 14.1^{c,d}	4.2 ± 1.5^{c,d}	4.6 ± 0.7^{d}
1.0	-	60.0 ± 14.1^{c,d}	69.1 ± 14.2^{c}	5.2 ± 2.4^{b,c}	5.1 ± 0.7^{c,d}
3.0	-	78.3 ± 15.8^{b}	82.5 ± 13.7^{b}	7.0 ± 1.1^{a}	6.6 ± 1.5^{b}
5.0	-	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	7.4 ± 0.9^{a}	8.2 ± 0.9^{a}
-	0.2	23.4 ± 11.2^{g}	23.3 ± 11.2^{g}	2.6 ± 0.5^{f}	2.4 ± 0.5^{f}
-	0.5	32.5 ± 11.0^{e,f,g}	36.7 ± 3.9^{f}	3.5 ± 1.1^{d,e,f}	4.1 ± 1.4^{d,e}
-	1.0	41.7 ± 12.6^{e,f}	50.8 ± 12.6^{d,e}	4.0 ± 0.9^{d,e}	5.2 ± 0.8^{c,d}
-	3.0	60.8 ± 23.2^{c,d}	69.2 ± 14.2^{c}	5.5 ± 1.4^{b}	5.9 ± 0.9^{b,c}
-	5.0	70.0 ± 24.5^{b,c}	83.4 ± 19.2^{b}	5.7 ± 1.2^{b}	6.6 ± 1.3^{b}

[2]Percentages and means with a different superscript letters within a *column* are significantly different at P≤0.05. Each value represents the percentages and means±SD from four replicates.

and Bonila (Li et al., 2009a). Interestingly, only 70.0 and 83.4% shoot regeneration was observed in Kaw and Earl, respectively, on MS medium containing 5.0 mg l^{-1} BA. An average of 7.4 shoots in Kaw and 8.2 shoots in Earl were regenerated on regeneration medium containing 5.0 mg l^{-1} kinetin, while an average of 5.7 shoots in Kaw and 6.6 shoots in Earl were regenerated on MS with 5.0 mg l^{-1} BA.

Rooting and plant establishment in greenhouse

More than 90% of the regenerated shoots were successfully rooted on half strength MS without the addition of growth hormones. A similar percentage of rooting was achieved in big bluestem cultivars, Bison and Bonila (Li et al., 2009a). Fully rooted plants were transferred initially to peat pellets for hardening for one week and then to the greenhouse for further growth and development.

In conclusion, we have successfully established an improved regeneration system in two cultivars of big bluestem, Kaw and Earl, which are grown in the southern US. Both the cultivars showed enhanced embryogenic calli formation (39.1% in Kaw and 39.8% in Earl) on MS medium containing 2,4-D and L-proline. In addition, shoots were successfully regenerated (100% in Kaw and Earl) on MS medium containing kinetin (5.0 mg l^{-1}). Because of high-frequency embryogenic calli formation and efficient plant regeneration, this protocol may be used for the application of genetic engineering technologies in big bluestem cultivars, Kaw and Earl.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The author, Muthusamy Manoharan thank Dr. Lynn Dahleen, L&Js Homegrown Exchange LLC, Albany, Wisconsin, for critical review of the manuscript and the Plant Powered Production (P3) Center, which was funded wholly or in part by the National Science Foundation (NSF) EPSCoR Program and the Arkansas Science and Technology Authority (ASTA) for providing funding support for this study. The NSF EPSCoR award number is: EPS-1003970.

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Vol. 15(39), pp. 2172-2175, 28 September, 2016
DOI: 10.5897/AJB2016.15505
Article Number: 7C53ECD60681
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Short Communication

A novel sampler for limnological investigation in developing world

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Received 1 June, 2016; Accepted 7 September, 2016

A simple pump sampler for shallow water bodies, constructed with materials that are affordable and readily available in the local market is presented. It is simple to operate and no electric motor is required. The challenge of cost of acquisition of conventional samplers for sampling the lentic habitat, especially small water bodies such as small natural lakes, rivers, streams and reservoirs for fisheries and water treatment facilities is minimised. The device can collect samples with ease, from various depths, up to five metres. The sampler consists of a manual suction pump produced from a hand bicycle pump-connected to a series of sample bottles by plastic delivery tubes. Samples were taken into the sample bottles by suction via a calibrated transparent plastic hose lowered to the required sampling depth.

Key words: Sampler, lentic, limnology, shallow water.

INTRODUCTION

There are various customized water samplers that have been designed for sampling of various water bodies for various aspects of limnological studies viz: plankton, zoological, phycological, bacteriological and physicochemical studies; some of which are towing nets for plankton (Pal and Choudhury, 2014; Alhassan, 2015); Irwin, Van Dorn and Kemmerer (Henny and Nomosatros, 2016; Anonymous, 2016), Meyer, Niskin and Friedinger (APHA, 1995; Pal and Choudhury 2014) water samplers to mention a few. These samplers though efficient are not readily available to many investigators or aquatic scientists in developing countries because of high costs of procurement and sometimes some may be unsuitable for sampling shallow water bodies, especially those less than one meter depth because they may induce turbulence.

The genesis of the development of this sampler was the loss of a Kemmerer sampler owned by the Hydrobiology Unit of the Department of Zoology and Environmental Biology at the University of Nigeria, Nsukka during an investigation on Agulu Lake, Anambra State, Nigeria in 1985. There was then an immediate need to develop a sampler for below water surface samples.

Irwin sampler (Welch, 1952) was originally designed to sample shallow water bodies such as puddles, etc. This new sampler adapted the principles behind the mode of operation of Irwin's sampler to design a sampler for water

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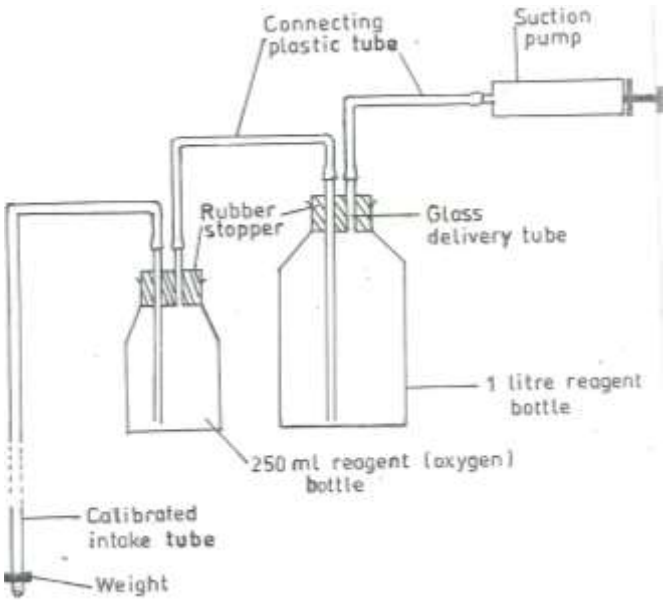

Figure 1. Sampler for shallow water bodies (Nweze, 1992).

bodies of about 5 m depth. It solves the problem of cost of acquisition of conventional samplers for sampling small water bodies such as small natural lakes, rivers, streams; and reservoirs for fisheries and water treatment facilities, that need to be studied in order to document the biodiversity and study of various local aquatic ecosystems.

Design

The sampler under presentation utilises suction pressure induced by a suction pump to collect water from various depths from a shallow water body. The sampler consists of a manual suction pump produced from a hand bicycle pump connected to a series of sample bottles by plastic delivery tubes. A calibrated plastic hose from the last bottle in the series away from the pump is dropped into the depth of sampling from a boat, dingy or raft. Samples from appropriate depths are sucked into the sample bottles by operating the bicycle pump (Nweze, 1992) (Figure 1).

Merits of the sampler

Materials for its construction are affordable and readily available in any local market. Samples from various depths can be collected with ease. It is simple to operate and no electric motor is required.

MATERIALS AND METHODS

1. Bicycle pump with air outlet at the tip: The base of the bicycle pump was unscrewed and the plunger was pulled out. The valve was removed, turned inside out (inverted) and greased with petroleum jelly. The valve of the pump was inverted to create a suction pump.

2. Reagent bottles of various volumes with plastic/ground glass caps: Their mouths (in-let openings) were wide enough to fit a rubber stopper with two openings. The number of bottles used depends on the replicates needed and the variables under investigation.

a. Two 250/300 ml oxygen bottles, preferably with ground glass caps. More bottles may be needed for biochemical oxygen demand (BOD) and chemical oxygen demand (COD).

b. Two 500 ml bottle for sample's phycological, bacteriological and physico-chemical analyses: temperature, colour, pH, alkalinity, phosphates, etc.

c. One 1000 ml bottle for chlorophyll, etc.

d. One 2500 ml bottle to receive the initial water that passed through the system. This could be used for investigations of integrated samples from the water column.

3. Glass delivery tubes

Dimension: diameter of 4 or 4.5 mm; minimum length required = 100 cm; Number = 12 (a pair for each bottle). Using a glass cutter, six tubes 8 cm long for attachment of the inlet hose were cut neatly, followed by another set of six tubes measuring 7 cm long, for attachment of the outlet hose. The glass delivery tubes could fit perfectly into the openings in the stopper and the plastic hose without air leaks. Lager diameter tubes require more suction pressure and the bicycle pump may not be adequate.

4. Plastic rubber stoppers to fit each bottle (six in number): The stoppers have 2 holes bored, each with 4 or 4.5 mm diameter depending on the size of available glass delivery tube.

5. Transparent plastic delivery tube/hose. The quantity required depends on the number of bottles used and the maximum depth to be sampled. A minimum of 7 m should be purchased for a water body of about 5 m depth.

a. For each bottle, there will be a long inlet tube (about the dimension of the depth of the bottle) cut as follows: 8 cm length: for the oxygen bottles = 16 cm for two bottles; 12 cm length: for 500 ml reagent bottles = 24 cm for two bottles; 14 cm length: for 1000 ml reagent bottles = 14 cm; m length for 2.500 ml bottle =18 cm.

Plate 1. Photograph of assembled pump sampler for shallow water bodies.

b. Connecting tubes for the bottles and pump cut as follows: 30 cm length: six pieces for 5 bottles and the suction pump = 180 cm; used plastic tube (total length) = 252 cm.
Note: The long inlet tube in the sample bottle will let in water to the bottom of the sample bottle and displace the air out of the bottle into the next bottle. Inlet glass delivery tubes could be long enough to reach the bottom of the sample bottles but they are not as durable as the plastic tubes.
c. Inlet tube: The remaining plastic tube is calibrated in centimetres and meters starting from one end, the 'zero' (0 cm) end, that will be dropped into the water under investigation.
6. Petroleum jelly: This acts as grease for lubricating the valve of the suction pump and sealing of junction between glass delivery tube and rubber stopper.
7. Bunsen burner/gas lighter (optional): For flaming plastic tubes to fit into the glass delivery tubes.
8. Wooden frame to accommodate bottles (optional).

Assembling steps

A schematic representation of the basic composition of the sampler is presented in Figure 1.

Step 1: One piece of 60 cm length plastic tube was flamed at one end and attached to the valve end of the suction pump.
Step 2: Two glass delivery tubes were passed through the openings on each of the rubber stoppers. The intake glass tube (8 cm) was extended a few centimetres into the specimen bottle, while the outlet glass delivery tube stopped at the opening of the rubber stopper.
Step 3: The remaining five (5) 30 cm lengths of plastic tubes are used to connect the rubber stoppers in series starting from the first oxygen bottle (A) to the next (B), to the 500 ml bottles (C and D), 1000 ml bottle (E) and lastly to the 2500 ml bottle (F) (outlet of bottle A to inlet of bottle B; outlet of bottle B to inlet of bottle C and so on to the last bottle- 2500 ml).
Step 4: The 2 500 ml bottle is connected to the 60 cm plastic tube from the suction pump.
Step 5: A weight is attached at the 'zero' end of the calibrated intake hose that will be dropped into the water during sampling (a needle and nylon thread may be used). The weight will make the tubing to be erect in water. The other end to the intake tube is attached to the intake glass delivery tube of the first oxygen bottle.
Step 6: The bottles are placed in the wooden rack (optional) and covered with their appropriate rubber stoppers with connecting tubes.
The sampler is ready for use (Plate 1).
Note: If the setup is not air tight, the junctions between glass tubes and stoppers are greased with petroleum jelly or water proof sheet/tape applied to make the system air tight.

Procedure for using the sampler

1. The weighted end of the calibrated intake tube (zero end) was dropped into the water to the depth to be sampled. Water is pumped up into the bottles until the 2500 ml bottle is filled. On initial filling, the contents of the bottle were discarded if a composite sample of the water column is not required. It contains water displaced from the depth of sampling to the surface that flushed out the air in the oxygen bottles. It gives a composite sample for the entire column, from the depth of collection to the surface and cannot be taken as a component of the depth sampled.
Note: The volume of the submerged tube in the water should be less than the volume of the last bottle in the series to ensure complete displacement of air in the sampling bottles and water from unwanted depths. The plastic tubes of 4 and 4.5 mm diameter have the capacity of 12.57 and 15.91 ml, respectively for one metre length.
2. The rubber stoppers of the oxygen bottles are removed and the bottles capped with the ground glass stoppers. The hose is removed from the water; the stoppers with tubes on the other bottles are removed and the bottles covered with their caps.
3. The samples were fixed or analysed as directed for the methods of determination of the various parameters under investigation. For instance, the dissolved oxygen was recorded immediately if using a field meter or fixed with reagents for the appropriate titrimetric method. Alkalinity and colour determinations should not be delayed. Also, other parameters that can be recorded immediately with *in situ* probes should be attended to.

DISCUSSION

The limitations faced by limnologists in developing countries in having access to standard samplers are enormous. Those that have the opportunity of working with standard samplers in laboratories overseas may not afford to purchase them and bring them home due to high cost of procurement, hence the need to develop an affordable alternative.

The use of this sampler eliminates the challenge of the investigator being restricted to subsurface and net samples only because of unavailability of standard underwater samplers. Studies have shown that even in shallow water bodies, there could be vertical variations in population and water quality parameters; hence, reports based on sub-surface samples only may not give a true picture of the characteristics of the water body. This shortcoming can be eliminated by sampling below the surface at various depths.

The sampler has the advantage of ensuring that there would not be the need for turning out water samples for oxygen and BOD determinations into other vessels/bottles, and samples for biological (plankton and bacteriological) and physicochemical studies are also collected simultaneously.

Apart from the low cost of assembling the components which are readily available locally, the sampler will not induce turbulence in the water; it is highly adaptable and its light weight makes it easy to transport.

Conflict of interest

The author has not declared any conflict of interest.

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academicJournals

Vol. 15(39), pp. 2176-2183, 28 September, 2016
DOI: 10.5897/AJB2016.15462
Article Number: 4B5BD8F60683
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation of the simultaneous effects of a heat stabilized starter concentration and the duration of fermentation on the quality of the opaque sorghum beer

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Received 12 May, 2016; Accepted 19 August, 2016

This study evaluates the simultaneous effect of dried starter concentration and fermentation duration on the quality characteristics of the African opaque sorghum beer using response surface methodology. The aim was to improve the beer quality and to optimize its fermentation process. Results show that the granule starter concentration and the fermentation duration have significant effects on the dry matter, refractive index, titrable acidity, glucose, raffinose and fructose contents of the beer. The lactic acid bacteria, yeasts and total mesophilic aerobic bacteria counts were significantly modified as a result of these fermentation parameters. The pH was only affected by the fermentation duration. The optimum beer quality could be reached between 10 and 15 h at starter concentration in the interval of 100 to 120 g/L. The use of the dried starter granules revealed to be an efficient alternative to produce sorghum beer with stable quality at a shorter fermentation time.

Key words: starter, kpete-kpete, sorghum beer**,** tchoukoutou.

INTRODUCTION

Opaque sorghum beers significantly contribute to the diet of millions of people in Africa due to their relatively low alcoholic content and high dry matter and nutrients concentration (Novellie and De Schaepdrijve, 1986). The beers are known as *tchoukoutou* in Benin (Kayodé et al., 2005), *dolo* in Burkina-Faso (Dicko et al., 2006), *burukutu* or *otika* in Nigeria (Odunfa, 1985), *bili bili* in Tchad (Maoura et al., 2005), *Kaffir* in South Africa (Novellie and De Schaepdrijver, 1986), *doro* or *chibuku* in Zimbabwe (Chamunorwa et al., 2002) and *ikigage* in Rwanda (Lyumugabe et al., 2010). *Tchoukoutou,* the Benin opaque sorghum beer, is produced from guinea corn (*Sorghum bicolor*) by women using various processes. In general, as in the conventional lager beer process, the manufacturing process consists of three main phases: malting, mashing and fermentation. Grains of sorghum are soaked in water overnight (9 to 12 h), germinated (72 to 85 h), sun dried (7 to 15 h), ground in a disc mill, mixed

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with water, decanted and divided into slurry and supernatant. The slurry is mashed under gradual heating until the boiling point is reached after 2 h, mixed with supernatant and allowed to ferment overnight, then filtered, boiled (6 to 9 h), cooled, and inoculated with a starter called *kpete-kpete*, which is generally harvested from the bottom of a previous fermenting beer (resulting from 13 to14 h overnight fermentation). The fermentation step is a critical step in the process, and its success depends on the accurate knowledge of the processor in terms of the starter handling. Several researches reported that the microorganisms contained in the traditional starters of African opaque beers mainly consist of yeasts and lactic acid bacteria (LAB) (Van der Aa Kühle et al., 2001; Demuyakor and Ohta, 1991; Sefa-Deheh et al., 1999; Sanni and Lönner, 1993). The microorganisms are kept alive by replacing the supernatant on a daily basis. The preservation of such starter is a tedious and a risky business since it is common that the starter loses its fermenting properties and therefore fails to make the beer effervescent, as a result of the death of the involved microorganisms.

Preliminary data demonstrated that rural and urban women's groups in the processing chain of opaque sorghum beers derive a direct benefit from increased marketing opportunities (Kayodé et al., 2007). Thus, innovations in the traditional brewing technology and the product quality could significantly improve the income and livelihood of rural households involved in this activity. Recently, Kayodé et al. (2012) defined a granule starter for the fermentation of opaque sorghum beer; but still, the effective dose and the fermentation duration for this granule are not determined.

The aim of the present study was to determine the optimum doses of the granule starter for the fermentation of the African opaque sorghum beers. More specifically, the objective was to evaluate the effect of the starter concentration and the fermentation duration on several beer quality in determining various factors such as dry matter, refractive index, pH, titratable acidity, ethanol, glucose, raffinose, fructose, maltose, yeast, LAB and total mesophilic aerobic bacteria counts of the fermenting beer. It is quite likely that these factors are interdependent. However, interactions between factors cannot be detected using the one-factor-at-a-time approach (Giovani, 1983). Therefore, we used the response surface method in applying a central composite design.

MATERIALS AND METHODS

Production of the granule starter and wort

The granule starters were produced using a tannin-free sorghum variety, yeasts and lactic acid bacteria harvested from the traditional starter of opaque sorghum beer. The processing procedure was according to the method described by Kayodé et al. (2012). Sorghum wort was produced according to the traditional brewing practices, as follows: sorghum grain were soaked overnight (10 h), germinated (72 h), sun to dried (12 h), ground in a disc mill, mashed in water by gradually heating until the boiling point was reached after 2 h, soured during an overnight rest, filtered, boiled (8 h) and cooled.

Experimental design

Response surface methodology is a statistical method that uses quantitative data derived from an appropriate experimental design, with quantitative factors use to estimate the relationship between a response and the factors in order to optimize processes or products (Giovani, 1983). In this study, an orthogonal rotatable central composite design (Montgomery, 2001) for K = 2 factors was used, to estimate the simultaneous effect of two process variables on physico-chemical and microbiological characteristics of sorghum fermenting beer in a quadratic function. The variables (factors) were the starter concentration (0-150 g/L), and the fermentation duration (0 to 24 h). In this experimental design, dry matter, refractive index, pH, titratable acidity, ethanol, glucose, raffinose, fructose, maltose, yeast, LAB and total mesophilic aerobic bacteria counts are considered as the responses. The design generated 14 observations which are distributed as follows: 4 kernel points, 4 star points and 6 replications at the central point. The design matrix and variable combinations are presented in Table 1.

Experimental fermentation

Sorghum wort (14 L) was inoculated with the granule starter at concentrations varying between 0 and 150 g/L. The inoculated wort was allowed to ferment at room temperature for an indicated time as specified in the experimental design (Table 1). When the predefined times were reached at a given starter concentration, samples were withdrawn, divided into two parts. The first part was immediately analyzed for microbiological characteristic while the second part was kept at -4°C for physicochemical analyses.

Counts of microorganisms

Total counts of mesophilic aerobic bacteria, LAB, yeasts and molds were enumerated according to the method described by Nout et al. (1987). Duplicate samples of stabilized starter (10 g) were diluted in 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5g NaCl, and 1000 mL distilled water, pH = 7.0) and homogenised with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Total mesophilic aerobic bacteria counts were determined on plate count agar (PCA, oxoid, CM 325, Hampshire, England) after incubation at 30°C for 72 h. Viable counts of LAB were determined on the Man, Rogosa and Sharpe agar (MRSA, CM 361, Oxoid, Hampshire, England) containing 0.1% (w/v) natamycin (Delvocid, DSM, The Netherlands) after incubation at 30°C for 72 h in anaerobic jar (Anaerocult A, Merck KGaA, Germany). Viable yeasts were determined on Oxytetracyclin glucose yeast extract agar (OGYA, Oxoid CM 0545, Basingstoke, Hampshire, England) after incubation at 25°C for 72 h.

Physico-chemical analysis

Titratable acidity and pH were determined as described by Nout et al. (1989). Dry matter was determined according to the American Association of Cereal Chemists (AACC) Approved Methods (AACC, 1984). The refractive index was measured using a refractometer (Sopelem 9596, France).

Table 1. Matrix of the model and combination of variables

Numbers	Level codes		Level of the variable	
	Quantity of starter	Fermentation duration	Quantity of starter (g/ L of wort)	Fermentation duration (h)
1	0	0	75	12
2	0	0	75	12
3	0	0	75	12
4	0	0	75	12
5	0	0	75	12
6	0	0	75	12
7	-1	-1	29.92	4.79
8	+1	-1	120.07	4.79
9	-1	+1	29.92	19.21
10	+1	+1	120.07	19.21
11	0	-α	75	0
12	0	+α	75	24
13	-α	0	0	12
14	+α	0	150	12

HPLC analysis of sugars and alcohol

Ethanol and soluble sugars were determined following the method developed by Mestres and Rouau (1997) using the column Aminex HPX87H-Biorad (Hercules, USA) thermostated at 37°C. Elution was done with sulfuric acid 5 mM at a flow rate of 0.6 mL/min. Detection was at 210 nm. Analyses were performed in triplicate.

Statistical analysis

Data were analyzed using the Minitab 14 statistical program. A second order polynomial model was proposed to establish the relationship between the responses (*Y*) and the variables (*X*) as follows:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_1^2 + b_4x_2^2 + b_5x_1x_2$$

Where, b_0 is constant, b_1 and b_2 are linear effect coefficients, b_3 and b_4 are quadratic effect coefficients, and *b5* is an interaction affect coefficient. The fitted polynomial equations were expressed in a 3D response surface in which the response is presented on the vertical axis and two factors at the two horizontal perpendicular axes.

RESULTS AND DISCUSSION

Effects of starter concentration and fermentation duration on dry matter and refractive index

The response values for the different treatments are presented in Table 2. The linear regression coefficients estimated are presented in Table 3. After the different treatments applied, the dry matter and the refractive index ranged between 3.42 and 15.15%, 9.50 and 15.00 (Table 2), respectively. The analysis of variance showed that the starter concentration (X_1) and the fermentation duration (X_2) significantly affect ($P < 0.001$) the dry matter content and the refractive index of the wort. Particularly, the linear effects of X_1 and X_2 on the dry matter content of wort are significant whereas the refractive index of the wort is significantly affected both by the linear and the quadratic effects of these factors (Table 3). Figure 1b shows the trend in refractive index as function of the starter concentration, the fermentation duration and their mutual interaction. Increase in the starter concentration and the fermentation duration results in a decrease in the refractive index values. This trend could be explained by the fact that the increase of starter concentration raised the level of microorganisms and then facilitated the degradation of soluble sugars. This degradation was marked for starter concentration in the range between 0 and 120 g/L and for the fermentation duration between 0 and 10 h. In opposite, we observed that when the starter concentration increased, the dry matter content of the wort also increased. Indeed, the high value in dry matter content (92.5 %) of granule starter used would have significantly contributed to the beer dry matter content.

Interestingly, one of the most important characteristic of African sorghum beers is their relatively high dry matter content which range between 5 to 13 g/100 ml (Agu and Palmer, 1998; Briggs et al., 2004). In the present experiment, it could be seen from the response surface plot that such value of dry matter content could be achieved at starter concentration between 100 and 120 g/L and fermentation duration at an interval of 20 and 22 h.

Effects on pH and titratable acidity

Acidity is one of the most important quality criteria to measure the acceptability as well as the stability and the conservation of the fermented foods (Kayodé et al., 2005). The acidity of the beer is measured through pH

Table 2. Response of the model for the physicochemical and microbiological characteristics of the wort.

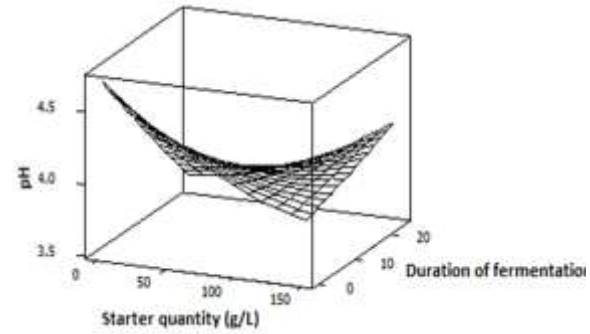
Code	Dry matter (%)	Refractive index	pH	Titratable acidity (% acide lactique)	Ethanol (%)	Glucose (mg/100g)	Raffinose (mg/100g)	Fructose (mg/100g)	Maltose (mg/100g)	Yeast (log_{10} CFU ml^{-1})	Lactic acid bacteria (log_{10} CFU ml^{-1})	Total count (log_{10} CFU ml^{-1})
1	8.78	9.50	3.98	1.20	2.45	0.66	0.48	0.00	0.00	8.24	8.86	9.98
2	8.18	9.80	3.99	1.30	2.45	0.62	0.48	0.00	0.00	8.07	8.61	9.79
3	8.73	9.80	3.99	1.30	2.45	0.60	0.36	0.00	0.00	8.91	8.2	9.88
4	8.46	9.70	3.98	1.40	2.45	0.57	0.44	0.00	0.00	8.21	8.86	9.95
5	8.26	9.80	3.97	1.40	2.45	0.77	0.50	0.00	0.00	8.92	8.19	9.91
6	8.74	9.80	3.91	1.30	2.45	0.74	0.62	0.00	0.00	8.93	8.89	9.96
7	6.19	13.80	4.21	1.30	2.45	3.36	2.05	0.40	8.92	7.18	6..91	7.92
8	11.28	12.00	4.15	0.80	1.65	2.40	1.41	0.15	4.07	7.72	7.56	7.82
9	5.07	9.80	3.77	2.40	2.31	0,35	0.51	0.00	0.00	8.13	8.11	8.36
10	9.41	10.00	4.19	1.40	2.25	0.38	0.28	0.00	0.00	8.21	8.08	8.65
11	12.58	15.00	4.18	0.90	1.06	3.10	1.92	0.40	0.00	6.22	6.08	6.32
12	6.70	10.00	3.73	2.10	2.81	0.42	0.41	0.00	0.00	8.43	8.38	9.76
13	3.42	14.00	4.26	2.10	0.45	2.96	2.09	0.65	0.00	6.40	7.23	7.42
14	15.15	9.80	3.84	0.90	2.57	0.60	0.34	0.00	0,00	7.80	7.84	8.26

Table 3. Values of the coefficients in the model and their significance

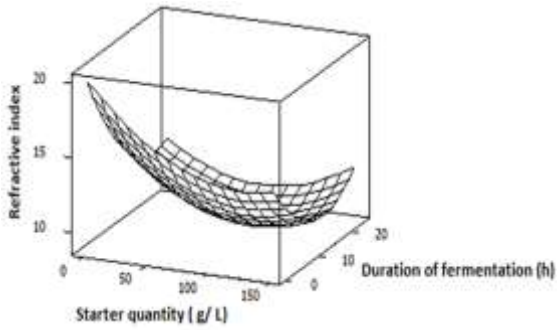
Coefficient	Dry matter (%)	Refractive index	pH	Titratable acidity	Ethanol	Glucose	Raffinose	Fructose	Maltose	Yeast	Lactic acid bacteria	Total count
b_o	8.382***	9.720***	3.980***	1.319***	2.478***	0.677**	0.487***	-0.006	0.382	8.573***	8.595***	9.876***
b_1	5.050***	-1.498***	-0.059	-0.610***	0.465	-0.846**	-0.661***	-0.231***	-0.846	0.514*	0.285	0.277
b_2	-2.229***	-2.499***	-0.200*	0.645***	0.588	-1.65***	-0.904***	-0.213***	-2.266	0.893**	0.968***	1.220***
b_3	0.345	2.127***	0.109	0.192**	-0.858	1.174**	0.758**	0.308***	1.109	-1.369**	-1.085**	-2.174***
b_4	0.701	2.727***	0.014	0.192**	-0.433	1.154**	0.707**	0.184*	1.108	-1.144**	-1.391***	-1.974***
b_5	-0.519	1.385	0.332	-0.346**	0.512	0.686	0.282	0.169	3.357	-0.318	-0.471	0.270
R^2	86.4	93.8	39.4	97.8	35.5	89.5	90.1	86.2	0.550	76.4	86.4	86.5

b_0: constant; b_1 and b_2: coefficients for starter concentration; b_3 and b_4: coefficients for duration of fermentation; and b_5: coefficient for interaction (starter concentration x duration of fermentation). *Significant at $p < 0.05$. **Significant at $p < 0.01$. ***Significant at $p < 0.001$. Data reported in this table are the measured (fitted) values of the coefficients b_0, b_1, b_2, b_3, b_4 and b_5, which are explained in detail in the statistical analysis section.

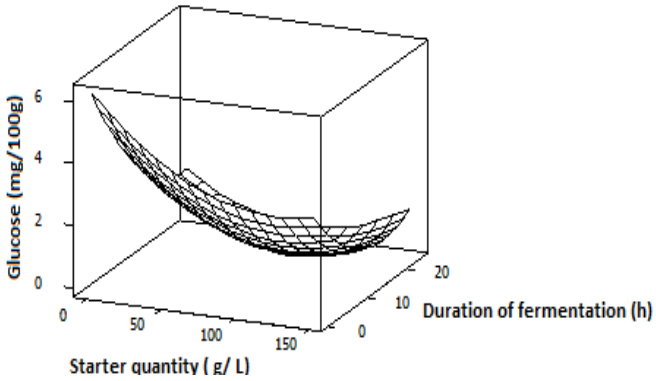
and titratable acidity. After the different treatments applied, pH ranged between 3.73 and 4.26 whereas titratable acidity ranged between 0.80 and 2.40 g kg^{-1} d.w (Table 2). The analysis of variance showed that, starter concentration did not significantly affect the pH but the linear effect of the fermentation duration is significant ($p < 0.05$). Figure 1a shows the trends in pH of wort which functions as starter concentration, the



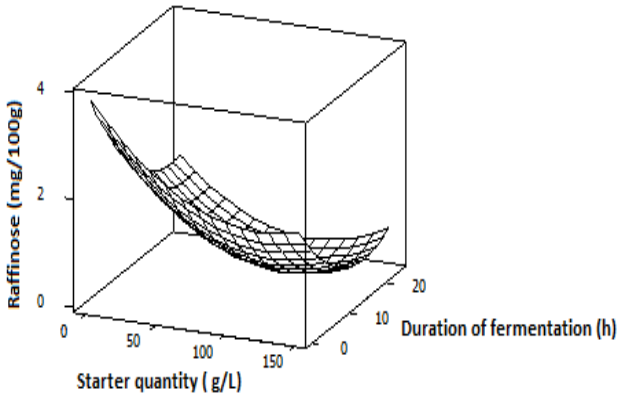
a) Effects on pH



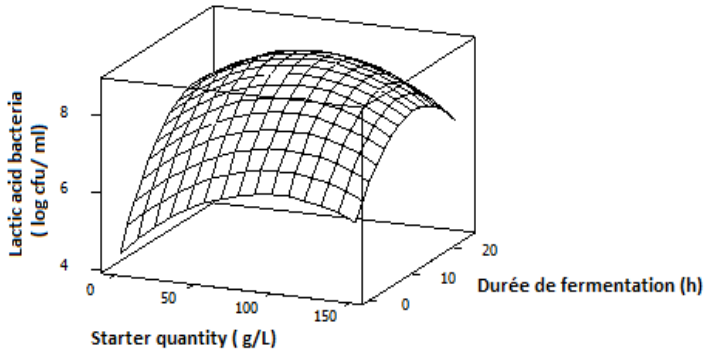
b) Effects on refractive index



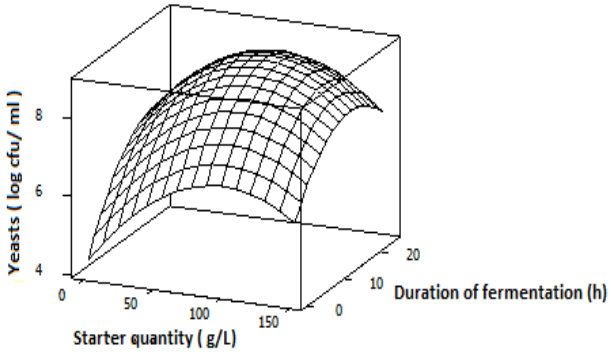
c) Effects on glucose concentration



d) Effects on raffinose concentration



e) Effects on lactic acid bacteria content



f) Effects on yeasts content

Figure 1. Response surface curves showing the effects of starter concentration (g L^{-1}starter in wort) and duration of fermentation on (a) pH, (b) refractive index, (c) glucose, (d) raffinose, (e) lactic acid bacteria and (f) yeasts content of sorghum wort.

fermentation duration and their mutual interaction. At fermentation duration > 20 h the pH was around 3.73 for starter concentrations between 120 and 150 g/L. For the titratable acidity, it was significantly ($p < 0.001$) affected by the two factors (X_1 and X_2). The linear and quadratic effects of these factors were significant on the wort titratable acidity (Table 3). The titratable acidity increased with the fermentation duration whereas it decreased when the starter concentration increases. As expected, the fermentation duration significantly affects the pH and the titratable acidity. This effect was expressed by a decrease in the pH and an increase in the product acidity when the fermentation duration increases. Previous studies reported that the process of sorghum wort fermentation is characterized by the increase of titratable acidity and the decrease of the pH (Hounhouigan et al., 1999; Mugula et al., 2003; Annan et al., 2003) and such trend is a characteristic of cereals fermented foods.

Effects on alcohol and soluble sugars contents

Ethanol has been identified as an important metabolic compound along with the different treatments applied. Sanni and Lönner (1993) identified ethanol as an essential alcoholic compound obtained from the fermentation process during the production of local sorghum beers such as pito, burukutu, sekete and agadagidi. Ethanol concentrations of beers from the different treatments were ranged between 0.45 and 2.81% (Table 2). Studies reported that the ethanol content in various African sorghum beers range between 2 and 3% (Sanni and Lönner, 1993; Kayodé et al., 2005). The analysis of variance showed that none of the factors significantly affected the ethanol concentration. This trend is in line with the results reported by N'Gessan et al. (2008) who found that, the increase of the inoculum concentration did not affect the ethanol production during sorghum wort fermentation.

According to the Mateo et al. (2001), in such inoculums, various strains of yeasts could be in an ecological competition. In our case the yeast strains contained in the starter used were not selected. Though to the ecological competition, the effect of the starter concentration and the fermentation duration was not significant. It is necessary to isolate the main yeast strains which contained in the starter in order to assess the effects of both factors on the beer alcohol content.

The soluble sugars identified in the fermented wort were: glucose, raffinose, fructose and maltose. After the different treatments applied, the concentration of these soluble sugars ranged between: 0.35 and 3.36 mg/100 g for glucose; 0.28 and 2.09 mg/100 g for raffinose; 0.00 and 0.651 mg/100 g for fructose; and 0.00 and 8.921 mg/100 g for maltose (Table 2). The analysis of variance showed that none of the factors significantly affected the maltose content while, the starter concentration (X_1) and the fermentation duration (X_2) significantly ($P < 0.05$) affected glucose, raffinose and fructose contents. Particularly, the linear and the quadratic effects of these factors were significant ($p < 0.01$) on these sugars content (Table 3). Figure 1a and d show, respectively the evolution of glucose and raffinose contents as a function of the starter concentration, the fermentation duration and their mutual interaction. The trends were quite similar. The two factors increased while the soluble sugars contents (glucose, raffinose and fructose) decreased. This effect was significant at starter concentrations ranging from 0 to 120 g/ L and at fermentation duration from 0 to 20 h. Clearly, the increase of starter concentration increases the fermentation activities in the wort. During the alcoholic fermentation process, yeasts transform these soluble sugars into ethanol (Leyral and Vierlin, 2007). After 20 h of fermentation, the increase of the starter concentration did not result in the increase of soluble sugars consumption. The main incriminated factor could be nitrogen limitation (Barre et al., 1998; Manginot et al., 1998). It was also reported that the amount of assimilable nitrogen influences the synthesis of sugars transporters (Bisson, 1999).

Effects on microbial content

The major microorganisms involved in the fermentation of opaque sorghum beer are LAB and yeasts (Maoura et al., 2005; Lyumugabe et al., 2010). As a result of the treatments applied, the number of yeasts, lactic acid bacteria and total mesophilic aerobic bacteria varied significantly as a function of the two factors and are respectively in the range between 6.08 and 8.89 log CFU/ml ; 6.22 and 8.93 log CFU/ml; and 6.32 and 9.98 log CFU/ml. The analysis of variance showed that the starter concentration and the fermentation duration significantly ($P < 0.05$) affect the different microorganisms groups. Particularly, the quadratic effects of these factors on the microbial content were very significant (($p < 0.001$). Figure 1 shows the trends in the number of microorganisms as a function of the starter concentration, the fermentation duration and their mutual interaction. High level of the starter concentration and fermentation duration results in high concentration of microorganisms in the wort. This trend is more remarkable for values in starter concentrations ranging between 0 and 50 g/L, and for fermentation duration between 0 and 10 h. This observation could be explained by the intensive fermentation rates due to the load and the faster microflora development at the highest rates of inoculation (Gotcheva et al., 2001). After 20 h of fermentation, the yeasts counts decreased. This could probably be due to the inhibitory effect of ethanol on yeasts growth. It was reported that a high content of ethanol in the wort can inhibit the growth, the viability, the metabolic activity of fermentative yeasts (Aguilera et al., 2006 ; Canetta et al., 2006 ; Hu et al., 2006 ; Hirasawa et al., 2007; Kitagaki et al., 2007; Lei et al., 2007; Wang et al., 2007; Watanabe et al., 2007; Wei et al., 2007).

Relationship between beer quality parameters

The Pearson correlation matrix of variables (Table 4) showed a correlation between the pH and others parameters such as: Refractive index ($r = 0.722$; $p < 0.01$), ethanol rate ($r = - 0.680$; $p < 0.01$), glucose concentration ($r = 0.752$; $p < 0.01$), yeasts ($r = - 0.623$; $p < 0.05$) and LAB ($r = - 0.598$; $p < 0.05$). It also revealed a significant relationship between the refractive index and ethanol concentration ($r = - 0.780$; $p < 0.001$), glucose concentration ($r = 0.967$; $p < 0.001$), yeast counts ($r = - 0.899$; $p < 0.001$) and lactic acid bacteria content of the wort ($r = - 0.913$; $p < 0.001$). The pH was negatively correlated with the ethanol content, yeasts and the LAB. This is in accordance with the fermentation characteristics

Table 4. Pearson correlation matrix of variables.

Parameter	Refractive index	pH	Ethanol	Glucose	Yeasts
pH	0.722**				
Ethanol	-0.780***	-0.680**			
Glucose	0.967***	0.752**	-0.717**		
Yeasts	-0.899***	-0.623*	0.798***	-0.829***	
Bacteria	-0.913***	-0.598*	0.662**	-0.848***	0.836***

*Significant at 5%; **significant at 1%; ***significant at 1‰.

of the African traditional beer which is a mixed fermentation that is lactic fermentation and alcoholic fermentation (Valyasevi and Rolle, 2002). During the lactic fermentation the increase in LAB content results in the production of lactic acid which lowers the pH and favours the yeasts growth. The ethanol content was positively correlated with the yeasts and LAB counts.

The alcoholic fermentation is characterized by the increase in yeasts which transform soluble sugars into ethanol (N'Guessan et al., 2008). That could also explain the negative correlation between glucose concentration and ethanol ($r = - 0.717$; $p < 0.01$). Yeasts are positively correlated with LAB ($r = 0.836$; $p < 0.001$). A symbiotic relationship between yeasts and LAB was previously reported (Nout, 1991; Savova and Nikolova, 2002). LAB create an acid environment which are favorable to yeasts growth (Yao et al., 2009) and produce vitamins to increase other factors, such as amino acids, to aid the growth of LAB (Lyumugabe et al., 2010).

Conclusion

The use of dried starter granules derived from the traditional starter brought significant improvements in the beer quality at relatively short fermentation duration. The application of the response surface methodology revealed the linear effects of the processing parameters as well as their mutual interactions. We recommend the use of the granule starter in the fermentation of African opaque sorghum beers for the production of stable beer at shorter fermentation duration.

Conflict of interests

The authors have not declared any conflict of interests.

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Vol. 15(39), pp. 2184-2191, 28 September, 2016
DOI: 10.5897/AJB2015.15177
Article Number: 2D18A5160685
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Preliminary investigation into the chemical composition of the invasive brown seaweed *Sargassum* along the West Coast of Ghana

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Received 17 December, 2015; Accepted 8 August, 2016

The arrival of the invasive brown seaweed *Sargassum* in the Western Region of Ghana was first reported in 2009. This impacted negatively on biodiversity, tourism and the livelihoods of the coastal communities. The objectives of the study among others were to identify and determine the nutritional and toxicological contents of the seaweed. Twenty four samples collected from 6 zones along the Western Region were analysed. Nutritional and toxicological analyses were carried out using an Atomic Absorption Spectrophotometer (AAS) 900T. Results of the study indicated that the *Sargassum* samples analyzed contained low concentrations of nitrogen. However, nitrogen utilized by plants, namely, nitrate and ammonia were very high, together with phosphates. This makes the *Sargassum* a good source organic fertilizer. However, the high concentrations of toxic heavy metals in the *Sargassum* defeat this assertion. Heavy metals have implications in both the growth and metabolic activities of plants. Most heavy metals, especially arsenic and lead are carcinogenic and are capable of causing skin, lung, liver and bladder cancers and miscarriages. Indiscriminate domestic and industrial wastes disposal, oil and gas activities, mining and high shipping traffic may have contributed to the heavy metal concentrations in the seaweeds.

Key words: Seaweed, *Sargassum*, nutrients, toxins, health.

INTRODUCTION

Invasion of the brown seaweeds *Sargassum* species on the beaches and in estuaries in the coastal regions of Ghana especially the Western Region was reportedly first recorded in 2009. Since then there have been several other invasions which have impacted negatively on biodiversity, tourism and the livelihoods of coastal communities especially the fisher folks whose lives are dependent on the fishing industry.

Sargassum belongs to the Phylum Ochrophyta, class Phaeophyceae and constitutes a distinct taxonomic group due to their morphological, anatomical and physiological complexities. It is the most diverse genus of marine macrophytes (Xie et al., 2013), including 265 genera and roughly about 1500 species worldwide.

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Sargassum spp. are known to have a broad geographical distribution from Central America, through Australia, New Zealand, Asia, Europe and Africa (Guiry and Guiry, 2011). *Sargassum* is highly tolerant to environmental parameters such as desiccation, full sunlight and variations in salinity and temperature. This enables it to occupy a broad range of habitats from the upper intertidal, mainly rock pools to the sub tidal and substrata from exposed rock to Eel-grass beds. The *Sargassum* spp. normally all reproduce sexually except for the halopelagic *Sargassum natans* and *Sargassum fluitans* which reproduce only by fragmentation (Dawes and Mathieson, 2008; Rogers, 2011).

In Ghana, two indigenous species of *Sargassum* have been recorded, namely *Sargassum vulgare* C.Agardh 1820, occurring in the intertidal zone and *Sargassum filipedula* C.Agardh 1824 which occurs on the offshore reefs.

The species *Sargassum* invading Ghana is believed to originate from the "Sargasso Sea" situated in the Western Atlantic tropical region of the Northern Hemisphere (Szechy et al., 2012). It is encircled by a gyre formed the Gulf Stream, The North Equatorial Current and the North Atlantic Drift (Luning, 1990).

Seaweeds are marine resources of various economic uses. The economic importance of seaweeds or algae lies on its utilization as a source food and in the pharmaceutical and medicine industries. For example *Sargassum* is known to form about 10% of the average diet in Japan where it is eaten raw as salad or cooked in coconut milk (Oyesiku and Egunyomi, 2014). It is also used as in Chinese medicine as an expectorant for bronchitis and to treat laryngitis, hypertension, infections, fever and goiter (Hou and Jin, 2005). The major products derived from the utilization of seaweeds are agar, alginic acid, also called alginin or alginate, fucoidan and carrageenan (McHugh, 2003; Namvar et al., 2013). Fucoidans are sulphated polysaccharides unique only to brown algae such as the *Sargassum* that have been found to have anti-tumor, anti-metastatic, anti-viral, anti-coagulant and anti-bacterial properties in laboratory trials (Khotimchenko, 2010).

Seaweeds are exported either in their raw forms (fresh or dried seaweeds) or in processed forms (carrageenan and kelp powder). They also provide energy to grazers and contribute immensely to the benthic detritus food chain. The seaweed beds also form important habitat for fishes and invertebrates. They are also known to be useful indicators of climate change and can be used to determine the diversity patterns and especially for planning conservation and sustainability of inshore marine resources (John and Lawson, 1991; Bolton et al., 2003).

Recently, several authors have published studies on the chemical composition of the *Sargassum* including their nutrients and heavy metal content. This is because of the usually heavy invasions and the need to put it to some economic use. Sudharsan et al. (2012) worked on seaweeds, including *Sargassum* and *Padina*. They observed these brown seaweeds were more tolerant to Zinc and recorded the highest level of zinc. Oyesiku and Egunyomi (2014) also did proximate analysis and chemical composition analysis especially nitrogen, phosphorous and potassium on *Sargassum* samples found offshore in Ondo State, Nigeria. Manivannan et al. (2009) reported that the brown algae *Padina gymnospora* showed the maximum content of mineral composition such as copper, chromium, iron, lead, sulphur, and calcium content than other seaweed. Nazni and Deepa (2015) also recently looked at minerals and heavy metal present in five selected red seaweed of South Coast Region of Tamilnadu with the aim of evaluating the minerals and heavy metals present in the seaweeds present in the south coastal line. They concluded that seaweeds are a potential source of essential minerals which can be used to promote good health.

The objectives of this study were to identify the invasive *Sargassum* on the coast of Ghana and to determine the chemical composition in order to promote its use as food or fertilizer to improve the livelihood of the coastal communities.

MATERIALS AND METHODS

Study area

Samples (24) collected offshore and onshore at low tide at specific locations along the coast of the Western Region of Ghana delineated into 6 zones (Figure 1).

Identification of *Sargassum*

The Seaweeds samples were identified using keys described by John et al. (2001, 2003). The Brown alga *Sargassum* spp. was identified in all the samples from the 6 zones, whilst the rest of the algae were only identified from zone 6 samples.

Chemical analysis of *Sargassum* spp.

Thirteen chemical parameters were analyzed. These were grouped in two major components; nutritional and toxicological parameters. The nutritional parameters were nitrogen, phosphate, ammonia, nitrate and potassium. The toxicological parameters were copper, zinc, iron, lead, cadmium, mercury, arsenic and chloride.

Preparation of seaweed sample for chemical analysis

Samples were washed under a jet of tap water and rinsed with distilled water to remove any minerals particles and organisms attached to the seaweed.

Principle

Heavy metals in plants are measured in a digest obtained by treating samples with an acid mixture made from concentrated nitric

Figure 1. Map of the study area in the Western Region of Ghana.

acid, concentrated sulphuric acid, and perchloric acid (Anderson and Ingram, 1989; Maiti, 2003).

Analytical procedure

Dried sample weighing about 1.0 g were placed into a 125 ml Erlenmeyer flask which has been previously washed with acid and distilled water. Ten milliliters of Ternary mixture (20 ml $HClO_4$: 500 ml HNO_3: 50 ml H_2SO_4) were added under a fume hood. The contents were thoroughly mixed and heated gently at low to medium heat on a hot plate under a perchloric acid fume hood. Heating was continued until dense white fumes appear (that is, fumes of sulphuric acid). After the mixture was heated strongly for half a minute, it was allowed to cool and 40 to 50 ml distilled water was added, boiled for half a minute on the same plate at medium heat. The solution was then cooled again and filtered using Whatman filter paper (No. 42, 9 cm) and made up to 100 ml in a Pyrex volumetric flask. Make up to the mark with distilled water. The solution was stored for heavy metal determination. Analysis was carried out using an Atomic Absorption Spectrophotometer (AAS 900T). Table 1 shows the various wavelengths used in the analysis.

Table 1. Wavelengths used in the AAS 900T for the determination of each metal.

Parameter	Wavelength
Mercury	253.7
Arsenic	242.8
Cadmium	228.8
Lead	283.3
Iron	248.3
Zinc	213.9
Potassium	766.5
Copper	324.8

RESULTS AND DISCUSSION

Identification of *Sargassum*

The Brown alga *Sargassum* spp. was identified in all the 24 samples from the 6 zones. *Sargassum* spp. identified using physical characteristics (in the identification guide courtesy of Dr. Jim Franks and the University of Southern Mississippi Gulf Coast Research Laboratory, Figure 2b) were not known to occur naturally in the Gulf of Guinea and especially Ghana. These were *S. fluitans* and *S. natans* (Figure 2).

The two identified species of *Sargassum*, namely, *S. fluitans* and *S. natans* are Holopelagic remaining free-floating through their entire life cycle. They reproduce vegetatively and never attach to the sea floor during their life cycle. Thus, they do not need a holdfast, an organ at the base of the seaweed that attaches the weed to a surface. In West Africa, three *Sargassum* spp. have been identified. These are *Sargassum cymosum* C.Agardh 1820 occurring mainly in Serra Leone and Gabon with *Sargassum filipendula* C. Agardh 1824 occurring in

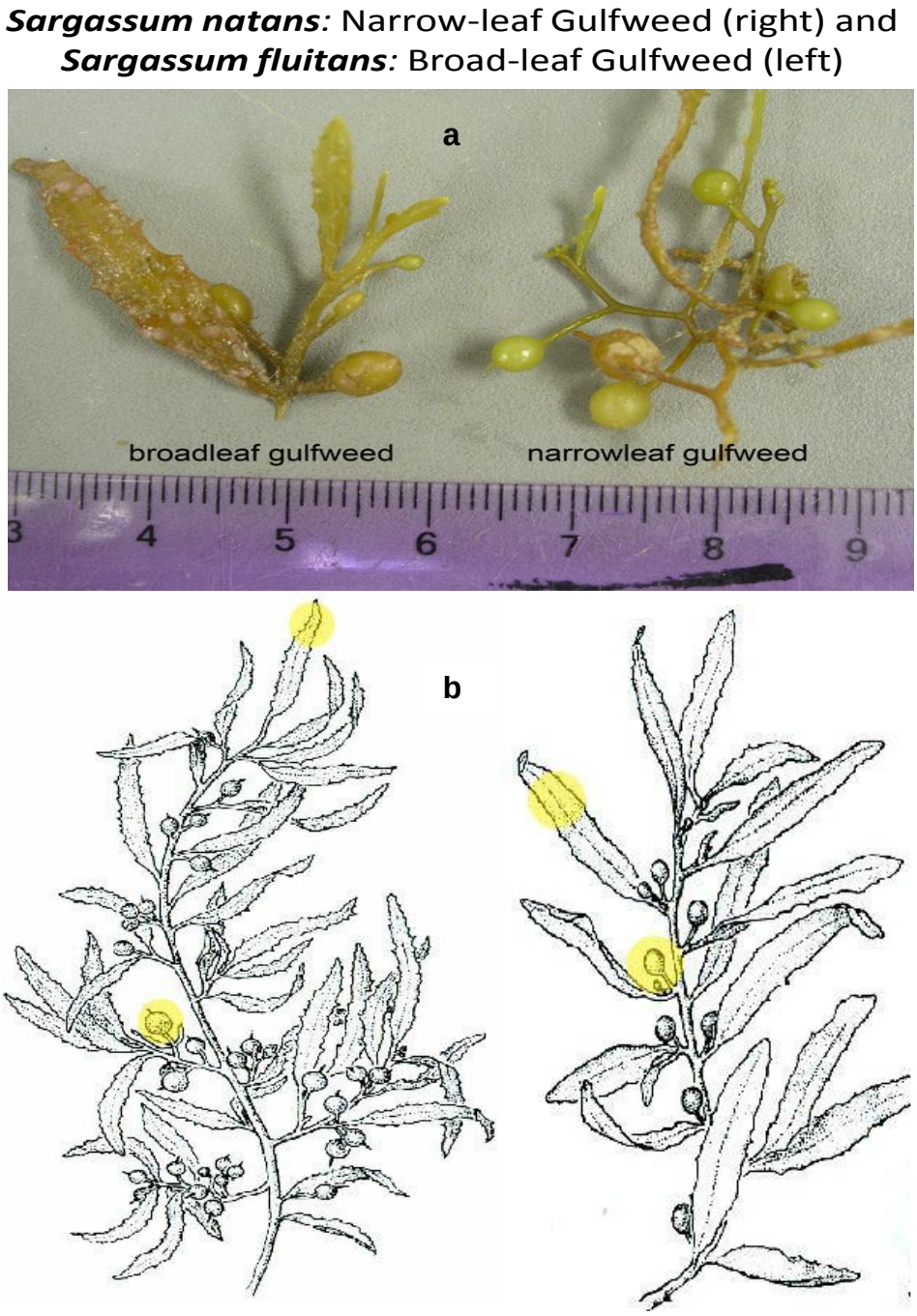

Figure 2. (a) *Sargassum fluitans* and *Sargassum natans*; (b) *Sargassum natans* (Pods – usually tipped with spikes or small leaves; Leaves – long-stalked, narrow) and *Sargassum fluitans* (Pods – usually not tipped with spikes or small leaves; Leaves – short-stalked, broad).

Ghana and Gabon, whilst the third *S. vulgare* C.Agardh 1820, the most common, is found from Senegal to Gabon.

As reported in Ghana, only *S. vulgare* found usually growing attached to inshore rocks and *S. filipendula* found usually growing attached under water offshore reefs.

Chemical analysis

The results of the chemical analysis of the *Sargassum* (Tables 2 and 3) were broken down in two broad sections; nutrients and heavy metals. The nutrients are among the elements known as essential nutrients needed for normal plant growth. Essential nutrients must be

Table 2. Concentrations of nutrients (mg/kg) recorded in the Invader *Sargassum* species from the various sampling zones along the coast of the Western Region, Ghana.

Parameter (ppm)	Zone 1 /onshore	Zone 2 /onshore	Zone 2	Zone 2 /onshore	Zone 3 /onshore	Zone 3 /onshore	Zone 3 /onshore	Zone 5	Zone 6 /onshore	Acceptable limits (ppm)
	Half Assini	Egbazo	Assiama	Asunda	Atuabo	Miemia	Axim/Agyan	Africa Beach	Essipon	
Nitrogen (N)	0.67	0.87	0.84	0.95	0.87	1.04	0.62	N/A	0.62	1 to 6
Phosphate $(PO_4)_2$	1.02	1.37	1.53	1.16	0.83	1.43	1.52	N/A	1.55	0.05 to 1
Amonia $(NH_4)_3$	460.8	878.4	568	354.4	734.4	424.8	741	N/A	554.4	1 to 6
Nitrate (NO_3)	208.8	201.6	180	187.2	410	237.6	288	N/A	216	1 to 6
Potassium (K)	2.28	1.88	1.42	0.72	2.48	2.41	1.08	N/A	1.86	0.3 to 6

Table 3. Concentrations of heavy metals (mg/kg) recorded in the Invader *Sargassum* species from the various sampling zones along the coast of the Western Region, Ghana.

Parameter (ppm)	Zone 1 /onshore	Zone 2 /onshore	Zone 2	Zone 2 /onshore	Zone 3 /onshore	Zone 3 /onshore	Zone 3 /onshore	Zone 5	Zone 6 /onshore	Acceptable limits (ppm)
	Half Assini	Egbazo	Assiama	Asunda	Atuabo	Miemia	Axim/Agyan	Africa Beach	Essipon	
Copper $(CU)^{++}$	27	27	28	30	29	36	24	N/A	22	20 to 100
Zinc (Zn)	79	16	37	57	100	44	22	N/A	37	100 to 400
Iron $(Fe)^{++}$	1413	1952	5910	1209	1530	1226	2284	N/A	2550	10 to 100
Lead $(Pb)^{++}$	248	329	169	105	225	335	190	N/A	86	30 to 300
Cadmium $(Cd)^{++}$	102	95	119	78	97	111	98	N/A	80	37
Mercury $(Hg)^{++}$	1	1	1	2	1	1	1	N/A	2	1 to 3
Asemic $(As)^{++}$	27.3	13	17	53.5	53.5	20	36	28.8	24	5 to 20
Chloride $(Cl)^{+}$	1353.2	1240.4	1353.2	225.54	61	1353.2	22.54	N/A	830.14	0.05 to 3

specific and cannot be replaced. A heavy metal is defined as a metallic element which is toxic and has a high density, at low concentrations can excite some biological processes, but at threshold concentrations becomes toxic (He et al., 1998).

Nutrients

The results of nutrient analysis (Figure 3) of the samples showed they contain a low concentration of nitrogen. However, plants utilized nitrogen in the form of nitrate (NO_3) and ammonia (NH_4) which occurred in very high concentration, together with phosphate (Table 2). Potassium a key element for plant growth was relatively high falling within the range expected in plants (0.3 to 6 ppm) (Peters and Laboski, 2011). This will facilitate the potential use of *Sargassum* as an organic fertilizer or manure. Oyesiku and Egunyomi (2014) also recommended the use of *Sargassum* as fertilizer after obtaining a good percentage ratio of N-P-K of 1-10-3. However, further enhancement of nutrient levels by addition of big slurry, chicken droppings or cow dunk will be needed. High nutrient in water bodies are associated mainly with domestic wastes both liquid and solids, industrial pollution, agricultural waste waters including inorganic fertilizer.

The nutrition content and utility of the seaweed

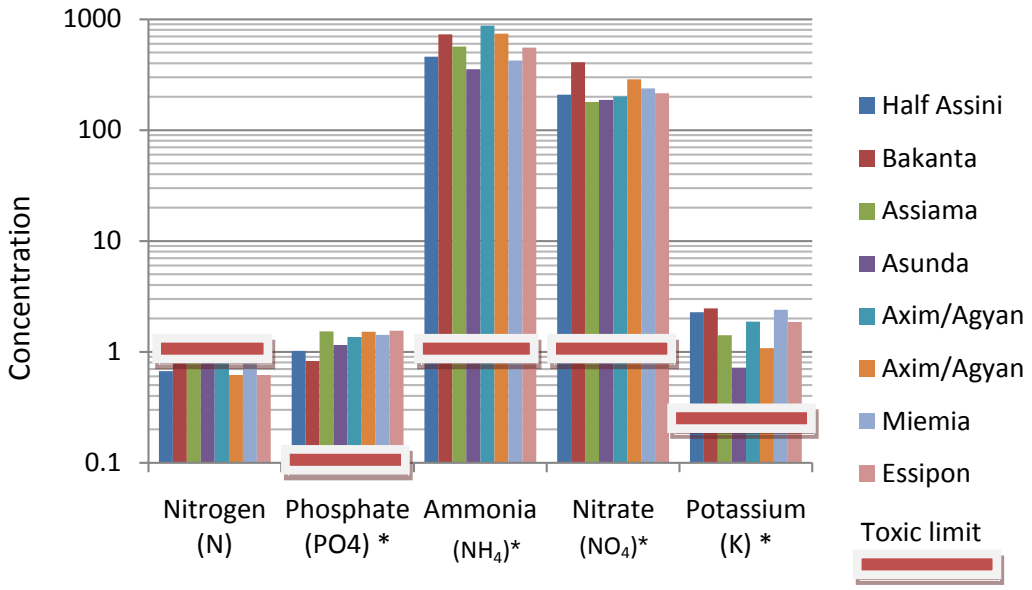

Figure 3. Histogram of the concentrations of nutrients (ppm) measured in the *Sargassun* samples analysed.

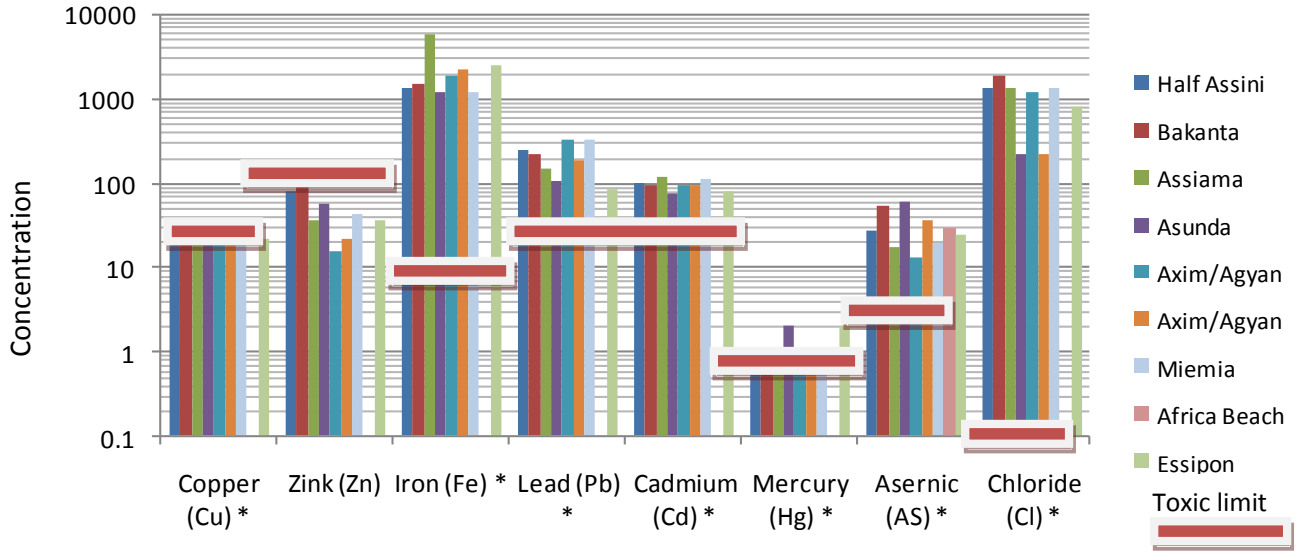

Figure 4. Histogram of the concentrations of heavy metals (ppm) measured in the *Sargassun* samples analysed.

cannot be considered alone without incorporating the results of the heavy metal concentrations found in all the samples evaluated in this study.

Heavy metals

Heavy metals being non-biodegradable can accumulate at various trophic levels through the food chain and can cause human health problems (He et al., 1998). In humans, these metals build up in living tissues and thus increase the dangers they pose by causing physical distress and life threatening illness such as damage to vital body systems ((Sudharsan,et al., 2012). Seaweeds are excellent agents for filtering heavy metals such as zinc, cadmium, copper, nickel and iron from the sea. Seaweeds are able to accumulate these metals from their environment into body cells to as much as 4,000 to 20,000 times more than in the surrounding waters (Sudharsan et al., 2012).

As noted in Figure 4, the concentrations of heavy metals from the evaluated samples are all broadly the

same. The minimum acceptable limit of these heavy metals in plants is shown by the red bars. From the analysis, six (Cu, Fe, Pb, Cd, Hg and As) out of the seven heavy metals measured were found to be in the toxic range (Kabata-Pendias, 2001). Only zinc was found to be below the toxic range. Sudharsan et al. (2012) reported that zinc was moderately absorbed by seaweeds and seagrasses. Veroy et al. (1980) explained that the ability for uptake of heavy metals in any species was based on the nature of their cell wall and polysaccharides. Other factors may be due to competition and the ability by the metals to bind with such polysaccharides which has been reported to be preferential (Paskins-Hurlburt et al., 1976). This may explain the low concentration of zinc as compared to the other metals.

Quari (1976) reported that red seaweeds have more ability to uptake these metals when compared to brown and green seaweeds. Heavy metals have implications in both the growth and metabolic activities of plants and humans. Sathya and Balakrishnan (1988) have reported that Cadmium even at very low concentrations can cause physiological disturbance like protein, carbohydrate and pigment concentration. Cadmium levels above 20 ppm in seaweeds have been designated for polluted environment (Lozano et al., 2003). In our current studies, a higher cadmium range of 78 and 119 ppm were recorded across the sampled locations. High Cadmium concentrations have been attributed to influx of domestic sewage and content of local gold bearing rock formations.

According to Al-Abdali et al. (1996), calcium carbonate ($CaCO_3$) in rock formation contains much cadmium impurities. In addition, steel used in port and industrial facilities contain cadmium coatings which can end up in surrounding waters. In a study conducted by Lozano et al. (2003), cadmium was reported to be more concentrated in red and brown seaweed than green seaweed. *Sargassum* is a brown alga and this may explain the high cadmium concentration recorded in the plant in this study.

Copper which also fell within the toxic range is an essential micronutrient in plants but at high concentrations can affect photosynthesis and lead to de-pigmentation as well as depressing growth in plants (Sathya and Balakrishnan, 1988).

Organisms which accumulate contaminants in their tissues can be used to assess the health of coastal environments, including their presence, levels and changes in contaminants. Seaweeds are such organisms which have been used as bio-monitor of heavy metals (Al-Homaidan, 2007; Kamala-Kannan et al., 2008).

It is possible that the sources of Pb, Fe and As all of which fell in the toxic range (Table 3) could be coming from the same source as many studies have shown a strong correlation between these three metals (Santos-Santos et al., 2006). These sources may include paints, dyes, certain fertilizers, metals and pesticides as well as industrial wastes. Combustion of fossil fuels and oil pollution could also account for the high concentrations of these three metals. Nazni and Deepa (2015) recorded high levels of lead in five seaweeds and attributed it to combustion of fossil fuels and oil pollution. The Western Region has for a long time been a hub of the gold industry and the coastal areas are at the terminus of the inland drainage system from renowned mining locations as Prestea, Tarkwa and Obuasi. It is now also the hub of oil exploration and drilling activities and all efforts must be made to thoroughly investigate the sources of these heavy metals so as to be clear as to where they originate.

Most heavy metals, especially arsenic and lead are carcinogenic and are capable of causing skin, lung, liver and bladder cancers. Low levels of arsenic are suspected to cause nausea and vomiting, damage to blood vessels and sensations (pin and needles) in the hands and feet. Exposure to high levels of lead can lead to damage of the kidney and brain. In pregnant women, it can lead to miscarriages and in men can damage the organ responsible for sperm production (Centre for Hazardous Substance Research, Kansas State University, Issue 15th March, 2009).

Iron recorded the highest concentration of 5,910 ppm at Essiama (Zone 2) (Figure 4). The air dried sample was taken from a wire fence, which is another likely source of the iron. Ali et al. (2011) had earlier also reported iron to be the highest in their study of seaweed species in the Strait of Hormuz, Iran. As mentioned earlier, accumulation of metals in seaweed depends on the type of polysaccharides in the seaweed and since various elements have different electronegativity, these can affect the type of metals to be taken actively, hence, the high uptake of iron by *Sargassum*.

Studies by Abdallah and Abdallah (2008), reported that variation of concentrations of metals in seaweed species from different sampling locations may be related not only to different metal levels, but also to factors such as tidal range, temperature, salinity regimes, dissolved nutrients, tissue type, age of plants, its nutritional history and the geological structure of the study area.

In this study, the highest concentrations of copper, lead and cadmium were all recorded in zones 2 and 3 (Table 3 and Figure 4) and Miemia (an important tourists hub situated in a cove west of Cape). Three points has the highest concentrations of all three metals out of all the locations sampled. Although, zones 2 and 3 stand out for having the highest toxic levels of these three metals, it should be noted that insufficient sampling of zones 4, 5 and 6 prevents further conclusions to be drawn as to whether the highest observed toxicity is focused on the western half of the study area, although intensive mining and industrial activity is associated with this area. Ali et al. (2011) recorded similar trends in an area with intense human and industrial activities. Gold mining is very much associated with these types of heavy metal load in the

water which can travel down river into the sea. Seabed trawling by offshore fishing vessels may lead to agitation and mixing of the sediments with the overlaying waters, which increase the heavy metals load in the water and hence make them available for uptake by the seaweed for example the *Sargassum*.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENT

The authors heartily acknowledge Lukoil Overseas Ghana Limited for providing the funds for this research.

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